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Clinical Associations and Prognostic Significance of Serum Anti-p53 Antibodies in Thai Patients with Hepatocellular Carcinoma

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SUMMARY **Mutations of the *p53* gene have been reported to be of prognostic significance in hepatocellular carcinoma (HCC). However, the clinical associations and prognostic value of anti-p53 antibodies, known to be products of the host immune response to these mutations, have been controversial. Serum anti-p53 antibodies were measured in 121 Thai patients diagnosed with HCC using a specific enzyme-linked immunosorbent assay (ELISA) kit. The clinical/pathological characteristics of the patients were compared with respect to the presence of serum anti-p53 antibodies. Cox regression analysis was performed to assess factor interaction and association with survival. Anti-p53 antibodies were detected in 13.2% (16 of 121) of our patients. There were no differences between groups with regard to age, sex, viral markers (HBsAg or anti-HCV), severity of liver disease and tumor advancement. The median survival rates for patients positive and negative for anti-p53 antibodies were 4.0 and 3.0 months, respectively (p = 0.443, by log-rank test). Multivariate analysis demonstrated that an advanced Okuda stage, lack of therapy and presence of portal vein thrombosis were independent factors related to the prognosis of the patients. Nonetheless, the presence of anti-p53 antibodies did not constitute a predictive variable associated with a poorer prognosis. Serum assay of anti-p53 antibodies, although rapid and easily performed, may not be suitable as an alternative to molecular detection of mutations in assessing tumor advancement and prognosis of patients with HCC.**

Hepatocellular carcinoma (HCC) is a worldwide cancer with high prevalence in sub-Saharan Africa and Southeast Asia where hepatitis B virus (HBV) infection and dietary exposure to the carcinogenic aflatoxins are common.[1,2] In Thailand, as in other Southeast Asian countries, HCC represents one of the most common malignancies, more predominant among men as compared to women.[3] Currently, the best therapeutic modality for HCC is surgical resection, but due to disease progression already manifest at presentation and rapid clinical deterioration, it is an option among only a minority of cases. Without treatment, the prognosis of most patients with HCC is rather poor with an overall median survival of approximately two months following diagnosis.[4] Assessment of the clinical/pathological and biological severity of HCC may help in determining treatment strategies and predicting outcome of the patients.

As generally accepted, the development of cancer is a multistep process with alterations of various regulatory genes playing a major role. Among these changes, the functional inactivation of the *p53* gene by missense point mutation is the most frequently encountered genetic alteration in human malignancies,[5,6] including HCC.[7-9] The *p53* gene is located

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on the short arm of chromosome 17 in the region 17p13 and encodes a 53 kDa nuclear phosphoprotein that serves as a transcription factor.[10] Loss of *p53* gene function accelerates the process of oncogenesis and could alter the phenotype of cancer cells, as well as the response of cells to agents that damage DNA.[11] Thus, p53 alterations have a crucial impact on tumor biology and have been considered potential markers of poor prognosis in different types of cancer.[12]

Detection of *p53* mutations has been widely performed by sequence analysis of the *p53* gene in tumor DNA or by detecting accumulated p53 protein by immunohistochemistry. The gene mutations resulting in excessive accumulation of p53 proteins with prolonged half-life are principally responsible for the detection of such mutant proteins.[5] Nonetheless, the above-mentioned methods require tumor tissue obtained from needle biopsy or surgical specimens and hence, are not suitable for routine clinical applications. Recently, it has been demonstrated that serum anti-p53 antibodies are products of the host immune response to mutated p53 proteins detectable in patients with a variety of cancers.[3,14] Previous studies have provided initial evidence that the presence of such antibodies is specific for malignant diseases.[14-16] Furthermore, detectable anti-p53 antibodies in serum seem to indicate a more aggressive behavior of the tumor. For example, studies in breast cancer patients have shown that the occurrence of such antibodies may be a useful determinant with regard to poor prognosis.[17-18] This has also been demonstrated in studies on patients with colorectal cancer, as well as gastric cancinoma.[19-21]

Nonetheless, the clinical implications of anti-p53 antibodies in HCC are still controversial. According to a recent study performed in Japan, the overall survival was significantly shorter among HCC patients who displayed the antibody than among those without this marker.[22] In contrast, another study from Europe has demonstrated that the presence of such antibodies does not seem to be associated with a poor prognosis.[23] The aim of the present study has been, therefore, to evaluate the clinical correlation and survival rates of Thai HCC patients with respect to the presence of serum anti-p53 antibodies.

