Pergamon

European Journal of Cancer, Vol. 34, No. 11, pp. 1789–1795, 1998

Printed in Great Britain
0959-8049/98/$—see front matter

PII: S0959-8049(98)00189-0

Original Paper

Infrequent Mutations and No Methylation of *CDKN2A* (*P16/MTS1*) and *CDKN2B* (*p15/MTS2*) in Hepatocellular Carcinoma in Taiwan

Y.-W. Lin,[1] C.-H. Chen,[2] G.-T. Huang,[2] P.-H. Lee,[3] J.-T. Wang,[2] D.-S. Chen,[2] F.-J. Lu[1] and J.-C. Sheu[2]

[1]Department of Biochemistry, College of Medicine, National Taiwan University, Taipei; [2]Department of Internal Medicine; and [3]Department of Surgery, College of Medicine, National Taiwan University Hospital, No. 1, Chang-Te Streetcine,Taipei, Taiwan, Republic of China

***CDKN2A* (*p16^{INK4A}/MTS1*) and *CDKN2B* (*p15^{INK4B}/MTS2*) have recently been shown to be potent inhibitors of the cyclin D/cyclin-dependent kinase-4 complex. Both genes are candidates for the putative tumour suppressor genes located at chromosome 9p21 and are frequently inactivated in many human cancers through homozygous deletion. More recently, another reported pathway of inactivation involves loss of transcription associated with *de novo* methylation of the 5′ CpG island of *p16/MTS1* and *p15/MTS2* in human cancers. We examined a total of 34 tumours from 30 hepatocellular carcinoma (HCC) patients for deletion, mutation and DNA methylation of these two genes by polymerase chain reaction (PCR) amplification, sequence analysis and Southern blot. Homozygous deletions of *P16/MTS1* exon 1 were only identified in 1 of 30 cases (3%). Homozygous deletions of *p15* exon 1 or exon 2 were found in 7 of 30 cases (13%). Automated sequencing analysis of *p16* exon 1 and 2 and *p15* exon 1 and 2 failed to demonstrate mutations in either *p16* or *p15* in any of these specimens. No aberrant 5′ CpG island hypermethylation of *p16* or *p15* was found in any of the primary tumours by Southern blot. These data suggest that the *p16/MTS1* gene has a limited role in HCC. However, deletions of the *p15/MTS2* gene are found in 13% HCC and might be involved in a subset of HCC.** © **1998 Elsevier Science Ltd. All rights reserved.**

Key words: *CDKN2/p16/MTS1*, *p15/MTS2*, hepatocellular carcinoma, deletion, methylation
Eur J Cancer, Vol. 34, No. 11, pp. 1789–1795, 1998

INTRODUCTION

THERE IS a high incidence of hepatocellular carcinoma (HCC) in Asia and South Africa [1, 2]. In Taiwan, it is the most common cause of cancer mortality. The disease is known to be closely associated with chronic hepatitis B or C viral infection [2, 3], or exposure to alfatoxin B_1 and environmental factors [4]. The molecular mechanism of hepatocarcinogenesis remains to be clarified.

Mutational activation of oncogenes and mutational inactivation of tumour suppressor genes have been shown to play important roles in multistage human carcinogenesis [5]. In contrast to oncogenes, tumour suppressor genes function as negative regulators of cell growth. Thus far, more than a dozen tumour suppressor genes have been found. For example, linkage analysis of melanoma families initially pointed to chromosome region 9p13-22 as the site of a familial melanoma gene [6]. Previous studies have demonstrated that homozygous deletions of chromosome 9p21-22 are common in leukaemia, melanoma, glioma, lung cancer, mesothelioma and bladder carcinomas, suggesting that a critical tumour suppressor gene resides in this region [6–10].

Recently, a putative tumour suppressor gene, *CDKN2/p16/MTS1* has been mapped to 9p21-22 [11]. Functionally, the *CDKN2/p16/MTS1* gene is a cell cycle-related gene encoding a protein that binds to cyclin dependent kinase (CDK4) and inhibits the ability of CDK4 to interact with cyclin D and stimulate passage through the G1 phase of the cell cycle [11].

Correspondence to J.-C. Sheu.
Received 4 Sep. 1997; revised 23 Feb. 1998; accepted 5 Mar. 1998.

It has been found that the *p16* gene is frequently homozygously deleted in many cell lines [12, 13]. In addition, mutations of this gene have been observed in cell lines including melanoma, lung cancer, liver cancer, colon cancer, and pancreatic cancer [12, 13]. In primary tumours, the frequency of *p16* gene alterations (deletions and mutations) varies [14–20]. The *p15*/*MTS2* gene is located approximately 25 kb centromeric to the *CDKN2* gene [13], and is structurally homologous to *CDKN2*/*p16*/*MTS1* and is also capable of inhibiting cyclin D/CDK4 kinase activity. Although *p15*/*MTS2* has been studied less intensively than *CDKN2*/*p16*/*MTS1* in malignancies, by virtue of its location and its structural and functional homology to *CDKN2*/*p16*/*MTS1*, it is also a candidate tumour suppressor gene in this chromosomal region.

