• BRIEF REPORTS •

Effect of c-*myc*, Ki-67, MMP-2 and VEGF expression on prognosis of hepatocellular carcinoma patients undergoing tumor resection

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Abstract

AIM: To explore the effect of c-*myc*, Ki-67, MMP-2 and VEGF expression on prognosis of hepatocellular carcinoma (HCC) patients undergoing tumor resection.

METHODS: Primary HCC patients underwent tumor resection were retrospectively analysed. The maximum size of the tumor was less than 5 cm, there was only one nodule in each patient. No chemoembolization was performed before resection. They were followed up after resection, and the time of recurrence was recorded. They were divided into 2 groups: group A (15 cases): tumor recurrence within 1 year after tumor resection, and group B (15 cases): with or without tumor recurrence 2 years after tumor resection. Pathological slices were made with tumor wax-sample. Immunohistochemistry staining was performed with c-*myc*, Ki-67, MMP-2 and VEGF monoclonal antibodies. Staining intensity was quantitatively analysed with a pathological diagram-writing analyzing system. The expressing intensity differences of stained molecules in cancer tissue and para-cancer were analysed.

RESULTS: c-*myc*, Ki-67, MMP-2 and VEGF expressing intensities in cancer tissue in group A were higher than those in group B (*P* values were 0.010, 0.030, 0.022 and 0.004, respectively), but they were not significantly different in para-cancer tissue in groups A and B (*P* values were 0.334, 0.343, 0.334 and 0.334, respectively).

CONCLUSION: The expression of c-*myc*, Ki-67, MMP-2 and VEGF in cancer tissue is related to the recurrence of HCC after tumor resection.

INTRODUCTION

Recurrence is the main factor influencing prognosis of hepatcocellular carcinoma (HCC) after tumor resection. The therapeutic measures for patients before and after operation are similar, but their prognosis after operation differs largely.

Some patients had tumor recurrence within 1 year after operation, while others had or did not have tumor recurrence 2 years after operation. The reason why there is such a difference is not clear. Researches on molecular biology have demonstrated that the prognosis of HCC is related to the activation of proto-oncogene, inactivation of tumor suppressor gene, abnormal expression of growth factors and/or their receptors[1]. Four kinds of molecules related to biologic characteristics of HCC were studied in this experiment to clarify their relationship with recurrence of HCC after resection.

MATERIALS AND METHODS

Patients

Thirty primary HCC patients undergoing resection at Department of Hepatobiliary Surgery in General Hosptial of PLA (301 Hospital) were retrospectively analysed. All resected samples were proved as HCC with pathologic examination. The selecting standards were as follows: solitary nodule with its maximum size less than 5 cm, no transarterial chemoembolization (TACE) or local thermal therapy (such as microwave coagulation or radiofrequency) before resection, no other specific treatment after resection. There were 28 males and 2 females. Their mean age was 51.5 (range, 27-75) years. The mean size of tumors was 3.0 cm. The patients were divided into two groups according to follow-up results: group A, which had tumor recurrence within 1 year after resection, group B, had or did not have tumor recurrence 2 years after resection. 15 cases were in group A (14 males and 1 female) and 15 cases in group B (14 males and 1 female). The differences of clinical data (sex, age, tumor size, liver function, serum AFP, transaminase, HBV infection) were not significant (Table 1).

Table 1 Clinical data of patients in groups A and B

Item	Group A (*n*=15)	Group B (*n*=15)
Sex	14 males, 1 female	14 males, 1 female
Age (yr)	54.7±14.3	48.2±8.4
Mean diameter (cm)	3.2±1.0	2.9±1.1
Tumor volume (cm³)	22.0±21.4	18.2±17.6
Liver function	grade A	grade A
Serum AFP[a] (grade)	0.9±1.0	0.6±1.0
ALT (U/L)	58.0±54.1	64.9±56.6
AST (U/L)	49.9±46.8	42.0±37.7
HBV infection rate	87% (13/15)	100 (15/15)

[a]serum AFP was graded as follows: 0: 0-200 µg/L, 1: 201-400 µg/L, 2: >400 µg/L.

Instrument and reagent

HPIAS-1000 high acuity color pathologic diagram-writing analyzing system (produced by Wuhan Champion Image Technology Corporation Limited). SP and DAB kit, monoclonal antibodies of c-*myc*, Ki-67, MMP-2 and VEGF were all purchased from Beijing Zhongshan Biological Technology Coporation Limited. The characteristics of antibodies are listed in Table 2.

