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• LIVER CANCER •

Is p53 gene mutation an indicatior of the biological behaviors of recurrence of hepatocellular carcinoma?

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Abstract

AIM: To evaluate mutant p53 gene in primary hepatocellular carcinoma and to investigate the correlation between it and the recurrence of hepatocellular carcinoma.

METHODS: Mutations of p53 gene were examined using anti-human p53 monoclonal antibody and immunohistochemical staining in 79 resected hepatocellular carcinomas. The correlations among variables of p53 positivity and invasiveness, disease free interval and survival were studied. In addition, in those who developed recurrence, the correlation among p53 positivity, clinical features and post-recurrence survival were also studied.

RESULTS: Of these 79 cases, 64 (81 %) had p53 mutation. Those patients with mutant p53 positivity had significantly more tumor recurrence (76.6 % *vs* 40.0 %, *P*=0.0107). However, the COX proportional hazards model showed that p53 overexpression had only weak correlations with recurrence free interval and survival time (*P*=0.088 and 0.081), which was probably related to the short duration of follow-up. The invasiveness variables may be predictors of HCC recurrence. On univariate analysis, more patients with mutant p53 positivity had vascular permeation [78.1 *vs* 40.0 %, *P*=0.0088, O.R. (odds ratio) =5.3], grade II-IV differentiation (98.4 *vs* 80.0 %, *P*=0.0203, O.R. =15.7), no complete capsule (82.8 *vs* 53.3 %, *P*=0.0346, O.R. =4.2) and daughter nodules (60.9 *vs.* 33.3 %, *P*=0.0527, O.R. =3.1) than patients with negative p53 staining. On multivariate analysis, only vascular permeation and grade of differentiation remained significant (*P*=0.042 and 0.012). There was no statistically significant correlation between the status of p53 in the primary lesion and the clinical features of recurrent hepatocellular carcinomas examined, including extrahepatic metastasis (*P*=0.1103) and the number of recurrent tumors (*P*=1.000) except for disease over more than one segment in the extent of recurrent tumors (*P*=0.0043). The post-recurrence median survival was lower in patients in whom p53 mutation had been detected in the primary lesion with a weak significance (3.42 months *vs* 11.0 months, *P*=0.051).

CONCLUSION: Our findings suggest that p53 mutation correlates significantly with invasiveness including vascular permeation, grade of cellular differentiation, incomplete capsule and multinodular lesions. Hepatocellular carcinomas with p53 mutations had more tumor recurrence and p53 mutation may also influence disease recurrence interval and survival time. Hepatocellular carcinomas with p53 mutations recur more extensively with a shorter survival. Therefore, p53 mutation in the primary lesion is useful as an indicator of the biological behavior of recurrent hepatocellular carcinomas.

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INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common types of malignant tumors that carry a poor prognosis. During the last 10 years, efforts have been made worldwide toward earlier detection and safer surgical resection of HCC. However, despite these recent diagnostic and therapeutic advances, postoperative recurrence is still common[1-4]. How to predict recurrence before resection is a challenging problem for surgeons. Certain characteristics related to HCC recurrence have been reported widely and variably in the literatures[1-11]. Risk factors which have been mentioned include vascular permeation, absence of capsule, presence of daughter nodules, histological grade of tumor differentiation, tumor size, associated cirrhosis, hepatitis B virus (HBV) infection, hepatitis C virus (HCV) infection, daughter nodules, and adequate section margin, etc.

The cellular wild-type p53 gene on chromosome 17p is an established tumor suppressor gene[12-14]. It regulates the cell cycle of DNA repair and synthesis, and also programmed cell death. Once it is mutated, loss of normal function leads to the evolution of neoplasm. Moreover, the speed of tumor growth and invasion may also be enhanced. When mutated, this gene may have transforming properties and can be stained immunohistochemically[15-21]. Its prognostic significance in some types of human cancer has been reported. The relationship between hepatocellular carcinoma (HCC) and overexpression of the mutant p53 gene have been studied in different countries[15-32]. The results are varied. In addition, mutation of the p53 gene was emphasized in advanced but not in early hepatocellular carcinoma[33,34]. However, the correlation between the clinical significance of such p53 mutations and the clinical recurrence of HCC has rarely been clarified.

