ヒト消化器癌のp53依存性アポトーシス関連分子異常の解析及びその治療応用

（研究課題番号14570466）

平成14年度～平成15年度 科学研究費補助金

基盤研究（C）（2）研究成果報告書

平成16年3月

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（研究課題番号）１４５７０４６６

平成１４年度～平成１５年度科学研究費補助金

（基盤研究（Ｃ）（２））研究成果報告書

平成１６年３月

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はしがき

本研究は、科学研究費基盤研究（Ｃ）（２）として補助を受け、平成１４年度より２年間に渡って行われたものである。本研究代表者は平成９～１０年度にわたり科学研究費、奨励研究（代表、０９７７０４０３；ＴＧＦ-βシグナル伝達に関わる分子の消化器癌における異常の解析及び癌治療への応用）により、ヒト肝発癌過程における癌関連遺伝子の異常を解析し、その成果を肝癌の診断や治療に応用する研究を推進してきた。本研究では従来の研究結果をもとに、さらにDNA損傷における細胞周期の停止、アポトーシス誘導の中心的役割を担うｐ５３経路の各分子の異常が解析され、肝発癌における役割が検討されたものである。

腫瘍の発生、進展には複数の遺伝子変化が必要であり、ヒト肝細胞癌（肝癌）においても現在までに多様な遺伝子／染色体変化が報告されている。一方、癌抑制遺伝子であるｐ５３遺伝子産物およびその経路に関わる各分子が、DNA損傷における細胞周期停止、アポトーシス誘導において主要な役割を担っていることが明らかとなり、また多種のヒト腫瘍においてｐ５３経路分子の異常が報告されている。

本研究において、肝発癌におけるｐ５３経路異常の全体像が明らかになり、肝発癌における役割が明確になったと思われる。その特徴の一つとして、肝癌では発癌初期より染色体の異常が観察されるが、ｐ５３経路異常が生じるのはより進行した段階であり、発癌初期に出現した異常染色体をもつ細胞にｐ５３経路異常が加わり、染色体異常がより拡大すると考えられた。今後はこれらの成果を、遺伝子診断や治療に結びつけたいと考えている。

最後に、本研究を行うにあたり御協力頂いた共同研究者、大学院生、実験助手や秘書の方々に深謝致します。

平成１６年３月

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交付決定額（配分額）　　（金額単位：千円）

	直接経費	間接経費	合計
平成１４年度	2，１００	0	2，１００
平成１５年度	1，４００	0	1，４００
平成　　年度			
平成　　年度			
平成　　年度			
総　　計	3，５００	0	3，５００

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研究成果による工業所有権の出願・取得状況

なし

Alteration of the *p14*^{ARF} gene and p53 status in human Hepatocellular Carcinomas

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Short title; The p14^{ARF} gene and p53 status in HCC

Abstract

Background

The INK4a/ARF locus encodes $p16^{INK4a}$ and $p14^{ARF}$, both 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 *p14*^{ARF} gene in hepatocellular carcinoma (HCC) from 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 *p14*^{ARF} gene, and alterations of p53 were also analyzed in the same series of HCCs.

Results

Homozygous deletion spanning from exon 1 β to exon 2 was found in one, and mutations within exon 2 were found two cases, but no promoter hypermethylation was detected. All three HCCs with $p14^{ARF}$ alteration were well differentiated. Twelve of the 44 HCCs (27.2%) showed immunohistochemical evidence of p53 alteration, however only one revealed to be well differentiated among tumors with p53 alteration. TaqMan PCR indicated that expression of $p14^{ARF}$ of HCCs was higher than all but three of the corresponding non-tumorous tissues ($P < 0.001$), and increased expression of $p14^{ARF}$ seemed to be associated with poorly differentiated phenotype. Absence of expression was seen in only one with homozygous deletion of the *p14*^{ARF}.