Hypermethylation of gene promoter regions, the CpG islands, resulting in loss of transcription, has recently been shown as a cause of inactivation of tumour suppressor genes, as demonstrated in the *VHL* gene in human renal cell cancer [21, 22]. In other neoplasms, such as non-small cell lung cancer, gliomas, and head and neck squamous cell carcinoma, *de novo* methylation of the 5′ CpG island of the *p16* gene is frequently found [22, 23]. This aberrant methylation correlated with the loss of expression of the normal p16, p15 or VHL mRNA. Reactivation of each gene has been obtained by treatment of aberrant methylated cell lines with the demethylating agent, 5-deoxyazacytidine [22–24], suggesting that hypermethylation of the gene is an alternate mechanism of inactivation.

So far, a few papers have reported the status of *p16*/*MTS1* gene alterations in HCC [25–27], but the role of the *p15*/*MTS2* gene in HCC has not been studied. In this paper, we examined 34 HCC samples for deletion and mutation of the *p16*/*MTS1* and *p15*/*MTS2* genes by polymerase chain reaction (PCR) amplification, Southern blot, and sequence analysis. In addition, 5′ CpG methylation of *p16*/*MTS1* and *p15*/*MTS2* was also analysed by Southern blot.

MATERIALS AND METHODS

Patients

HCC patients seen consecutively from 1987 to 1994 in National Taiwan University Hospital, who received surgical resections and for whom frozen liver tissue was available, were randomly selected for this study. The diagnosis of HCC was confirmed by histology. A total of 30 patients were selected (Table 1). 1 patient received two operations for primary and recurrent HCCs and there were two tumours in each operation (case 29). Another patient had two small tumours (case 30). Therefore, a total of 34 tumours were

Table 1. *Clinical data of hepatocellular carcinoma patients who had* p16 *and* p15 *deletions*

Case no.	Sex	Age	HBsAg	Anti-HCV	Tumour diameter (cm)	*p16* exon 1	*p16* exon 2	*p15* exon 1	*p15* exon 2
1	F	55	N	P	3.6	N	N	N	N
2	M	41	P	N	7	N	N	N	N
3	M	59	P	N	2.6	N	N	N	N
4	F	67	N	P	1.8	N	N	N	N
5	M	60	P	N	5	N	N	N	N
6	M	64	P	?	5	N	N	N	N
7	M	46	P	N	2.2	N	N	Deletion	N
8	M	67	P	N	3.6	N	N	N	N
9	M	39	P	?	8	N	N	N	N
10	M	29	P	?	4.3	N	N	N	N
11	F	43	P	?	4.5	N	N	N	N
12	F	68	N	P	4.5	N	N	N	N
13	M	65	P	P	2.2	N	N	N	N
14	F	57	N	N	7.5	N	N	N	N
15	M	50	P	N	2.5	N	N	N	N
16	M	44	P	?	5	N	N	N	N
17	F	47	P	N	5	N	N	Deletion	Deletion
18	F	64	N	P	3	N	N	Deletion	Deletion
19	M	31	P	N	4	N	N	N	N
20	M	56	P	P	17	N	N	N	N
21	F	60	N	P	4.8	N	N	N	N
22	M	60	N	P	2.5	N	N	N	N
23	M	60	P	N	20	Deletion	N	N	N
24	M	63	N	P	2	N	N	N	N
25	M	63	N	P	3.2	N	N	N	N
26	F	65	P	N	7	N	N	N	N
27	M	52	P	N	4	N	N	N	N
28	M	54	P	N	13	N	N	N	N
29	M	57	P	N	$T_a = 3$	N	N	Deletion	Deletion
					$T_b = 2$	N	N	Deletion	Deletion
					$T_1 = 2$	N	N	Deletion	N
					$T_2 = 1.7$	N	N	Deletion	Deletion
30	M	64	N	P	$T_a = 2.8$	N	N	N	N
					$T_b = 2.2$	N	N	N	N

T_a, T_b, primary tumours; T_1, T_2, recurrent tumours; ?, not determined; HBsAg, hepatitis B surface antigen; anti-HCV, hepatitis C virus antibody.

studied. The tumour size was less than 3 cm in 12 cases, between 3 and 5 cm in 15 cases, larger than 5 cm in the remaining 7 cases. 21 patients were male and 9 were female. Patients' age ranged from 29 to 67 years. 20 were positive for hepatitis B surface antigen (HBsAg), and 10 were negative. Hepatitis C virus antibody (anti-HCV) was positive in 2 of 20 patients who were positive for HBsAg. However, in the 10 patients negative for HBsAg, 9 patients were positive for anti-HCV and only 1 was negative.

DNA and RNA isolation

To avoid contamination, cryosections of 5–7 μm were prepared from the tumour and non-tumour liver tissues, and sections containing predominantly neoplastic cells were used for the extraction of genomic DNA and RNA by conventional procedure [5].

Loss of heterozygosity (LOH) analysis

Tumours from 30 patients were examined for LOH using six markers (*D9S168*, *D9S162*, *D9S156*, *D9S171*, *D9S169*, and *D9S161*) on 9p. PCR was performed in a 25 μl reaction volume containing 5 μM of each primer, 0.125 mM dATP, 1.25 mM each of dGTP, dCTP, and dTTP, 3 μCi-^{35}S of dATP, 25 ng DNA, and 0.75 units of Taq DNA polymerase. The reactions were performed in 27 cycles under the following conditions: 30 sec at 94°C for denaturation, 75 sec at 55°C for primer annealing and 30 sec at 72°C for primer extension. Finally, the PCR product was further incubated at 72°C for another 6 min. After electrophoresis, the gels were dried at 80°C and exposed to X-ray film for 3 days. The band pattern was compared between tumorous and non-tumorous tissues for each pattern.