In this study, we did immunohistochemical staining to investigate the overexpression of p53 protein in HCC in a series of patients. The correlation of the clinical and pathological variables of HCC, recurrence of HCC and the biological behaviors of the mutant p53 gene were studied. The goal of this study was to elucidate the possible role of p53 mutations in the prediction of recurrent HCCs.

MATERIALS AND METHODS

Patients

One hundred patients with HCC who underwent hepatectomy at Mackay Memorial Hospital, between January 1993 and December 1997 whose tissue specimens (formalin fixed, paraffin wax embedded) were histopathologically found to have no degeneration or necrosis were selected for this study. Clinical details were available from medical records of all patients. Seventy-nine patients entered this study and twenty-one cases were excluded for the following reasons: (1) immediate operative mortality, (2) failure to obtain p53 results due to severe, extensive tumor necrosis, some of which probably resulted from preoperative transcatheter hepatic arterial chemoembolization (TACE), (3) incomplete follow-up, and (4) causes of death not related to liver disease. The mean age of patients was 52.4±16.6 years (range 16-82) with a male to female ratio of 2:1 (52:27). All received curative resections. The surgical operations included major resections (15 partial lobectomies, 31 lobectomies and 9 extended lobectomies) and minor resections (19 segmentectomies, 3 subsegmentectomies and 2 wedge resections). After resection, all patients were followed up at our out-patient-clinic receiving regular clinical assessment, periodic abdominal ultrasonography (every 2 to 3 months during the first 5 years, then every 4 to 6 months thereafter) to detect tumor recurrence, serum alpha-fetoprotein (AFP) and liver biochemistry (every 2 months during the first 2 years, then every 4 months during the following 3 years, and every 6 months thereafter). Abdominal computed tomography was also done (every 6 months during the first year, then every year).

Methods

Five-micron-thick formalin-fixed and paraffin embedded sections were cut, deparaffinized and rehydrated with graded alcohol and xylene. Endogenous peroxidase was blocked using 3 % H_2O_2 for 5 minutes, followed by a brief wash in Tris buffer, pH 7.2. Sections were rehydrated and heated in citrate buffer, pH6.0, in a microwave oven at 500 watts for 10 minutes to retrieve the antigen. The tissues were stained with a monoclonal mouse anti-human p53 antibody (DAKO-p53, clone DO-7, Dako Corp, Carpinteria, Calif. U.S.A.) and a labeled streptavidin-biotin staining kit (DAKO LSAB kit, alkaline phosphatase system 40). They were incubated with the antibody at a dilution of 1:100 (in Tris-HCl buffer) for one hour at room temperature. The peroxidase reaction used 3,3-diaminobenzidine tetrahydrochloride as chromogen and the slides were counterstained with hematoxylin. Two independent, blinded observers evaluated all tissue sections. Only nuclear staining was regarded as positive. Cases were scored as negative when no cell was stained even at a concentration as high as 1:10 in a triplicate study. A known colon adenocarcinoma with diffuse p53 nuclear accumulation was stained in parallel as the positive control. For negative controls, we used buffer instead of the specific primary antibody.

We used light microscopy to search for the highest concentration of reactive staining nuclei in each p53 staining section and counted 1 000 cells from the most aggressive area of the tumor to represent the tumor' s behavior and reduce count variability. Specific staining was identified by the presence of a red reaction product in the nuclei and was graded as negative (0), slight (+), moderate (++), or strong (+++) immunostaining, with the distribution as diffuse or focal. The percentage of nuclei immunostained was estimated and was scored without knowledge of the grade of tumor differentiation.