Conclusions

Compared with p53 alteration, $p14^{ARF}$ alteration does not occur frequently, but may play a role in a subset of Japanese HCC in early stage of hepatocarcinogenesis. On the other hand, overexpression of $p14^{ARF}$ was frequently observed in HCC, especially in poorly differentiated tumor probably reflecting oncogenic stimuli in these tumors.

Key words

$p14^{ARF}$, p53, INK4a/ARF locus, hepatocellular carcinoma

Introduction

The INK4a/ARF locus on chromosome 9p21 has a unique genetic organization, which codes two proteins with different functions. These two transcripts arising from different first exon, alternatively spliced into common second exon, give rise to two distinct and unrelated proteins with tumor suppressor 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 *p16*^{INK4a} gene is known to be inactivated by mutations, homozygous deletions, or promoter methylation in many tumors of diverse origin.[4-6] These features provide strong evidence that $p16^{INK4a}$ plays a critical role in various oncogenic processes. The principal inactivation mechanisms of the *p16*^{INK4a} are varying widely,[7-9] but its frequent elimination as a result of methylation of the promoter region in hepatocellular carcinoma (HCC) has been reported recently.[10, 11]

As for $p14^{ARF}$, deletion inactivation of the INK4a/ARF locus has been reported in several human cancers.[12-14] The promoter hypermethylation of the CpG islands of the *p14*^{ARF} gene as well as the *p16*^{INK4a} has been reported in primary colorectal,[15] gastric,[16] and esophageal cancers.[17] Moreover, alterations of $p14^{ARF}$ expression levels have been observed in lung cancer,[18, 19] breast carcinomas,[20, 21] colorectal tumors,[22] and hematological malignancies.[23] 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,[24] but the frequency and mechanism of inactivation of $p14^{ARF}$, the key trigger of p53 stabilization, has not yet been identified.

The focus of the study presented here is therefore on alterations of $p14^{ARF}$ of 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 investigate p53 alterations in the same series of HCCs. In this report, we describe alterations of $p14^{ARF}$ in HCCs from Japanese population with special attention to p53 inactivation and discuss the role of disruption of the p53/ARF pathway during human hepatocarcinogenesis.

Methods

Samples

HCC samples and their 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 and immediately stored at –80 °C. Eight patients were positive for hepatitis B surface antigen (HBsAg), 27 for hepatitis C virus antibody (HCVAb), two for both HBsAg and HCVAb, while seven 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 moderately differentiated and nine 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.[25] Of the 44 HCCs, 28 were subjected to quantitative RNA expression analyses. Informed consent was obtained from all patients.

Methylation analysis of the $p14^{ARF}$ gene promoter

The methylation status in the CpG islands of the $p14^{ARF}$ 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 $p14^{ARF}$ promoter. The details of the modification reaction, PCR conditions and sequences of the primers used have been described previously.[15] The primer sequences designed for the $p14^{ARF}$ promoter spanned six CpG within the 5' region of the gene. Primer sequences for the unmethylated $p14^{ARF}$ promoter were 5'-TTTTTGGTGTTAAAGGGTGGTGTAGT-3' (sense) and 5'-CACAAAAACCCTCACTCACAACAA-3' (antisense), which amplify a 132-bp product. The primer sequences for the methylated $p14^{ARF}$ promoter were 5'-GTGTTAAAGGGCGGCGTAGC-3' (sense) and 5'-AAAACCCTCACTCGCGACGA-3' (antisense), which amplify a 122-bp product. The annealing temperature for the PCRs of the unmethylated and methylated promoter was 60℃. DNA from KATO-III (HSRRB JCRB0611, Osaka, Japan) of a gastric cancer cell line, which is reported to contain the methylated promoter of the $p14^{ARF}$ gene,[16] was used as a positive control for the methylated sequence, and DNA from normal peripheral lymphocytes was used as a

negative control. The PCR products were electrophoresed on 1% agarose gels, stained with ethidium bromide and visualized under ultraviolet illumination.

