

Inhibitory effect of tumor suppressor p33^{ING1b} and its synergy with p53 gene in hepatocellular carcinoma

Zhi Zhu, Jing Lin, Jian-Hui Qu, Mark A. Feitelson, Can-Rong Ni, Fang-Mei Li, Ming-Hua Zhu

Zhi Zhu, Jing Lin, Jian-Hui Qu, Can-Rong Ni, Fang-Mei Li, Ming-Hua Zhu, Department of Pathology, Changhai Hospital, Second Military Medical University, Shanghai 200433, China
Mark A. Feitelson, Department of Pathology, Anatomy and Cell Biology, Thomas Jefferson University, Philadelphia, PA, USA
Supported by the National Natural Science Foundation of China (Grants No.30070344 and No.30070839)
Correspondence to: Ming-Hua Zhu, Department of Pathology, Changhai Hospital, Second Military Medical University, 174 Changhai Road, Shanghai 200433, China. mhzhu2000@hotmail.com
Telephone: +86-21-25074604 Fax: +86-21-25074604
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Abstract

AIM: To investigate the inhibitory effect of tumor suppressor p33^{ING1b} and its synergy with p53 gene in hepatocellular carcinoma (HCC).

METHODS: Recombinant sense and antisense p33^{ING1b} plasmids were transfected into hepatoma cell line HepG2 with lipofectamine. Apoptosis, G₀/G₁ arrest, cell growth rate and cloning efficiency in soft agar of HepG2 were analyzed after transfection. In three hepatoma cell lines with different endogenous p53 gene expressions, the synergistic effect of p33^{ING1b} with p53 was analyzed by flow cytometry and luciferase assay was performed to detect the activation of p53 downstream gene p21^{WAF1/CIP1}. In addition, the expression and mutation rates of p33^{ING1b} in HCC tissues were measured by immunohistochemistry and polymerase chain reaction-single strand conformation polymorphism (PCR-SSCP).

RESULTS: Overexpression of p33^{ING1b} inhibited cell growth of HepG2, induced more apoptosis and protected cells from growth in soft agar. Combined transfer of p33^{ING1b} and p53 gene promoted hepatoma cell apoptosis, G₀/G₁ arrest and elevated expression of p21^{WAF1/CIP1}. Immunostaining results showed co-localized P33^{ING1b} with P53 protein in HCC tissues and there was a significant relation between protein expression rates of these two genes ($P < 0.01$). Among 28 HCC samples, p33^{ING1b} presented a low gene mutation rate (7.1%).

CONCLUSION: p33^{ING1b} collaborates with p53 in cell growth inhibition, cell cycle arrest and apoptosis in HCC. Loss or inactivation of p33^{ING1b} normal function may be an important mechanism for the development of HCC retaining wild-type p53.

INTRODUCTION

Inhibitor of growth 1, ING1, is a newly cloned tumor suppressor^[1], and appears to play a role in programmed cell death and cell cycle arrest, whereas antisense ING1 protects cells from apoptosis in various experimental systems. Ectopic expression of ING1 cDNA or ING1 suppression by antisense RNA demonstrates that ING1 is a negative regulator of cell proliferation^[2-4]. ING1 protein has been reported to bind directly to p53 protein by immunoprecipitation *in vitro*, modulating the function of p53 as a transcription activator^[5]. ING1 gene is mapped on human chromosome, 13q33-34, a region that has been implicated in the progression of various tumors^[6,7]. Deregulated expression and mutation of ING1 gene are found in breast carcinoma, oral/esophageal squamous cell carcinoma, gastric carcinoma and malignant lymphoma, etc.^[9,12-16]. It was also found that ING1 gene encodes several differentially initiated and spliced mRNAs, which have common 3' exon and encode at least two distinct proteins in mice, and possibly three distinct proteins in human cells (p47^{ING1a}, p33^{ING1b}, and p24^{ING1c}). RT-PCR analysis showed that the ING1b form is a major transcript in human normal tissues. All the known ING1 protein isoforms share an identical C-terminal domain with a conserved PHD finger motif, which might directly interact with DNA^[17,18,33].

HCC is one of the 10 most common cancers in the world and is almost uniformly fatal. The genetic events leading to the development of hepatocellular carcinoma are not well documented. Gene mutation and dysfunction of wild-type tumor suppressor p53 are important molecular events in the process of hepatocarcinogenesis^[19-32]. p33^{ING1b} has a close relationship with p53 and neither p53 nor p33^{ING1b} alone can cause cell growth inhibition^[4,5], which prompted us to investigate their potential role in hepatocellular carcinogenesis. We examined whether genetic mutation and altered protein expression of p33^{ING1b} and p53 were responsible for the development and progression of human HCC.