The differences of p53 overexpression in diverse clinicopathologic parameters were evaluated. Parameters included the presence of associated liver cirrhosis (confirmed from the operative findings and also from the pathological examination of the specimen), HBV surface antigen (HBsAg), hepatitis C virus (HCV) infection (antibody to HCV, anti-HCV), Child-Pugh classification of liver reserve, serum alpha fetoprotein (AFP) titer, tumor size (<3 cm, 3-10 cm, >10 cm), cell differentiation grade (Edmondson and Steiner grade I *vs* II-IV), encapsulation (complete, infiltration by HCC or absent), vascular permeation (including vascular invasion and/or tumor thrombi within the portal vein or hepatic vein), and presence of daughter nodules (Table 1). The time lapse between the postoperation till the detection of recurrence is defined as the recurrence free interval. During the follow-up (median 3 years, range 2 to 5 years), 55 patients had tumor recurrence (48 intrahepatic, and 7 both intrahepatic and extrahepatic) and 43 patients died. We also correlated the p53 overexpression with the outcomes. In these 55 patients with recurrence, the correlation between p53 mutation of the primary lesion (presence or absence) and recurrence was studied. The following prognostic factors after recurrence were also analyzed: extrahepatic metastasis (presence or absence), the number (solitary or multiple) and the extent of recurrent tumors (affecting more than or less than one segment), treatment for recurrent tumor (surgical or nonsurgical treatment), and survival time after recurrence.

Table 1 Characteristics of 79 patients with HCC

Characteristics	*n* (%)
Age (years, mean±S.D.)	52.4±16.6
Male	52 (65.8)
Liver cirrhosis	57 (72.2)
Child class A:B	55:24 (70:30)
Tumor size small (<3 cm): median (3-10 cm): large (>10 cm)	24:25:30 (30.4:31.6:38.0)
HBsAg (+)	60 (75.9)
Anti-HCV (+)	41 (51.9)
AFP: normal: >1000ng / ml	30:20(38.0:25.3)
Edmondson grade: I:II:III:IV	4:30:42:3(5.1:38.0:53.2:3.8)
Capsule: absent: incomplete: complete	54:7:18(68.4:8.9:22.8)
Vascular permeation	56 (70.9)
Daughter nodules	44 (55.7)
Resection, major: minor	55:24 (69.6:30.4)

Notes: HCC: hepatocellular carcinoma; HBsAg: hepatitis B surface antigen; Anti-HCV: antibody to hepatitis C virus; AFP: alpha-fetoprotein; Edmondson grade: Edmondson-Steiner grade of cellular differentiation.

Statistical analysis

The data were tested with statistical programs (BMDP), Student' s *t*-test or Mann-Whitney test for continuous variables, chi-square test or Fisher' s exact test for categorical variables, and logistic regression and COX proportional hazards model for multivariate analysis. P value<0.05 was defined as statistically and significantly different.

RESULTS

Among the 79 patients, 64 (81 %) patients had a p53-positive result. Among the 64 patients with immunopositivity, 29 patients (45 %) had moderate (++) immunostaining and 3 patients (4.7 %) had strong (++++) immunostaining. The correlations between a positive oncoprotein p53 and patient characteristics are shown in Table 2. Age, gender, positivity of HBsAg or Anti-HCV, levels of AFP, liver cirrhosis, Child-Pugh class A or B, and size of the HCC showed no statistically

significant difference between p53 positive and negative groups. From univariate analysis, a significant correlation was found between p53 over-expression and vascular permeation (78.1 *vs* 40.0 %, *P*=0.0088, odds ratio (O.R.)=5.357), grade of differentiation (Edmondson-Steiner grade I *vs*. II to IV, 98.4 *vs* 80.0 %, *P*=0.0203, O.R.=15.750), complete capsule *vs* infiltration or absent capsule (82.8 *vs* 53.3 %, *P*=0.0346, O.R. =4.200), and presence of daughter nodules (60.9 *vs* 33.3 %, *P*=0.0527, O.R.=3.120) (Table 2). From multivariate analysis, only vascular permeation and grade of differentiation remained significant (*P*= 0.042 and 0.012, respectively).

