

Association of low p16INK4a and p15INK4b mRNAs expression with their CpG islands methylation with human hepatocellular carcinogenesis

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Abstract

AIM: To study the significance of p16 and p15 transcription suppression with hypermethylation of their genes' 5' CpG islands during human hepatocellular carcinogenesis.

METHODS: The mRNA expression levels of p16 and p15 genes were evaluated in cancerous, para-cancerous and non-cancerous tissues of 20 HCC, 3 normal liver tissues from 3 accidentally died healthy adults using semi-quantitatively Northern blot. The methylation status was also assessed with methylation specific PCR.

RESULTS: p16 mRNA expression level was decreased in the cancerous tissues in 60% (12/20) of HCC patients, of which 2 cases had no p16 mRNA detected, 5 cases (25%) displayed variation in the order of cancerous < para-cancerous < non-cancerous liver tissues. p15 mRNA expression level was decreased in the cancerous tissues in 50% (10/20) HCC patients, of which one case had no p15 mRNA detected, 4 cases (20%) displayed variation in the order of cancerous < para-cancerous < non-cancerous liver tissues. In cancerous, para-cancerous and non-cancerous tissues, p16 promoter CpG islands hypermethylation occurred 65%, 60% and 35%, while p15 promoter CpG islands hypermethylation occurred 50%, 40% and 25%. Of 12 HCCs with lower p16 mRNA expression level, 11 cases showed p16 promoter CpG islands methylation (91.6%). Hundred percent (10/10) HCCs with lower p15 mRNA expression level showed p15 promoter CpG islands methylation. Significant correlation between 5' CpG islands methylation and p16/p15 mRNA expression suppression was found. The decreased expression of p16/p15 mRNA or methylation of p16/p15 promoters 5' CpG island was significantly correlate with poor differentiation of HCC ($P=0.0083$, 0.0102 , 0.00271 , 0.0218 , respectively, $P<0.05$).

CONCLUSION: p16 and p15 genes transcriptional inactivation might play an important role in hepatocarcinogenesis. 5' CpG islands methylation might be the major mechanism of p16 and p15 genes inactivation in primary HCC in the studied

population. 5' CpG islands methylation of p16 and p15 genes might be an early event in hepatocarcinogenesis.

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INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common causes of death from cancer in China. The mechanisms of hepatocellular carcinogenesis are not yet expounded although alterations of some cell cycle tumor suppressor genes have been revealed to be involved in hepatocellular carcinogenesis. p16INK4a and p15INK4b are homologous cell cycle tumor suppressors, which are within 30 kb of one another on chromosome 9p21 region and in the same transcription orientation. p16INK4a has 3 exons whereas p15INK4b is encoded by 2 exons. They act as competitive inhibitors by binding directly to CDK4 and CDK6 and prevent their association with a cyclin, which in turn arrest the cells in late G1 phase of the cycle with pRB in a hypophosphorylated state^[1-4].

Although the high frequency of P16 protein expression deficiency or low expression in HCC was reported, the rate of p16 gene homozygous deletion and mutation is much lower than P16 protein expression deficiency. This suggested that there might be another mechanism inactivating p16 and p15 genes in human primary hepatocarcinoma^[5-9].

It has been demonstrated that aberrant methylation of CpG islands, which are CpG dinucleotide rich region located mainly in the promoter regions of many gene, serves as an alternative mechanism for inactivation of the tumor suppressor gene in cancers^[10]. Recently, aberrant methylation of the p16 and p15 promoters has been reported^[11-16]. The discordant events in which low P16 protein expression was not accompanied with deletion or mutation could be resulted from the low mRNA expression level of p16 gene due to hypermethylation of its CpG islands. To further study the association of hypermethylation of CpG islands in p16 and p15 genes with disrupted transcription of the corresponding genes in hepatocarcinogenesis, and to address the question whether the expression of p16 and p15 genes, as well as CpG island methylation of p16 and p15 genes is related to the differentiation degrees of HCC, the mRNA expression of p16 and p15 genes, as well as their CpG island methylation status in 20 HCCs was studied.