MATERIALS AND METHODS

Materials

Human hepatoma cell lines HepG2, PLC/PRF/5, and Hep3B were maintained in Dulbecco's MEM with 10% FCS (Hyclone), 2 mmol/L L-glutamine, and antibiotics at 37 °C in a 50 mL/L atmosphere. Plasmid pCI-ING1b (kindly provided by Dr. Karl Riabowol, Department of Medical Biochemistry, Calgary University, Canada) was digested with *EcoRI/XbaI* and *XhoI/BamHI*, and the resultant 900 bp fragment (ING1b cDNA full length) was ligated into the pcDNA3 vector (Invitrogen) containing the neomycin-resistance gene. This recombinant produced sense pcDNA3-p33ING1b plasmid (*EcoRI/XbaI*-digested) and antisense plasmid, pcDNA3- α p33ING1b (*XhoI/BamHI*-digested). Recombinant plasmids pCMV containing sense and antisense wild-type p53 gene were constructed in our laboratory (reported in other manuscripts, data not shown). Plasmid WWP-LUC containing the promoter of p21^{WAF1/CIP1} gene derived the expression of a luciferase reporter gene (kindly provided by Dr. B. Vogelstein, John Hopkins Oncology Center).

HCC and para-cancerous liver tissues were obtained from 57 patients with hepatocellular carcinoma who underwent surgery between 1997 and 2002 at the Department of Pathology, Changhai Hospital, Second Military Medical University. Twelve normal liver tissues were obtained from autopsy. All tissues were fixed in 10% formalin and paraffin-embedded.

Transient transfection

To determine the effects of p33^{ING1b} and p53 gene upon the growth of hepatoma cells, 1×10⁶ cells of HepG2, PLC/PRF/5, and Hep3B were plated overnight and co-transfected by Lipofectamine 2000 (Gibco BRL) following the manufacturer's instructions. The plasmids used in co-transfection were divided into four groups. HepG2 cells with endogenous wild-type p53 gene^[22] were transfected with 3 μ g of pcDNA3-p33ING1b, pcDNA3- α p33ING1b, pcDNA3-p33ING1b+pCMV- α wtp53, respectively, and pcDNA3 vector alone as a control. PLC/PRF/5 cells with endogenous mutant p53 and Hep3B with endogenous p53 completely deleted^[22] were treated with 3 μ g of pcDNA3-p33ING1b, pcDNA3-p33ING1b+pCMV-wtp53, pcDNA3- α p33ING1b+pCMV-wtp53, respectively, and pcDNA3 plain vector alone as control group. Cells were incubated overnight with the lipofectamine mix, then washed with PBS and maintained in complete medium.

Apoptosis and cell cycle arrest assay

Forty-eight hours after transfection, cell apoptosis was assayed using annexin V-FITC apoptosis detection kit (BD PharMingen, USA). Cells were collected, washed twice with cold PBS and resuspended in binding buffer (10 mmol/L HEPES/NaOH pH7.4, 140 mmol/L NaCl, 2.5 mmol/L CaCl₂, sterile filtered) at a concentration of 1×10⁶ cells/mL. One hundred microliters of the solution was transferred to a 5 mL culture tube, and 5 μ L of annexin V-FITC and 5 μ L of propidium iodide were added, then the cells were gently vortexed and incubated for 15 min at room temperature in the dark. Cell apoptosis was analyzed with FACSlibur.

CycleTEST plus DNA reagent kit (Becton Dickinson, CA) was applied to the cell cycle arrest assay. The number of each cell cycle phase was determined by FACSlibur following instructions of the manufacturer. All these assays were repeated at least twice.

Growth of HepG2-pcDNA3-p33ING1b, HepG2-pcDNA3- α p33ING1b, and HepG2-pcDNA3 in serum medium and soft agar

HepG2 cells were plated at 1×10⁶ cells per 100 mm dish and incubated overnight, then transfected with 3 μ g of pcDNA3-p33ING1b, pcDNA3- α p33ING1b, respectively, and pcDNA3 vector alone as a control by lipofectamine 2000 (Gibco BRL). After transfection, the cells were selected in 800 μ g/mL of G418 (Gibco BRL) for 3 wk. All resistant colonies were trypsinized and grown in complete medium. Resistant colonies of HepG2-pcDNA3-p33ING1b, HepG2-pcDNA3- α p33ING1b and HepG2-pcDNA3 cells were grown in complete medium and the number of viable cells was determined at daily intervals for 7 d after seeding. Cell viability was determined by trypan blue staining. All experiments were constructed in triplicate, and the results were evaluated blindly. For growth in soft agar, 1×10⁴ cells/well were seeded in triplicate into six-well plates and allowed to grow for 21 d. The colonies were counted under a phase contrast microscope.

