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Immunohistochemical study on p53, H-rasp21, c-erbB-2 protein and PCNA expression in HCC tissues of Han and minority ethnic patients

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Abstract

AIM To find out the difference of human primary liver carcino genesis between Han and minority ethnic patients in Xinjiang.

METHODS Expression of p53, c-erbB-2, H-rasp21 protein and proliferating cell nuclear antigen (PCNA) in tumor tissues of 50 patients (Han 38, minority 12) with primary hepatic carcinoma was detected by immunohistochemistry (LSAB).

RESULTS The positive frequency of p53, c-erbB-2, H-rasp21 an d PCNA expression was 46.0% (23/50), 70.0% (35/50), 68.0% (34/50) and 8 2.0% (41/50) in tumor tissues; 4.0% (2/50), 22.0% (11/50), 64.0% (32/50) and 52.0% (26/50) in peritumors respectively and a significant difference, except for H-rasp21, of oncogene alteration was found ($P<0.05$) be tween tumor and non-tumorous tissues. Combined the three oncogenes alteration, 26% (13/50) tumor tissues had positive immunoreactivity, but in peritumor and normal livers it was negative.The positive rate of p53, c-erbB-2 and H-rasp21 protein expression was 39.5% (15/38), 60.5% (23/38) and 39.5% (15/ 38) in tumors of Han patients; 66.7% (8/12), 100% (12/12) and 75.0% (9/12) in minorities respectively, with statistical difference ($P<0.05$).

CONCLUSION Overexpression of p53, c-erbB-2 and H-rasp21 in human primary liver carcinoma is an important biomarker of genetic alteration. The different frequency of these oncogenetic changes may reflect some environmental or/and ethnic hereditary factors affecting the liver carcinogenesis. The special life style of Han, Uygur, Kazak and Mongolia nationalities in Xinjiang may also be related to the etiopathogenesis of this disease.

INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the major cancers with highest mortality in the world. There is a striking variety of HCC incidence rates between countries, with a highest to lowest of 112.5 for males and 54.7 for females. The high-risk populations are clustered in Sub-Sahara Africa and eastern Asia[1].Although many etiological factors, e.g., HBV, aflatoxin B1, heavy alcohol drinking, etc. have been established[2-4], the exact mechanisms of HCC carcinogenesis is still poorly understood. The natural environment, multiple ethnic groups with different hereditary background, and special life style in Xinjiang are quite different from other areas in China, which may implicate the etiopathogenesis of HCC.

A great number of studies have demonstrated that human HCC hepatocarcinogenesis is a multistepwise and multigenetic alteration induced by various pathogens with the involvement of multifactorial etiology. Alterations of cytogenes, including mutation, amplification, allelic loss of oncogenes and tumor suppressor genes, are most common in many forms of human cancers, including HCC. Overexpression of p53 protein, c-erbB-2, c-myc and H-ras oncoprotein and proliferating cell nuclear antigen (PCNA) were observed in the tumor tissues of patients or experime ntal abnormals with HCC[1-10]. In order to understand the possible mechanism of hepatocarcinogenesis and if there is any difference among various ethnic patients with HCC, we detected the expressions of *p*53 protein, c-erbB-2 and H-rasp21 oncoprotein, and PCNA in

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tumor, peritumor and normal liver tissues by immun ohistochemistry.

MATERIALS AND METHODS

Patients

A series of 50 patients with HCC, including 38 Han, 12 minorities (Uygur 8, Kazak 2 and Mongolia 2); males 34, femals 16; aged from 18 to 72 (average 50.1) years, was collected from the 1st Teaching Hospital of Xinjiang Medical University from 1991 to 1995.

Specimens

All the tumor and peritumor tissues of 50 cases and 10 specimens of normal liver tissue as a control were fixed in 100mL/L formalin and embedded in paraffin for routine histopathologic examination, and these paraffin blocks were obtained from the pathologic record. The blocks including 50 tumors, 50 peritumors and 10 non-tumors were cut into five sections of 4μm in thickness and underwent H.E. and immunohistostaining, respectively.