MATERIALS AND METHODS

Samples and extraction of total RNA and DNA

With informed consent of patients and ethics committee approval, surgically resected tumor, adjacent cancerous as well as non-cancerous tissue samples from 20 HCC patients, and

normal liver tissues from 3 accidentally died healthy adults were collected in West China Hospital of Sichuan University. The diagnosis of HCC was confirmed histologically in all cases.

Total RNA was extracted from 100 mg frozen tissue specimen with one-step guanidine thiocyanate-phenol method and dissolved in 5 g/L SDS solution. Genomic DNA was isolated from 100 mg frozen tissue specimen by digestion with proteinase K, followed by standard phenol-chloroform extraction and ethanol precipitation. The concentration of RNA and DNA was calculated according to their A_{260} values.

The genomic DNA of human plasmacytoma cell line HS-Sultan, which had p16 and p15 CpG islands methylation, was kindly gifted by Dr. Lo YM in Chinese University of Hong Kong. It was used as a methylation control for methylation specific PCR.

Northern blot hybridization

The plasmid that contained complete sequences of p16 cDNA or p15 cDNA was kindly gifted by Dr. D Beach in Cold Spring Harbor Laboratory. The 0.8 kb cDNA fragment of p16 and 2 kb cDNA fragment of p15 were nick translated with non-radiated digoxin using Boehringer Mannheim random primer DNA labeling kit. A 0.9 kb cDNA fragment of γ -actin was used as control probe. The total RNA (20–30 μ g) was fractionated on 1% agarose-formaldehyde gel and blotted onto nylon membrane (Bio-Rad) by capillary transfer. The hybridization was carried out in a solution containing 70 g/L SDS, 500 g/L formamide, 20 mL/L blocking reagent, 50 mmol/L sodium phosphate, pH7.0, 1 g/L sarcosyl, 5 \times SSC, 80 ng/mL probe. The hybridization and the subject signal chemiluminescent imaging were performed according to the manufacturer's manual. Every membrane was sequentially hybridized with p16, p15 and γ -actin cDNA probe after the bound probe was washed with 5 g/L SDS.

The intensity of the hybridization signal was scanned with UVP GDS8000 and quantited with Gelworks of UVP. To calculate the relative expression level of p16 or p15 gene, the hybridization signal intensity of γ -actin was employed as internal control to correct the unidentical amount of RNA loaded in electrophoresis, because γ -actin gene has a stable expression in various tissues.

Bisulfite conversion of DNA

Bisulfite modification and methylation specific PCR were conducted based on the principle that bisulfite treatment of DNA would convert unmethylated cytosine residues into uracil, whereas methylated cytosine residues would remain unmodified. Thus, the sequence-specific primers would distinguish the bisulfite converted unmethylation sequences and the unmodified methylation sequences by U and C.

The chemical modification was performed essentially as described previously with minor modification^[17]. DNA (1–2 μ g) in a volume of 50 μ L was denatured by NaOH (final concentration, 0.2 mol/L) for 10 min at 37 °C. Totally 520 μ L of 3 mol/L sodium bisulfite at pH5.0 and 30 μ L of 10 mmol/L hydroquinone, both freshly prepared, were added. After being mixed, the samples were incubated under the cover of mineral oil at 56 °C for 16 h. The modified DNA was desalted with the Wizard DNA purification resin and eluted in 50 μ L of water. For alkali desulphonation, an equal volume of 0.6 mol/L NaOH was added to an eluted DNA and incubated for 5 min at room temperature, modified DNA was precipitated with sodium acetate and ethanol and resuspended in 30 μ L of water. The DNA was stored at -70 °C.

