Cell proliferation, apoptosis and the related regulators p27, p53 expression in hepatocellular carcinoma

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AIM: To investigate the expression of cell apoptosis, proliferation and the related regulators p27, p53 in hepatocellular carcinoma (HCC).

METHODS: The expression of p27, p53, proliferating cell nuclear antigen (PCNA) and apoptosis in 47 HCC specimens and 42 surrounding non-cancerous tissues were detected by the immunohistochemistry and terminal deoxy-nucleotidyl transferase-mediated nick end labeling (TUNEL) technique. Meanwhile, the clinical significance of them was analyzed combining with the clinicopathological factors and follow-up data.

RESULTS: (1) The average proliferating index and apoptotic index in HCC were significantly higher than that in adjacent liver tissues. The proliferating index was associated with extrahepatic metastasis. The apoptotic index was significantly lower in TNM stage I-II than in stage III-IV. The proliferating index of groups with p53-/p27+ was significantly lower than that in group with p53+/p27- (P = 0.030); (2) The level of p27 in the cytoplasmic fraction was higher in non-tumoral liver tissues and was associated with clinical stage; (3) Survival analysis showed advanced stage (P = 0.031) and with extrahepatic metastasis (P = 0.045) was significantly associated with shorter survival. In addition, the prognosis of patients with p53-/p27+ was longer than that of patients with p53+/p27- (P = 0.0356).

CONCLUSION: The p53 mutation and decreased p27 expression might be involved in the imbalance of proliferation and apoptosis in HCC. Cytoplasmic displacement might lead to the inactivation of p27 protein in HCC cells and acts early during carcinogenesis of HCC. The combined examination of p27, and p53 expression allows reliable estimation of prognosis for patients with primary hepatic carcinoma.
collected from patients in First Hospital of Xi’an Jiaotong University and 42 samples had adjacent nontumoral liver tissues. Thirty-eight patients were men, and 9 were women. The median age was 47 years, with age-range from 29 to 77 years. Thirty-seven of 47 HCCs arose in cirrhotic liver. All tumors were histologically diagnosed. Tumor gross type and stage were diagnosed using Edmondson’s classification and Union Internationale Contre le Cancer (UICC) criteria. Tumor cellular differentiation was identified by Edmondson’s classification. Forty patients (85%) had HBV infection, and 2 (4%) had a history of alcohol abuse. The remaining 5 (11%) had chronic liver diseases unrelated to HBV or alcohol. No patient received pre-operative chemotherapy or chemoembolization. The clinicopathologic features of 47 patients with HCC are listed in Table 1. After curative surgery, all patients were followed up every 3 mo till death. They were followed from 2 to 36 mo (mean 15 mo). Actuarial survival was measured from the day of surgical operation to the date of death or last follow-up.

### Table 1: Clinicopathological characteristics of 47 patients

| Clinicopathological parameters | Number | %  |
|-------------------------------|--------|----|
| Gross type                    |        |    |
| Massive                       | 21     | 44.7|
| Nodular                       | 26     | 55.3|
| Tumor size                    |        |    |
| ≤5 cm                         | 19     | 40.4|
| >5 cm                         | 28     | 59.6|
| Cellular differentation       |        |    |
| I+ II                         | 39     | 82.9|
| III+IV                        | 8      | 17.1|
| Stage                         |        |    |
| I+ II                         | 13     | 27.7|
| III+IV                        | 34     | 72.3|
| Capsular infiltration         |        |    |
| Present                       | 19     | 40.4|
| Absent                        | 28     | 59.6|
| Extral hepatic metastasis     |        |    |
| Present                       | 15     | 31.9|
| Absent                        | 32     | 68.1|