Homozygous deletion analysis of the $p14^{ARF}$ gene

We used comparative multiplex PCR to detect homozygous deletions in exon 1 β and exon 2 of the $p14^{ARF}$ gene as previously described.[11, 17] Two primer sets of the $p14^{ARF}$ exon 2 and the *b-actin*, as well as exon 1 β and the *b-actin*, were amplified simultaneously in a single reaction for each comparative multiplex PCR. PCR was performed at a final volume of 25 μl with a pH of 8.4 including 10 mM Tris-HCl, 50 mM KCl, 50 μg/ml BSA, 1.5 mM $MgCl_2$, 5% DMSO, 0.2 mM of each deoxynucleotide triphosphate, 0.5 units of Taq polymerase (Applied Biosystems, Foster City, CA), 50 ng of the template DNA and 20 pmol of each primer. All reactions were hot-started and cycle parameters were initially denatured at 95°C for 5 minutes followed by 25 cycles at 95℃ for 0.5 minutes, 60℃ for 0.5 minutes and 72℃ for 0.5 minutes. 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-SSCP and sequencing of the $p14^{ARF}$ gene

PCR-single strand conformation polymorphism (PCR-SSCP) analyses for exon 1 β and exon 2 of the $p14^{ARF}$ gene were performed, by using primer pairs designed to cover the entire coding region and under the conditions described by Kita *et al.*[7] After denaturation at 99 °C for 5 minutes, the PCR products were loaded onto 6% polyacrylamide gels with or without 10% glycerol and resolved by electrophoresis at 20 °C. 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) on an ABI 310 genetic analyzer (Applied Biosystems).

Relative quantitative real-time RT-PCR of $p14^{ARF}$ mRNA

cDNA was generated from total RNA by using Super-Script™ reverse transcriptase (Gibco BRL, Rockville, MD) according to the manufacturer's instructions. Oligonucleotide primers and TaqMan probes were designed using Primer Express,

version 1.0 (Applied Biosystems). The sequences of the PCR primer pair and the TaqMan probe 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 minutes and at 95 °C for 10 minutes, followed by 40 cycles at 95 °C for 15 seconds and at 60 °C for 1 minute. The standard curve was constructed with serial dilutions of cDNA from the HLF cell line (HSRRB JCRB 0405, Osaka, Japan) for analysis of $p14^{ARF}$ mRNA expression, which clearly expressed the mRNA of the target genes. In order to compare the 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)*, 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 averaged from the values of duplicate reactions.

Immunohistochemistry for p53

The primary antibodies used in this study were 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 minutes with biotinylated anti-mouse IgG (1:200 dilution; Vector Laboratories, Burlingame, CA), and incubated for 30 minutes together with the avidin-biotin peroxidase complex reagent with the aid of a Vectastain ABC kit (Vector Laboratories). 0.05% diaminobenzidine was used as the final chromogen, and hematoxylin was used as the nuclear counterstain.

Statistical Analyses

The χ^2 test, Fisher's exact test, Mann-Whitney U-test or Kruskal-Wallis test was used for analyses of the relationship between expression of the mRNA and various data. For analysis of relationship between grade of differentiation and alteration of $p14^{ARF}$ or p53, the 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 methylated at the $p14^{ARF}$ CpG island, while DNA from normal peripheral lymphocytes was unmethylated at the same site as previously described.[16] On the other hand, no hypermethylation of the $p14^{ARF}$ promoter was observed in either the 44 HCCs or their non-tumorous tissues (Fig. 1A). Fig. 1B shows representative findings of comparative multiplex PCR of the $p14^{ARF}$ gene. One HCC (case 1) showed homozygous deletion in exon 1β and exon 2 of the $p14^{ARF}$ gene. All DNA samples were also screened with PCR-SSCP for mutation of the $p14^{ARF}$ 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 frame shift mutation (case 3; Fig.1C).

