p53 expression in hepatocellular carcinoma in a population in Singapore with endemic hepatitis B virus infection

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Abstract

Aims—To study the expression and clinical significance (if any) of p53 protein in hepatocellular carcinomas (HCC) arising in a population with endemic hepatitis B virus (HBV) infection.

Methods—Immunohistochemical staining was performed on formalin fixed, paraffin wax embedded histological sections of 46 HCC cases using an antihuman p53 monoclonal antibody; serial sections were also stained for hepatitis B surface antigen (HBsAg), hepatitis B core antigen (HBcAg) and alpha fetoprotein (AFP). Nuclear p53 staining was assessed according to intensity (absent, weak or strong) and extent (<5%, 6–25%, 26–50%, and >50%) of positive cells. Tissue HBsAg, HBcAg and AFP were recorded as absent or present.

Results—The p53 protein was expressed in 35% (16 of 46) of HCCs; the positive rate in grade III/IV tumours (13 of 31; 42%) was higher than in grade I/II tumours (three of 15; 20%) but this was not statistically significant. HBsAg positive tumours showed almost the same proportion of p53 staining (11 of 29; 38%) as HBsAg negative ones (five of 17; 29%).

Conclusions—The p53 protein was expressed in 35% of HCC cases. There was no statistically significant correlation between HBV infection and p53 protein expression. Similarly, there was no definite correlation between p53 positivity and tumour size, histological grade or vascular invasion.

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Mutation of the p53 tumour suppressor gene represents the most common molecular change in human cancer[1–9] and has been identified in hepatocellular carcinomas (HCC). The frequency rates, however, show geographical variation. In HCC prevalent countries such as Mozambique and the Qidong area in China, regions exposed to high aflatoxin concentrations, the rates of p53 mutation have been reported to be as high as 59 and 50%, respectively.[10–13] At the other end of the spectrum, these high rates are rarely found in HCC cases from countries such as Japan (19%) and in whites resident in the United Kingdom (20%).[14–16]

Singapore, with a predominantly Chinese population, has a high incidence of HCC.[17] It also has a significant hepatitis B virus (HBV) carrier population with most cases of HCC arising in patients with HBV infection.[18] Integration of HBV DNA into the hepatocytic genome is frequently associated with the presence of HCC, with chromosome 17 being one of the targets.[19] In some cases the viral insert was mapped to chromosome 17p near the p53 gene chromosomal location,[20] raising the possibility of a link between p53 gene mutation and HBV induced HCC. There have been some recent data showing that the prevalence of p53 positive HCC was significantly higher in patients with serological markers of hepatitis B or C viral infection than in those without.[21]

With this in mind, we decided to analyse immunohistochemically the frequency and clinical significance (if any) of p53 protein expression in HCC occurring in Singapore where HBV infection is endemic.

Methods

Hepatocellular carcinoma liver resection specimens taken between January 1988 and March 1994 were retrieved from the archives of the Department of Pathology, National University Hospital, Singapore. This retrospective review yielded 46 cases, all of whom were of Asian origin (41 Chinese, three Malay and two Indonesian).

All of the HCC specimens had been fixed in 10% buffered formalin and routinely processed. Paraffin wax embedded histological sections stained with haematoxylin and eosin were evaluated. The tumour size was noted. HCC specimens were graded from I to IV according to Edmondson and Steiner's classification.[22] Vascular invasion was recorded. Adjacent non-tumorous liver parenchyma was examined for the presence of cirrhosis.

p53 protein immunostaining was performed using a Streptavidin biotin immunoperoxidase method. Deparaffinised 4 μm sections were treated with 3% hydrogen peroxide for 10 minutes to block endogenous peroxidase. The sections were then incubated with 5% bovine serum albumin (BSA) in TRIS-buffered saline (TBS) for 30 minutes to eliminate any non-specific staining. Excess BSA was drained from the slides before the addition of an antihuman p53 monoclonal antibody (DO-7; Dako, Glostrup, Denmark; M7001) at an optimal dilution of 1 in 50 in 0·1% BSA and incubated overnight at 4°C. The tissue sections were subsequently washed with TBS and incubated with biotinylated