Table 2 Characteristics of antibodies used in this study

Name of antibody	Specificity	Dilution	Secondary antibody
c-*myc*	Monoclonal Mouse anti human	1:50	Goat anti mouse
Ki-67	Monoclonal Mouse anti human	1:50	Goat anti mouse
MMP-2	Monoclonal Mouse anti human	1:50	Goat anti mouse
VEGF	Monoclonal Mouse anti human	1:50	Goat anti mouse

Immunohistochemistry staining

Serial 4 μm thick sections were made with wax sample of resected tumors. Immunohistochemistry staining was performed with SP three-step method using the monoclonal antibodies listed in Table 2.

Quantitative analysis of positive cells

Quantitative analysis of the examined molecules was performed with HPIAS-1000 high acuity color pathologic diagram-writing analyzing system. Molecules in cancer and para-cancer tissues were analysed. Three fields of view (FOV) were randomly selected in cancer and para-cancer tissues to quantitatively analyse the expressing intensity. One hundred cells were observed in each FOV, positively stained cells were calculated, finally the average positively stained cells in 100 observed cells were determined. The cells were determined as positive-staining only if they were stained without considering their staining intensity. The medium optical density(MOD) of plasma or nuclei in positively stained cells was calculated with a pathologic diagram-writing analyzing system. The product of multiplication of average positively stained cells and MOD was calculated, which was considered as the expressing intensity of positively stained molecules.

Comparison of staining

The difference of expressing intensity of examined molecules in cancer and para-cancer tissues was compared in the same group (group A or B), the difference of expressing intensity in groups A and B was compared in the same tissue (cancer tissue or para-cancer tissue).

Statistical analysis

Data were presented as mean±SD. Paried-sample t test was used to compare the difference, the statistic software SPSS 10.0 was used. $P<0.05$ was considered statistically significant.
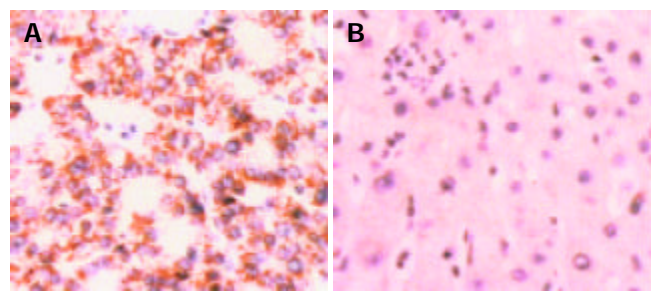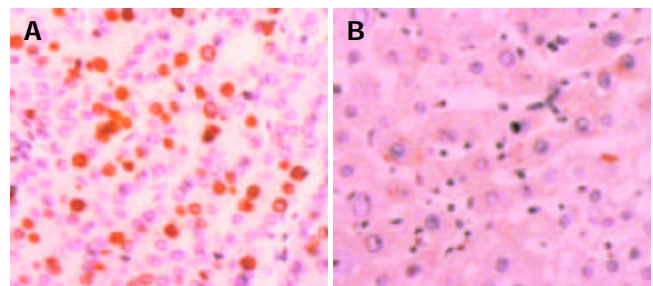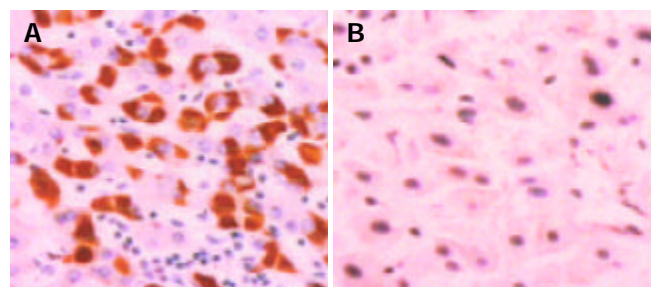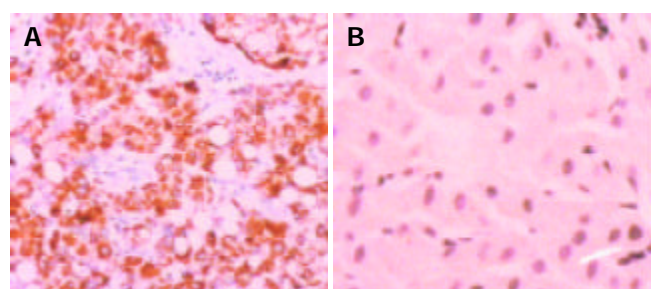
RESULTS

Expressing rate and intensity of examined molecules in cancer and para-cancer tissues in group A (Table 3)

Table 3 Expressing rate(%) and intensity of examined molecules in cancer and para-cancer tissues in group A

Item	Cancer tissue	Para-cancer tissue
c-*myc*	80 (12/15)[b]; 3.95±2.81[b]	0 (0/15); 0.00±0.00
Ki-67	60 (9/15)[b]; 1.57±2.20	7 (1/15); 0.57±2.21
MMP-24	7 (7/15)[a]; 3.70±4.13[a]	7 (1/15); 0.80±3.10
VEGF	67 (10/15)[b]; 5.44±4.20[b]	7 (1/15); 0.04±0.15

[a]$P<0.05$, [b]$P<0.01$ vs Para-cancer tissue.