Luciferase assay

HepG2, PLC/PRF/5 and Hep3B cells were added to 24-well plates (1×10⁵ cells/well) and incubated overnight in complete medium, then cotransfected using lipofectamine method. The plasmids transfected into each cell line were the same as in the apoptosis assay but plus 0.2 μ g of the reporter plasmid WWP-LUC and 10 ng of an internal control renilla luciferase plasmid, pSV40 (Promega, Madison, WI) in each well. Cells were harvested 48 h after transfection. The activities of firefly and renilla luciferase were measured with a luminometer simultaneously using the dual-luciferase reporter assay kit (Promega) and normalized for the variation in transfection efficiency. All tests were done in triplicate.

Ethanol treatment

After transient transfection, the cells were treated with 60 mL/L ethanol in complete medium or PBS as control to induce apoptosis. Four hours after treatment, all cells (adherent or floating) were collected by trypsinization. Then apoptosis assay and luciferase assay were repeated again as described above. All tests were done in duplicate.

Immunohistochemistry

Protein staining of p33^{ING1b} and p53 of 57 HCC and para-cancerous tissues was determined using EnVision method (DAKO). Histological sections (4- μ m thick) on 0.02% poly-L-lysine coated slides (Sigma Chemical Co.) were deparaffinized and rehydrated, and the endogenous peroxidase activity was blocked by incubation with 2% H₂O₂ in phosphate buffer, followed by microwave antigen retrieval in citrate buffer (pH6.0). Nonspecific binding was blocked with goat serum, and sections were incubated with p33^{ING1b} antibody and p53 antibody (clone DO-7, purchased from American antibody Corp. USA) overnight at 4 °C, respectively. Having washed

thrice to eliminate the residual antibody, the sections were incubated with EnVision complex. The reaction was developed by incubation with 3,3'-diaminobenzidine tetrahydrochloride (Sigma Chemical Co.), washed and counterstained with hematoxylin.

Genomic DNA extraction and PCR-SSCP analysis

Genomic DNA from 28 cases of HCC was isolated from paraffin-embedded tissues by SDS/proteinase K treatment, phenol-chloroform extraction and ethanol precipitation^[8]. The coding region of ING1b gene exon-1a was amplified by PCR with primers S1(5'ATGTTGAGTCCTGCCA-ACGGGGA3') and AS1 (5'CTCTTGGTATTTTCGCGTC-GAT3'). Exon-2 was amplified as four overlapping fragments with four primer sets: S2 (5'ATCCTGAAGGAGCTAG-ACGAGTGC3') and AS2 (5'CTTGCCGCTGTTGC-CCGCTG3'), S3 (5'TTCGAGGCGCAGCAGGAGCT3') and AS3 (5'CTTGGCCTTCTTCTCCTTGGG3'), S4 (5'CAGCAACCACGACCACGACG3') and AS4 (5'TGA-GCCCCACGCACGAGAAG3'), and S5 (5'CCTCC-CCATCGACCCAACG3') and AS5 (5'CTACCTGTT-GTAAGCCCTCTC3'). PCR mixture contained 100 ng of genomic DNA as the template, with 1.2 mmol/L MgCl₂, 1×PCR buffer, 200 μmol/L of each deoxynucleotide triphosphate, 20 pmol of each primer and 1 unit of rTth DNA polymerase XL (Perkin Elmer, Foster city, CA) in a 50 μL volume. After 35 cycles, 1 μL of PCR product was mixed with 8 μL of loading dye (95% formamide, 20 mmol/L EDTA, 0.05% bromophenol blue, and 0.05% xylene cyanol), heat denatured, chilled on ice, and applied onto 8% polyacrylamide gel with 5% glycerol. The bands were then detected by silver staining. Aberrantly migrating bands were excised, re-amplified with the same sets of primers, then cloned into EcoRV-digested PMD18-T vector (TaKaRa Corp. Japan) and sequenced (completed by TaKaRa Corp. Japan).

Statistical analysis

The relationship between p33^{ING1b} and p53 protein positive staining was evaluated using Wilcoxon signed rank test. The growth difference of HepG2-p33ING1b and HepG2-α p33ING1b in soft agar was analyzed by Student's *t*-test. All data were represented as mean±SD. *P*<0.05 was considered statistically significant.