MATERIAL AND METHODS

Patients

Serum samples were collected from 121 patients with HCC in Chulalongkorn University Hospital from January 1997 to July 1999. They comprised 103 males and 18 females with their age ranging from 23 to 89 years (mean 55.6 ± 12.4 years). HCC was diagnosed based on liver tumor characteristics detected by ultrasound/CT scan and confirmed by histology and/or serum alpha-fetoprotein (AFP) levels above 400 IU/ml. The clinical/pathological data of the patients were recorded including sex, age, biochemical liver function tests, severity of liver disease graded as Child-Pugh score, AFP level, tumor staging and tumor characteristics, type of therapy and patients' survival time defined as the period from initial presentation to death.

The prognostic indicators responsible for overall survival were determined by univariate and multivariate analysis. The following variables were entered into the models: age (0, younger than 50 years; 1, older than 50 years), sex (0, male; 1, female), HBsAg (0, negative; 1, positive), anti-HCV (0, negative; 1, positive), serum AFP (0, below 400 IU/ml; 1, above 400 IU/ml), number of tumors (0, single; 1, multiple), tumor size (0, below 5 cm in diameter; 1, above 5 cm in diameter), Okuda stages (0, stage I; 1, stages II and III), poorly differentiated histological type (0, negative; 1, positive), anti-p53 antibodies (0, negative; 1, positive), portal vein thrombosis (0, negative; 1, positive), extrahepatic metastasis (0, negative; 1, positive), therapy for HCC (0, yes; 1, no).

In our study, the following etiologies of HCC were established: 67 patients with chronic hepatitis B infection (HBsAg-positive), 9 patients with chronic hepatitis C infection (anti-HCV-positive), 30 patients with alcoholic cirrhosis (7 patients were seropositive for HBsAg and 3 were positive for anti-HCV) and among the remaining 25 cases the etiology was unknown. There were 12 patients who had undergone surgical liver resection, 31 patients with transarterial chemoembolization (TACE), 6 patients with systemic chemotherapy and 72 patients without any specific treatment due to the patients' advanced stages or refusal of therapy.

Serological assay for anti-p53 antibodies

Sera were collected at the initial presentation and stored at

–70°C until subjected to the respective test. The detection of anti-p53 antibodies in sera was performed by using a commercially available enzyme-linked immunosorbent assay (ELISA) kit (Pharmacell, France). The assay was performed according to the manufacturer's instructions.

Hepatitis markers

All sera were tested for HBsAg using a commercially available kit (Auszyme II; Abbott Laboratories, North Chicago, Ill, USA), and for anti-HCV by third generation enzyme-linked immunosorbent assay (ELISA) (Recombinant c22-3, c200, and NS5) obtained from Ortho Diagnostic Systems (Chiron, Emeryville, CA, USA).

Liver function test

Serum aspartate aminotransferase (AST), alanine aminotransferase (ALT) and alkaline phosphatase (AP) levels were determined from each specimen by automated chemical analyzer (Hitachi 911) at the central laboratory Chulalongkorn Hospital. The normal levels obtained in healthy adults are within the range of 0-38 U/I for AST/ALT and 98-279 U/I for AP, respectively.

Statistical analysis

Data were presented as percentage, mean and standard deviation. The Chi-square test and unpaired *t* test were performed to compare clinical data according to the presence or absence of anti-p53 antibodies as appropriate. Survival curves were constructed using the Kaplan-Meier method and differences between curves were established using the log-rank test. The Cox regression analysis was performed to identify which independent factors would have a significant influence on the overall survival. *P* values below 0.05 were considered statistically significant.

RESULTS

Serum anti-p53 antibodies and clinical/pathological characteristics

Serum anti-p53 antibodies were detected in 16 (13.2%) of 121 patients with HCC. Among those sera positive for anti-p53 antibodies, mean serum AFP, total bilirubin and aspartate aminotransferase (AST) were significantly lower when compared with the sera negative for these antibodies. There were no differences between groups with regard to other factors such as age, sex, the other biochemical liver function tests, viral markers (HBsAg or anti-HCV), Child-Pugh stage, Okuda stage, tumor size, number of tumors, degree of tumor cell differentiation, portal vein thrombosis and extrahepatic metastasis (Table 1).