Methylation specific PCR

The primers were designed for the CpG islands in promoter

and the first exon region of both p16 and p15 genes. All bisulfite-converted DNA samples were amplified with primers specific for methylated p16 or p15 sequence, and also amplified with primers specific for unmethylated p16 or p15 sequence. The sense and antisense primers for methylated p16 sequence were 5' TTATTAGAGGGTGGGGCGGATCGC3' and 5' GACCCGAACCGCGACCGTAA3'. The sense and antisense primers for unmethylated p16 sequence were 5' TTATTAGAGGGTGGGGTGGATTGT3' and 5' CAACCCCAAACCACAACCATAA3'. The sense and antisense primers for methylated p15 sequence were 5' GCGTTCGTATTTTGCGGTT3' and 5' CGTACAATAACCGAACGACCGA3'. The sense and antisense primers for the unmethylated p15 sequence were 5' TGTGATGTGTTTGTATTTTGTGGTT3' and 5' CCATACAATAACCAAACAACCAA3'. The 25 μ L of PCR mixture contained 50 ng of modified DNA, 200 μ mol/L each dATP, dGTP, dCTP and dTTP, 1 μ mol/L each primer, 50 g/L DMSO, 2.5 mmol/L MgCl₂, 1 \times reaction buffer and 1.5 U Taq polymerase. The PCR conditions were as follows: one cycle of at 95 °C for 5 min, 35 cycles of at 95 °C for 30 s, at 65 °C for 30 s (p16 methylation primers) or at 60 °C for 30 s (p16 unmethylation primers, p15 methylation and unmethylation primers), at 72 °C for 45 s, and 1 cycle of at 72 °C for 7 min. A 10 μ L of PCR products was then electrophoresed on a 25 g/L agarose gel, stained with ethidium bromide, and visualized under UV illumination in UVP GDS8000 gel imaging system^[17].

Statistical analysis

The difference in the p16 and p15 mRNA expression between cancer and noncancer tissue was analyzed using *t* test. The correlation of p16 or p15 gene mRNA expression level with its corresponding methylation of CpG islands was analyzed using Fisher's exact test. The correlation of between mRNA expression, and methylation of p16 and p15 genes with pathologic characteristics was also analyzed with Fisher's exact test. A *P* value <0.05 was considered statistically significant.

RESULTS

p16 and p15 mRNA expression in human hepatocarcinoma

After Northern blot hybridization, the 1.3 kb transcript of p16 mRNA, 3.8 kb transcript of p15 mRNA, 2.1 kb transcript of γ -actin mRNA were detected.

As shown in Figure 1, there was a decrease in p16 mRNA expression level in the cancerous tissues in 60% (12/20) of HCC compared with their corresponding para-cancerous and non-cancerous liver tissues, and p16 mRNA signal was not detected in 2 cases (10%). Five cases (25%) displayed variation in the order of cancerous <para-cancerous <non-cancerous tissues. The mRNA expression level of p16 gene in cancerous tissues was significantly lower than that of non-cancerous liver tissues (*P*<0.05).

p15 mRNA expression level was decreased within the cancerous tissues in 50% (10/20) of HCC, and p15 mRNA was not detected in 1 case. Four cases displayed variation in the order of cancerous <para-cancerous <non-cancerous tissue. The mRNA expression level of p15 gene in cancerous tissues was significantly lower than that of non-cancerous liver tissues (*P*<0.05).

Methylation status of the 5'CpG islands of p16 and p15 genes

Hypermethylation of p16 promoter CpG islands occurred in cancerous tissues (65%, 13/20), and also in para-cancerous tissues (60%, 11/20,) and non-cancerous tissues (35%, 7/20). Four cancerous tissues exhibited only the methylation band,

while the others showed methylation and unmethylation bands, suggesting partial methylation (Figure 2)^[11,13,18].

Aberrant promoter methylation of p15 gene was detected in 50%(10/20) of patients with HCC. Among them, the methylation was found only in cancerous tissues in 2 cases, in both cancerous and para-cancerous tissues in 3 cases, in all of cancerous, para-cancerous and non-cancerous tissues in 5 cases. The partial methylation occurred in all of the non-cancerous tissues. None of the 3 healthy subjects displayed p16 or p15 methylation, whereas they displayed unmethylated p16 and p15 alleles (Figure 3).