RESULTS

Drastically enhanced apoptosis and G₀/G₁ arrest in hepatoma cells by synergy of p33^{ING1b} with p53

In HepG2 cells with endogenous wildtype p53 gene, the apoptosis rate of HepG2-pcDNA3-p33ING1b (22.53±1.4%) was significantly higher than those of other HepG2 experiment groups (*P*<0.01, Figures 1A-C). The proportion of HepG2-pcDNA3-p33ING1b cells arrested at G₀/G₁ phase (67.45±9.6%) was much more than cells transfected with vector or other control plasmids (*P*<0.05, Table 1) (Figure 1D-G). To further investigate the synergistic effect of p33^{ING1b} with p53, HepG2 cells were treated by 6% ethanol. After treatment, the apoptosis rate of HepG2 experiment groups was elevated, and the elevation extent of HepG2-pcDNA3-p33ING1b was the most significant (48.92±1.6%, *P*<0.01, Figures 1A-C).

In PLC/PRF/5 cells with endogenous mutant p53, the difference in apoptosis rate between cells cotransfected with pcDNA3-p33ING1b and pCMV-wtp53 (7.81±0.3%) and control plasmid was not significant (*P*>0.05). After 6% ethanol treatment, apoptosis of all PLC/PRF/5 experiment groups was enhanced. The apoptosis rate of cotransfection with p33^{ING1b} and wtp53 (42.8±1.3%) was significantly higher than that of other groups (*P*<0.01). In addition, the apoptosis induced by cotransfection with p33^{ING1b} and wtp53 (1.59±0.2%) in Hep3B cells without endogenous p53

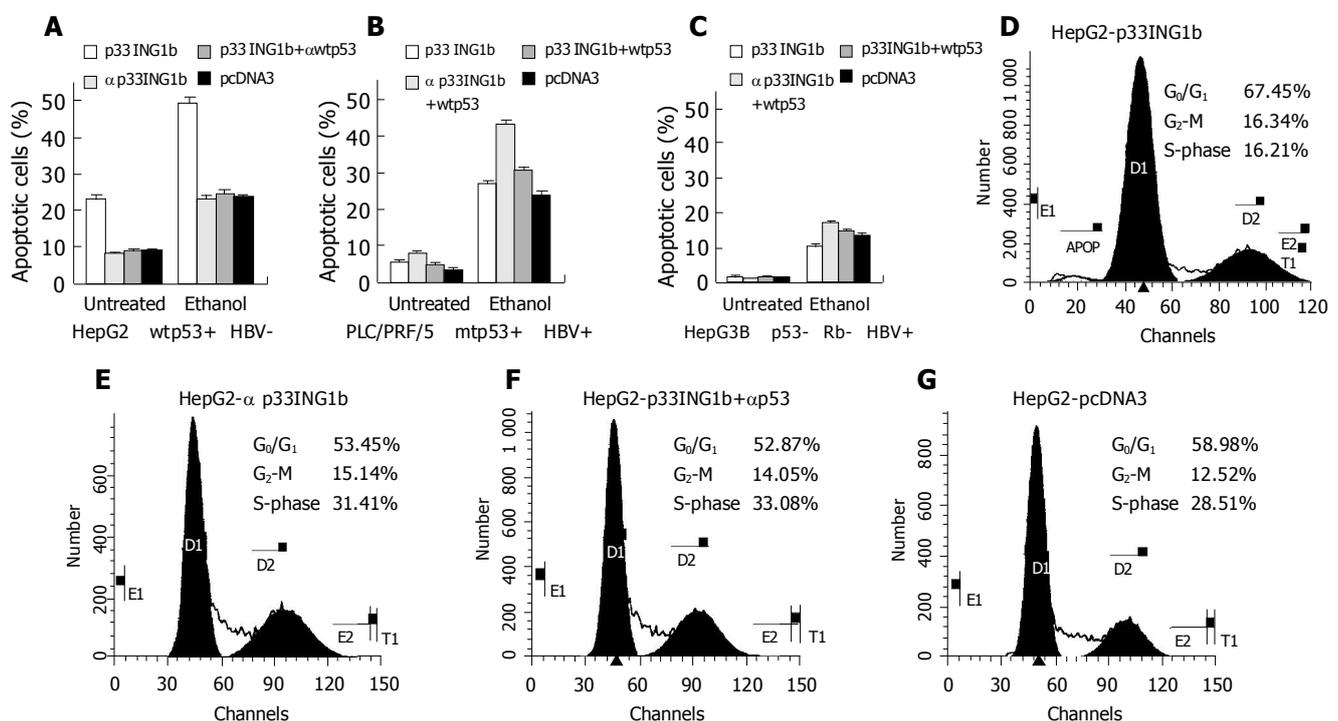


Figure 1 FACS analysis of cell apoptosis in three hepatoma cell lines (A-C) and arrest of HepG2 cells at G₀/G₁ cell cycle (D-G).