PCR amplification of p16/MTS1 *and* p15/MTS2 *genes*

All 34 HCC samples were examined for deletions of exon 1 and exon 2 of the *p16/MTS1* and *p15/MTS2* genes by the PCR amplification method. The primers used for PCR amplification were designed from the published *p16* and *p15* sequences [13, 15, 19] and are listed in Table 2.

Purified DNAs (100 ng) were amplified in a 50 μl solution containing a 0.6 μM concentration of each primer, 50 μM of each deoxynucleotides triphosphate, 5% dimethylsulphoxide (DMSO), and 2 units of Taq polymerase. All PCR reactions were performed in 96-well microplates. The reaction mixtures were prepared either manually or using a robotic workstation (Biomek-1000, Beckman, U.S.A.). The PCR conditions used for *p16* and *p15* gene amplification were one cycle at 94°C (5 min), two cycles at 94°C (30 sec) with the annealing temperature (T_{ann} of 62°C (50 sec); and extension at 72°C (50 sec); two cycles with $T_{ann} = 60$°C (50 sec) and 72°C (50 sec); 25 cycles with $T_{ann} = 56$°C (15 sec) and 72°C (30 sec); and finally one cycle at 74°C (10 min). The PCR products were electrophoresed on 1.5% agarose gels. The absence of visible bands amplified by PCR indicated homozygous deletions. For each run of PCR amplification, non-tumoral liver tissue was used as a normal control.

PCR conditions were controlled including the amounts of template and the number of cycles, so that equal amounts of template should have produced equal amounts of amplified product. We demonstrated that our PCR assay was in the linear part of the amplification process, i.e. before product saturation.

The quality of genomic DNAs was confirmed by PCR amplification of a similar sized fragment (400 bp) of the β-actin gene using the following PCR primer: sense 54 (5′-GAAACTACCTTCAACTCCATC-3′); and antisense AS4 (5′-CTAGAAGCATTTGCGTGGACGATGGAGGGGCC-3′).

Sequence analysis

To investigate whether somatic mutations of *p16/MTS1* or *p15/MTS2* genes were present in any of these HCCs, we screened the coding region of exon 1, 2 of *p16/MTS1* and exon 1, 2 of *p15/MTS2* using automated fluorescence-based sequencing analysis. A sequence analysis of the PCR products was performed to screen for *p16* and *p15* mutations in all the HCC samples which had no *p16* and *p15* deletions. After PCR, 40 μl of the specific amplification product (exon 1 and exon 2 of *p16* and *p15*) was used to generate a template for sequencing using Wizard PCR purification kits (Promega, Madison, Wisconsin, U.S.A). The purified PCR products were directly sequenced using a cycle sequencing protocol and reagents supplied with the Taq Dye Terminator Cycle Sequencing Kit (ABI, Foster City, California, U.S.A). The thermal cycling conditions of sequencing were 25 cycles at 94°C for 50 sec, 60°C for 50 sec, and 72°C for 50 sec after the initial denaturation step of 94°C for 5 min. After PCR, the reaction mixtures were purified and stored at −20°C until

Table 2. Primers used in polymerase chain reaction (PCR) and sequencing

Name	Sequences	Primary use
p16-2F(S)	5′GAAGAAAGAGGAGGGGCTG3′	Amplify *p16* exon 1
p16-1108R(A)	5′GCGCTACCTGATTCCAATTC3′	Amplify *p16* exon 1
p16-P45(S)	5′CGGAGAGGGGGAGAGCAG3′	Sequence *p16* exon 1
p16-42F(S)	5′GGAAATTGGAAACTGGAAGC3′	Amplify *p16* exon 2
p16-449R(A)	5′GGAAGCTCTCAGCGTACAAA3′	Amplify *p16* exon 2
p16-P25(S)	5′AGGGGGCTCTACACAAGCTT3′	Sequence *p16* exon 2
p16-P410R(A)	5′TCTCAGATCATCAGTCCT3′	Sequence *p16* exon 2
p15-1F(S)	5′TAATGAAGCTGAGCCCAGGT3′	Amplify *p15* exon 1
p15-1R(A)	5′AATGCACACCTCGCCAACG3′	Amplify *p15* exon 1
p15-S15(S)	5′GAAAGAAGGGAAGAGTGTCG3′	Sequence *p15* exon 1
p15-2F(S)	5′CTTTAAATGGCTCCACCTGC3′	Amplify *p15* exon 2
p15-2R(A)	5′CGTTGGCAGCCTTCATCG3′	Amplify *p15* exon 2
p15-S2(S)	5′TGGCTCTGACCACTCTGC3′	Sequence *p15* exon 2
p15-AS4(A)	5′CAAGTCCACGGGCAGACG3′	Sequence p15 exon 2

S, sense primer; A, antisense primer.

electrophoresis. Dried pellets were resuspended in 4 μl of loading buffer [5:1 (v/v) de-ionised formamide/50 mM ethylenediamine tetra-acetic acid (EDTA) pH 8.0], and were heated at 90°C for 5 min prior to loading on a 6% (w/v) polyacrylamide gel containing 7 M urea. Electrophoresis was performed on an ABI 373 automated sequencer.