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Correlation between clinical and histological features and p53 status in hepatocellular carcinoma

Characteristics	Total	p53 positive	p53 negative
Number of patients	46	16	30
Sex (M:F)	37:9	13:3	24:6
Age (years)	30–76	30–76	34–76
	(mean, 54·3)	(mean, 54·6)	(mean, 54·2)
Race			
Chinese	41	15	26
Others	5	1	4
HBV infection	29	11	18
AFP elevation	21	11	10
Tumour			
size of tumour (cm)	1·5–14·5	2–13	1·5–14·5
		(mean, 8·1)	(mean, 7·6)
Edmondson's grade			
I	7	1	6
II	8	2	6
III	24	9	15
IV	7	4	3
vascular invasion	38	16	22
HBsAg positive	6	1	5
HBcAg positive	1	0	1
Non-tumourous liver			
cirrhotic	19	8	11
HBsAg positive	29	11	18
HBcAg positive	4	2	2

rabbit antimouse immunoglobulin (Dako, E354), diluted 1 in 200 in 0·1% BSA for one hour at room temperature. After washing with TBS, the sections were incubated with per-oxidase conjugated Streptavidin (Dako, K9000), diluted 1 in 400 in TBS (pH 7·6), for one hour at room temperature. Finally, the sections were developed in diaminobenzedine in hydrogen peroxide substrate with 50 mg of imidazole (Sigma, St Louis, Missouri, USA; 1-0250) for 10 minutes. The sections were counterstained with Gill's haematoxylin. Sections in which the primary antibody was replaced with non-immune rabbit serum served as negative controls. A p53 positive gall bladder carcinoma served as a positive control. On analysis of p53 immunoreactivity only nuclear staining was regarded as specific. The intensity of staining was graded as either absent (0), weak (+) or strong (+ +). The extent of staining was semiquantitatively evaluated and categorised as
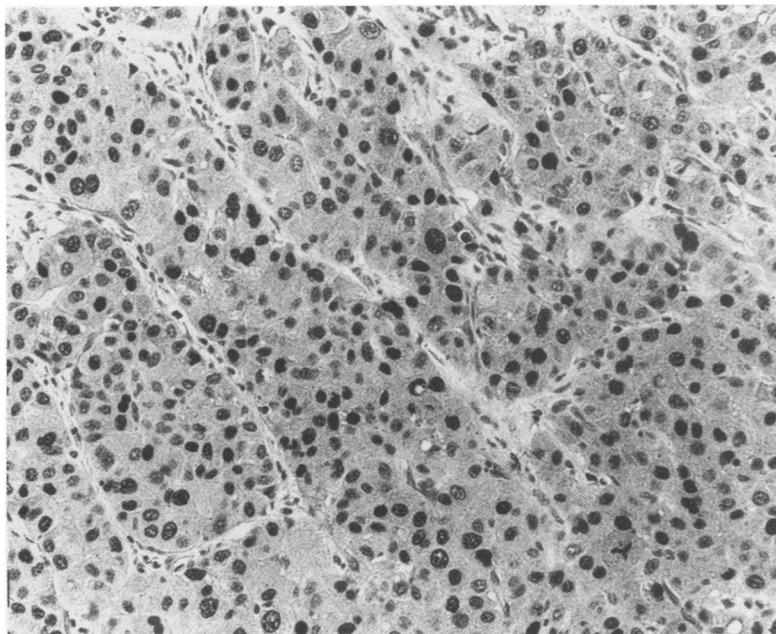
<5%, 6–25%, 26–50%, and >50% of positive cells.

Serial sections of the tumour and non-tumorous tissue were also stained for hepatitis B surface antigen (HBsAg) (Calbiochem, San Diego, California, USA), hepatitis B core antigen (HBcAg) (Dako) and alpha fetoprotein (AFP) (Dako) using Streptavidin biotin immunoperoxidase methods. HBsAg, HBcAg and AFP were recorded as absent or present. Categorical data were assessed using the χ^2 test.

