Cell Proliferation Index and the Expression of p53 and Bcl-2 in Tumorous and Non-Tumorous Lesions of Hepatocellular Carcinoma and Metastatic Liver Cancer

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In the development of a cancer, unlimited cell proliferation has been believed to play an important role. In addition, a programmed cell death called apoptosis, which is regulated by several oncogenes and tumor suppressor genes, has been suggested to be another important different pathway of carcinogenesis. Recently, several reports on cell proliferation capacity and apoptosis in the development of human liver disease have been published, but the cell proliferation index and its relationship between the expression of the bcl-2 and p53 genes involving apoptosis has not yet been discussed in view of the clinical differences of primary and metastatic liver cancer. In this study, we investigated the cell proliferation index and expression of p53 and bcl-2 in the tumorous and non-tumorous portions of both hepatocellular carcinoma and metastatic liver cancer. The expression of p53 was observed in both hepatocellular carcinoma and metastatic liver cancer, but bcl-2 expression was observed neither in hepatocellular carcinoma nor in metastatic liver cancer. In hepatocellular carcinoma, the p53 positive group showed a higher Ki-67 score (cell proliferation index) and more tumor numbers than the p53 negative group (p <0.05). In metastatic liver cancer, the results were the same as in hepatocellular carcinoma (p <0.05). However, we could not correlate the p53 expression and its prognostic significance in hepatocellular carcinoma.

Unlimited cell proliferation has been the central concept in the pathogenesis of cancer. The role of bcl-2 in the development of B-cell lymphoma prompted an interest in the inhibition of pro-

grammed cell death as a contributing concept in cancer pathogenesis. Many reports have linked this concept to breast, prostate and colon cancer, and a number of genes including Fas, TGF β-1, bcl-2, bax and p53 have been suggested as playing important roles. There have been some studies on the role of these genes in the development of liver cancer, but studies on the relationship between the genes and cell proliferation, or the expression of these genes and metastatic liver cancer are limited so far. The authors of the present study investigated the cell proliferation of tumor and peritumoral normal tissues in both hepatocellular carcinoma and metastatic liver cancer, and the expression of bcl-2

and p53 as well as the clinical differences according to the expression of bcl-2 and p53.

MATERIALS AND METHODS

This study included 37 patients with either hepatocellular carcinoma (22 patients) or metastatic liver cancer (15 patients) diagnosed and treated with hepatic resection between January 1987 and December 1995 at Yongdong Severance Hospital. Mean age of the subjects was 54.7 years, ranging from 34 to 78. There were 25 men and 12 women.

We selected the tumorous and non-tumorous normal tissue adjacent to the tumor from the re-sected specimen which had been well preserved and chose one paraffin block for each case. The cell proliferation index was designated as "Ki-67", which was measured by the following method; anti-Ki-67 antigen (Immunotech, Westbrook, ME, USA) was used for staining the paraffin blocks and a nucleus stained a dark-brown color was considered as positive (Fig. 1, 2). 1,000 cells were observed under a high- power field ($\times 400$) and the number of positive cells indicated the Ki-67 index. p53 immu-nostaining was performed using a streptavidin-biotin immunoperoxidase method. Four-micron-thick sec-tions were deparaffinized in xylene and dehydrated with graded alcohol. The sections were placed in citrate buffer (pH 6.0), and a 10-minute microwave pretreatment was used to facilitate antigen retrieval.
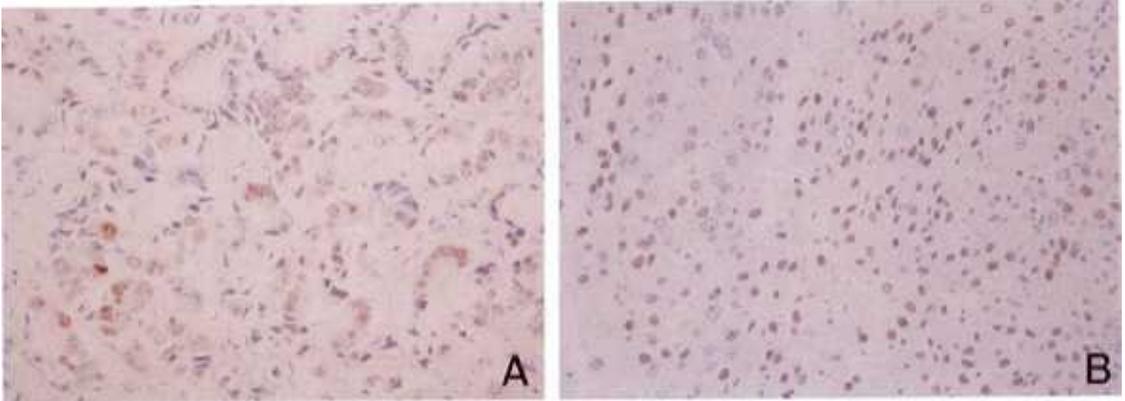