Southern blot analysis

A Southern blot was performed for further confirmation of homozygous deletions of the *CDKN2B* gene. Ten micrograms of DNA was digested with EcoRI restriction enzyme (BM, GmbH, Germany), separated by electrophoresis in a 1.0% agarose gel and transferred to a Nylon membrane, PCR-derived probes of exon 1 and exon 2 of *p16*/*MTS1* and *p15*/*MTS2* were used individually for hybridisation [22, 23].

Methylation analysis

To determine whether 5′ CpG methylation of *p16*/*MTS1* and *p15*/*MTS2* was present in HCCs, these samples were analysed by Southern blot analysis [22]. Genomic DNA was restricted with a non-methylation-sensitive restriction enzyme, such as Hind III or EcoRI, and combined with a methylation-sensitive enzyme (such as EagI, SacII, SmaI). Methylation status was analysed by the fragment sizes recognised by a probe for *p16* or *p15* exon 1 [22, 23].

Genomic DNA (10 μg) was digested with excess restriction endonuclease overnight according to the conditions specified by the manufacturer (BM, GmbH, Germany), followed by ethanol precipitation. Restricted DNA was fractionated on a 1% agarose gel, and transferred to a Nylon membrane. Filters were hybridised with a random, primed-labelled, PCR-generated probe corresponding to exon 1 of *p15* or *p16*, as described previously [22–24], at 65°C overnight, followed by stringent washes, including a final wash at 70°C for 30 min with 0.1×SSC–0.1% (sodium dodecylsulphate (SDS). The blots were exposed to X-ray film.

Reverse transcription–PCR (RT–PCR)

RT–PCR was performed as described previously [22], using 1 μg total cellular RNA to generate cDNA. These cDNA products were amplified by PCR, using a primer for exon 1 (1F) and exon 2 (2R) of *p15* or exon 1 (p45) and exon 2 (410R) of *p16* (Table 2). To control for cDNA quality, β-actin was used as an internal control. The PCR products were analysed on 1.5% agarose gels and stained with ethidium bromide.

RESULTS

LOH of chromosome 9p in HCC

Thirty-four tumours from 30 HCC patients were analysed for LOH at 9p. LOH was detected in 3 of 20 (15%) informative cases at *D9S168*, 1 of 19 (5%) informative cases at *D9S162*(9p21), 1 of 20 (5%) informative cases at *D9S171*(9p21), and 4 of 21 (19%) informative cases at *D9S169*(9p21). There were no LOHs in *D9S156*(9p22-23) and *D9S161*(9p13-21). The highest frequency of LOH was found at locus *D9S169*(9p21). In the four tumours of case 29, two tumours (T_a and T_1) had LOH at locus *D9S169*, the other two had none.

Homozygous deletions of the p16 *and* p15 *genes*

By PCR amplification, the absence of a specific band of the *p16* exon 1 gene, indicating homozygous deletions, was found in only one tumour (case 23; 3%) (Table 1, Figure 1). In the remaining 33 tumours which showed specific bands, the band intensity was similar to each other as well as to that of the non-tumour tissue. For the *p16* exon 2 gene, all 34 HCC samples showed a specific band, indicating the absence of homozygous deletions. The band intensity was almost the same as that of non-tumour tissues. The patient (case 23) with *p16* exon 1 deletions was a male, 60 years old, negative for HCV, and positive for HBsAg. The tumour was approximately 20 cm in diameter. Cases 20 and 23 are brothers, and have a familial cancer risk. However, no deletion was noted in case 20 (Table 1).

A homozygous deletion of *p15*/*MTS2* exon 1 was found in seven tumours (4 patients) of the 34 HCCs (Table 1), while deletions of *p15*/*MTS2* exon 2 were identified in five tumours (3 patients) of 34 HCCs (Table 1, Figure 1). In the remaining specimens which showed a specific band, the band intensity was almost the same as that of the non-tumour tissue. In the 4 patients who had a *p15* exon 1 or 2 deletion, the tumours were no larger than 5 cm. In case 29 who had four tumours, all the primary and recurrent tumours had a deletion of *p15* exon 1. While in exon 2, although the primary tumours had exon 2 deletion, only one of the two recurrent tumours had deletion of *p15* exon 2 (Table 1, Figure 1). For quality control, the results showed that the β-actin gene could be detected in all 34 HCC samples and the intensity of the band was similar.

To rule out the possibility of overestimating or underestimating the frequency of *p15* and *p16* gene deletions, we also performed Southern blot analysis to confirm our results. Intragenic deletions of the *p15* or *p16* gene were detected in 5 cases (data not shown).

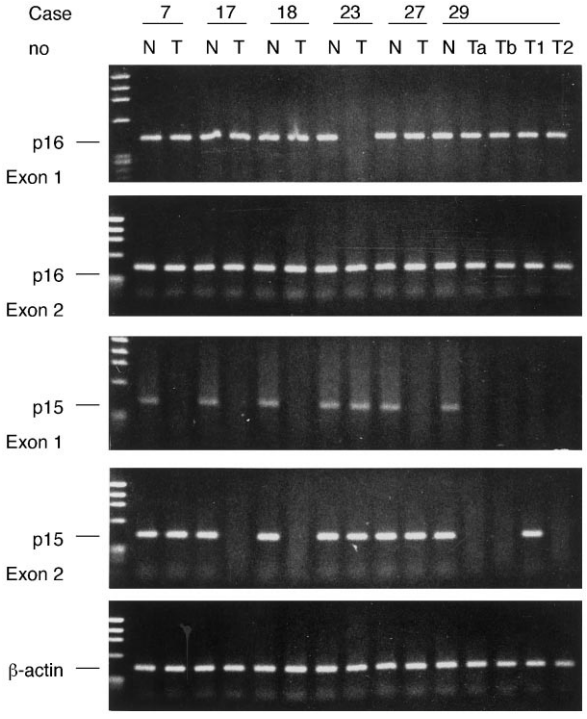

Figure 1. Representative polymerase chain reaction (PCR) amplification of the *p16* and *p15* gene in hepatocellular carcinoma. T, tumour; N, non-tumour tissue. T_a, T_b, primary tumours; T_1, T_2, recurrent tumours.

