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• LIVER CANCER •

Expression of p27, cyclin E and cyclin A in hepatocellular carcinoma and its clinical significance

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Abstract

AIM: To investigate the expression of p*27*, cyclin E and cyclin A in hepatocellular carcinoma (HCC) and its potential clinical significance.

METHODS: Expression of p27, cyclin E and cyclin A in 45 HCC specimens and 30 adjacent noncancerous lesions obtained from 45 patients during surgery was examined by immunohistochemical SABC assay. The diameter of tumor ranged from 1 cm to 19 cm (d≤5 cm, 9 samples; 5 cm<d≤ 10 cm, 19 samples; d>10 cm, 17 samples). The tumors were graded according to the criteria described by Edmondson-Steiner: well-differentiated HCC group (Grade I+II), 26 samples; poorly-differentiated HCC group (Grade III+IV), 19 samples. According to the clinical-pathologic features, 19 samples were poorly encapsulated, 15 samples had portal invasion of cancer, 11 samples had extrahepatic metastasis, and 12 samples had intrahepatic metastasis. All of the samples were classified as the invasive and metastatic group, while the remaining was classified as the non-invasive and non- metastatic group.

RESULTS: The average labeling index (LI) of p27 in HCC lesions was significantly higher than that in adjacent noncancerous lesions (45.87±14.21 *vs* 33.77±12.92, t=3.745, P<0.001). The LI of p27 was associated with differentiation, invasiveness and metastasis of the tumors (34.46±12.29 *vs* 52.80±11.36, t=5.17; 41.42±12.86 *vs* 51.44±14.10, t=2.48; P<0.05). Cyclin E was overexpressed in 16 cases (35.6 %) while cyclin A was overexpressed in 21 cases (46.7 %) in HCC lesions. No overexpression of cyclin E or cyclin A could be observed in adjacent non-carcinoma lesions and normal liver tissues. The overexpressions of cyclin E and cyclin A were correlated with differentiation, tumor thrombus, invasiveness and metastasis (P<0.05). Expression of cyclin E was significantly correlated with expression of cyclin A (r=0.329, P<0.05). The LI of p27 was significantly decreased in cyclin E, cyclin A positive groups (40.33±11.91 *vs* 49.50±13.76, t=3.05; 38.86±11.19 *vs* 52.57±12.62, t=3.89; P<0.05).

CONCLUSION: p27, cyclin E, cyclin A play cooperative roles in HCC tumorigenesis, differentiation, invasiveness and metastasis. Detection of their expression may be helpful in prediction of tumor progression.

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INTRODUCTION

Hepatocellular carcinoma is one of the most common malignant tumors in China and has poor prognosis due to its high incidence of recurrence and metastasis. Recent studies on HCC have been focused on tumorigenesis, progression, invasiveness as well as novel strategy of therapeutics.

It has been implicated that the activity of cell proliferation is directly associated with tumorigenesis, progression and invasiveness. Therefore estimation of cell proliferative activity is important in prediction of the biological aggressiveness of tumor cells. The regulatory molecules of cell cycle are parameters for the prediction of cell proliferative activity[1]. Cell cycle progression is controlled by protein complexes such as cyclins, cyclin dependent kinases (CDKs) and cyclin dependent kinase inhibitors (CDKIs). The sequential activation and subsequent inactivation of cyclin-CDK complexes govern the progression of eukaryatic cells throughout cell cycle[2]. In cell cycle, the period from the late G1 to S phase is the most important restriction point for cell proliferation. Whether cells pass the G1/S restriction point determines the continuity of cell proliferation[3]. The most direct protein at G1/S point is retinoblastoma protein (pRb)[4]. Phosphorylated pRb can bind to transcription factor (E2F) that regulates cell cycle by activation of DNA synthesis. Both cyclin E and cyclin A play important roles in G1/S restriction point[5,6]. Cyclin E dramatically increases from the late G1 phase to the early S phase[5] which binds to CDK2 and phosphorylates pRb. When cell enters S phase, cyclin E and cyclin A-CDK2 complex cooperate continuously for the phosphorylation of pRb until the end of M phase[6]. As one of CDKIs, p27 can prevent pRb from phosphorylation and arrest the cell cycle at G1 phase[7]. Cyclin E and cyclin A are direct substrates of p27. Recent researches have shown that the activity of p27 protein can be up-regulated by multiple tumorigenesis related factors such as transforming growth factor β[8], interferon[9] and cAMP[10]. The expression of p27 has been implicated in the tumorigenesis of many kinds of tumors[11-15]. However, little is known about the association between cyclins and hepatocellular carcinoma. In the present study, we detected the expression of p27, cyclin E and cyclin A in 45 HCC samples and 30 adjacent noncancerous lesions by immunohistochemical assay to elucidate their correlation with tumorigenesis, progression and metastasis of HCC.