Figure 1 Positive and negative c-*myc*, A: Positive c-*myc*; B: Negative c-*myc*.

Figure 2 Positive and negative Ki67, A: Positive Ki67; B: Negative Ki67.

Figure 3 Positive and negative MMP-2, A: Positive MMP-2; B: Negative MMP-2.

Figure 4 Positive and negative VEGF, A: Positive VEGF; B: Negative VEGF.

Expressing rate and intensity of examined molecules in cancer and para-cancer tissues in group B (Table 4)

Table 4 Expressing rate (%) and intensity of examined molecules in cancer and para-cancer tissues in group B

Item	Cancer tissue	Para-cancer tissue
c-*myc*	40 (6/15); 1.34±2.74	7 (1/15); 0.26±1.00
Ki-67	40 (6/15); 0.18±0.38	7 (1/15); 0.01±0.04
MMP-2	20 (3/15); 0.61±1.70	0 (0/15); 0.00±0.00
VEGF	27 (4/15); 1.04±3.38	0 (0/15); 0.00±0.00

Expressing rate and intensity of examined molecules in cancer tissues in groups A and B (Table 5)

Table 5 Expressing rate (%) and intensity of examined molecules in cancer tissues in groups A and B

Item	Group A	Group B
c-*myc*	80 (12/15); 3.95±2.81[b]	40 (6/15); 1.34±2.74
Ki-67	60 (9/15); 1.57±2.20[a]	40 (6/15); 0.18±0.38
MMP-2	47 (7/15); 3.70±4.13[a]	20 (3/15); 0.61±1.70
VEGF	67 (10/15); 5.44±4.20[b]	27 (4/15); 1.04±3.38

[a]$P<0.05$, [b]$P<0.01$ *vs* Group B.

Expressing rate and intensity of examined molecules in para-cancer tissues in groups A and B (Table 6)

Table 6 Expressing rate (%) and intensity of examined molecules in para-cancer tissues in groups A and B

Item	Group A	Group B
c-*myc*	0 (0/15); 0.00±0.00	7 (1/15); 0.26±1.00
Ki-67	7 (1/15); 0.57±2.21	7 (1/15); 0.01±0.04
MMP-2	7 (1/15); 0.80±3.10	0 (0/15); 0.00±0.00
VEGF	7 (1/15); 0.04±0.15	0 (0/15); 0.00±0.00

Micrographs of c-myc, Ki-67, MMP-2 and VEGF
The microgaghs of c-*myc*, Ki-67, MMP-2 and VEGF positive and negative expression were listed in figures 1-4.

DISCUSSION

Relationship of c-myc expression with prognosis of HCC
Activation of c-*myc* oncogene plays important role in cancer occurrence, gene location on chromosome of 8q24. c-*myc* oncogene can be activated through two ways. One is that it is confluenced by light chain sequence of immunoglobulin through chromosomal translocation, the other is through DNA amplication. The protein coded by c-*myc* oncogene contains 439 amino acids, and can be combined specifically with intranuclear DNA to play transcription regulating function. c-*myc* oncogene is not expressed in the resting phase of cells, while it is rapidly expressed under the induction of mitoses, then it promotes cell proliferation and infiltration. It was reported in literature[2] that HBV interpolation could promote amplication and over-expression of c-*myc* oncogene, then normal cellular genetic regulation was disturbed. Genetic mutation related with cancer occurrence could be induced through this mechanism, it was related with occurrence of HCC. c-*myc* oncogene codes phosphoric acid protein whose molecular weight is 62 KD. The protein is located in the nuclei of normal hepatocytes and HCC cells, as well as in plasm of some cells. It was demonstrated *in vitro* that the blockage of c-*myc* expression could suppress the growth of HCC cells[3-5]. Some researchers demonstrated that the expression of c-*myc* in HCC tissue was related with the prognosis of HCC patients[6]. Wang *et al.*[7] reported that amplification of c-myc oncogene was correlated with a poor prognosis of HCC. Niu *et al.*[8] reported that the positive expressing rate of c-*myc* in HCC was correlated with the histological differentiation, and was significantly higher in the poorly differentiated samples than in well differentiated samples. Zhang *et al.*[9] reported that c-*myc* gene amplification was closely related to the development and progression of HCC. This study showed that c-*myc* expressing rate and intensity in cancer tissue in patients of group A were higher than those in para-cancer tissue, while the c-*myc* expressing rate and intensity in cancer tissue in patients of group B were not significantly different from those in para-cancer tissue. c-*myc* expressing intensity in cancer tissue in patients of group A was higher than that in patients of group B. It demonstrated that the expression of c-*myc* in cancer tissue could reflect the malignancy of HCC. HCC with high c-*myc* expression in cancer tissue had a high recurrence rate. c-*myc* expressing rates in cancer tissue in the two groups of patients were significantly different, suggesting that c-*myc* over-expression occurs in HCC, its expressing intensity is related to the prognosis of HCC.