Survival of patients with respect to anti-p53 antibodies

Kaplan-Meier survival curves demonstrated that the median survival for patients positive and negative for anti-p53 antibodies were 4.0 and 3.0 months, respectively (Fig. 1). However, upon comparison of the median overall survival time by using the log-rank method, statistical significance could not be established between these two groups (p = 0.443).

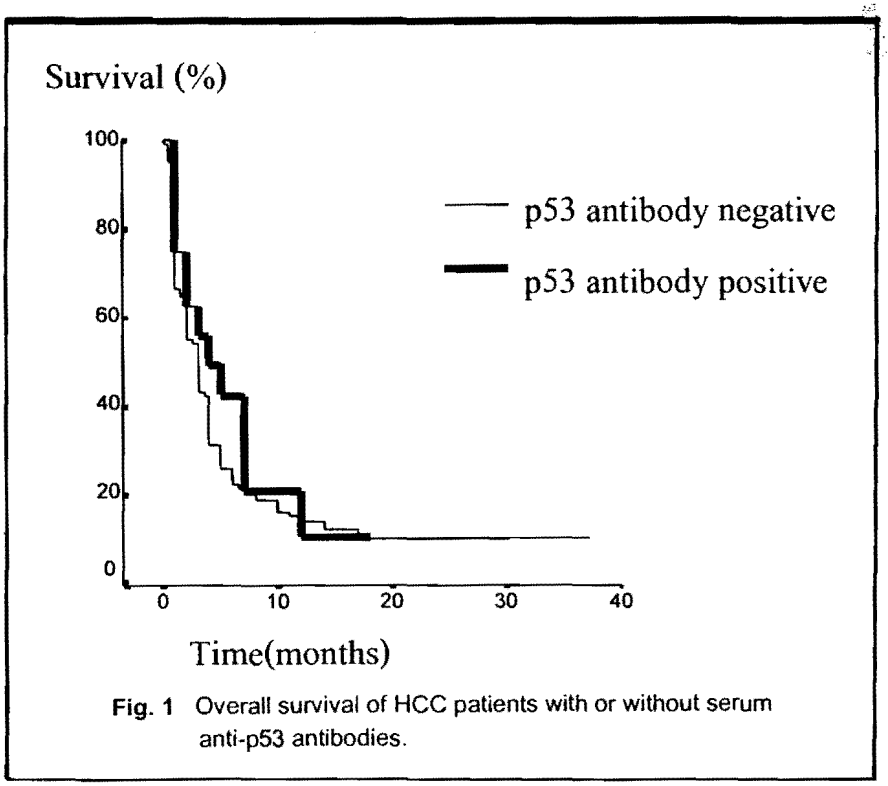

Fig. 1 Overall survival of HCC patients with or without serum anti-p53 antibodies.

Table 1 Clinicopathologic parameters in HCC with or without serum anti-p53 antibodies

Parameters	Anti-p53 antibodies positive (n = 16)	Anti-p53 antibodies negative (n = 105)	*P* value
Age (years)	53.1 ± 16.3	56.0 ± 11.7	NS
Sex (male/female)	14/2	89/16	NS
Viral marker* (positive/negative)	12/4	64/41	NS
Child-Pugh classification (A/B/C)	13/3/0	57/42/6	NS
AFP (IU/ml)	5,391.3 ± 11,858.9	31,553.9 ± 8,4070.2	0.013
Biochemical liver function tests			
Total bilirubin (mg/dl)	1.1 ± 0.5	3.0 ± 3.9	<0.001
Alkaline phosphatase (IU/L)	406.5 ± 264.0	556.9 ± 368.3	NS
AST (IU/L)	98.9 ± 99.7	160.9 ± 142.6	0.048
ALT (IU/L)	69.0 ± 59.3	88.7 ± 106.3	NS
Albumin (g/dl)	3.7 ± 0.7	3.5 ± 0.7	NS
Prothrombin time (seconds)	13.4 ± 1.4	14.5 ± 3.5	NS
Number of tumors (single/multiple)	4/12	64/41	NS
Size of tumor** (≤ 5 cm/5 cm-50%/>50%)	0/9/7	11/34/60	NS
Okuda's staging (I/II/III)	2/14/0	21/70/14	NS
Tumor cell differentiation			
(well/moderately/poorly)	3/5/1	5/22/12	NS
Vascular invasion (yes/no)	6/10	25/80	NS
Extrahepatic metastasis (yes/no)	1/15	14/91	