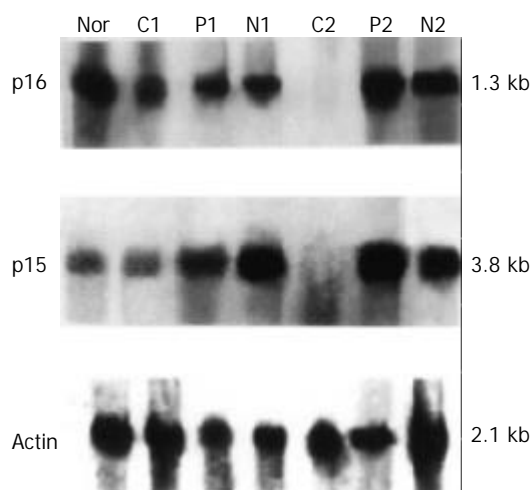


Figure 1 Northern blot analysis of the p16 and p15 genes in human primary hepatocarcinoma. Total RNA from tissues of human primary hepatocarcinoma was hybridized with cDNA fragment probes of p16, p15 and γ -actin labeled with non-radiated digoxinin. N: Normal liver tissue control; C, P, N represent RNA from cancerous, para-cancerous, non-cancerous liver tissues respectively; 1, 2 represent the No. of HCC patient.

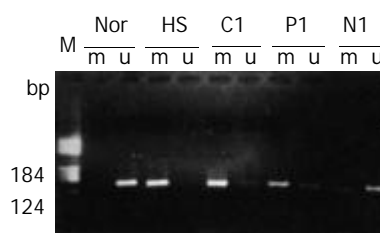


Figure 2 Methylation analysis of p16 gene in human primary hepatocarcinoma. MSP product of p16 gene from HCC tissues was electrophoresed on a 25 g/L agarose gel. M: pBR322/HeaIII DNA molecular marker; N: Normal liver tissue DNA; HS: HS-Sultan DNA (positive control); C, P, N represent RNA from cancerous, para-cancerous, non-cancerous liver tissue respectively; 1, 2 mark the HCC patient number; m: PCR products from methylation specific primers, u: PCR products from unmethylation specific primers.

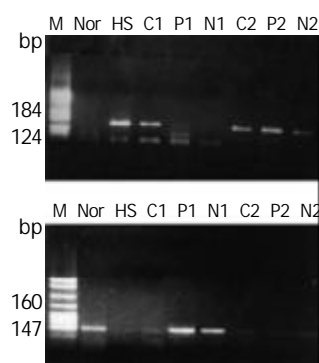


Figure 3 Methylation analysis of p15 gene in human primary hepatocarcinoma. MSP product of p15 gene from HCC tissues was electrophoresed on a 2.5% agarose gel. M: pBR322/HeaIII DNA molecular marker (in graph m) or pBR322/Msp I DNA marker (in graph u); N: Normal liver tissue DNA; HS: HS-Sul-

Table 1 CpG island methylation status and expression of p16 and p15 genes and clinical information of 20 HCC patients

Cases	p16 methylation ¹	p16 expression	p15 methylation ¹	p15 expression	HBsAg ⁴	Cirrhosis ⁵	Differentiation ⁶
1	±	D	+	D	+	Y	L
2	+	D	+	D	+	Y	L
3	±	D	+	D	+	N	M
4	±	N	±	D	+	Y	M
5	±	D	+	D	+	N	L
6	+	D	-	N	+	N	L
7	-	N	-	N	-	N	H
8	±	N	±	D	-	N	M
9	±	D	±	D	+	Y	M
10	-	N	-	N	-	N	H
11	+	D	N ²	N	+	Y	L
12	±	D	-	I	+	Y	M
13	-	D	±	D	+	Y	M
14	-	N	-	I	-	N	M
15	+	D	-	N	+	N	L
16	±	N	±	D	-	Y	L
17	-	D	-	N	-	N	H
18	-	I	-	I	+	N	H
19	±	D	±	D	-	Y	L
20	-	N	-	N	+	N	H

¹+Positively amplified with methylation primers only; - positively amplified with unmethylation primers only; ± positively amplified with methylation and unmethylation primers; ²N: Negatively amplified with methylation or unmethylation primers, ³D: Decreased; I: Increased; N: No significant difference; compared with non-cancer tissue, ⁴+: Positive; -: Negative; ⁵Y: Cirrhosis; N: No cirrhosis, ⁶H: High differentiation; M: moderate differentiation; L: Low differentiation.

tan DNA(positive control); C, P, N represent RNA from cancerous, para-cancerous, non-cancerous liver tissue respectively; 1, 2 mark the HCC patient number; m: PCR products from methylation specific primers, u: PCR products from unmethylation specific primers.