Table 2 Comparison of characteristics between p53 positive and negative groups

Characteristics	P53 Positive (*n*=64)	P53 Negative (*n*=15)	*P* (UV)
Age (years)	52.8	48.3	n.s.
Male	65.6 %	66.7 %	n.s.
Liver cirrhosis	76.6 %	53.3 %	n.s.
Child class A	68.8 %	73.3 %	n.s.
Tumor ≤3 cm	34.4 %	13.3 %	n.s.
>10 cm	35.9 %	40.0 %	n.s.
HBsAg (+)	78.1 %	66.7 %	n.s.
Anti-HCV (+)	53.1 %	46.6 %	n.s.
AFP <20 ng/mL	37.5 %	40.0 %	n.s.
>1 000 ng/mL	21.9 %	40.0 %	n.s.
Edmondson grade I[a]	1.6 %	20.0 %	0.0203
Capsule complete	17.2 %	46.7 %	0.0346
Daughter nodules	60.9 %	33.3 %	0.0527
Vascular permeation[b]	78.1 %	40.0 %	0.0088

Notes: *P* (UV): The *P* value by univariate analysis; In multivariate analysis, the significant variables of [a] and [b]: *P* values were 0.0120 and 0.0420 respectively; HBsAg: hepatitis B surface antigen; Anti-HCV: antibody to hepatitis C virus; AFP: alpha-fetoprotein; Edmondson grade: Edmondson-Steiner grade of cellular differentiation; n.s.: no statistical significance; O.R.: odds ratio.

Table 3 shows that patients with p53 positivity had more tumor recurrence (76.6 % *vs* 40.0 %, *P*=0.0107) and more death (59.4 % *vs* 33.3 %, *P*=0.0683). After analysis with the COX proportional hazards model, p53 overexpression had only a weak correlation with recurrence free interval and survival time (*P*=0.088 and 0.081). Factors influencing HCC recurrence and time lapse to recurrence were vascular permeation (*P*=0.0002, O.R.=5.36), complete capsule (*P*=0.0160, O.R. =3.10), and p53 positivity (*P*=0.088, O.R.=2.29) (Table 4). The significant variables affecting death resulting from recurrence included vascular permeation (*P* <0.0001, O.R.=8.35) and p53 positivity (*P*=0.081, O.R.=2.38).

Table 3 Correlation of p53 with the outcome of patients with HCC

Outcome	p53 positive (*n*=64)	p53 negative (*n*=15)	*P* value
Morbidity of surgery (%)	6.3	6.7	n.s.
Recurrence (%) (number)	76.6 (49)	40.0 (6)	0.0107
Death[a] (%)	59.4	33.3	0.0683
Recurrence free interval (median, months)	8.3	39.1	0.0880
Duration of survival (median, months)	11.8	41.9	0.0810

Notes: n.s.: no statistical significance; Death[a]: patients died of recurrence.

Table 4 Factors influencing tumor recurrence and death of patients in multivariate analysis

Variables	*P*	O.R.
Recurrence		
Vascular permeation	0.0002	5.36
Complete capsule	0.0160	3.10
*p*53 positivity	0.0880	2.29
Death		
Vascular permeation	<0.0001	8.35
*p*53 positivity	0.0810	2.38

Note: O.R: odds ratio.

In 55 patients with recurrent HCCs, there was no statistically significant correlation between the status of mutant p53 positivity in the primary lesion and the treatment for recurrent tumors, and the clinical features of recurrent HCCs examined, i.e. the existence of extrahepatic metastasis (*P*=0.113), and the number of recurrent tumors (*P*=1.000), except for the extent of recurrent tumors over one hepatic segment (*P*=0.0043). The median survival after recurrence was shorter (3.42 months *vs* 11 months) in those with p53 mutation with a weak significance (*P*=0.051) (Table 5).