### Immunohistochemical study

Liver samples were routinely fixed in 40 g/L formaldehyde solution and embedded in paraffin. After slicing into 4 μm-thick sections, IHC was performed using Dako Elivision™ plus two-step System. In brief, the sections were dewaxed in xylene and rinsed in alcohol and graded alcohol/water mixtures. Then, 30 mL/L hydrogen peroxide was applied to block endogenous peroxidase activity. The sections were subsequently treated in a microwave oven twice for 5 min in citrate buffer (pH6.0) at high power. After blocking with goat serum, the mouse monoclonal antibodies against p53 (ZM-0408), p27 (ZM-0340) and proliferating cell nuclear antigen (PCNA) (ZM-0213) (Zymed Biotechnology, Zymed, CA) were applied on the slides at the dilution of 1:50, 1:120 and 1:50, respectively. After rinsing, staining was performed with the Elivision™ plus two-step System (kit-9902. Dako, Carpinteria, CA). The color was developed by reacting with 3,3-diaminobenzidine. Slides were then counterstained with hematoxylin, dehydrated, cleared and mounted. A human breast cancer specimen was used as a positive control for p53 and p27, while the reactive tonsil was used for PCNA. Negative controls were performed by replacing the primary antibody with non-immune mouse serum or PBS.

### Detection of apoptosis

Apoptosis detection was performed using In Situ Cell Death Detection kit, AP (Boehringer-Mannheim, Germany). After a standard procedure of deparaffinization and rehydration, all specimens were heated with microwave in 0.05 mol/L citric acid solution for antigen retrieval and washed twice with PBS. The slides were incubated with TUNEL reagent for 1 h at 37 °C, washed and incubated for 45 min with Converter-AP reagent at 37 °C. After washing the sections were counterstained with Fast Red. As positive control, tissue sections were pre-treated 60 min with 1 μg/mL DNA-Se I. For negative control only TdT buffer without deoxynucleotidyl transferase was used.

### Immunostaining evaluation

Slides were mounted independently by two investigators without notifying any clinical or pathological information. For p53 and p27, the tissue sections with more than 10% immunoreactivity in at least 1 000 HCC cells from at least five randomly selected fields were defined as positive, and those with less than 10% immunoreactive tumor cells were defined as being negative. The proliferating and apoptotic index were defined as the percentage of tumor cells displaying immunoreactivity from at least five randomly selected microscopic fields.

### Statistical analysis

Values were expressed as mean±SD, the statistical evaluation was performed using the statistical program for social science for windows (SPSS version 10.0). $X^2$ Fisher’s exact test, Student’s $t$ test and one-way ANOVA were used. Actuarial survival curves were constructed by the Kaplan-Meier method. The cumulative survival rate c between groups was compared, using the Log-rank test. Relevant prognostic factors were identified by univariate and multivariate Cox proportional hazard regression analyses. Tests were considered significant when the $P$ values were <0.05.

### RESULTS

#### Proliferating and apoptotic activity in HCC

Figures 1 and 2 respectively showed the PCNA and apoptotic cell expression in HCC and adjacent nontumoral liver tissues. The mean PI was $30.34±4.46\%$ in HCC, which was significantly higher than that in adjacent non-tumoral liver lesions ($27.88±5.89\%$, $t = 2.233$, $P = 0.028$). The mean AI was significantly higher in HCC ($8.62±2.28\%$) than that in adjacent non-tumoral liver lesions ($7.38±2.61\%$, $t = 2.383$, $P = 0.019$). Table 2 shows the relationship between PI, AI and clinicopathological features. The proliferating index was associated with extrahepatic metastasis ($P = 0.019$). The apoptotic index was significantly lower in those of TNM stage I+II than in those of stage III+IV ($P = 0.030$).
Expression of p53 and p27 proteins in HCC

The positive rate of p27 protein expression was significantly lower in HCC (40.4%, 19/47) than that in the adjacent liver tissue (64.3%, 27/42) ($\chi^2 = 5.057, P = 0.025$) (Figure 3A). Table 3 shows the relationship between p27 expression and clinicopathological features. The positive rate of p27 in the groups with tumor diameter $>5$ cm and capsular infiltration was significantly lower than that in the groups with tumor $\leq 5$ cm ($P = 0.044$) and without capsular infiltration ($P = 0.026$). The positive rate of p53 expression was significantly higher in HCC (38.3%, 18/47) than in the adjacent liver tissues (16.7%, 7/42) ($\chi^2 = 5.138, P = 0.023$) (Figure 4). The positive rate of p53 was not associated with the analyzed factors in HCC ($P > 0.05$).