MATERIALS AND METHODS

Data of patients

From 1998 to 1999, 45 HCC specimens and 30 adjacent noncancerous lesions were obtained from 45 patients with HCC during surgery in our hospital. A senior pathologist made the final diagnosis on the basis of histological examination. No patient had received radioactive therapy, chemotherapy,

transcatheter arterial chemoembolization or immunotherapy before operation. There were 36 (80 %) males and 9 (20 %) females, aged from 30 to 65 with an average age of 45±11. A total of 30 (66.67 %) patients had AFP levels over 400 ng/ml, 32 (71.1 %) patients were HbsAg positive. The diameter of tumor in this group ranged from 1 cm to 19 cm (less than 5 cm in 9 samples, 5 cm to 10 cm in 19 samples, more than 10 cm in 17 samples). These tumors were graded based on the criteria of Edmondson-Steiner (Grade I+II in 26 samples, grade III+IV in 19 samples). According to the clinical-pathologic features, 19 specimens had no or little capsule, 12 samples had portal vein invasion of the tumors, 11 samples had extrahepatic metastasis and 12 samples had intrahepatic metastasis. The criteria for invasiveness or metastasis of tumor were the tumor tissue with no or poor capsule, or portal vein invasion, or intrahepatic or extrahepatic metastasis[16]. Twenty-five cases (55.56 %) were defined as invasive/metastatic group.

Tissue sampling

Fresh surgical tissue samples were fixed immediately in formaldehyde solution for 12-24 h and paraffin-embedded for immunohistochemical assay.

Immunohistochemical assay

Immunohistochemical study was performed using avidin-biotin-complex method. Briefly, 4 μm slices of tissue section were deparaffinized and rehydrated. Endogenous peroxidase activity was blocked with 0.3 % hydrogen peroxide for 10 min. Sections were then incubated with 0.03 mol/L citrate buffer (pH=6.0) and heated in microwave oven for 10 min. After three times of rinsing with phosphate-buffered saline (PBS) (pH=7.4), the slides were incubated with 10 % normal goat serum at room temperature for 20 min to block the nonspecific reaction, and incubated overnight with primary antibody (p27 monoclonal antibody 1:50, cyclin E polyclonal antibody 1:50 and cyclin A polyclonal antibody 1:100) at 4 ℃. After rinsed in PBS, they were incubated with second antibody (1:100) for 30 min at room temperature, and reacted with the avidin-biotin peroxidase complex at a concentration of 1:100 for 30 min after washed in PBS. The peroxidase reaction was visualized by incubating the section with 0.01 % 3,3'-diaminobenzidine tetrahydrochloride and hydrogen peroxide mixture. The slides were counterstained with hematoxylin. In negative control, primary antibody was replaced by normal mouse serum.

Immunohistochemical evaluation

The protein reaction (p27, cyclin E or cyclin A) was considered as positive when nuclei showed staining signals. At least 500 *p*27 positive cells from at least 5 randomly selected fields (×400) were counted[17]. When the positive rate for each protein of carcinoma cells was over 5 %, overexpression of cyclin E and cyclin A was defined according to the report[18,19].

Statistical analyses

The correlation of *p*27, cyclin E or cyclin A with carcinogenesis, differentiation, invasion and metastasis of HCC was analyzed with SPSS8.0 software. The χ^2 test and Student *t* test were employed for analyses, *P* value less than 0.05 was regarded as statistically significant.

