MUTATION AND ABNORMAL EXPRESSION OF P16^{INK4a} IN HEPATOCELLULAR CARCINOMA

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ABSTRACT

Objective: To investigate the relationship between p16^{INK4a} and primary hepatocellular carcinoma (HCC), especially hepatitis B-related HCC. Methods: p16^{INK4a} and its protein in HCC were analyzed with PCR-SSCP and the immunohistochemistry methods respectively. Results: The positive incidence of p16^{INK4} protein expressing in HCC was lower than that of normal liver tissue (P<0.05), and the absence of p16^{INK4} protein was associated with HCC metastasis (P<0.05). The low frequency of mutation of p16^{INK4} exon1 and exon2 upstream fragment was found in HCC. Conclusion: Absence of p16^{INK4} protein in HCC was not associated with HBV-infection.

Key words: Liver cancer, Tumor suppressor gene, p16^{INK4a} gene

p16^{INK4a} is a representative element of CDKI family. p16^{INK4a} protein binds to CDK4 thus inhibiting cyclin D1-CDK4 to phosphorylating of Rb, thereby halting cell progression at G1. Abnormal expression of p16^{INK4a} results in cell progression uncontrolled at G1/S, which makes cell malignant transformation. In many tumors, alteration of p16^{INK4a} gene and absence of p16^{INK4a} protein had been reported.[1] However, the relationship between p16^{INK4a} and primary hepatocellular carcinoma, especially hepatitis B-related HCC, has been little understood. In this study, we analyze the p16^{INK4a} protein and mutation of p16^{INK4a} gene in HCC using immunohistobiochemistry and PCR-SSCP respectively, in order to investigate the mechanism of p16^{INK4a} alteration in the progression of HCC.

MATERIALS AND METHODS

Sample

Thirty-seven cases of HCC tumor tissues (paraffin-embedded or frozen) were diagnosed by the First and Second Affiliated Hospital to Dalian Medical University. Among them, the tumor tissues and adjacentnoncancerous liver tissues of 10 cases were surgically resected, then immediately stored at -30°C to prepare for DNA analyses. Histopathological diagnosis as HCC was ascertained for all tumors. 13 cases of HCC were diagnosed as HCC with metastasis (the tumor invaded to other organs such as lung, diaphragm and greater omentum etc.). 14 cases of HCC were found without metastasis. HBV-infection incidence of HCC was 82.35%. The normal liver tissues of 13 cases as control were obtained from pathology specimens of patients undergoing surgical resection for the other disease or at autopsy, it was confirmed to be assured no HCC cells in these liver tissues under microphotograph.

p16 Immunohistochemistry

All tissues were fixed with 10% formallin and in embedded paraffin, then cut to 4 μm thick sections. After the sections were deparaffinlized and to Hydrated, immunohistochemistry was performed according to the Sp kit protocol (purchased from ZYMEMED Company). p16 antibody (purchased from Santa Cruz Company) were diluted to 1:50 for immunostaining p16 protein. The cells with nuclear and/or plasma immunostaining were considered as p16 protein reaction positive. The tissue in which the percent of positive cells was more than 10% was considered a p16 protein positive sample.

PCR-SSCP Analysis

Tissue DNA extractions were obtained followed the method previously described. PCR-SSCP analysis was performed on paired DNA samples from a patient's tumor and adjacent noncancerous tissues. The exon1 and exon2 upstream fragment primers were designed according to the method previously described[2, 3] and the reagent used was provided by Takara Company.

The primers for exon1:

MTS1E1F:5'GAAGAAAGAGGAGGGGCTG3'

MTS1E1R: 5'GCGCTACCTGATTCCAATTC3'

The primers for the upstream of Exon2:

F1: 5'ACACAAGCTTCCTTTCCGT3';

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F2: 5'AAGCCCTCCCGGGCAGCG3'

PCR was performed in reaction of 50 μl composed of DNA 0.5 μg 10×PCR buffer (Takara Company) 5 μl, 20 mmol/1 dNTP (Takara Company) 4 μl, 25 pmol each primer for exon1 (for exon2, 20 pmol), and Taq polyzenzyme (Takara Company) 1.25 U. The PCR conditions comprised 3 min of denaturation at 94°C, followed by 30 cycles of 1 min at 94°C, 50 sec at 62°C for exon1 (or 50 sec at 58°C for exon2), and 1 min at 72°C, then 5 min of extension at 72°C. 10 μl PCR reaction mixture were denatured in 5 μl stop buffer at 95°C for 5 min, and then flash-cooled on ice. Denatured products were loaded on 10% nondenatured polyacrylamide gel (acrylamide: bisacrylamide was 29:1), gel were run at 50 V in 0.6×TBE buffer for 13 h at room temperature. After electrophoras, the gel was stained by silver staining methods.