The number of p53 positive and p27 negative cases (p53+/p27- group), p53 negative and p27 positive cases (p53-/p27+ group), p53 negative and p27 negative cases (p53-/p27- group), p27 positive and p53 positive cases (p53+/p27+ group) were 11, 12, 17 and 7 cases, respectively. There was no correlation between the expression of p53
and p27 (χ² = 0.038, P = 0.845).

**Altered subcellular localization of p27 in HCC**

The cytoplasmic expression of p27 was found in the HCC and adjacent non-tumoral liver tissues (Figures 3B and C). So we detected the p27 localization in 10 normal liver tissues from patients, who died due to accidents, to define whether the cytoplasmic localization is a specific phenomenon for tumor cells. We also found the cytoplasmic localization of p27 in normal liver tissues (Figure 3D). Therefore, the cytoplasmic displacement of p27 might not be a specific phenomenon to tumor cells. However, the cytoplasmic sequestration of p27 was more frequent in HCC and adjacent non-cancerous lesions.

The average LI of cytoplasmic p27 was 27.6±14.1% in adjacent non-cancerous lesions, which was higher than that in HCC 20.5±15.9% (t = 2.058, P = 0.043). Altered subcellular localization of p27 was correlated with clinical stage (P = 0.021), shown in Table 3.

**Relationship between the p27 or p53 expression and proliferating, apoptotic index**

Cell proliferative and apoptotic activity were compared between p27 positive and negative cases and between p53 positive and negative cases. The mean PI and AI were 29.26±4.81% and 8.42±1.84% in p27 positive cases, while 31.07±4.15% and 8.75±2.56% in p27 negative cases. No significant difference in AI and PI were found between the two groups. The mean PI and AI also had no significant difference between the p53 positive and negative groups.

The patients were divided into two groups according to the median LI of cytoplasmic p27. The mean AI was significantly lower in high cytoplasmic p27 cases (7.16±1.90%) than in low cytoplasmic cases (8.42±2.44%, P = 0.044), shown in Table 4.

### Table 2 Relationship between PI, AI and clinicopathological features

| Parameters | AI (%) | P   | PI (%) | P   |
|------------|--------|-----|--------|-----|
| Gross type |        |     |        |     |
| Massive    | 8.29±2.26 | 0.376 | 30.10±3.81 | 0.739 |
| Nodular    | 8.88±2.30 | 0.54±4.99 |        |     |
| Tumor size |        |     |        |     |
| ≤5 cm      | 8.11±2.03 | 0.208 | 29.79±4.85 | 0.492 |
| >5 cm      | 8.96±2.41 | 0.71±4.23 |        |     |
| Cellular differentiation |        |     |        |     |
| Well or moderate | 8.87±2.38 | 0.091 | 30.46±4.46 | 0.686 |
| Poor       | 7.38±1.19 |        | 29.75±4.74 |     |
| Stage      |        |     |        |     |
| I + II     | 7.46±2.30 | 0.030^a | 28.85±3.85 | 0.306 |
| III+IV     | 9.06±2.15 |        | 30.91±4.60 |     |
| Capsular infiltration |        |     |        |     |
| Present    | 8.79±2.68 | 0.674 | 31.79±4.10 | 0.066 |
| Absent     | 8.50±2.01 |        | 29.36±4.50 |     |
| Extrahepatic metastasis |        |     |        |     |
| Present    | 9.00±1.89 | 0.436 | 32.53±3.93 | 0.019^a |
| Absent     | 8.44±2.45 |        | 29.31±4.38 |     |

^aP<0.05 vs Stage III+IV.