RESULTS

*p*27 was expressed both in HCC and in adjacent noncancerous lesions (Figures 1,2). The average LI of p27 in HCC lesions was significantly higher than that in adjacent noncancerous lesions (45.87±14.21 *vs* 33.77±12.92, *P*<0.001). The LI of p27 was associated with differentiation, tumor size, invasiveness and metastasis of HCC (*P*<0.05). No correlation was found between the LI of p27 and the number of cancer foci (Table 1).

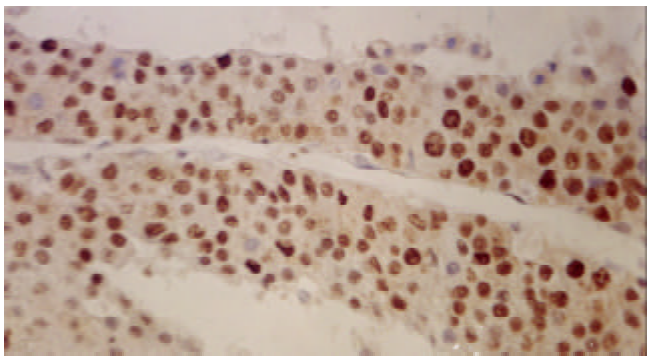

Figure 1 Nuclear staining of p27 protein in HCC (SABC, 400×).

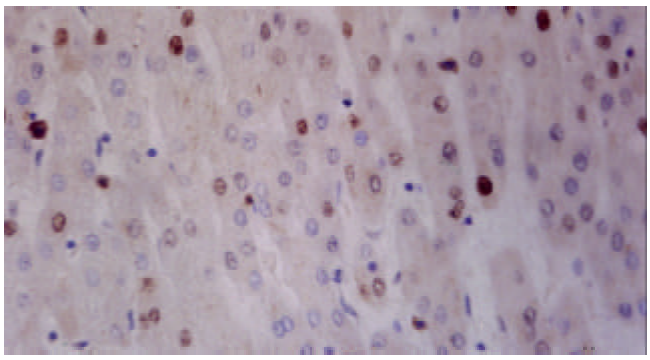

Figure 2 Nuclear staining of p27 protein in adjacent noncancerous lesions (SABC, 400×).

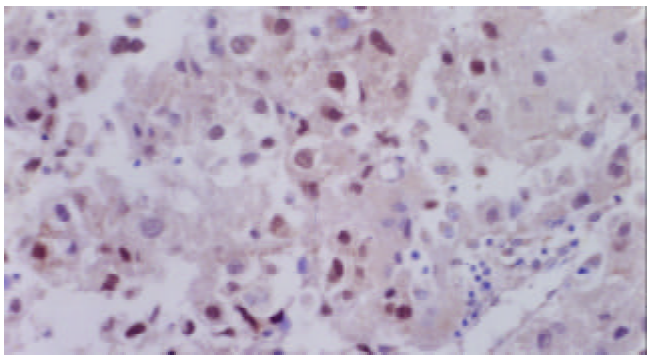

Figure 3 Nuclear staining of cyclin E protein in HCC (SABC, 400×).

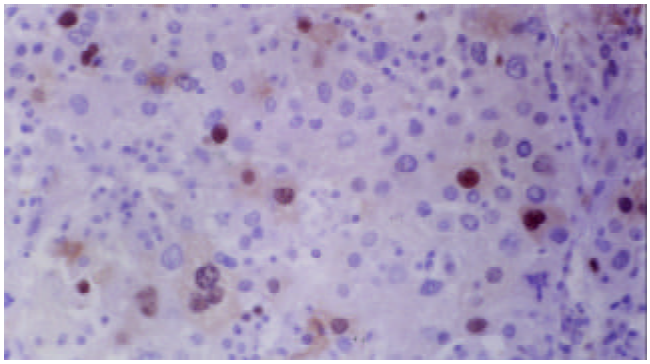

Figure 4 Nuclear staining of cyclin A protein in HCC (SABC, 400×).