Immunohistochemical staining

Four-μm thick sections were dewaxed, and rehydrated according to routine procedures.Nonspecific binding was blocked by incubation with bovine albumin for 10 minutes and endogeneous peroxidase activity was blocked with 30mL/L hydro gen peroxide for 5 minutes. The sections were pretreated in a microwave oven at 750W for 10 minutes in citrate buffer (pH 6.0), and then incubated for 5 minutes in microwave oven with primary antibodies, including monoclonal anti-p53 protein (Do-7), anti-H-rasp21 oncoprotein, anti-PCNA (P-c 10) (1:40) and polyclonal anti-c-erbB-2 oncoprotein (1:200) (Dako), respectively. We used the LSAB kits (Dako) for immunostaining, the secondary antibodies and reagents w ere treated by the routine procedures in microwave oven. The colour was developed using 3-amino-9-ethyicarbazol (AEC). The sections were counterstained with Meyer's hematoxylin.

Assessment of staining reaction

Only tumor cells with distinct nuclear immunostaining in both *p*53 protein and PCNA were considered positive. Tumors were recorded as positive for c-erbB-2, H-rasp21 oncoprotein if more than 5% of tumors showed distinct membrance and cytoplasm staining. The immunoreactivity was registered semiquantitatively. The positive cells were scored as (-) negative; (+) 1-5 positive cells per high magnification (400 fold); (++) 6.20 positive cells; (+++) more than 21 positive cells found in *p*53 protein and PCNA immunostaining respectively.

Statistics

The immunoreactivity for p53, c-erbB-2, H-rasp21 and PCNA parameters were considered to represent discrete values in various lesions. The Chi square test (χ^2) was used and $P<0.05$ was accepted as a statist ically significant difference.

RESULTS

The immunostaining *p*53 protein and PCNA appeared red-brown in nucleus and tumor or peritumor cells with various densities were distribuled unevenly (Figures 1, 2). The immunoreactivity of c-erbB-2 and H-rasp21 oncoprotein showed distinct membrance and cytoplasma staining (Figures 3, 4). Table 1 summarizes the different frequencies of immunostaining positive cells for *p*53 protein, H-rasp21, c-erbB-2 and PCNA in tumor and non-tumor tissues.

There was a significant difference among three oncogenes expression respectively except PCNA between Han and minorities ($P<0.05$). The onc oprotein expression was lower in Han than in the three minorities. Table 2 sum marizes the three oncogenes expression in tumor and non-tumor tissues and distr ibution of different nationalities.

Table 3 shows the combined expression of three oncogenes in HCC of different nationalities. The frequency was higher in minorities (41.7%) than in the Hans (21.1%), without statistical difference ($P>0.05$).

Table 1 Expression of p53 protein, c-erbB-2, H-rasp21 and PCNA in various liver lesions

Histology	*n*	*p*53		c-erbB-2		H-ras		PCNA	
		n	%	*n*	%	*n*	%	*n*	%
Tumor (A)	50	23	46.0	35	70.0	34	68.0	41	82.0
Peritumor (B)	50	2	4.0[a]	11	22.0[a]	32	64.0[c]	26	52.0[a]
Cirrhosis	33	2	6.0	9	27.3	23	70.0	18	54.5
Non-cirrhosis	17	0	0	2	11.8	9	53.0	8	47.0
Normal liver (C)	10	0	0	0	0	3	30.0	0	0

[a]$P<0.05$, *vs* A, C; [c]$P<0.05$, *vs* C; χ^2 test.

Table 2 Expression of p53, c-erbB-2, H-rasp21 and PCNA in HCC of different nationalities

Nationalities	*n*	*p*53		c-erbB-2		H-ras		PCNA	
		n	%	*n*	%	*n*	%	*n*	%
Han	36	15	39.5	23	60.0	15	39.5	32	84.2
Minority	12	8	66.7[a]	12	100.0[a]	9	75.0[a]	9	75.0
Uygur	8	5	62.5	8	100.0	6	75.0	5	62.5
Kazak	2	1	50.0	2	100.0	2	100.0	2	100.0
Mongolia	2	2	100.0	2	100.0	1	50.0	2	100.0

[a]$P<0.05$, *vs* Han, χ^2 test.