Relative mRNA level of $p14^{ARF}$

Target quantities of mRNA were determined from the standard curve and expressed as an n-fold difference relative to the standard sample, that is the 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, even although the quantity of GAPDH mRNA was adequate. In this HCC, homozygous deletion in exon 1β and exon 2 of the $p14^{ARF}$ 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 $p14^{ARF}$ gene showed mRNA expression (Cases 2 and 3; Table 1). In all but three HCCs, the expression of $p14^{ARF}$ mRNA was higher than that of the surrounding non-tumorous tissues ($P< 0.001$; Fig. 2A). $p14^{ARF}$ overexpression appeared to be associated with a poorly differentiated phenotype, although the association was not statistically significant ($P = 0.108$ by the Mann-Whitney U-test, well-differentiated vs. poorly-differentiated; Fig. 2B).

Alteration of $p14^{ARF}$, and p53 and grade of differentiation of HCCs

To investigate the relationship between alteration of the $p14^{ARF}$ gene and p53, we performed immunohistochemistry of p53 on 44 paraffin-embedded HCC tissues. Typical strong expressions of p53 are shown in Fig. 3. 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 *p14*^{ARF} gene, one also showed p53 alteration (Case 1; Table 1). All but one HCC with alteration of p53 were moderately or poorly differentiated ($P < 0.05$ by the Fisher's exact test; Table 2). On the other hand, all three with alteration of the *p14*^{ARF} gene were well-differentiated HCCs ($P < 0.05$ by the 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 *p14*^{ARF} gene, and compared the results with those of immunohistochemistry of p53 in the same HCC samples. Previously, Baek *et al.*[26] reported that comparative RT-PCR analysis showed no expression of p14^{ARF} in five of 20 HCCs. Of these five, three showed homozygous deletion of the *p14*^{ARF} gene, but the contribution of promoter hypermethylation of the *p14*^{ARF} gene to HCC formation was not examined. Another report showed that no promoter hypermethylation of the *p14*^{ARF} gene was detected in any of 20 HCCs.[27] Peng *et al.* also analyzed the alteration of the *p14*^{ARF} gene in 40 HCCs and found that homozygous deletion was the predominant mechanism of p14^{ARF} inactivation.[28] On the other hand, Tannapfel *et al.* identified that 9 % of HCCs showed hypermethylation of the *p14*^{ARF} promoter.[29] Herath *et al.* also reported that 46% of Australian HCC and 29% of South African HCC carried hypermethylation of the *p14*^{ARF} promoter.[30] In our series of 44 HCCs, promoter hypermethylation of the *p14*^{ARF} gene was not detected either, suggesting that it is not the major mechanism of p14^{ARF} inactivation for HCC in Japan. The fact that p14^{ARF} expression was absent in only one case with homozygous deletion in exon 1 β and exon 2 also supports this idea. The *p14*^{ARF} promoter methylation has been shown to be somewhat complex and heterogeneous methylation pattern may result in the difference of frequency of the *p14*^{ARF} promoter methylation.[22] In this respect, we might overlook some CpG methylation of the *p14*^{ARF} promoter. However, both Herath's and our studies applied identical MS-PCR using the same sequence of primer,[30] which probed most frequently methylated site among CpGs examined in colorectal cell line.[22] In addition, almost every HCC showed more p14^{ARF} expression than their corresponding non-cancerous tissue. Then, so far as Japanese HCC was concerned, we could conclude that methylation of the p14^{ARF} promoter was not so frequent. According to Herath's report, risk factors other than hepatitis B or C virus, such as hemochromatosis and aflatoxin, were identified in 24 of 37 (65%) Australian and 21 of 24 (88%) South African HCC cases.[30] On the other hand, thirty-seven of 44 HCC cases (84%) in our study showed hepatitis B or C virus infection. Although, we could not describe the clear reason for the difference in frequencies of the methylation, it might be, in part, accounted for by differences in the etiology of the patients.