*Viral marker: HBsAg or Anti-HCV; NS: not significance
**The size was expressed in diameter in relatively small and solitary tumors (≤ 5 cm in diameter) and in the remaining cases the sum of the tumor areas in their largest areas relative to the whole liver area was taken as the size of the tumor

Table 2 Risk factors associated with overall survival in HCC

Variables	Risk ratio (95% CI)	*P* value*
Female	0.43 (0.23-0.80)	0.0075
Age (> 50 yrs)	0.90 (0.60-1.34)	NS
AFP (> 400 IU/ml)	1.30 (0.81-2.10)	NS
HBsAg-positive	1.06 (0.71-1.58)	NS
Anti-HCV positive	0.53 (0.21-1.31)	NS
Okuda stage II and III	2.39 (1.33-4.30)	0.0037
Multiple tumor masses	1.32 (0.87-1.99)	NS
Tumor diameter > 5 cm	2.70 (1.57-6.26)	0.0202
Poorly differentiated histological type	0.51 (0.24-1.10)	NS
Portal vein thrombosis	1.90 (1.22-2.96)	0.0043
Extrahepatic metastasis	1.56 (0.88-2.76)	NS
Anti-p53 antibodies	0.81 (0.45-1.46)	NS
Absence of therapy	2.30 (1.48-3.58)	0.0002

95% CI: 95% confidence interval; NS: not significance
**P* value based on univariate Cox regression analysis

Analysis of factors related to overall survival

Univariate analysis was used to determine significant risk factors associated with the overall survival. The clinical/pathological variables are depicted in Table 2. In addition to female sex, Okuda's stage I, tumor size below 5 cm in diameter and absence of portal vein thrombosis, a better overall survival rate was observed with patients who received therapy of HCC.

Stepwise Cox regression multivariate analysis of all factors listed in Table 2 revealed that Okuda's stage II and III (risk ratio [95% confidence interval, CI] = 2.85 [1.08-7.53], p = 0.0342), presence of portal vein thrombosis (risk ratio [95% CI] = 2.91 [1.44-5.86], p = 0.0028) and absence of therapy (risk ratio [95% CI] = 1.93 [1.06-3.50], p = 0.0312) were independent factors related to the prognosis of patients.

In our study, the presence of anti-p53 antibodies was not a predictive variable associated with a poorer prognosis of HCC in univariate and multivariate analysis.

DISCUSSION

Mutations of the *p53* gene have been widely investigated in HCC among different populations worldwide. For instance, in some geographic areas such as Southern Africa and some regions of China, a high prevalence of a specific G to T transversion mutation affecting the third nucleotide of codon 249 is detected in approximately 30-50% of cases.[7,24] This 'hot spot' mutation is thought to be caused by exposure to dietary aflatoxins prevailing in these regions where HBV infection is also endemic. On the other hand, in Thailand where the prevalence of HBV infection is comparable but aflatoxin contamination is reported to be much lower, the G to T mutations of codon 249 are not common (6.7%).[25] Likewise, in countries where dietary aflatoxin contamination is negligible such as the United States, Europe and Japan, the mutation at codon 249 is rare, even though p53 mutations are still prevalent but occur at sites distinct from this codon.[9,26,27] These discrepancies in *p53* mutations support the concept of geographical variations as to the etiology of hepatocarcinogenesis.

The presence of anti-p53 serum antibodies has been reported in 2-25% and 32% among Caucasian and Japanese HCC patients, respectively.[14,15,22,23] In our study performed among Thai patients, elevated p53 antibody levels were detected in 16 of 121 cases (13.2%). This prevalence rate is in accordance with the mutation rate of the *p53* gene, which has been reported to amount to 13.3% in a previous study on Thai patients.[25] In that report, direct sequencing of exon 5-8 revealed 2 mutations, an AGG to AGT transversion at codon 249, and an ATC to AAC at codon 254, respectively. Although we have not directly examined *p53* gene mutations, it is conceivable to conclude that the presence of circulating anti-p53 antibodies in our study correlates with *p53* gene mutations or p53 protein over-expression in tissue specimens, as has been suggested by previous reports.[22,23,28] Also, as mentioned above, such a relationship between *p53* mutations or protein over-expression in the tumor and presence of serum anti-p53 antibodies has already been documented in other types of cancer.