Statistical Analyses

Used x^2 test in this study.

RESULTS

$p16^{INK4a}$ Protein Expression in HCC and Normal Liver Tissues

$p16^{INK4a}$ proteins are located in nuclear or/and cytoplasm and the cells with $p16^{INK4a}$ protein were located in the livers. $p16^{INK4a}$ protein was stained weakly in the tumor tissues and compared with that in adjacent noncancerous tissues (Figure 1). Statistical analyses (Table 1) shows that the rate of $p16^{INK4a}$ protein expression has a significant difference between HCC and normal tissues ($P<0.05$). Expression rate of $p16^{INK4a}$ protein in HCC with metastases was lower than that of HCC without metastases ($P<0.05$).

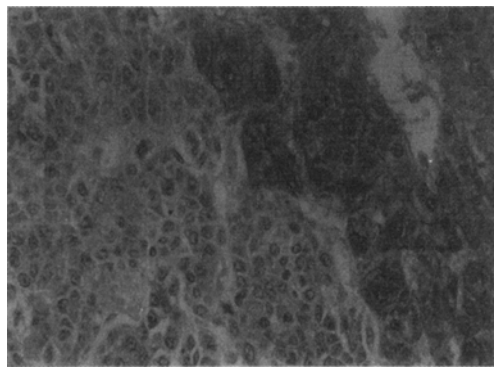


Fig. 1. $p16^{INK4a}$ protein expression in HCC (SP staining, ×200)

Table 1. $p16^{INK4a}$ expression in liver tissue

	$p^{16INK4a}$ positive	$p^{16INK4a}$ negative
HCC (total)	51.35% (19/37)*	48.65% (18/37)
with metastasis	23.08% (3/13)**	76.92% (10/13)
without metastasis	71.43% (10/14)	28.57% (4/14)
Normal liver tissue	100% (13/13)	0% (0/13)

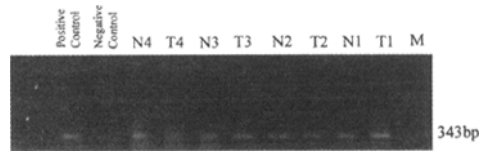
*Compared to normal liver tissue, $P<0.05$ **compared to HCC without metastasis, $P<0.05$

The Relationship between p16 Protein Expression and HBV-infection in HCC

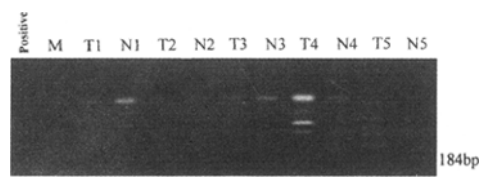
HBV immunologic markers were detected in 28 out of the 34 cases studied (82.35%) HCC. $p16^{INK4a}$ protein was detected in 15 out of 28 (53.75%) HCC with HBV infection and 3 out of 6 (50.00%) HCC without HBV infection. There was no significant difference between them ($P>0.05$).

PCR-SSCP Analyses of p16 Exon1 and Exon2

For 10 cases, tumors tissues and adjacent noncancerous liver tissues were screened for mutation of $p16^{INK4a}$ by PCR-SSCP. All showed the presence of PCR product at 343 bp (exon1) and 184 bp (exon2) (Figure 2). An SSCP abnormal mobility shift was noted in exon2 of $p16^{INK4a}$ in 1 of 10 of the HCC (Figure 3), compared with its adjacent normal tissue, whereas all of the other paired tumor and normal DNA yield normal results by SSCP analysis of $p16^{INK4a}$ exon 1 and 2.



(a) PCR product of $p16^{INK4a}$ exon1



(b)PCR product of upstream fragment in $p16^{INK4a}$ exon2
There were several nonspecial bands in N1 and T4.