Fig. 1. Hepatocellular carcinoma cells with a positive nucleus staining for Ki-67(A) and p53(B)
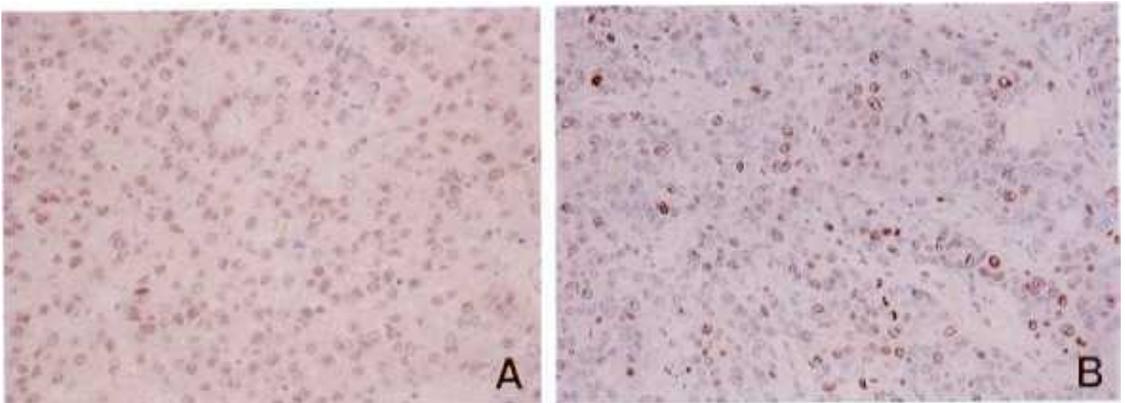

Fig. 2. Metastatic liver cancer cells with a positive nucleus staining for Ki-67(A) and p53(B)

These sections were subsequently incubated with normal horse serum (Vector Laboratories Inc., Burlingame, CA, USA) for 5 to 10 minutes to block endogenous peroxidase. Primary mouse MAbs for p53 (clone 124, DAKO, Glostrup, Denmark) was applied to the sections at a dilution of 1 : 80 and incubated overnight at 4°C in a humidified chamber. After washing in tris-buffered saline (TBS), horse biotinylated antimouse immunoglobulin was applied for 30 minutes. After a thorough washing in TBS, the sections were treated with streptavidin-peroxidase conjugate (Vector Laboratories, Burlingame, CA, USA) for 30 minutes at room temperature. The p53 staining was visualized as dark-brown with incubation in 3,3'-diaminobenzidine (Polyscience Inc., Warrington, PA, USA) for 5 minutes. The sections were counterstained with slight Mayer hematoxylin, dehydrated, and coverslipped with permanent mounting media. A specimen with colon carcinoma known to be strongly p53 positive was used as a positive control. Immunostaining for bcl-2 expression was done by the same method and cytoplasmic immunoreactivity with primary mouse MAbs for bcl-2 (clone 124, DAKO, Glostrup, Denmark) was observed. A specimen with follicular B-cell lymphoma known to be strongly p53 positive was used as a positive control.

p53 and bcl-2 overexpression were scored by a semiquantitative method evaluating the intensity of positive-stained cells. The intensity was graded as (−): no staining, (+): slight staining on some cells or in most cells, (+ +): moderately strong staining,

(+ + +): strong staining in almost all cells. (+), (+ +) and (+ + +) stainings were regarded as positive in both p53 and bcl-2 expression (Fig. 1, 2).

We compared the cell proliferation index related to the expression of p53 and bcl-2 and demonstrated the different clinical manifestations in both positive and negative groups. The differences in the bcl-2 expression rate and histologic differentiation according to p53 expression in hepatocellular carcinoma and metastatic liver cancer were analyzed through χ^2-test. Mean Ki-67 score, mean number of tumors and mean number of metastasized lymph nodes were analyzed through t-test. Survival and disease-free survival rates according to p53 expression in hepatocellular carcinoma patients were analyzed through Kaplan-Meier method and log-rank test. A p-value of less than 0.05 was considered statistically significant.