Among the 44 HCCs we examined, mutation of the *p14*^{ARF} gene was found in two HCCs, although mRNA of the *p14*^{ARF} was also detected with TaqMan PCR in these tissues. Recent studies indicate that the C-terminal domain encoded by exon 2 of the *p14*^{ARF} gene, where two mutations were detected in our study, contains an important nucleolar localization signal.[31] Mutations in exon 2 may therefore affect the nuclear localization of p14^{ARF} and thereby interfere with p14^{ARF} -regulated Mdm2-dependent stabilization of p53.[32] On the other hand, mutation in exon 1 β has not been found in any of the HCCs, nor in any other malignant tumors except for melanoma.[33,34]

Evidence supporting direct biochemical interactions between p14^{ARF} and p53 has been obtained,[35] leading to the hypothesis that p14^{ARF} inactivation and the *p53* mutation in human cancers must be mutually exclusive in the mutational sense because both act on the same pathway. Such a relationship between alterations of the *p14*^{ARF} and the *p53* gene has been reported in some human cancers including HCC.[12,19,30] On the other hand, non-small-cell lung cancers and gastric cancers were reported lacking an inverse correlation between the *p14*^{ARF} alteration and the *p53* mutations.[13,16] In the present study, the population of HCCs with p14^{ARF} inactivation appears to be too small for an analysis of this relationship. However, all three HCCs with alteration of the *p14*^{ARF} gene were well differentiated tumors but only one with p53 alteration revealed to be well differentiated. These results indicated that alteration of the *p14*^{ARF} gene emerge in 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, p14^{ARF} overexpression seemed to be associated with a poorly differentiated phenotype. In addition, mRNA levels of p14^{ARF} in most cases were much higher than those of surrounding non-tumorous tissues. Patients with follicular lymphoma characterized by a high level of p14^{ARF} expression had a significantly shorter overall survival time from the time of diagnosis than other patients.[23] Several mitogenic stimuli such as E1A, myc, oncogenic ras, and E2F-1 are known to upregulate the *p14*^{ARF} gene leading to p53 stabilization.[36,37] Therefore, the high expression of p14^{ARF} mRNA observed in HCCs may reflect oncogenic stimuli and/or inactivation of other tumor suppressor pathways in these tumors, such as that of RB. Recently, we reported that 81% of human HCCs harbored alteration of one of the RB pathway molecules,[11] resulting in deregulation of E2F-1. Therefore, disruption of the RB pathway may contribute to overexpression of p14^{ARF} in

HCCs. $p14^{ARF}$ overexpression and the subsequent p53-dependent cell cycle arrest or apoptosis appear as normal cellular responses when hyperproliferative signals are present. Therefore, downstream effectors of the p53/ARF pathway may also be involved which cause elusion of cell cycle control or apoptosis in HCCs.

In the study presented here, we found that alterations of the *$p14^{ARF}$* gene are not frequent in HCCs from Japanese population. However, deletions or mutations of the *$p14^{ARF}$* gene were detected in some well differentiated HCCs, suggesting that disruption of the p53/ARF pathway with inactivation of $p14^{ARF}$ may play a role in earlier stage than that with p53 alteration during HCC formation. In addition, overexpression of $p14^{ARF}$ of 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

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Table 1

Clinicopathological findings and Alterations of $p14^{ARF}$ and p53 in 44 patients

Clinicopathological parameter				$p14^{ARF}$			p53 IHC
Case no.	Age/Sex	Virus	Differentiation	Genetic/Epigenetic Alteration	TaqMan PCR T	TaqMan PCR N	
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	1bp 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	-

Table 1

Clinicopathological findings and Alterations of $p14^{ARF}$ and p53 in 44 patients (cont.)