Table 3 The nationality distribution of the combined expression of three oncogenes in HCC

Nationalities	*n*	*p*53+c-erbB-2+H-rasp21	
		n	%
Han	38	8	21.1
Minority	12	5	41.7

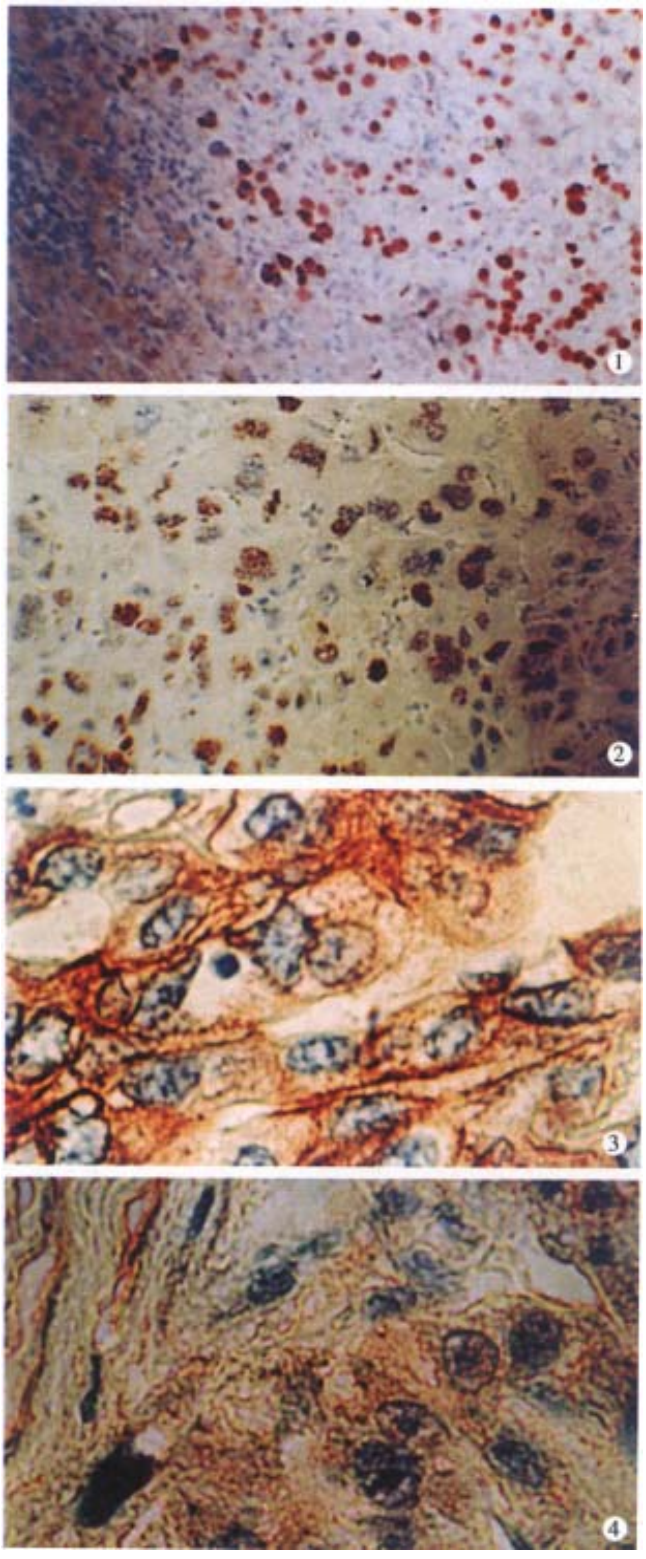

Figure 1 Strongly positive immunoperoxidase staining of the *p*53 protein with the anti-p53 mAb Do-7 in HCC, predominantly in nuclei. Absence of staining in nontumorous liver. LSAB method, ×100

Figure 2 HCC with diffuse PCNA staining using mAb PC10. LSAB method, ×200

Figure 3 Cholangiocellular carcinoma showing extensive stron gly membrance and plasma positivity for c-erbB-2 using poly-Ab code N. A485. LSAB method, ×400

Figure 4 Diffuse H-rasp21 using mAb Ncc-Ras-001 staining in HCC and nontumorious tissues. LSAB method, ×400