RESULTS

In hepatocellular carcinoma, p53 expression was noted in 12 out of 22 cases (54.5%) while bcl-2 expression was noted in none. In metastatic liver cancer, p53 expression was noted in 9 out of 15 cases (60.0%) while bcl-2 expression was noted in none. In areas adjacent to a tumor, neither p53 nor bcl-2 was expressed. The Ki-67 scores of tumor and adjacent tissue were 72.6 ± 73.6 and 3.1 ± 4.3 for

Table 1. Expression rate of p53, bcl-2 and Ki-67 score in hepatocellular cancer and metastatic liver cancer

		HCC[+] (N=22)		Met. Ca.[†] (N=15)	
		Tumor	NTL[‡]	Tumor	NTL[‡]
p53	(+)	12	0	9	0
	(−)	10	22	6	15
bcl-2	(+)	0	0	0	0
	(−)	22	22	15	15
Ki-67 score[§]		72.6±73.6	3.1±4.31	22.6±107.6	4.7±6.8

[+]: Hepatocellular cancer, [†]: Metastatic cancer, [‡]: Notumorous liver, [§]: No. of positive cells/1000 cells

Table 2. Tumoral characteristics according to p53 expression in hepaticellular carcinoma (N=22)

	p53		
	Positive	Negative	
bcl-2 (+)	0	0	p>0.05
(−)	12	10	
Ki-67 score[+]	109.5±82.3	28.4±19.8	p<0.05
No. of tumor	1.6±0.9	1.1±0.3	p<0.05
Tumor grade[†] I/II/III	0/4/8	3/4/3	p>0.05

[+]: No. of positive cells/1000 cells
[†]: Edmondson and Steiner grade

hepatocellular carcinoma, and 122.6 ± 107.6 and 4.7 ± 6.8 for metastatic liver cancer (Table 1).

In hepatocellular carcinoma, the Ki-67 score and the number of tumors were significantly larger in the p53 positive group than in the p53 negative group. Histologic differentiation was generally poorer in the p53 positive group than in the p53 negative group, although the difference was not statistically significant (Table 2).

In metastatic liver cancer, the Ki-67 score and the number of tumors were significantly larger in the p53 positive group than in the p53 negative group. There was no significant difference in tumor size or the number of involved lymph nodes between the p53 positive and p53 negative groups (Table 3).

Table 3. Tumoral characteristics according to p53 expression in metastatic liver carcinoma (N=15)

	p53		
	Positive	Negative	
bcl-2 (+)	0	0	p>0.05
(−)	9	6	
Ki-67 score[+]	157.1 ± 117.9	70.8 ± 69.2	p<0.05
No. of tumor	1.8 ± 1.4	1.0 ± 0.0	p<0.05
Size of tumor	3.5 ± 2.9	2.7 ± 2.0	p>0.05
No. of LN[†]	2.2 ± 2.4	2.6 ± 1.4	p>0.05

[+]: No. of positive cells/1000 cells
[†]: No. of metastatic lymph node

The 1-, 3- and 5-year survival rates were 77.8, 55.6 and 41.7% respectively in the p53 negative group, and 100.0, 53.3 and 53.3% respectively in the p53 positive group with no significant difference. The 1-, 3- and 5- year disease-free survival rates were 77.8, 55.6 and 41.7% respectively in the p53 negative group, and 100.0, 53.3 and 53.3% respectively in the p53 positive group, with no significant difference (Fig. 3).

DISCUSSION

As with many other tumors, liver cancer is caused by a complex interaction of various factors, which may be involved in clinical patterns and prognosis. With the concept of apoptosis being suggested in the pathogenesis of cancer, the focus of studies has been on the abnormalities in the control of programmed cell death. As the role of bcl-2 in breast cancer and prostate cancer has been reported, involvement of genes in the pathogenesis and clinical expression of other forms of cancer is being investigated.

A few oncogenes and tumor suppressor genes control apoptosis. The bcl-2 gene family involved in apoptosis consists of two subgroups with different functions. Bcl-2 and others (Bcl-XL, MCLI, AI, mbcl-X, Ced-9, BHRFI, LMW5-HL) as a cell death suppressor, and Bax, Bcl-Xs, Bak and Bad as a cell death promoter, control programmed cell death (Hockenberry, 1994; Reed, 1994; Lu et al. 1996).
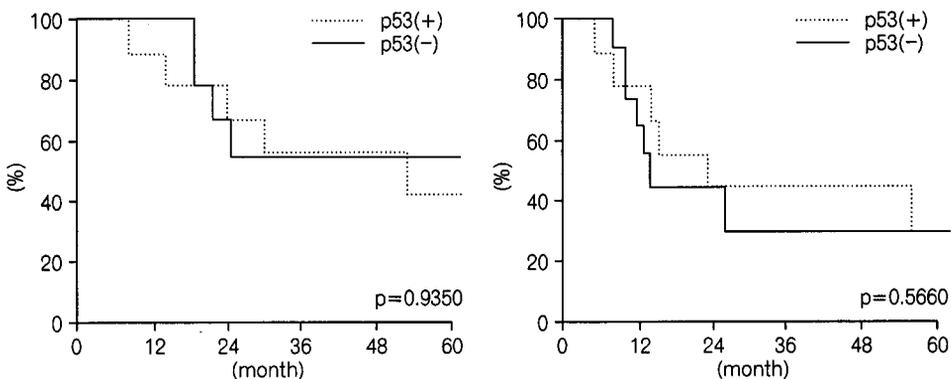