($1.59 \pm 0.2\%$) was significantly lower than that in HepG2-pcDNA3-p33ING1b group, but the difference in apoptosis rate between each experiment group of Hep3B was not significant ($P > 0.05$). After 6% ethanol treatment, the difference was still not significant although the apoptosis rate of each Hep3B experiment group had a slight increase (Figure 1A-C). The proportion of PLC/PRF/5 cells cotransfected with p33^{ING1b} and wtp53 arrest at G₀/G₁ phase ($78.16 \pm 10.6\%$) was significantly higher than those in other control groups ($P < 0.05$), but the difference between each Hep3B experiment group was not significant ($P > 0.05$, Table 1).

Overexpression of p33^{ING1b} inhibited hepatocellular growth and survival

The growth curve of HepG2 cells showed that HepG2-pcDNA3-p33ING1b cells grew significantly slower than HepG2-pcDNA3 and HepG2-pcDNA3- α p33ING1b cells ($P < 0.01$, Figure 2). Additional experiments were conducted to test whether p33^{ING1b} overexpression correlated with anchorage-independent growth in soft agar. Results showed that the growth in soft agar of HepG2-pcDNA3-p33ING1b cells with p33^{ING1b} overexpression was inhibited compared with that of HepG2-pcDNA3 cells ($P < 0.01$). However, HepG2 cells stably transfected with pcDNA3- α p33ING1b significantly promoted the anchorage-independent growth in soft agar ($P < 0.01$, Table 2).

Synergistic effect of p33^{ING1b} with p53 on activating p53 downstream gene, p21^{WAF1/CIP1}

The results of luciferase assay showed that the activity of

Table 1 G₀/G₁ cell cycle arrest analysis of hepatoma cells after cotransfection with p33^{ING1b} and wtp53 gene or control plasmids (mean \pm SD, %)

Cell line	Transfection plasmids	G ₀ /G ₁ arrest
HepG2	p33 ^{ING1b}	67.45 \pm 9.6 ^a
	α p33 ^{ING1b}	53.45 \pm 10.1
	p33 ^{ING1b} + α wtp53	55.61 \pm 8.3
	pcDNA3	58.78 \pm 10.3
PLC/PRF/5	p33 ^{ING1b}	64.79 \pm 11.1
	p33 ^{ING1b} +wtp53	78.16 \pm 10.5 ^c
	α p33 ^{ING1b} + wtp53	60.17 \pm 9.8
	pcDNA3	56.56 \pm 8.8
Hep3B	p33 ^{ING1b}	48.90 \pm 7.6
	p33 ^{ING1b} +wtp53	55.91 \pm 10.1 ^e
	α p33 ^{ING1b} + wtp53	50.92 \pm 8.5
	pcDNA3	52.87 \pm 7.0

^a $P < 0.05$, ^c $P < 0.05$, and ^e $P > 0.05$ vs the control groups.

Table 2 Cloning efficiency of HepG2 cells after transfection of sense and antisense p33^{ING1b} gene (mean \pm SD)

Experiment	Number of colonies		
	HepG2-p33ING1b ^b	HepG2- α p33ING1b ^d	HepG2-pcDNA3
1	65 \pm 7	149 \pm 12	86 \pm 9
2	68 \pm 15	151 \pm 10	93 \pm 11
3	72 \pm 10	148 \pm 11	94 \pm 10

^b P and ^d $P < 0.01$, vs HepG2-pcDNA3 control.

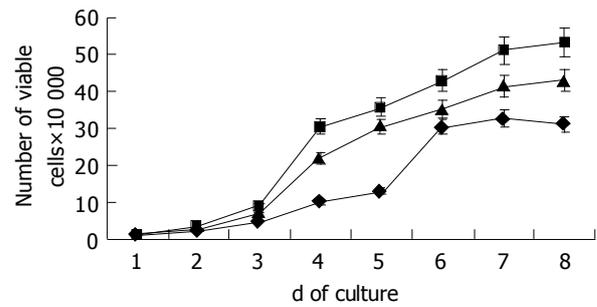


Figure 2 Growth curves for HepG2 cells stably transfected with pcDNA3 (▲), pcDNA3-p33ING1b (●), or pcDNA3- α p33ING1b (■) in complete medium containing 10% serum.