Reciprocal relationship between mRNA expression of p16/p15 and their promoter methylation status

Significant correlation between 5' CpG island methylation and low mRNA expression of p16/p15 was found in the 20 HCCs ($P < 0.05$). Among 12 HCCs, which displayed lower p16 mRNA expression level in cancerous tissue, 11 cases showed p16 promoter 5' CpG island methylation (91.6%). Similar event took place in p15 gene. All of 10 HCCs with decreased p15 mRNA expression in cancerous tissue had 5' CpG island hypermethylation (100%), while unmethylation occurred in non-cancerous liver tissue. Interestingly, 1 case displayed no detectable transcript p15 gene in cancerous tissue with hypermethylation (Table 1).

The relationship between mRNA expression, or methylation and pathologic characteristics

Significant correlation either between decreased p16/p15 mRNA expression and poor cellular differentiation of HCC, or between p16/p15 promoters 5' CpG islands methylation and poor differentiation of HCC was found ($P = 0.0083$, 0.0102 , 0.00271 , 0.0218 , respectively, $P < 0.05$). However, there was no significant correlation between p16/p15 mRNA expression and HBV infection or cirrhosis, or between their promoter methylation and HBV infection or cirrhosis (Table 1).

DISCUSSION

Our study showed that a decreased p16 mRNA expression level was detected within cancerous tissues in 60% of HCCs compared with their corresponding non-cancerous tissues, 5 of which displayed in turn variation in cancerous tissue less than in para-cancerous tissue, then in non-cancerous liver tissues. It was interesting that among the 12 cases with lower p16 mRNA expression, 9 cases were accompanied with p16 gene promoter partial methylation in cancerous tissues, while 2 cases were accompanied with complete methylation. In contrast, methylation was not detected in normal liver tissues from 3 accidentally died subject. The partial methylation of p16 gene promoter may explain why the p16 mRNA expression just decreased but not loss in majority of HCCs. It has been reported that partial loss of expression of p16 was associated with low level of methylation rather than complete loss^[11,19,20]. The relationship between p16 mRNA expression and its promoter CpG island methylation has been demonstrated in other studies. Kaneto *et al.*, using methylation specific PCR and immunohistochemistry, had detected methylation of p16 promoter in HCC (72.6%, 16/22) and loss of expression in all methylation positive HCC^[13]. Roncalli *et al.* reported that methylation of p16 promoter with complete loss of immunoreactivity occurred in 27 of 33 HCC (82%)^[15]. Thus, our result, which was consistent with other reports, suggested that association of p16 gene 5' CpG island methylation with transcription inactivation might play an important role in hepatocarcinogenesis, and 5' CpG island methylation was the major mechanism of p16 gene inactivation.

Like the alteration of p16 gene, a decreased p15 mRNA expression level was found within cancerous tissue in 50% of HCC compared with their corresponding non-cancerous liver tissues. All cases that had a lower transcription level of p15 were in conjunction with the hypermethylation of p15 promoter CpG island. Roncalli *et al.* detected methylation of p15 islands in 42% of HCC (14/33), and methylation positive HCC had a

complete loss of immunocytochemical expression of p15. Wong *et al.* reported a high frequency of p15 methylation (64%, 15/25) in HCC. Yang *et al.* showed methylation of p15 in 49% of HCC^[14-16]. Based on Northern blot analysis, our study found that a complete loss of p15 mRNA expression accounted for a small portion of the p15 methylation positive cases, while the majority had decreased transcription level. This discrepancy of our result with other studies might be due to the high sensitivity of Northern blot analysis. Nevertheless, our result provided further evidence from different aspect for the contribution of the epigenetic alteration to inactivation of p15 gene in hepatocarcinogenesis.