Relationship of Ki-67 expression with prognosis of HCC
Tumor cells consist of proliferating cells (S, G2, M, G1 stage), temporarily non-porliferating cells (G0) and non-proliferating cells. Ki-67 can label proliferating cells in any stage except those in stage G0, while it is not expressed in cells in silent stage. Ki-67 is rapidly degraded or its antigenic determinant is disappeared after mitosis. So Ki-67 is considered as a kind of objective marker reflecting proliferating activity of cells. Ki-67 is located in cell nuclei, and always in particle shape in immunohistochemistry staining. Ki-67 labelling index has been considered as a marker of cellular proliferative activity, the higher the Ki-67 labelling index, the lower the cellular differentiation and the poorer the prognosis of HCC[10-16]. It was demonstrated in this study that Ki-67 expressing rate in cancer tissue in group A was higher than that in para-cancer tissue, while the expressing intensity was not significantly different. Ki-67 expressing rate and intensity in cancer tissue in group B were not significantly different from those in para-cancer tissue. Ki-67 expressing intensity in cancer tissue in group A was much higher than that in group B, suggesting that Ki-67 expression could reflex malignancy of HCC. HCC with a high Ki-67 expression had a high recurrence rate. Ki-67 expressing rate in cancer tissue of the two groups was not significantly different, suggesting that Ki-67 is abnormally expressed in HCC, its expressing intensity is related to the prognosis of HCC.

Relationship of MMP-2 expression with prognosis of HCC
A main step of invasion and metastasis of malignant tumor is to degrade extracellular matrix (ECM) and basement membrane. There are much collagen IV in ECM and basement membrane, collagen IV is very important for maintaining the integrity of ECM and basement membrane. Collagenase IV could degrade collagen IV and destroy the integrity of basement membrane. Matrix metalloproteinase II (MMP-2) is a kind of collagenase IV, its expression is related to tumor recurrence and metastasis. Positive particles of MMP-2 are located in cell plasm. Some researchers reported that MMP-2 expression in cancer tissue was related with prognosis of HCC, HCC with high MMP-2 expression had high malignancy, easy recurrence and metastasis[17-23]. This study showed that MMP-2 expressing rate and intensity in cancer tissue in group A were higher than those in para-cancer tissue, MMP-2 expressing rate and intensity in cancer tissue in group B were not significantly different from those in para-cancer tissue. MMP-2 expressing intensity in cancer tissue in group A was much higher than that in group B, suggesting that MMP-2 expression could reflex malignancy of HCC. HCC with a high MMP-2 expression had a high recurrence rate. MMP-2 expressing rate in cancer tissue of the two groups was not significantly different, suggesting that MMP-2 is abnormally expressed in HCC, its expressing intensity is related to the prognosis of HCC.

Relationship of VEGF expression with prognosis of HCC
Vascularization is an important link in tumor growth, invasion and metastasis. Tumor blood vessels not only provide nutrition needed for tumor growth, but also provide pathways for spreading tumor cells. Vascular endothelial growth factor (VEGF) could promote cell proliferation and vascularization, and is

closely related to growth, invasion and metastasis of HCC. Expression of VEGF in HCC tissue is positively correlated with growth and metastasis of HCC, it could be a marker for determining the prognosis of HCC. Positive particles of VEGF are located in plasm of tumor cells. Some researchers reported that HCC patients with high VEGF expression had poor prognosis[24-28]. This study showed that VEGF expressing rate and intensity in cancer tissue in group A were higher than those in para-cancer tissue, VEGF expressing rate and intensity in cancer tissue in group B were not significantly different from those in para-cancer tissue. VEGF expressing intensity in cancer tissue in group A was much higher than that in group B, suggesting that VEGF expression could reflex malignancy of HCC. HCC with a high VEGF expression had a high recurrence rate. VEGF expressing rate in cancer tissue of the two groups was not significantly different, suggesting that VEGF is abnormally expressed in HCC, its expressing intensity is related to the prognosis of HCC.

Clinical value of this study

Ultrasound guided biopsy becomes a kind of mature diagnostic technique for HCC. It was shown in this study that characters of HCC with recurrence within 1 year after tumor resection were c-*myc*, Ki-67, MMP-2 and VEGF high expression in cancer tissue. If prognosis of HCC patients could be determined through quantitative analysis of biopsied tissue stained with immunohistochemistry before treatment, then theoretical fundamentals for HCC patients to select therapy method could be provided.

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