### Table 3 Relationship among p27, cytoplasmic p27 and p53 staining with clinicopathological features

| Parameters | p27 | p53 | P   | P   | Cytoplasmic p27 LI (%) | P   |
|------------|-----|-----|-----|-----|------------------------|-----|
| Gross type |     |     |     |     |                        |     |
| Massive    | 8   | 13  | 0.770 |     | 10  | 11  | 0.237 | 26.85±21.61 | 0.349 |
| Nodular    | 11  | 15  |     | 8   | 18  |     | 17.11±14.62 |     |
| Tumor size |     |     |     |     | 0.044^a | 7   | 12  | 0.866 | 25.48±24.52 | 0.068 |
| ≤5 cm      | 11  | 8   |     | 0.044^a | 7   | 12  | 0.866 | 25.48±24.52 | 0.068 |
| >5 cm      | 8   | 20  |     |     | 11  | 7   | 19.93±15.71 |     |
| Cellular differentiation |     |     |     |     | 0.855 | 13  | 26  | 0.126 | 20.13±16.34 | 0.688 |
| Well or moderate | 16  | 5   | 0.855 | 13  | 26  | 0.126 | 20.13±16.34 | 0.688 |
| Poor       | 3   | 23  | 0.246 | 6   | 7   | 0.498 | 29.24±23.59 | 0.021^a |
| Stage      |     |     |     |     |      |     | 15.58±10.46 |     |
| I + II     | 7   | 6   | 0.246 | 6   | 7   | 0.498 | 29.24±23.59 | 0.021^a |
| III+IV     | 12  | 22  | 0.026^a | 6   | 13  | 0.435 | 18.13±14.52 | 0.276 |
| Capsular infiltration |     |     |     |     |      |     | 24.12±20.87 |     |
| Present    | 4   | 15  | 0.188 | 5   | 10  | 0.632 | 14.73±9.15 | 0.141 |
| Absent     | 15  | 17  |     |     |      |     | 23.82±20.39 |     |

^aP<0.05 vs HCC.

### Table 4 Relationship among p27, cytoplasmic p27 and p53 staining with PI and AI

| Parameters | p53 (+) | P   | PI (%) | P   | p53 (–) | P   | PI (%) | P   |
|------------|---------|-----|--------|-----|---------|-----|--------|-----|
| AI         | 8.61±2.87 | 0.989 | 29.50±4.93 | 0.314 |
| PI (%)     | 8.62±1.88 | 0.633 | 29.26±4.81 | 0.176 |
| p27 (+)    | 8.42±1.84 | 0.044^a | 30.33±5.17 | 0.708 |
| p27 (–)    | 8.73±2.56 | 31.07±4.15 |     |     |
| Cytoplasmic p27 |      |       |        |     |
| High       | 7.16±1.90 | 0.044^a | 30.33±5.17 | 0.708 |
| Low        | 8.42±2.44 |       | 29.81±4.37 |     |

^aP<0.05 vs cytoplasmic p27 low.
Survival analysis
The patients were divided into two groups according to the median PI or AI of the tumors. The survival analysis was performed on 47 patients and took into account the following variables: age, gender, tumor size, UICC tumor stage, Edmondson grade, extrahepatic metastasis, capsular infiltration, p53, p27, cytoplasmic p27 LI and PI, AI. In univariate analysis a significant correlation with short survival was found only for TNM stage ($P = 0.002$), present extrahepatic metastasis ($P = 0.001$), capsular infiltration ($P = 0.014$), and low p27 expression ($P = 0.042$). Multivariate analysis was performed by Cox proportional hazard regression model, TNM stage ($P = 0.031$) and present extrahepatic metastasis ($P = 0.045$) were significantly associated with shorter survival. For other factors, no significant correlations with overall survival were found.