Sequence analysis of the p16 *and* p15 *genes*

Sequence analysis of exon 1 and exon 2 of *p16/MTS2* and *p15/MTS2* failed to demonstrate mutations in the remaining tumours which had no homozygous deletions.

Methylation analysis

Exon 1 of *p16* lies in a typical CpG island, which is unmethylated in all normal tissues tested. Restriction with a non-methylation-sensitive restriction enzyme, such as Hind III or EcoRI, provides a convenient flanking cut which, when combined with a methylation-sensitive enzyme (such as EagI, SacII, SmaI), allows rapid determination of the methylation status of this CpG island. Except for the tumours with deletions, restriction with EcoRI and the methylation-sensitive restriction enzyme SacII produced a pattern of unmethylation in all 33 tumours (two restriction fragments of 3.3 kb and

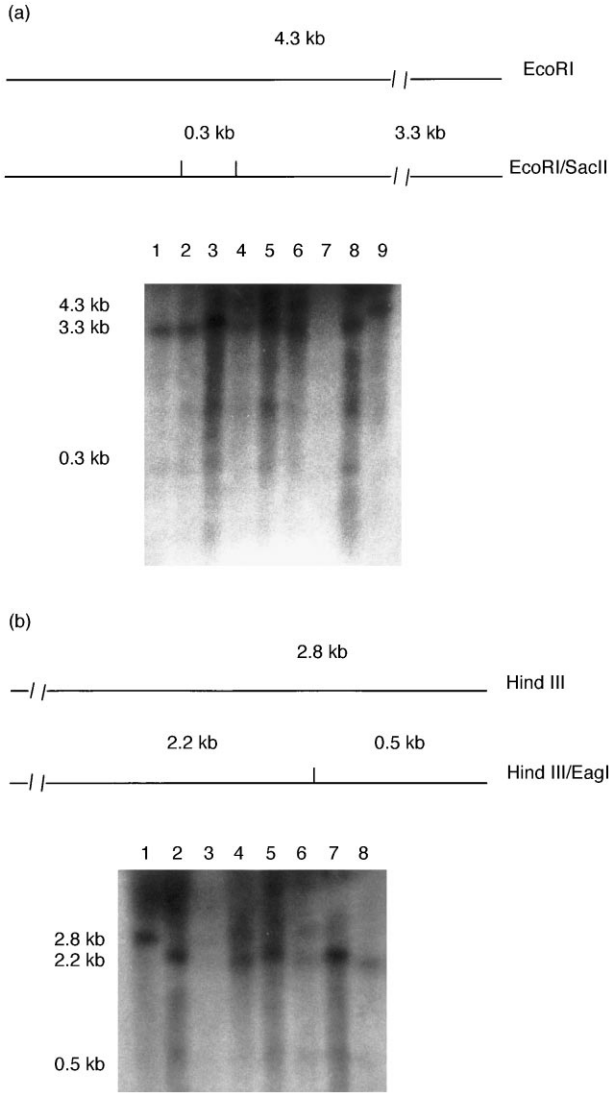

Figure 2. Methylation analysis of genomic *p16*(a) and *p15*(b) by Southern blot analysis. (a) The predicted sizes of restriction fragments used to analyse the methylation status of *p16* are shown at the top. DNA from hepatocellular carcinoma (HCC) patients restricted with EcoRI and SacII (lanes 1–8). Lanes 1–8, all HCC samples; lane 9, EcoRI-restricted DNA for control. All the HCC samples without *p16* deletion showed an unmethylation pattern. Lane 1, case 1; lane 2, case 3; lane 3, case 7; lane 4, case 13; lane 5, case 17; lane 6, case 18; lane 7, case 23; lane 8, case 27. (b) The predicted sizes of restriction fragments used to analyse the methylation status of *p15* are shown at the top. DNA from HCC patients restricted with Hind III and EagI (lanes 2–8). Lane 1, Hind III-restricted DNA for control; lanes 2–8, all HCC samples. Case 13 (lane 4) showed a pattern of hemi-methylation. All the other samples produced an unmethylation pattern. Lane 2, case 3; lane 3, case 7; lane 4, case 13; lane 5, case 16; lane 6, case 20; lane 7, case 23; lane 8, case 27.