In our present study, partial methylation of p16 and/or p15 promoter was detected in 50% of non-cancerous liver tissues (10/20). However, the p16/p15 positive liver tissue still expressed mRNA of p16/p15 genes. It was very interesting to note that a complete methylation of p16 promoter CpG island was present in cancerous tissue while partial methylation in non-cancerous tissues. A similar phenomenon of p15 gene alteration occurred in 2 cases. This supported the hypothesis that methylation of cell cycle inhibitor gradually accumulate in the transition from cirrhosis or chronic hepatitis to HCC^[13,15]. Yu *et al.* detected that hypermethylation of the p16 promoter CpG islands occurred overwhelmingly in HCC samples (56.6%, 17/29) and less frequently in adjacent non-cancerous tissues (21.9%, 6/29) after analyzing the methylation profiling of 20 promoter-CpG islands of genes in human primary hepatocellular carcinoma. Therefore, they thought that hypermethylation of p16 promoter CpG islands may occur at very late stage of the hepatocarcinogenesis^[21]. Edamoto *et al.* showed that higher rate of promoter methylation of p16 in HCCs (59%) than in cirrhotic liver (21%) or chronic hepatitis lesions (0/10)^[22].

Our study also showed that in the non-cancerous liver tissues, the frequency of p15 partial methylation was higher in cirrhosis (44%, 4/9) than non-cirrhosis (9%, 1/11). Also the rate of p16 partial methylation was higher in cirrhosis (44%, 4/9) than non-cirrhosis 27%, 3/11), although these results had no statistical significance probably due to the few studied cases (Data not shown). The percentage of p16 methylation in cirrhosis was reported 29.4% by Kaneto *et al.*, and 39% by Roncalli *et al.*^[13,15]. The frequency of p15 methylation reported by Roncalli *et al.* was 33%^[15]. Roncalli *et al.* noted that p15 was responsible for the early methylation pattern in cirrhosis but not in HCC. They proposed that p15 but not p16 was necessary for the inactivation of other genes in the transition from cirrhosis to HCC^[15]. Nevertheless, the methylation of p16 and p15 might be the major mechanism to inactivate cell-cycle inhibitor in preneoplastic stage.

The correlation between either decreased p16/p15 mRNA expression or p16/p15 promoter CpG island methylation and poor differentiation of HCC was determined in our present study. Interestingly, the correlation of loss of p16 protein expression with the cellular differentiation of HCC and gastric cancer has been reported^[23,24]. Park *et al.* also noted that inactivation of p16 exon 1 by DNA hypermethylation occurred during the progression of tumor cells to poorly differentiated HCC, which was induced by diethylnitrosamine plus thioacetamide in Fischer 344 rats^[25]. These studies suggested that the aberrant alteration of mRNA expression and methylation of p16 and p15 genes might be not only involved HCC carcinogenesis but also associated with its progress.

Although the mechanism of methylation in cancers remains to be explained clearly, it has been generally recognized that two methyltransferases, Dnmt1 and Dnmt3b cooperatively maintain DNA methylation and gene silencing in human cancer cells^[26-28]. It has been reported that higher expression of methyltransferases was associated with methylation of p16

and p15 genes in HCC^[29]. It has been noted that either the inhibitors of methyltransferase, such as 5-aza-2'-deoxycytidine and tea-polyphenol (-)-epigallocatechin-3-gallate or antisense inhibitors of DNA methyltransferases, reactivated silenced tumor suppressor genes and inhibited the growth of cancer cells^[30-33].

Thus, methylation of 5' CpG islands of p16 and p15 genes might be an early event in hepatocarcinogenesis. The methylation of p16 and p15 genes could be a biomarker for monitoring HCC and other tumors, which was demonstrated by recent studies^[14,34,35,36]. It also provides an insight into therapy of the high risky patients and prevention of HCC.

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