DISCUSSION

Alterations of oncogenes and tumor suppressor genes were critical events in hepatocarcinogenesis induced by HBV/HCV, chemical carcinogens, etc. have been demonstrated by biopathological and experimental researches[7-10]. Cerutti[11] and Tong [12] reported that the mutagenesis of H-ras proto-oncogene and *p*53 tumor suppressor gene may be the most important events change in hepatocellular carcinomas. Fujimoto *et al*[13] observed some allelic loss of tumor suppressor genes in Chinese patients with hepatocarcinomas. Scor sone *et al*[14] demonstrated that *p*53 gene mutations clustered at codon 249 in HBV positive hepatocarcinoma patients in China. Recently, Ming *et al*[15] also found that frequency of 249 codon mutation of *p*53 gene was much higher in high prevalent area of hepatocellular carcinomas than that in the low-risk area in China.More than 95% (20/21) cancer specimens exhibited strong intranuclear accumulation of *p*53 protein, which can be detected by immunohistology. However, Biersing *et al*[16] and Vesey *et al*[17] found little or no point mutations of *p*53 gene in human hepatocarcinoma in Swedish and Aust ralian patients.Therefore, the overexpression of *p*53 protein in hepatocarcinoma spe cimens can be used as the mutant *p*53 biopathological mark in tumor tissues. Liu *et al*[18] and Zu *et al*[19] found that the frequency of intranuclear positive immunoreactivity of *p*53 protein was significantly different between high-risk and low-risk HCC areas, and HBxAg can enhance *p*53 protein accumulation, suggesting that HBxAg was capable of binding *p*53 and formed a protein-protein complex which might reduce or inactivate the antiproliferative activity of *p*53 and played an important role in the pathogenesis of HBV-associated hepatocarcinomas . In this study, the overexpression of *p*53 protein revealed very significant difference between tumor (46%) and peritumor (4%) tissues, and negative in normal liver samples ($P<0.05$), indicating that *p*53 gene mutations played similar roles in hepatocarcinogenesis in Xin Jiang regardless of the etiological factors. Su *et al*[20], Nakapoulou-*et al*[21] and Yu *et al*[22] have reported that c-erbB-2 oncogene was related to the dysregulation of proli feration and differentiation of the hepatocytes in hepatotumorigenesis, and over expression in various pathological lesions had different frequencies. We observed that overexpression of c-erbB-2 oncoprotein in tumor (70%) and in peritumor (22%) and in normal liver tissues also showed very distinct difference ($P < 0.05$), suggesting that c-erbB-2 oncogene and tumor suppressor genes jointly participate in the occurrence and development of HCC, and related to

the abnormal regulation even malignant transformation of hepatocytes induced by various pathogenes. Ushijima *et al*[23], Imai *et al*[24], Tamano *et al*[25] found that mutations of ras oncogene may be the early events, and the expression in tumor or non-tumor tissues can be detected with different rates. Lin *et al*[7] found that the expression of N-ras in liver tumor was correlated to the diff erentiated status of altered liver cells. In this study, there was no statistical signif icance between tumor (68%) and peritumor (64%) tissues in the expression of H-ras oncoprotein. Positive cells were also found in 30% of normal liver specimens, reasonably which can not be explained. Proliferating cell nuclear antigen (PCNA), as an index of cellular proliferative status was determined in various lesions. Waga *et al*[26], Maeda *et al*[27] found that *p*53 tumor suppressor gene can control the cyclin-dependent kinases to regulate DNA replication involving PCNA interaction by *p*21 protein pathway. The overexpre ssion of PCNA with high frequency was usually used as a reliable marker for assessment of tumor progre ss, premalignant evolution and clinical prognosis of patients with various malig nancies. We found that the positive frequency of PCNA in tumor (82%) was significantly higher than in peritumor (52%) tissues ($P<0.05$), indicating that the non-neoplastic tissues surrounding the hepatocarcinomas may have premalignant lesions including cellular hyperplasia, dysplasia, even *in situ* carcinoma. Therefore, these multiple molecular indexes in combination could be used as the biomolecular standard for diagnosis of malignant and benign lesions. The inactivation of tumor suppressor gene *p*53, Rb, *p*16, etc. was demonstrated in different forms, and implied the pathogenesis of human malignant diseases. Slagle *et al*[28] and Shao *et al*[29] found the loss of heterozygosity on chromosome 17p near the *p*53 gene or 1p in HBV-positive HCC in China.