Table 5 Correlation between the clinical features of recurrent hepatocellular carcinoma and the presence of a p53 mutation in the primary lesion

The clinical features	P53 Positive (*n*=49)	P53 Negative (*n*=6)	*P*
Extrahepatic metastasis (number) (%)	24 (49.0)	3 (50.0)	0.1130
Multiple-recurrent tumors (number) (%)	34 (69.4)	4 (66.7)	1.0000
Extent of recurrent tumors:	34 (69.4)	5 (83.3)	0.0043
More than one segment (number) (%)			
Median survival after recurrence (Months)	3.4	11.0	0.0510
Treatment for recurrent tumors			
Surgery (number) (%)	0	1[a] (16.7)	n.s.
Non-surgical[b](number) (%)	20 (40.8)	3 (50.0)	
No treatment (number) (%)	29 (59.2)	2 (33.3)	

Notes: n.s.: no statistical significant; [a]: A 55 year old man had a resection of segment II and III; [b]: The Non-surgical treatments included transcatheter arterial chemoembolization and percutaneous ethanol injection.

DISCUSSION

P53 gene mutation has been identified in over half of human tumors, including HCC, and is the most common genetic abnormality in human cancers[13, 17, 20, 25, 34-42]. Its inactivation by mutation is thought to be a fundamentally important step in carcinogenesis. In addition, the correlation among p53 gene alteration and diagnosis, assessment of tumor progression, recurrence, or cancer prognosis has been investigated and reported[24,28,33]. Recently, Nagao *et al*[43] and Saegusa *et al*[44] found p53 overexpression to be strongly associated with proliferation activity of HCC cells by immunohistochemical studies. Lowe *et al*. demonstrated that a few point mutations on p53 which thus inactivated the gene produced treatment-resistant tumors[45,46]. They suggested p53 status was an important determinant of tumor response to therapy. This indicates that recurrent HCCs with p53 mutation therefore either have a high proliferation rate or are resistant to treatment. Our results supported theirs from a clinical point of view, and also suggested that the high malignant potential was caused by the

p53 mutation. The positive rate of the mutant p53 gene in our HCC patients was 81 %. A wide range in the incidence of p53 mutations from 0 to over 70 % has been reported, with a lower frequency than in other types of cancer, except for special populations in China and Africa. Factors related to the wide variation in positivity may include different thresholds of positivity adopted, different anti-p53 antibodies used, geographical variations and differences in the molecular mechanisms of hepatocarcinogenesis, such as aflatoxin exposure. Some authors have raised a question of whether p53 protein over-expression can represent p53 gene mutation in neoplasms[47]. Hall mentioned a very close correlation between p53 expression and mutations of the p53 gene and found that most antibodies gave the same results[48]. The high recurrence rate after resection is one of the main factors in the poor outcome for HCC patients[1-6, 10, 11]. Tumor recurrence limits the long-term survival. However, tumor recurrence is well correlated with tumor invasiveness. Tumor invasiveness may be determined from vascular permeation, the grade of cell differentiation, infiltration or absence of capsule and presence of daughter lesion. According to our study, they are also all compatible with oncoprotein p53 positivity. In our study, p53 protein over-expression correlated well with tumor recurrence (P=0.0107). To analyze the factors relating to HCC recurrence and death, vascular invasion, complete capsule, and p53 positivity correlated well with recurrence and only vascular permeation and p53 positivity correlated with death. A weak association with both recurrence free interval and duration of survival with mutation of the p53 gene was found. The weak correlation may be attributed to the short duration of follow-up (2 to 5 years, median 3 years) in this study.

Vascular permeation indicating tumor invasiveness, consists of either tumor invasion of the hepatic vein, portal vein and/or hepatic artery, or tumor thrombi within the vessels. It may be detected preoperatively by ultrasonography, arteriography or portography, intraoperative ultrasonography or direct observation, or postoperative pathological examination of surgical specimens. Vascular permeation is the most consistent significant prognostic factor of postoperative tumor recurrence[10]. In our univariate analysis, the positive p53 status was significantly related to vascular permeation and in the COX model, patients with vascular permeation had significantly shorter recurrence free intervals and survival periods.