p21^{WAF1/CIP1} promoter in HepG2-pcDNA3-p33ING1b (13.81 ± 1.0), or PLC/PRF/5 (12.99 ± 1.1) and Hep3B cells (10.32 ± 0.6) cotransfected with p33^{ING1b} and wtp53 genes was significantly stronger than that in control cells transfected with insert-free vector (3.027 ± 0.4) ($P < 0.01$). Whereas after transfection with antisense-p33^{ING1b} and antisense-wtp53 plasmids, the activity of p21^{WAF1/CIP1} promoter activated by p33^{ING1b} or p53 gene alone was significantly lower than that activated by p33^{ING1b} and p53 in combination ($P < 0.01$), and similar to that activated by transfection with plain vector ($P > 0.05$). After 6% ethanol treatment, the activity of p21^{WAF1/CIP1} promoter was elevated in all experiment groups, especially in HepG2-pcDNA3-p33ING1b (17.82 ± 0.8), Hep3B and PLC/PRF/5 cells cotransfected with p33^{ING1b} and wtp53 genes (18.128 ± 1.0 and 16.82 ± 0.7 , respectively) ($P < 0.01$, Figure 3). These results indicate that the function of p53 as a transcriptional activator depends on the presence of p33^{ING1b}, and the synergistic action of p33^{ING1b} with p53 might enhance the activation of p21^{WAF1/CIP1}.

Expression of p33^{ING1b} and p53 and mutation analysis of p33^{ING1b} gene in HCC

Immunohistochemistry results revealed that both p33^{ING1b}

Table 3 Relation of p33^{ING1b} and p53 protein expression in HCC tissues

p33 ^{ING1b} expression	Cases (n)	p53			
		-	+	++	+++
HCC					
-	33	19	7	3	4
+	10	4	3	2	1
++	10	0	0	3	7
+++	4	1	1	2	0
Para-cancerous tissues					
-	38	38	0	0	0
+	13	13	0	0	0
++	0	0	0	0	0
+++	0	0	0	0	0
Normal liver tissues					
-	10	10	0	0	0
+	2	2	0	0	0

Note: Staining was estimated as follows: -, negative; +, weak; ++, intermediate; +++, strong positive staining.

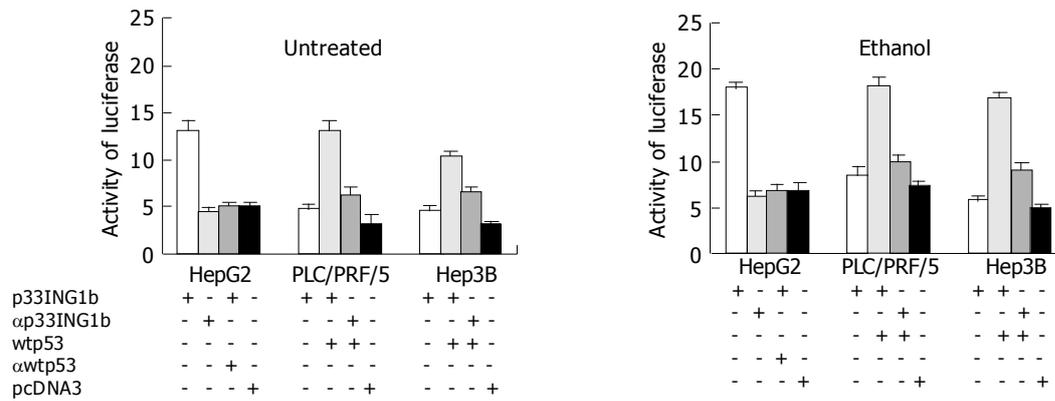


Figure 3 Analysis of p21^{WAF1/CIP1} promoter activation in three hepatoma cell lines.

and p53 proteins were all nuclear positive in paraffin-embedded tissues from 57 cases of HCC. The positive rate of p33^{ING1b} and p53 was 42.1% and 57.9%, respectively. Both had the same positive localization in HCC tissues, and their expression had a positive correlation (Wilcoxon test, $r = 0.783$, $P < 0.01$). In 13 out of 51 para-cancerous liver tissues and 2 out of 12 normal liver tissues, p33^{ING1b} protein presented weak staining, whereas p53 protein was negative in all para-cancerous liver tissues and normal liver tissues (Table 3 and Figure 4).

No aberrant PCR-amplification products were found in tumor and para-cancerous tissues compared with normal liver tissues, suggesting that ING1b gene did not have a fragmental frame shift or more than a dozen of base pairs deletion in HCC. Five HCC cases presenting abnormal band shifting were identified by SSCP, two of them showed a G to C transversion at the middle nucleotide of codon 215 with an amino change in ING1b gene exon2 by DNA sequencing, resulting in a cysteine to serine substitution. p33^{ING1b} gene did not exhibit mutation in para-cancerous

tissues. All the point mutations were confirmed by repeated PCR amplification and sequencing from both ends (Figure 5). The frequency of missense mutation of the ING1b gene in HCC was low (7.1%). Our results were consistent with those of head and neck squamous cell carcinoma (13%) reported by Gunduz *et al.*^[18], and esophageal squamous cell carcinoma (12.7%) reported by Chen *et al.*^[16].