Coexpression of p53 and p27 in HCC
The PI of p53-p27+ group was 27.50±5.61%, which was significantly lower than that of the p53+p27- group ($P = 0.030$) and tended to be lower than that of the p53-p27- group or of the p53+p27+ group (Table 5). The prognosis of patients with p53-p27+ was longer than that of patients with p53+p27- ($P = 0.0356$, log-rank test, Figure 5). There was no significant difference between the apoptotic index (Table 5) and clinocopathological factors (Table 6).

DISCUSSION
Nowadays, despite a variety of therapeutic strategies, HCC remains a significant cause of cancer death. The distant and lymph nodal metastasis are not very common in HCC; therefore to study the parameters for evaluating the biological behavior is very important for the prevention and treatment of this disease. The balance between cell proliferation and apoptosis determines the velocity of tumor growth, while the balance between regulators of proliferation and apoptosis is an important determinant of tumor behavior.

Most precious investigations about the relationship between proliferation and apoptosis are based on staining of tumoral samples without comparing them with surrounding non-tumoral liver, which should be the best control tissue to be used for comparison. Pizem et al[9], recently investigated apoptosis and proliferation in HCC and non-neoplastic liver samples, they found that neo-plastic and non-neoplastic liver tissues differ in proliferative but not in apoptotic activity. Uncontrolled tumor cell proliferation plays an important role in HCC growth. In our study, the proliferating and apoptotic index in neoplastic hepatocytes were higher than that in pericancerous liver tissue. The results in lines with earlier observations[10,11], indicating that during multi-stage

Table 5 Relationship between coexpression of p27 and p53 staining with PI and AI

| p53 (-) p27 (+) | p53 (+) p27 (-) | p53 (+) p27 (+) | p53 (-) p27 (-) |
|----------------|----------------|----------------|----------------|
| PI 27.50±5.61  | 31.93±4.17     | 30.43±4.39     | 30.13±3.94     |
| AI 7.85±2.43   | 8.23±2.31      | 7.48±1.36      | 7.05±2.17      |

Table 6 Correlation between the expression of p27, p53 and clinocopathological factors

| Parameters                  | p53-p27+ | p53+p27- | p53+p27+ | p53-p27- | $P$  |
|-----------------------------|----------|----------|----------|----------|------|
| Gross type                  |          |          |          |          |      |
| Massive                     | 4        | 6        | 4        | 7        | 0.546|
| Nodular                     | 8        | 5        | 3        | 10       |      |
| Tumor size                  |          |          |          |          |      |
| ≤5 cm                       | 6        | 2        | 5        | 6        | 0.359|
| >5 cm                       | 6        | 8        | 2        | 12       |      |
| Cellular differentiation    |          |          |          |          |      |
| Well or moderate            | 11       | 8        | 5        | 6        | 0.119|
| Poor                        | 1        | 3        | 2        | 2        |      |
| Stage                       |          |          |          |          |      |
| I+II                       | 5        | 2        | 3        | 3        | 0.127|
| III+IV                     | 7        | 8        | 4        | 15       |      |
| Capsular infiltration       |          |          |          |          |      |
| Present                     | 4        | 5        | 1        | 9        | 0.999|
| Absent                      | 8        | 6        | 6        | 8        |      |
| Extrahepatic metastasis     |          |          |          |          |      |
| Present                     | 3        | 3        | 2        | 7        | 0.220|
| Absent                      | 9        | 8        | 5        | 10       |      |
hepatocarcinogenesis cell proliferation is a key determinant of the velocity of the process. Though a corresponding increase in apoptotic activity was also found, the increased cell proliferation could not completely be balanced by increased apoptosis. The proliferating index was associated with extrahepatic metastasis and the apoptotic index was significantly lower in those of TNM stage I-II than in those of stage III-IV.

Many methods have been used for apoptotic activity assessment[12,13]. The TUNEL methods have been proven to provide a means for early detection of cells undergoing apoptosis even before the onset of gross apoptotic morphology[14]. The earlier studies showed too long proteinase K pre-treatment could cause false positive TUNEL staining by stimulating endogenous endonuclease. So we used microwave antigen retrieval substituting the long proteinase K pre-treatment could cause false positive results can be excluded.