Fig. 3. Survival & disease-free survival rates according to p53 expression in hepatocellular carcinoma patients

Bcl-2 is a proto-oncogene encoding 26 Kda oncoprotein located in the mitochondrial inner surface. Bcl-2 was discovered while studying chromosomal translocation (14;18) frequently observed in follicular B-cell lymphoma (Yunis *et al.* 1982; Tsujimoto *et al.* 1984). As well, Bcl-2 has been observed in immunohistochemical analysis of such cancers as nasopharyngeal cancer (80%) (Lu *et al.* 1993), breast cancer (75%) (Joensuu *et al.* 1994) and prostate cancer (76%) (Colombel *et al.* 1993). The Bcl-2 gene inhibits apoptosis and it is expressed in some self-renewing tissues and liver tumor cells. In liver diseases, bcl-2 expression has been observed in bile ductule and small bile duct epithelium, while the expression is low in hepatocyte or large bile duct epithelium (Charlotte *et al.* 1994). The expression rate of bcl-2 varies from 2.1% to 13.5% (Zhao *et al.* 1994; Hamazaki *et al.* 1995). In metastatic liver cancer, however the expression rate is high (Skopelitou *et al.* 1996).

p53 is a main tumor suppressor gene involved in repair after DNA damage. p53 extends the progression from the G1 phase to S-phase in DNA damage, allowing more time for DNA repair. Once DNA repair fails, p53 triggers apoptosis, removing abnormal cells through programmed cell death (Lane, 1992). Mutation of p53 results in the loss of such functions, causing neoplastic transformation (Lane, 1992). Such mutation was found in 38% of human malignant tumors, and in hepatocellular carcinoma the mutation rate has reached 25~50% (Greenblatt *et al.* 1994).

In this study, the expression rates of p53 in hepatocellular carcinoma and metastatic liver cancer were 54.5% and 60.0% respectively, with no expression in the normal tissues adjacent to the tumor. Bcl-2 was not expressed in hepatocellular carcinoma, metastatic liver cancer and normal tissue adjacent to the tumor. The results for p53 expression in hepatocellular carcinoma were in accordance with those in other similar studies (Greenblatt *et al.* 1994). And it is suggested that the mutation of wild-type p53 results in genetic instability and causes neoplastic transformation (Lane, 1992). As for bcl-2, the results were in contrast to the study by Skopelitou, where the bcl-2 expression rate was 40~60% in metastatic liver cancer (Skopelitou *et al.* 1996).

The Ki-67 Index was significantly greater in the p53 positive group than in the p53 negative group. The number of tumors was also significantly greater in the p53 positive group than in the p53 negative group. These results are in accordance with reports showing the positive relationship between p53 expression and tumor size, tumor differentiation, capsular infiltration, portal vein thrombosis and intrahepatic metastasis (Okuda *et al.* 1996). p53 positive tumors have also shown a high proliferation index and high growth index, indicating the central role of p53 in cell proliferation (Soini *et al.* 1996). The degree of histologic differentiation was poorer in the p53 positive group, although the difference was not statistically significant. Hino (Hino *et al.* 1996) reported that cell proliferation in apoptosis was related to hepatocellular carcinoma growth, and that cell differentiation is low in active cell proliferation induced by p53 expression. The p53 and bcl-2 expression were reported to be in reverse relationship, and p53 may downregulate the expression of bcl-2 (Haldar *et al.* 1994; Joensuu *et al.* 1994). Although high p53 expression was noted in hepatocellular carcinoma and in metastatic cancer, the relationship between p53 and bcl-2 expression is not clear in the present study since bcl-2 expression was not noted.

As the results show, p53 expression is high in tumor cells suggesting that p53 expression may be related to neoplastic transformation. p53 expression also seems to result in multiple metastasis and more cell proliferation in the tumor. Therefore, expression of tumor proteins related to apoptosis influences the clinical outcome of hepatocellular carcinoma and metastatic liver cancer, ultimately affecting prognosis. It is not clear in the present study how p53 expression relates to prognosis in hepatocellular carcinoma. With more cases, however, it may be possible to postulate how those independent indicators function as comprehensive biochemical prognostic factors in both primary and metastatic liver cancer.

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