Clinicopathological parameter				$p14^{ARF}$			p53 IHC
Case no.	Age/Sex	Virus	Differentiation	Genetic/Epigenetic Alteration	TaqMan PCR T	TaqMan PCR N	
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	-

Table 1

Clinicopathological findings and Alterations of $p14^{ARF}$ and p53 in 44 patients (cont.)

Clinicopathological parameter				$p14^{ARF}$			
Case no.	Age/Sex	Virus	Differentiation	Genetic/Epigenetic Alteration	TaqMan PCR		p53 IHC
					T	N	
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	-

Abbreviations: B, positive for HBsAg; C, positive for 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 *GAPDH* gene was used as an internal control of TaqMan PCR. Data of $p14^{ARF}$ mRNA expression were normalized to that of GAPDH.

Table 2
Alteration of $p14^{ARF}$, p53 and grade of differentiation in HCC

$p14^{ARF}$ alteration	Grade of differentiation (no. of cases)		Total no.
	Well	Moderately or Poorly	
With	3	0	3
Without	13	28	41
Total no.	16	28	

p53 alteration	Grade of differentiation (no. of cases)		Total no.
	Well	Moderately or Poorly	
With	1	11	12
Without	15	17	32
Total no.	16	28	

All HCCs with $p14^{ARF}$ alteration were well-differentiated ($P < 0.05$ by the Fisher's exact test). On the other hand, eleven of 12 HCCs with p53 alteration were moderately or poorly differentiated phenotype ($P < 0.05$ by the Fisher's exact test).

Figure legends

Figure 1. Examples of analysis of the $p14^{ARF}$ in HCC.

(A) Representative findings of MS-PCR analysis of the $p14^{ARF}$ gene promoter. Asterisk: molecular weight marker of ΦX174/HaeIII digest; N: denotes non-tumorous tissue; T: HCC tissue; U and M:PCR products from, respectively unmethylated and methylated DNA of the $p14^{ARF}$ promoter. KATO-III which contains the methylated $p14^{ARF}$ 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 show PCR products from unmethylated DNA, but no product from methylated DNA, indicating that the $p14^{ARF}$ promoter was unmethylated.

(B) Representative findings of homozygous deletions in exon 1 β and exon 2 of the $p14^{ARF}$ gene. The homozygous deletions were identified by comparative multiplex PCR analysis. β -actin was used as an internal control. The tumor of case 1 (1T) exhibits homozygous deletion in exon 1 β and exon 2 of the $p14^{ARF}$.

(C) Direct sequencing of two cases with an abnormal mobility shift in PCR-SSCP. In the tumor of case 2, a missense mutation was detected in codon 119. In the tumors of case 3, one base pair insertion resulting in a frameshift mutation was detected in codon 69.

Figure 2. Relative mRNA levels of $p14^{ARF}$ in HCC.

$p14^{ARF}$ expressions are shown in relation to those of *GAPDH* and the mean (± SE) for each group is also shown.

(A) The mRNA levels of $p14^{ARF}$ of 28 HCCs and corresponding non-tumorous tissues. N: non-tumorous tissue; T: HCC tissue. In HCC of case 1 (△) with homozygous deletion of the $p14^{ARF}$, no $p14^{ARF}$ mRNA was detected.

(B) The mRNA levels of $p14^{ARF}$ for each grade of differentiation. The mRNA level of case 1 with homozygous deletion was excluded. Although, not statistically significant, expression of $p14^{ARF}$ was correlated with poorly differentiated phenotype (P = 0.108 by the Mann-Whitney U-test; Well vs. Poorly differentiated)

Figure 3. Immunohistochemical staining of p53 in HCC.

Strong nuclear staining of p53 in tumor cells.