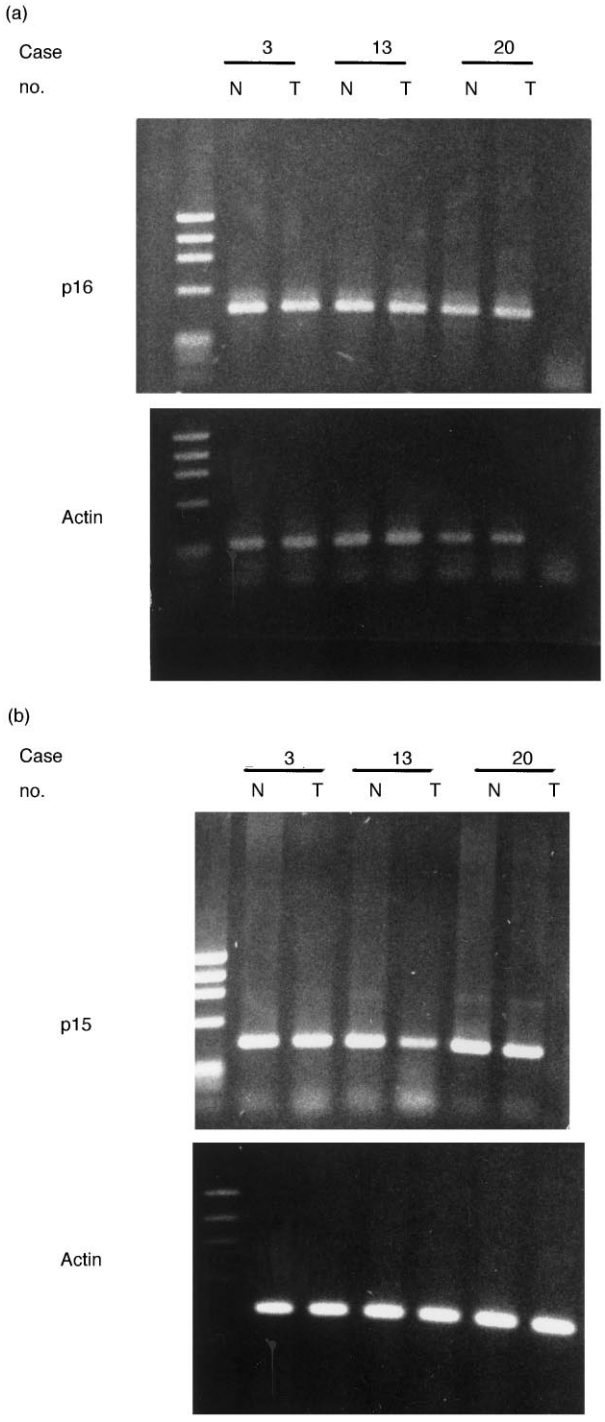

Figure 3. Reverse transcription–polymerase chain reaction (RT–PCR) analysis for mRNA expression of *p16*(a) and *p15*(b). The mRNA expression of *p16* and *p15* is normal in all the samples which had no deletion of the *p15* and *p16* gene. Only case 13 showed a weaker band. To control for cDNA quality, β-actin was used as a control. T, tumour; N, non-tumour tissue.

0.3 kb) (Figure 2a). For control, DNA was only digested with EcoRI and the sample produced a fragment of 4.3 kb (Figure 2a, lane 9). Southern blot analysis confirmed homozygous deletion of *p16* exon 1 in case 23, since when hybridised with exon 1 it produced no visible bands (Figure 2a, lane 7). However, the background of lane 7 is markedly reduced. To eliminate the possibility of underloading the DNA sample, we not only repeated the experiment three times, but also used a control probe to show equal loading of the DNA samples (data not shown).

Structurally, *p15* is a candidate of hypermethylation-associated inactivation, because a 5′ CpG island is located around the transcription start site. The 760 nucleotide region around exon 1 of *p15* also contains sites for a number of methylation-sensitive restriction endonucleases with the CpG dinucleotide in the recognition sequence [24]. Restriction of genomic DNA from tumours with Hind III, plus the methylation-sensitive enzyme, EagI, on Southern hybridisation with *p15* exon 1 probe provided an initial assessment of the methylation status of this 5′ CpG island. The *p15* gene was unmethylated at this EagI site in normal tissues. For a control, DNA was only digested with Hind III and the sample produced a fragment of 2.8 kb (Figure 2b, lane 1). Twenty-five of the 34 HCC samples showed a pattern of unmethylation (two restriction fragments of 2.2 kb and 0.5 kb) (Figure 2b). Only one tumour produced a pattern of hemimethylation (three restriction fragments of 2.8 kb, 2.2 kb, and 0.5 kb (Figure 2b, lane 4, case 13)). In case 7, which showed deletion of *p15* exon 1 by PCR amplification, homozygous deletion of *p15* exon 1 was also confirmed by Southern blot (Figure 2b, lane 3, case 7).

mRNA expression of p16 *and* p15

By RT–PCR, the presence of a specific band indicating normal mRNA expression was found in all the HCCs which had no deletion in *p16* exon 1 and *p15* exon 1 (Figure 3). The control RT–PCR product of β-actin, was readily detectable (Figure 3). In the patient (case 13) who showed a hemimethylation pattern at the *p15* gene (Figure 2b, lane 4), a weaker mRNA band was found (Figure 3b, lane 4).