Whether the grade of differentiation of HCC is a determinant of recurrence after resection has been debated for a long time. The association of grade of anaplasia (Edmondson-Steiner' s classification) with p53 positivity also varied in reports[28,49,50]. In our series, less overexpression of p53 and less recurrence were found in patients with well differentiated tumors (Edmondson-Steiner' s grade I) than in those with grade II to IV tumors. The histological differentiation of the HCCs in this study correlated with p53 mutations, and the incidence of p53 mutations increased with increased dedifferentiation. Our findings were consistent with previous reports showing p53 mutation to be associated with the progression of HCC as a late event in hepatocarcinogenesis[33, 34].

The exact mechanism of capsular formation is not known. A tumor capsule may act as a barricade preventing the spread of cancer cells and has a positive role in the prognosis of HCC. The invaded capsule was regarded as incomplete in our series. We found the overexpression rate of p53 was similar in patients with no capsule and incomplete capsule (87.1 % *vs* 85.7 %), but was significantly lower in those with a complete capsule (17.2 %, P=0.0346) (Table 2). Other authors had different findings[23,24,49,50]. Multifocal HCCs are also a controversial issue. Some consider them an early metastasis via the portal vein, but some consider them multicentric. The former is a poor prognostic factor but the latter might not be[51]. Without the aid of molecular biology, it is difficult to differentiate daughter nodules, intrahepatic metastatic nodules and multicentric HCC. In the present study, we selected daughter nodules as intrahepatic metastasis according to the criteria of the Liver Cancer Study Group of Japan in order to assess the clinical outcome after recurrence. As for the evaluation of prognosis after recurrence, Ikeda *et al*.[1] reported that the most significant factor affecting the survival time of patients with intrahepatic recurrence was the number of tumor nodules at the time of recurrence. Those with daughter nodules showed a higher mutant p53 positive rate than the group with solitary HCC (P=0.0527). This might suggest that most daughter nodules favor intrahepatic metastasis.

Tumor size has been emphasized as one of the significant prognostic factors[2-5] because vascular invasion and daughter lesions may increasingly develop as the tumor grows. In our study, no significant correlation between p53 positivity and tumor size was found. In addition, tumor size also had no significant correlation with histological grade, vascular invasion, recurrence free interval or survival in our patients. From our experience, some large HCCs may be the result of expansive growth and may have slow intraportal or distant spread.

The implications of our results, nevertheless, are that the immunohistochemical detection of p53 is a valuable tool for prediction of recurrence in patients after resection or for identifying subgroups of patients who may be at higher risk. There is some discrepancy between our results and the findings of previous studies on the role of p53 expression in determining the prognosis of patients with HCC. These discrepancies, however, might reflect important variables of selection, such as number of patients, histological type of tumors, tumor stage, period of follow-up, and type of antibody used. All the patients entering our study had received curative resections.

Prognosis after recurrence in relation to p53 mutations in the primary lesion is rarely reported in the literatures. Our findings suggest that HCCs with p53 mutations have a higher malignant potential. Matsuda *et al*.[52] found that the postrecurrence survival of patients with repeat surgery was better than that of patients who were treated conservatively. However, from our study, the type of treatment for recurrent HCCs did not affect the postrecurrent survival because the choice of treatment was closely related to the number and extent of recurrent tumors and the liver function of the remnant. The majority of our patients had diffused multiple recurrent nodules over the liver remnant. Repeat surgery was undertaken on only one patient. In our study, the postrecurrent survival was weakly lower (P=0.051) in patients with p53 mutations in their primary lesion than in those without them.

We thus consider the status of p53 mutations in the primary lesion to be useful as a predictor affecting both the recurrence after resection and the prognosis after recurrence, even before the pathologic findings of recurrent HCCs are known. Therefore, it is important in the follow-up of patients after resection of HCCs. In conclusion, patients with p53 mutations have a worse prognosis than patients without such mutations, including survival after recurrence. Therefore, p53 mutation in the primary lesion is considered useful as an indicator of the biological behavior of recurrent HCCs.

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