It is noteworthy that the alteration of *p*53 tumor suppressor gene and c-erbB-2 and H-ras oncogenes in different ethnic patients with HCC revealed significant difference. The expression of *p*53 protein and c-erbB-2 and H-ras oncoprotein in carcinoma specimens was higher in the minority than in Han patients by immunohistochemical detection ($P<0.05$). Moreover, the three combined abnormal expression of *p*53 protein and c-erbB-2 and H-rasoncoprotein in tumor tissues was higher in minority (41%) than in Han (21%) patients. This may reflect, to a certain extent, the differences of ethnic gene-susceptibility to various carcinogenes and effect on hepatocarcinogenesis. The pathogenesis of primary hepatic carcinoma is a multistage process with the involvement of multifactorial etiology, and the gene-environment interactions are involved in the develop ment of HCC in humans. In order to understand the exact difference concerning the etiopathogenesis of HCC among different ethnic groups, further studies with more samples are needed, especially in minority ethnic patients.

REFERENCES

1 Chen CG, Yu MW, Liaw YF. Epidemiological characteristics and risk factors of primary hepatocellular carcinoma. *J Gastroenterol Hepatol*, 1997;12:9-10,S294-308
2 Wu X, Gu J, Ptt Y, Hassan M, Spitz MR, Beasley RP, Hwang LY. Mutagen sensitivity as a susceptibility marker for human hepatocellular carcinoma. *Cancer Epidemiol Biomarkers Prev*, 1998;7:567-570
3 Brotodihardjo AE, Tait N, Weltman MD, Liddle C, Little JM, Farrell GC. Hepatocellular carcinoma in western Sydney. Aetiology, changes in incidence, and opportunities for better outcomes.*Med J Aust*,1994;161:433-435
4 Kim H, Lee H, Yun Y. X-gene product of hepatitis B virus induces apoptosis in liver cells. *J Biol Chem*,1998;273:381-385
5 Ozturk M. p53 mutation in hepatocellular carcinoma after aflatoxin exposure. *Lancet*,1991;338:1356-1359
6 Yu MW, Chen CJ, Luo JC, Brandt Rauf PW, Carney WP, Santella RM. Correlations of chronic hepatitis B virus infection and cigarette smoking with elevated expression of neu oncoprotein in the development of hepatocellular carcinoma. *Cancer Res*,1994;54:5106-5110
7 Lin YZ, Din L, Chen JY. The expression of c-myc, N-ras mRNA and its relations to the differentiation of preneoplastic altered hepatocytes. *Zhonghua Zhongliu Zazhi*,1993;15:97-100
8 Zheng H, Shank RC. Changes in methyl sensitive restriction sites of liver DNA from hamsters chronically exposed to hydrazine aulfate.*Carcinogenesis*, 1996;17:2711-2717
9 Gressani KM, Rollins LA, Leone KS, Cline JM, Miller MS. Induction of mutations in Ki-ras and INK4a in liver tumors of mice exposed in utero to 3-methylcholanthrene. *Carcinogenesis*, 1998;19:1045-1052
10 Li DC, Liu TW, Wang DT. Expression of cellular oncogenes in human primary liver cell carcinoma. *Zhonghua Binglixue Zazhi*, 1990;19:116-118
11 Cerutti P, Hussanin P, Pourzand C, Aguilar F. Mutagenesis of the H-ras protooncogene and the p53 tumor suppressor gene. *Cancer Res*, 1994;54(7Suppl):1934s-1938s
12 Tong ZY. Advances in primary hepatocarcinoma research. *Zhongguo Zhongliu Linchuang*,1998;25:137-142
13 Fujimoto Y, Hampton LL, Wang NJ, Xie JP. Alteration of tumor suppressor genes and allelic losses in human hepatocellular carcinoma in China. *Cancer Res*,1994;54:281-285
14 Scorsone KA, Zhou YZ, Butel JS, Slagle BL. p53 mutations cluster at codon 249 in hepatitis B virus positive hepatocellular carcinomas from China. *Cancer Res*,1992;52:1635-1638
15 Ming LH, Yuan BJ, Thorgerisson SS, Zu YR, Wu ZY, Sun ZT. Characteristics of high frequency 249 codon mutation of p53 gene in hepatocellular carcinoma in prevalen area of China. *Zhonghua Zhongliu Zazhi*, 1999;21:122-124
16 Biersing L, Andersson C, Lithner F. Hepatocellular carcinoma in patients from northern Sweden with acute intermittent porphyria: morphology and mutations.*Cancer Epidemiol Biomarkers Prev*,1996;5:393-397
17 Vesey DA, Hayward NK, Cooksley WG.p53 gene in hepatocellular carcinoma from Australia. *Cancer Detect Prev*,1994; 18:123-130
18 Liu H, Zou SQ, Xia SS. Relationship between expression of mutant *p*53 and clinicopathological features in hepatocellular carcinoma. *Zhongguo Zhongliu Linchuang*,1997;24:122-