Table 1 Expression of p27 and pathological features ($\bar{x}\pm s$)

Groups		*n*	p27 LI($\bar{x}\pm s$)		*P*
Histological grade	I+II	26	52.80±11.36	*t*=5.17	<0.001
	III+IV	19	34.46±12.29		
Tumor size (d)	≤5 cm	9	53.28±15.17	*F*=5.934	<0.005
	5 cm<d≤10 cm	19	49.50±10.96		
	>10 cm	17	37.59±13.12		
Envelope	Full	26	48.82±13.35	*t*=1.76	>0.05
	Rupture	19	41.54±14.15		
Tumor thrombi	+	13	36.83±12.74	*t*=2.77	<0.01
	-	32	49.16±13.41		
Metastasis	Positive	11	35.52±11.70	*t*=3.02	<0.05
	Negative	34	49.22±13.43		
Number	<2	33	47.65±15.08	*t*=1.41	>0.05
	≥2	12	40.98±10.52		
Non-invasive and non- metastatic group		20	51.44±14.10	*t*=2.48	<0.02
Invasive and metastatic group		25	41.42±12.86		

Table 2 Expression of cyclin E, cyclin A and pathological features

Group		*n*	Cyclin E		Cyclin A	
			+	χ^2 / *P*	+	χ^2 / *P*
Histological grade	I+II	26	5 (19.23 %)	7.16 / <0.05	8 (30.77 %)	6.25 / <0.05
	III+IV	19	11 (57.89 %)		13 (68.42 %)	
Tumor size (d)	≤5 cm	9	1 (11.11 %)	1.58 / >0.05	5 (55.56 %)	1.29 / >0.05
	5<d≤10 cm	19	7 (36.84 %)		7 (36.84 %)	
	>10 cm	17	8 (47.06 %)		9 (52.94 %)	
Envelope	Full	26	7 (26.92 %)	2.00 / >0.05	10(38.46 %)	1.67 / >0.05
	Rupture	19	9 (47.37 %)		11 (57.89 %)	
Tumor thrombi	+	13	8 (61.54 %)	5.38 / <0.01	10 (76.92 %)	6.72 / <0.01
	-	32	8 (25.00 %)		11 (34.38 %)	
Metastasis	Positive	11	6 (54.55 %)	1.32 / >0.05	9 (81.82 %)	7.23 / <0.01
	Negative	34	10 (29.41 %)		12 (35.29 %)	
Number	<2	33	9 (27.28 %)	2.47 / >0.05	12 (36.36 %)	5.76 / <0.05
	≥2	12	7 (58.33 %)		9 (75.00 %)	
Non-invasive and non- metastatic group			4 (20.00 %)	3.86 / <0.05	4 (20.00 %)	9.95 / <0.01
Invasive and metastatic group			12 (48.00 %)		17 (68.00 %)	

The overexpression of cyclin E and cyclin A could be exclusively seen in HCC (Figures 3,4), the overexpression rate was 35.6 %(16/45) for cyclin E and 46.7 %(21/45) for cyclin A. The overexpression of cyclin E and cyclin A was associated with differentiation, invasiveness and metastasis of HCC (P<0.05). No correlation could be found between the LI of p27 and the tumor size (P>0.05)(Table 2).

The LI of p27 decreased significantly both in cyclin E and in cyclin A overexpressed tissues (40.33±11.91 *vs* 49.50±13.76, 38.86±11.19 *vs* 52.57±12.62, P<0.05). The overexpression of cyclin E was significantly correlated with that of cyclin A (P<0.05, r=0.329), (Table 3).

Table 3 Relationship between expressions of cyclin E and cyclin A

		Cyclin A		*P*
		+	-	
cyclin E	+	11	5	<0.05
	-	10	19	

DISCUSSION

Uncontrollable proliferation is the property of tumor cells. Cell proliferation activity involves in tumorigenesis and progression, and is one of the prominent parameters in evaluating the biological aggressiveness of carcinoma.

As one of the major CDK inhibitors, p27 can arrest cell cycle by blocking phosphorylation of pRB. Its substrate is G1 cyclins such as cyclin E and cyclin A. Low p27 protein levels were found in aggressive stomach[11], lung[12], prostate[13], breast[14] and pituitary[15] cancers, suggesting that p27 might suppress the progression of tumor[20].