Fig. 2. The PCR products of $p16^{INK4a}$ exon1 and exon2
Note: T: tumor tissue N: adjacent noncancerous liver tissue

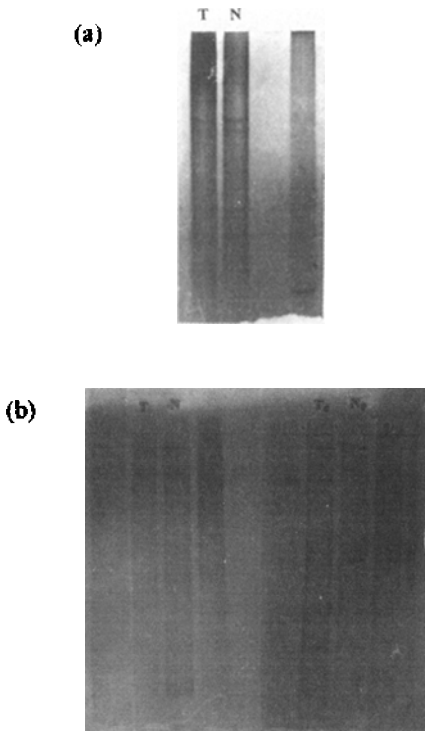


Fig. 3. PCR-SSCP analysis of $p16^{INK4a}$ exon1 and exon2

(a) PCR-SSCP analysis of $p16^{INK4a}$ exon1. Normal result was seen in T patient compared with its adjacent noncancerous tissues (N).

(b) PCR-SSCP analysis $p16^{INK4a}$ exon 2: One different mobility-shift pattern (m) was seen in the HCC tumor DNA from T_6 patient, compared with the banding pattern (N_6) DNA from its adjacent noncancerous tissues.

DISCUSSION

$p16^{INK4a}$ genetic alteration has been found in many tumor cell lines.[4] These alterations might result in the change of $p16^{INK4a}$ protein expression.[5] In our experiment, the positive incidence of $p16^{INK4a}$ protein in HCC was significantly lower than that in normal liver tissue (Table 1), suggesting that absence of $p16^{INK4a}$ protein is associated with hepatocarcinogenesis. (Moreover, $p16^{INK4a}$ protein was detected in tissues in two cases of cirrhosis but the data is not shown in this paper). The same result was also seen in all the adjacent noncancerous tissues of HCC with cirrhosis. Together with the fact that most of HCC examined were in their late stage, it is suggested that the loss (or inactivation) of $p16^{INK4a}$ protein may be involved in the progression of HCC.

$p16^{INK4a}$ genetic alterations were also found in many metastatic tumors,[6] the results of our experiment suggest the association of $p16^{INK4a}$ protein loss (or inactivation) with HCC metastasis. Absence of $p16^{INK4a}$ protein was detected in 76.92% of HCC with metastasis and 28.57% of HCC without metastasis. There were significant differences between them ($P<0.05$).

Considering the various inactivation mechanisms of $p16^{INK4a}$ in many human cancers, we performed not only protein analyses, but also examinated the $p16^{INK4a}$ gene status. No homozygous deletion of $p16^{INK4a}$ exon1 and exon2 upstream fragment was found by PCR in any case of the 10 HCC tumors examined (5 of which have $p16^{INK4a}$ protein loss). Only in one of the samples a mutation of $p16^{INK4a}$ exon2 was observed by SSCP analysis, accompanied with absence of $p16^{INK4a}$ protein expression. These results were similar to the report of Hui[7] and Kita,[8] suggesting that the mutation and homozygous deletion of $p16^{INK4a}$ exon1 and exon2 is infrequently seen in HCC. Together with the high frequency of $p16^{INK4a}$ protein loss in HCC, the result of PCR-SSCP implied that absence of $p16^{INK4a}$ protein is caused by other mechanisms such as methylation, rather than gene deletion or mutation.

HBV had been considered as a virus related to hepatocarcinogenesis. Hui[7] and Chaubert[9] both found $p16^{INK4a}$ mutation in HCC with HBV infection. In our examination, HBV infection had been detected in 83.25% of the HCC. These findings urged us to study further whether there is a relationship between $p16^{INK4a}$ protein loss and HBV infection in HCC. However, we can not find a significant difference between absence of $p16^{INK4a}$ protein and HBV infection ($P>0.05$), suggesting that HBV infection may not be responsible for the loss of $p16^{INK4a}$ protein in HCC.

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