Loss or reduced expression of p27 have been found in a variety of human cancers and associated with more aggressive tumor behavior[5-18]. The present study shows that p27 expression was significantly decreased in HCC. This finding of under-expression of p27 in HCC was in accordance with the previous reports[19,20]. In this study, we have shown that expression of p27 in HCC was associated with tumor diameter and capsular infiltration. It is therefore suggested that p27 should work as a negative regulator during hepatocarcinogenesis. The p53 gene is one of the most important tumor suppressor genes determined so far[24]. The p53 protein confirmed by immunohistochemical staining was considered as the mutation type. The facts that p53 protein expressed low in non-cancerous liver tissue and high in HCC, indicating that the high expression of p53 protein is probably associated with the cancerous transformation of HCC. In our study p27 expression did not correlate with p53. Bhardwaj et al[26], using Western blotting analyzed the p27 expression in different p53 defects. The results showed that p27 at high level only in hepatoma cell lines with normal p53 genes. The relationship between p27 and p53 needs further study.

However, contrary to expectations, p27 and p53 expression did not correlate with cell proliferating and apoptotic activity in our experiment. Similar observations were also reported in other carcinoma[26,27]. It may mean that proliferation and apoptosis of cancer cells are regulated not by a single cell-cycle regulator, but by a balance of negative and positive regulators. Further analysis found that the mean PI of p53+/p27- group was significantly higher than that of the p53-p27+ group. This suggests the mutated p53 and decreased p27 level may be involved in the imbalance of cell proliferation and apoptosis in HCC. The mutated p53 and decreased p27 may exert their carcinogenic effect in multi-stage of hepatocarcinogenesis by two ways: (1) promoting rapid proliferation and cell transformation; (2) inhibiting effect on the apoptosis. In addition, our study found the prognosis of patients with p53+/p27+ was longer than that of patients with p53+/p27-. Therefore, combination study of the expression of p53 and p27 was thought to be useful for predicting the prognosis of HCC.

Indeed, cytoplasmic localization of p27 immunostaining has been reported in various types of human cancers[28-31]. We also found the cytoplasmic localization of p27 was more frequent in HCC and surrounding noncancerous liver. Furthermore, the cytoplasmic staining for p27 was more frequent accompanied with nuclear staining in normal controls. However, the increase in the cytoplasmic staining for p27 was often observed in the absence of a concomitant increase in the nuclear staining and was sometimes associated with a decrease in the nuclear staining. Some tumors expressed an increased level of p27, mainly because of an increase in the cytoplasmic level of this protein. The increase in the amount of cytoplasmic p27 was more frequent in early stage (I and II) tumors. Altered subcellular localization of p27 was also reported in Barrett’s associated adenocarcinoma and colon cancer[32,29]. In agreement with our results, cytoplasmic localization of p27 was an early event during the carcinogenesis.

The protein p27 can bind and inhibit the active cyclin/CDK complexes in the nucleus, so the cytoplasmic displacement might play an important role in the inactivation of this protein in tumor cells and contribute to tumor development[33]. The apoptotic index is low in high cytoplasmic p27 groups supported this speculation, although the mechanisms responsible for the abnormal subcellular localization has not been known. It may be due to loss of the tuberous sclerosis complex gene-2 (TSC2); the HER/Grb2/MAPK pathway leads to nuclear export of p27[31], overexpression cyclinD3 contributes to retaining p27 in the cytoplasm[34], PKB/Akt phosphorylates p27 impairing its nuclear import[35-37].

In conclusion, our study shows that neoplastic and non-neoplastic liver tissues differ in proliferative and apoptotic activity. The mutated p53 and decreased p27 might play an important role in maintaining neoplastic hepatocytes survival. Cytoplasmic displacement is an alternative mechanism of inactivating p27 that acts early during hepatocarcinogenesis. The combined examination of p53 and p27 expression allows reliable estimation of prognosis for patients with HCC.

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