DISCUSSION

In this study, only one of 30 HCC cases (34 tumours) had a deletion in the *p16* exon 1 (1/30, 3%). No deletions were found in exon 2. Furthermore, no mutations or methylation of the *p16* exon 1 and exon 2 gene were found in any HCC samples. Our results indicate that alterations (mutations and deletions) of the *CDKN2A/MTS1/p16* gene are rarely found in HCC and might have a limited role in hepatocarcinogenesis. Although no mutations of *p15/MTS2* exon 1 or 2 were found in any primary tumours, homozygous deletions of *p15/MTS2* exon 1 or exon 2 were found in primary tumours (4/30, 13%). No aberrant 5′CpG island hypermethylation of *p16/MTS1/CDKN2* or *p15/MTS2* was found in any of the primary tumours by Southern blot. Therefore, the homozygous deletions within the *p15/MTS2* gene might be associated with the development of a subset of HCC.

To date, the two most common mechanisms for loss of *p16* function are homozygous deletion and loss of transcription associated with hypermethylation of the 5′ CpG island region [22–24, 28]. Our current results show that the frequency of *p16* gene alterations is low and no hypermethylation pattern of the *p16* gene is detected in HCC. Therefore, we suggest that the *p16* gene plays a limited role in the development of HCC in patients from Taiwan. In agreement with our data, Kita and colleagues demonstrated that the mutations or deletion of the *p16* gene are not frequent, but play a role in a subset of human HCC [25]. More recently, Hui and associates found that the HCC samples from their series had no p16 protein expression [26]. However, homozygous deletions and gene mutations were not detected in HCC samples, which showed strong p16 mRNA expression. They proposed that post-transcriptional inactivation of the *p16* gene occurs in multistage hepatocarcinogenesis. Our results also showed infrequent alterations of the *p16* gene in HCC and most of the tumours (97%) expressed p16 mRNA. However, we did not analyse p16 at the protein level. Currently, only one paper has reported that mouse skin tumours express p16 mRNA but not protein [27]. Therefore, the mechanism of *p16* gene inactivation by post-transcriptional regulation in HCC needs further investigation.

The clonality of HCC is still controversial. Microsatellite polymorphism analysis also provides information about clonality of multiple HCCs. In this study we found the two tumours (T_a and T_1) of case 29 had LOH and the other tumours had no LOH at *D9S169*. The three tumours (T_a, T_b and T_1) had deletion of exon 2 of *p15*, while the recurrent tumours (T_1) has no deletion of exon 2 of *p15*. These data provide evidence that multiple small HCCs might have different clonality. Our previous studies, using DNA fingerprinting methods demonstrated that multiple HCCs frequently have different clonalities [29]. However, the clonality of HCC needs further study.

Homozygous deletion of *p15* with retention of *p16* in some gliomas and leukaemia has been described [20, 23, 28], suggesting that *p15* may be an important target for inactivation in certain tumour types. In our studies, homozygous deletion of the *p15* gene was also found in 13% of HCCs. Therefore, we suggest that the *p15* gene may play a role in a subset of HCC and the mechanism of *p15* gene inactivation is homozygous deletion. By microsatellite analysis, we found that the highest frequency of LOH (19%) was at marker *D9S169* on chromosome 9p21 in HCC. At the same time, those cases with LOH on chromosome 9p21 have *p15* exon 1 or exon 2 deletions. These data suggest that the *p15* gene might be a tumour suppressor gene near 9p21. Nevertheless, the possibility of other coexistent target genes in addition to *MTS1* and *MTS2* on 9p21 cannot be completely excluded. Frequent LOH on chromosome 9p with a low incidence of mutation in the *MTS1* and *MTS2* genes has been reported in malignant mesothelioma and glioma [30, 31]. Additional studies to search for any LOH at 9p with more microsatellite markers and to determine the frequency of *MTS1* and *MTS2* alterations in other primary tumours are needed to elucidate the roles, if any, of these genes in carcinogenesis.

1. Linsell DA, Higginson J. The geographic pathology of liver cell cancer. In Cameron HM, Linsell DA, Warwick GP, eds. *Liver Cell Cancer*. New York, Elsevier Science, 1976, 1–16.
2. Beasley RP. Hepatitis B virus as the etiologic agent in hepatocellular carcinoma—epidemiologic considerations. *Hepatology* 1982, **2**, 21–26.
3. Chen DS. Hepatitis B virus infections, its sequelae, and prevention in Taiwan. In Okuda K, Ishak KG, eds. *Neoplasm of the Liver*. Tokyo, Springer, 1987, 71–80.
4. Sheu JC, Huang GT, Shih LN, *et al.* Hepatitis C and B viruses in hepatitis B surface antigen negative hepatocellular carcinoma. *Gastroenterology* 1992, **103**, 1322–1327.