DISCUSSION

Ectopic expression of the first isolated human ING1 cDNA is growth-suppressive in different cell lines^[1,2]. One of the reasons why this gene has gained increasing attention from the biological community is that it has no structural similarity with p53, but has many tumor suppressive functions including growth arrest, apoptosis, senescence and sensitization to drug treatment. p33^{ING1b}, one of the alternative transcripts of ING1 gene, is physically associated with p53, further proving its role in carcinogenesis^[4,5]. As an important member of p53-interacting proteins family, p33^{ING1b} inactivation

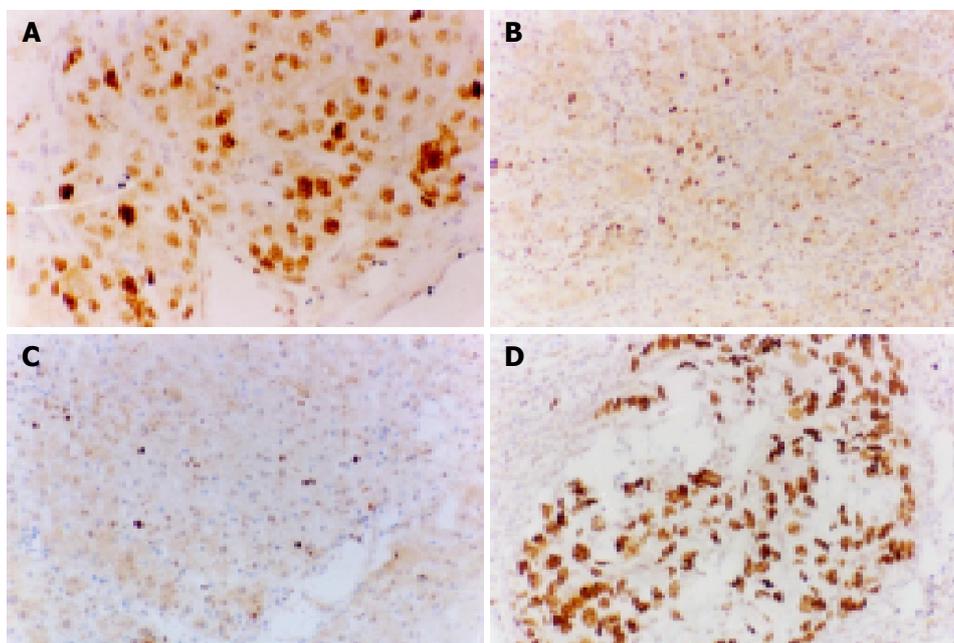


Figure 4 IHC staining for p33^{ING1b} on HCC (A), para-cancerous tissues (B), and normal liver tissues (C), and for p53 on HCC (D). A and D: p33^{ING1b} and p53 were nuclear-positive and located in the same area of HCC, $\times 400$; B and C: p33^{ING1b} presented weak IHC staining, $\times 200$.

might be another critical reason for the loss of tumor suppressive function of p53 in HCC. Moreover, the tumor suppressive activity of p33^{ING1b} requires an intact p53 gene, neither of these two genes can inhibit tumor cell growth when one of them is suppressed^[26,27,35].

We found that overexpression of p33^{ING1b} inhibited the growth of HepG2 cells and suppressed the cell anchorage-independent survival in soft agar, which is consistent with the tumor-suppression role of p33^{ING1b} in the development of HCC. However, the synergic effects of p33^{ING1b} and p53 on apoptosis and cell cycle arrest in hepatoma cell lines have not been well established. In this study, overexpression of p33^{ING1b} effectively inhibited the growth of hepatoma cells and protected cells from malignant transformation; cointroduction of p33^{ING1b} and p53 enhanced apoptosis of hepatoma cells, arrested more cells at G₁/G₀ phase and elevated the expression of p21^{WAF1/CIP1} gene compared with introduction of either p33^{ING1b} or p53 gene alone; the expression of p33^{ING1b} and p53 proteins had a close relation and exhibited the same positive staining localization in HCC; p33^{ING1b} gene had a low mutation rate (7.1%) in HCC. These results suggest that p33^{ING1b} plays an important role in refraining the process of hepatocarcinogenesis and the cooperation of p33^{ING1b} and p53 is essential to normal biological functions of these two genes^[29-31]. However, this cooperation in hepatoma cells might be influenced by other factors such as HBV infection or Rb gene deletion.