Results

p53 protein expression was detected in 16 (35%) of the 46 HCCs analysed (table). The average size of p53 positive tumours was 8·1 cm compared with 7·6 cm for the negative lesions. Vascular invasion was evident in 38 (83%) cases, 16 of which expressed p53 protein. Cirrhosis was documented in 19 (41%) patients; of these, eight had p53 expression in tumour cells. AFP was detected in the tumour cells of 21 (46%) patients, 11 of whom had p53 positive lesions. A higher proportion of grade III/IV tumours (13 of 31; 42%) than grade I/II tumours (three of 15; 20%) were p53 positive (p<0·26). Considering only p53 tumours showing extensive staining—that is, at least more than 25% of the tumour cells, p53 was expressed in five of 23 (22%) grade III/IV tumours compared with one of 13 (8%) grade I/II tumours (figure).

HBsAg was detected in 29 (63%) cases; staining was also present in the tumour cells in six of these. HBcAg was detected in four HBsAg carriers; one showed staining within tumour cells and this case also contained malignant HBsAg positive cells. Overall, no difference was noted in p53 positivity between the HBV positive tumours (11 of 29; 38%) when compared with HBV negative ones (five of 17; 29%)(p<0·80). Furthermore, p53 expression was absent in those cases where HBsAg (five of six) and HBcAg (one patient) was demonstrated in the malignant cells.

Discussion

Abnormalities in the structure and expression of the p53 tumour suppressor gene occur frequently in HCC cell lines.[8] So far, the clustering of p53 mutations at codon 249 seems to be HCC specific. In fact, G to T mutations at codon 249 have been identified at comparable frequency in HCC from the Qidong area of China, a region with high exposure to aflatoxins.[13] However, reports from other parts of the world show that p53 mutation rates in HCC vary from low aflatoxin, low HCC incidence populations to high aflatoxin, high HCC incidence populations.[12-16] These data indicate that there is a possible geographic variation in the cause and pathogenesis of HCC and that HCC may not be a homogeneous group of tumours as far as genetic mechanisms are concerned.

Apart from aflatoxins, HBV infection is also a known risk factor for HCC. In fact, integration of HBV DNA into the hepatocytic genome is a frequent event associated with

A hepatocellular carcinoma showing extensive p53 staining of the nuclei (immunoperoxidase; × 50).

hepatocarcinogenesis; viral integration into the chromosome occurs close to the p53 gene locus.[19 20] Therefore, a link between p53 gene mutation and HBV induced HCC is possible.[21]

Singapore has a population with endemic HBV infection and a high prevalence of HCC.[17 18] In our analysis 35% (16 of 46) of the patients with HCC examined were p53 positive on immunohistochemistry. This represents an intermediate rate between populations with high and low incidence of HCC.[10–16 21 23] We found a higher proportion of p53 protein expression in grade III/IV tumours but the numbers were too small for statistical analysis. There was no definite correlation with tumour size, cirrhosis or AFP status.

We were unable to demonstrate a positive correlation between HBV infection and p53 protein expression in HCC (p<0·80). Furthermore, we noted that of the six cases with HBsAg positive and the single case with HBcAg positive malignant cells, almost all developed HCC in the absence of p53 protein expression. These findings are at variance with a recent report showing that the prevalence of p53 positive HCC was significantly higher in patients with serological markers of HBV infection than in those without.[21] However, we must emphasise that we were indirectly examining p53 gene mutations through immunohistochemical detection of the more stable mutant p53 protein. Wynford-Thomas has pointed out the range of circumstances in which detectable p53 expression might not correlate with the presence of mutation of the p53 gene giving false positive or false negative results.[24] In viral induced carcinogenesis the viral protein may actually target the p53 protein to more rapid catabolism, as in the case with the human papilloma virus.[25] It would appear from the results of our study that either p53 mutations in HCC are not correlated with HBV infection or that viral infection acts by negating the increase in the half-life of the mutated p53 protein.

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