Our data showed that the LI of p27 was higher in HCC than in adjacent noncancerous tissues, and the expression of p27 was mainly localized in nuclei. This might suggest that p27 works as a positive regulator in tumorigenesis of HCC. Two possible mechanisms could be involved. First, p27 might be regulated by self-stabilization. It has been demonstrated[21] that expression of p27 was regulated primarily at the posttranscriptional level and its mRNA level was stable throughout the cell cycle. When cells are stimulated by

mitogen, p27 protein undergoes rapid degradation via the ubiquitin-proteasome pathway. However, this proteolysis was dramatically reduced in resting cells[22]. Thus, increased expression of p27 in some tumors may be resulted from self-stable regulating mechanism by which increased expression of cyclins attenuates the activity of the proteasome pathway for p27, and then causes an increased expression of free p27 protein that can counteract the increased cyclins in tumorigenesis. Second, gene mutation may also be responsible for this situation. Recent studies have revealed a gene deletion and polymorphism in primary breast cancer and leukemia[23,24]. Whether increased expression of p27 in HCC is caused by mutant protein remains to be elucidated.

In the present study, the expression of p27 was decreased in cases with biologically aggressive phenotypes such as poor differentiation, metastasis and invasiveness. It has been reported that cultured tumor cells expressed more p27 as they grew from single layer to tri-dimension and cell contact inhibition could be suppressed by p27 antisense oligonucleotide[25]. All these suggested that decreased expression of p27 was related with tumor progression and could be used as a potential prediction factor for HCC.

Although many researchers focused on the role of cyclin E and cyclin A in cell cycle in tumor cells[26,27], few studies have ever addressed on the aspect of HCC. Our data showed that cyclin E and cyclin A proteins were exclusively expressed in HCC but not in adjacent noncarcinous lesions. The expression of cyclin E and cyclin A was mainly localized in nuclei, suggesting that overexpression of cyclin E and cyclin A could promote cell cycle and cell proliferation, and therefore was associated with tumorigenesis. Cyclin E expressed in cytoplasm of tumor cell may be caused by increased synthesis, decreased degradation and failure to transportation. Our data also showed that overexpression rate of cyclin E and cyclin A was associated with low histological grade of tumors with high expression rate in poorly differentiated tumors, suggesting the overexpression of cyclin E and cyclin A was associated with poor differentiation. Overexpression of cyclin E was correlated with formation of tumor thrombi, while overexpression of cyclin A was associated with tumor thrombi, metastasis and satellite lesions. Thus overexpression of cyclin E and cyclin A is linked to tumor invasiveness and metastasis potency, suggesting a poor prognosis for patients with overexpression of cyclin E and cyclin A. Patients with cyclin E overexpression had a four-year survival rate[28] and overexpression of cyclin A had a positive relationship with the amount of cells at S-phase and a reverse correlation with the four-year survival rate[18]. Our data were partially similar to these findings. Unfortunately, we were unable to evaluate the correlation of overexpression of cyclin E and cyclin A with the prognosis due to incomplete follow-up data of the patients.

p27 suppresses cyclin/CDK complexes mainly by binding itself to cyclins. It has been reported that p27 could bind to CDK2 and played an inhibitory role in regenerating liver[29]. Zerfass-Thome[30] reported that p27 arrested cell cycle by blocking transactivation of cyclin-A gene which is dependent on cyclin-E gene expression, suggesting a mechanism of interaction among p27, cyclin E and cyclin A. The lower p27 LI in positive cyclin E and cyclin A group in our study might be resulted from the interaction of cyclin-CDK complexes that can suppress its expression. And expression of cyclin E had a positive relationship with expression of cyclin A. These suggest that p27, cyclin E and cyclin A play cooperatively important roles in tumorigenesis, differentiation, aggressiveness and metastasis of HCC.

Although a lot of studies on cyclins/CDKI have been done, many questions remain to be answered. It has been reported that proliferative tumor cells *in vitro* could be arrested in G1 phase by using antibody IgM against cyclin E and cyclin A in culture[31]. Other studies *in vivo* showed that invasiveness of tumor could be dramatically suppressed by down-regulation of G1 and S phase proteins[32]. It seems that cyclins may become potential targets for tumor therapy in the near future. Further studies are needed to elucidate the mechanism of interaction among cyclins and the pathway of regulation before they can finally be used as a novel strategy for prediction of prognosis and therapeutics of HCC.

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