5. Sheu JC, Huang GT, Lee PH, *et al.* Mutation of p53 gene in hepatocellular carcinoma in Taiwan. *Cancer Res* 1992, **52**, 6098–6100.
6. Cannon A, *et al.* Assignment of a locus for familial melanoma, MLM, to chromosome 9p13-p22. *Science* 1992, **258**, 1148–1152.
7. Diaz MO, Rubin CM, Harden A, *et al.* Deletions of interferon genes in acute lymphoblastic leukemia. *N Engl J Med* 1990, **332**, 77–82.
8. Cheng JQ, Jhanwar SC, Lu YY, Testa JR. Homozygous deletions within 9p21-p22 identify a small critical region of chromosomal loss in human malignant mesotheliomas. *Cancer Res* 1993, **53**, 4761–4763.
9. Fountain JW, Karayiorgou M, *et al.* Homozygous deletions within human chromosome band 9p21 in melanoma. *Proc Natl Acad Sci USA* 1992, **89**, 10557–10561.
10. Oplopade OI, Buchhagen DL, Malik K, *et al.* Homozygous loss of the interferon genes defines the critical region on 9p that is detected in lung cancers. *Cancer Res* 1993, **53**, 2410–2415.
11. Serrano M, Hannon GJ, Neach D. A new regulatory motif in cell-cycle control causing specific inhibition of cyclin D/CDK4. *Nature* 1993, **366**, 704–707.
12. Nobori T, Miura K, Wu DJ, Lois A, Takabayashi K, Carson DA. Deletions of the cyclin-dependent kinase-4 inhibitor gene in multiple human cancers. *Nature* 1994, **368**, 753–756.
13. Kamb A, Gruis NA, Weaver Feldhaus-J, *et al.* A cell cycle regulatory potentially involved in genesis of many tumor types. *Science* 1994, **264**, 436–440.
14. Igaki H, Sasaki H, Kishi T, *et al.* High frequent homozygous deletion of the *p16* gene in esophageal cancer cell lines. *Biochem Biophys Res Commun* 1994, **203**, 1090–1095.
15. Caldas C, Hahn SA, *et al.* Frequent somatic mutations and homozygous deletions of the *p16(MTS1)* gene in pancreatic adenocarcinoma. *Nat Genet* 1994, **8**, 27–32.
16. Hayashi N, Sugimoto Y, Tsuchiya E, Ogawa M, Nakamura Y. Somatic mutations of the MTS (multiple tumor suppressor) 1/CDK4I (cyclin-dependent kinase-4 inhibitor) gene in human primary non-small cell lung carcinomas. *Biochem Biophys Res Commun* 1994, **202**, 1426–1430.
17. Sruck CH III, Gonzalez Z, Ulueta M, *et al. p16* gene in uncultured tumors. *Nature* 1994, **370**, 183–184.
18. Cairns P, Mao L, Merlo A, *et al.* Rates of *p16(MTS1)* mutations in primary tumors with 9p loss. *Science* 1994, **265**, 415–417.
19. Okamoto A, Demetrick DJ, Spillare EA, *et al.* Mutations and altered expression of *p16*INK4 in human cancer. *Proc Natl Acad Sci USA* 1994, **91**, 11045–11049.
20. Washimi O, Nagatake M, Osada H, *et al. In vivo* occurrence of *p16(MTS1)* and *p15(MTS2)* alterations preferentially in non-small cell lung cancers. *Cancer Res* 1995, **55**, 514–517.
21. Herman JG, Latif F, Weng Y, *et al.* Silencing of the VHL tumor suppressor gene by methylation in renal carcinoma. *Proc Natl Acad Sci USA* 1994, **91**, 9700–9704.
22. Merlo A, Herman JG, Mao L, *et al.* 5′CpG island methylation is associated with transcriptional silencing of the tumor suppressor *p16*/CDKN2 in human cancers. *Nat Med* 1995, **1**, 686–692.
23. Herman JG, Merlo A, Mao L, *et al.* Inactivation of the CDKN2/*p16/MTS1* is frequently associated with aberrant DNA methylation in all common human cancers. *Cancer Res* 1995, **55**, 4525–4530.
24. Herman JG, Merlo A, Mao L, Baylin SB. Hypermethylation-associated inactivation indicated a tumor suppressor role for $p15^{INK4B1}$. *Cancer Res* 1996, **56**, 722–727.
25. Kita R, Nishida N, Fukuda Y, *et al.* Infrequent alterations of the $p16^{INK4A}$ gene in liver cancer. *Int J Cancer* 1996, **67**, 176–180.
26. Hui A-M, Sakamoto M, Kanai Y, *et al.* Inactivation of $p16^{INK4}$ in hepatocellular carcinoma. *Hepatology* 1996, **24**, 575–579.
27. Linardopoulos S, Street AJ, Quelle DE, *et al.* Deletion and altered regulation of $p16^{INK4}$ and $p15^{INK4b}$ in undifferentiated mouse skin tumors. *Cancer Res* 1995, **55**, 5168–5172.
28. Otsuki T, Clark HM, Wellmann A, Jaffe ES, Raffeld M. Involvement of CDKN2($p16^{INK4A}$/*MTS1*) and $p15^{INK4B}$/*MTS2* in human leukemias and lymphomas. *Cancer Res* 1995, **55**, 1436–1440.
29. Sheu JC, Huang GT, Chou HC, Lee PH, Wang JT, Chen DS. Multiple hepatocellular carcinoma at the early stage have different clonality. *Gastroenterology* 1993, **105**, 1471–1476.
30. Cheng JQ, Jhanwar SC, Klein WM, *et al.* p16 alterations and deletion mapping of 9p21-22 in malignant mesothelioma. *Cancer Res* 1994, **54**, 5547–5551.
31. Li YJ, Hoang-Xuan K, Delattre JY, *et al.* Frequent loss of heterozygosity on chromosome 9, and low incidence of mutations of cyclin-dependent kinase inhibitors *p15(MTS2)* and *p16(MTS1)* genes in gliomas. *Oncogene* 1995, **11**, 59—600.

Acknowledgements—This study was supported by grants from the Department of Health and National Science Council, Executive Yuan (NSC-87-2314-B-002-249), National Taiwan University Hospital (NTUH-S861532-A41) and Liver Disease Prevention and Treatment Research Foundation, Taiwan, Republic of China.