Fig 1

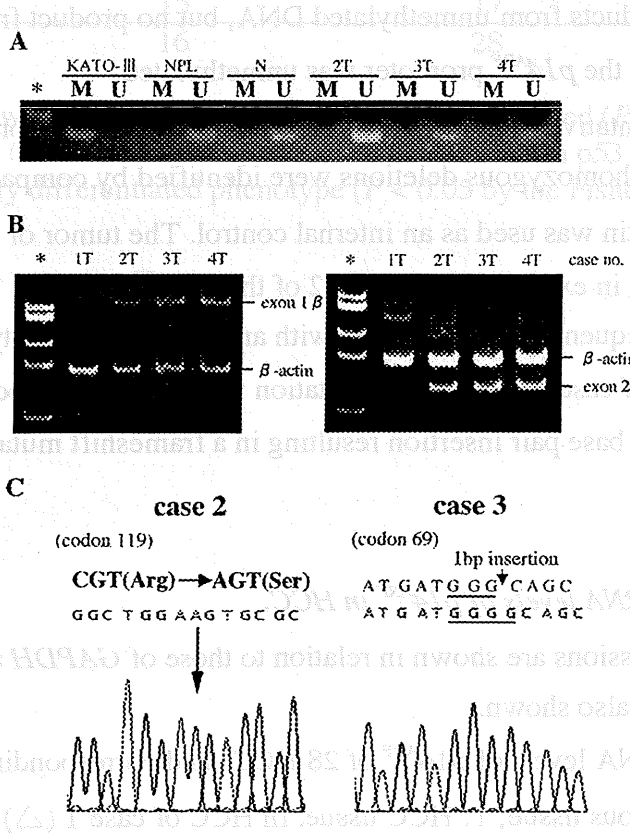
A
KATO-III
NPL
N
2T
3T
4T
* M U M U M U M U M U M U M U
B
* 1T 2T 3T 4T
exon 1β
β-actin
* 1T 2T 3T 4T case no.
β-actin
exon 2
C
case 2
(codon 119)
CGT(Arg)→AGT(Ser)
GGC T GG AAG T GC GC
case 3
(codon 69)
1bp insertion
AT GATGGGCAGC
AT G AT GGGGC AGC

Fig 2

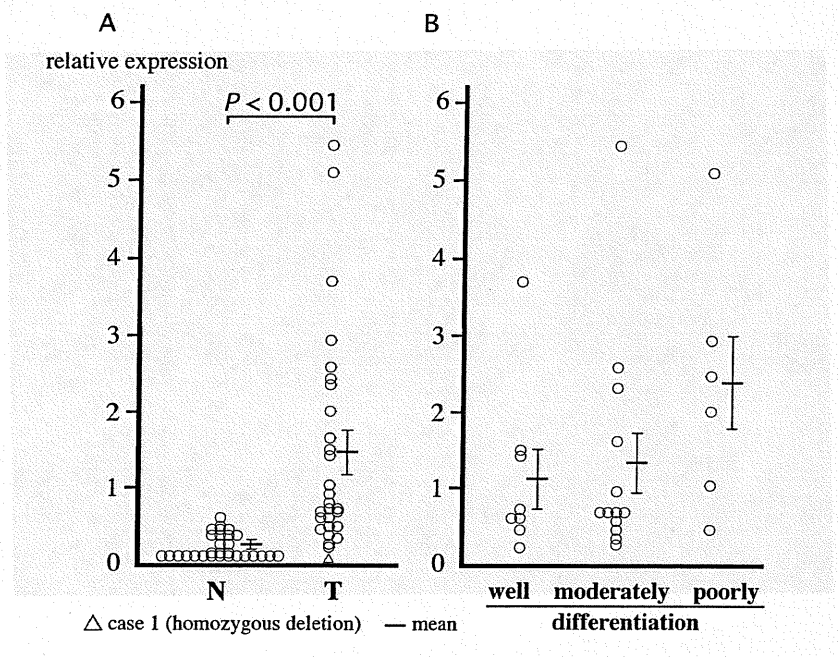
A
B
relative expression
P < 0.001
6
5
4
3
2
1
0
N
T
well
moderately
poorly
differentiation
△ case 1 (homozygous deletion)
— mean

Fig 3