There is evidence that HBV X antigen is capable of binding to wild-type p53 protein, leading to p53 inactivation and accumulation in HBV-infected liver cells, which in turn result in HBV-associated hepatocarcinogenesis^[25,32]. p33^{ING1b} protein also binds to p53 protein and forms a complex in vivo. It is hypothesized that HBxAg may interact with p53-p33^{ING1b} protein complex and influence the biological synergy of p33^{ING1b} with p53. After cotransfection of p33^{ING1b} and p53 into PLC/PRF/5 or Hep3B cells infected with HBV, apoptosis and G₀/G₁ arrest are elevated, but the extent is not as significant as that in HepG2 cells without HBV infection^[22]. These results suggest that HBV may play an important role in repressing the cooperation of p33^{ING1b} with p53. Whether HBV can directly bind to p33^{ING1b} protein and the mechanism of HBV inactivating p33^{ING1b} remain to be studied.

Tumor suppressors contribute to the regulation of mammalian cell cycle and apoptosis, largely through

interaction with multiple proteins, which form a complex gene network^[28,33]. P33^{ING1b} is an important member of p53-related gene network, and other downstream genes may influence the synergy of p33^{ING1b} with p53. It is interesting that in Hep3B cells with HBV infection and endogenous Rb gene complete deletion^[22], apoptosis and cell cycle arrest induced by combined transfection of p33^{ING1b} and p53 gene are much lower than those in HepG2 and PLC/PRF/5 cells, suggesting that loss of normal function of other important genes, such as Rb gene, may be a potential mechanism for inactivation of p33^{ING1b} and its cooperation with p53.

The data from other laboratories demonstrate that overexpression of p33^{ING1b} in human fibrosarcoma cell lines containing endogenous wild-type p53 greatly increases cell sensitivity to DNA damage caused by chemotherapeutic drug etoposide or γ irradiation^[5]. Our study showed that combined transfer of p33^{ING1b} and p53 enhanced cell sensitivity to ethanol, rendered more cell apoptosis and arrested in G₀/G₁ phase, and strongly activated the expression of downstream gene p21^{WAF1/CIP1}, suggesting that the synergy of p33^{ING1b} with p53 may enhance the tumor-suppressing effect of chemotherapy on HCC.

Although the sequence of ING1 gene is not altered frequently, previous study also noted the presence of p33^{ING1b} gene mutation in head and neck squamous cell cancer (HNSCC)^[18] and esophageal squamous cell cancer (ESCC)^[16], which are similar to our HCC results. There is accumulating evidence that gene mutation is not the main mechanism of biological inactivation of p33^{ING1b} in HNSCC^[18]. Many studies have shown that p33^{ING1b} protein is downregulated in other cancers such as breast cancer, gastric and colon cancer, malignant lymphoma, but methylation of the ING1 promoter region occurs in HNSCC^[8-16]. ING1 gene may serve as a "class II tumor suppressor" being inactivated at the level of RNA rather than DNA^[36,37]. As p33^{ING1b} and p53 have a close cooperation, low expression of p33^{ING1b} protein may be a mechanism underlying the inactivation of wild-type p53 in these cancers. Immunostaining in this study showed that p33^{ING1b} and p53 had a positive relation in HCC tissues and both had the same nuclear localization. Cells positive for p53 staining can express mutant p53 protein because of its prolonged half-life, and cells expressing wild-type p53 protein are usually negative for p53 staining. Our results demonstrate that p33^{ING1b} might also have a close relation with mutant p53 and wtp53. It is still not clear which domain of p53 protein binds to p33^{ING1b} protein. If mutation does not locate in the interaction domain of p53, physical interaction between p33^{ING1b} and p53 proteins would not be affected. Whether p33^{ING1b} binds to mutant p53 still has normal biological functions needs further study.

In conclusion, p33^{ING1b} cooperates with p53 in inhibiting hepatoma cell growth. Whether the interaction between these two genes occurs at other levels such as at p53 DNA binding level or p53 nuclear transport needs further investigation. The involvement of p33^{ING1b} in p53 signaling pathway indicates that p33^{ING1b} is essential for p53 function, loss or inactivation of p33^{ING1b} may contribute to malignant transformation of HCC retaining wild-type p53.

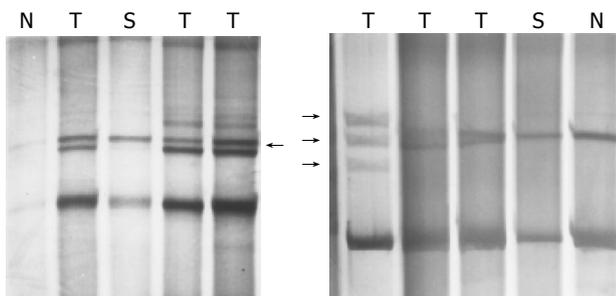


Figure 5 Altered migration pattern of PCR products of p33^{ING1b} gene from HCC genomic DNA by SSCP. N, normal DNA; T, tumor DNA; S, para-cancerous tissue DNA.

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