125
19 Zhu MH, Dai YM, Zhang RH. HBxAg enhanced *p*53 protein accumulation in hepatoma cells. *Zhonghua Binglixue Zazhi*,1999; 28:31-34
20 Su Q, Liu YF. Expression of c-erbB-2 protein and EGF receptor in hepatitis B, cirrhosis and hepatocellular carcinoma. *Zhonghua Binglixue Zazhi*, 1995;24:93-95
21 Nakopoulou L, Stefanaki K, Filaktopoulos D, Giannopoulou I. C-erbB-2 oncoprotein and epidermal growth factor receptor in human hepatocellular carcinoma: an immunohistochemical study.*Histol Histopathol*,1994;9:677-682
22 Yu MW, Chen CJ, Luo JC, Brandt Rauf PW, Carney WP, Santella RM. Correlations of chronic hepatitis B virus infection and cigarette smoking with elevated expression of neu oncoprotein in the development of hepatocellular carcinoma. *Cancer Res*,1994;54:5106-5110
23 Ushijima T, Makino H, Kakiuchi H, Inoue R, Sugimura T, Nagao M. Genetic alterations in HCA-induced tumors. *Princess Takamatsu Symp*,1995;23:281-291
24 Imai Y, Oda H, Arai M, Shimizu S, Nakatsuru Y, Inoue T, Ishikawa T. Mutational analysis of the *p*53 and k-ras genes and allelotype study of the Rb-1 gene for investigating the pathogenesis of combined hepatocellular-cholangiocellular carcinomas. *Jpn J Cancer Res*,1996;87:1056-1062
25 Tamano S, Ward JM, Diwan BA, Keefer LK, Weghorst CM, Calvert RJ, Hennman JR, Ramliak K, Rice JM. Histogenesis and the role of *p*53 and ras mutations in hepatocarcinogenesis by glyceryl trinitrate (nitroglycerin) in male F344 rats. *Carcinogenesis*,1996;17:2477-2486
26 Waga S, Hannon GJ, Beach D, Stillman B. The p21 inhibitor of cyclin-dependent kinases controls DNA replication by interaction with PCNA [see comments].*Nature*,1994;369:574-578
27 Maeda T, Takenaka K, Adachi E, Matsumata T, Shirabe K, Honda H, Sugimachi K, Tsuneyoshi M. Small hepatocellular carcinoma of single nodular type: a specific reference to its surrounding cancerous area undetected radiologically and macroscopically.*J Surg Oncol*,1995;60:75-79
28 Slagle BL, Zhou YZ, Butel JS. Hepatitis B virus integration event in human chromosome 17p near the p53 gene identifies the region of the chromosome commonly deleted in virus-positive hepatocellular carcinomas.*Cancer Res*, 1991;51:49-54
29 Shao JY, Li MH, Liew CT, Wu QL, Liang XM, Hou JH. A preliminary study of loss of heterozygossity on chromosome 1p in primary hepatocellular carcinoma.*Zhonghua Binglixue Zazhi*,1999;28:28-30

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