One of the most attractive characteristics of *p53* mutations is their clinical implication. With some types of solid tumors, *p53* mutations appear to have been associated with poor prognosis, suggesting that these mutations may occur in the later stages of tumor progression.[29] Likewise, in HCC, there have been increasing data on the association between the loss of *p53* function and poorer prognosis.[30-34] For example, Mise *et al.* [31] have recently reported that the expression of *p53* mutations was observed in more advanced HCC and could be an independent factor for determining the prognosis of the tumor. Hayashi *et al.*[30] reported that the presence of such mutations in HCC was associated with a shortened survival. Likewise, Honda *et al.*[32] found *p53* mutations to be a prognostic indicator for poor survival in patients undergoing liver resection. In contrast, the correlation between the presence of anti-p53 antibodies and the prognostic implications for HCC has been inconclusive. Shiota *et al.*[22] reported that the overall survival was significantly shorter among patients who exhibited such an antibody response than those without this marker. However, Saffroy *et al.*[23] have recently demonstrated that the development of anti-p53 antibodies does not display any prognostic significance regarding survival.

In our study, although the presence of anti-p53 antibodies was associated with lower levels

of AFP, total bilirubin and serum AST ($p < 0.05$), there was no correlation with other clinical/pathological features of the patients such as sex, age, clinical stage or histological type of the tumor, number and size of tumor, presence of vascular thrombosis and extrahepatic metastasis. In addition, the presence of these antibodies was not an independent predictor of outcome for patients with HCC. Our data demonstrated that upon univariate analysis, male gender, Okuda's stage II or III, larger tumor size, presence of portal vein thrombosis and absence of treatment were found to significantly contribute to an overall shorter survival. By multivariate analysis, we revealed that absence of therapy, Okuda's stage II and III and presence of portal vein thrombosis were independent factors related to the prognosis of the patients. Thus, in concordance with Saffroy *et al.*,[23] our data demonstrated that serum anti-p53 antibodies do not appear to be of importance for predicting tumor advancement and the prognosis in HCC.

Regarding the discrepancy between anti-p53 antibodies and p53 mutations as prognostic factors affecting survival, this is considered to be caused by the facts that tumors with *p53* gene mutations not always elicit an antibody response. Only tumors with *p53* gene missense mutations, as opposed to stop, splice/stop, splice or frameshift mutations, are able to induce antibodies, as the amount of protein produced in the latter cases is insufficient to elicit an immune reaction.[35] Furthermore, mutations in exons 7-8 are generally not immunogenic, in contrast with those of exons 5-6.[36] Also, the immune response of patients with p53 antibodies has been demonstrated to be restricted to but a small subset of peptides at the amino and carboxy termini of p53, irrespective of the type of cancer.[13] It has been suggested that such antibodies can be recognized in both wild-type and mutant conformations of the p53 protein.[34]

Moreover, regarding the association between etiologic risk factors and carcinogenesis, some studies have suggested that HBV infection itself could affect carcinogenic pathways, causing *p53* abnormalities independently. Unsal *et al.*[37] reported an apparent association between the presence of HBV-encoded X antigen (HbxAg) and wild-type p53 in HCC. Based on these data, a possible interference of HBV with wild-type p53 function has been suggested. Indeed, recent evidence has demonstrated that the carboxy-terminal domain of HBxAg complexes with p53 in the cytoplasm, thereby preventing its entry into the nucleus and subsequent induction of apoptosis, and thus contributing to hepatocarcinogenesis.[38-40] However, in our study, the rate of sera positive for HBsAg did not display any significant correlation with the presence of anti-p53 antibodies.

In conclusion, anti-p53 antibodies were found in approximately 13% of Thai patients with HCC. The presence of these antibodies did not appear to correlate with the clinical/pathological characteristics and advancement of the disease. Furthermore, its significance as an independent marker for predicting the prognosis of HCC is still unclear. Our results suggest that detection of anti-p53 antibodies, although easily performed in clinical settings, may not constitute a suitable alternative to molecular detection of mutations in assessing clinical implications and prognostic outcome of patients with HCC.

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