Expression of Heparanase and nm23–H1 Protein in Hepatocellular Carcinoma and Cirrhosis Tissues

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Abstract **Objective** To investigate the expression of heparanase and nm23−H1 protein in the tissues of hepatocellular carcinoma (HCC) and their clinical significance. **Methods** immunohistochemistry was employed to detect the expression of heparanase and nm23−H1 protein in 72 cases of HCC, 62 cases of cirrhosis and 23 cases of normal liver tissues with no preoperative treatment. **Results** The positive rate of heparanase in HCC tissues was significantly higher than that in cirrhosis and normal liver tissues, and it was obviously lower in HCC tissues of clinical TNM stage Ⅰ, Ⅱ and in cases without metastasis than that in the stage Ⅲ, Ⅳ, and the group with metastasis. The heparanase expression in the groups of AFP≥400μg/L, in portal vein tumor embolus and in multiple tumor nodes were significantly higher than that in the groups of AFP<400μg/L, without tumor embolus and single node. The positive rate of nm23−H1 in HCC tissues was significantly lower than that in cirrhosis and normal liver tissues, in HCC tissues in the clinical TNM stage Ⅰ, Ⅱ was obviously higher than that in the stage Ⅲ, Ⅳ, in the cases without metastasis it was significantly higher than in the group with metastasis. The nm23−H1 expression in the groups of AFP≥400μg/L and portal vein tumor embolus were lower than that in the groups of AFP<400μg/L and without tumor embolus. In addition, there was negatively correlation between the expressions of heparanase and nm23−H1. **Conclusion** The over−expression of heparanase and low expression of nm23−H1 may play important roles in the pathogenesis, development and metastases of HCC. Combination of the heparanase and nm23−H1 expressions may be helpful in diagnosing and predicating the biological behavior of HCC patients.

Key words Heparanase; Nm23−H1; Hepatocellular carcinoma; Immunohistochemistry

Hepatocellular carcinoma (HCC) is a common malignancy currently with the increasing occurrence both in the far eastern Asian countries and in the US [1]. The principal lethal factor of HCC dues to distant metastasis [2,3]. In the past decades, studies of HCC metastasis have been practically focused on some genes, for example heparanase and nm23−H1. The roles of heparanase in tumor invasion, metastasis and angiogenesis have been extensively established [4~8], and nm23−H1 is a candidate gene for the suppression of cancer metastasis. Several studies[9~11] showed that reduced nm23−H1 expression was closely related to metastatic progression with poor prognosis. However the relationship between the expressions of heparanase and nm23−H1 protein is poorly understood in HCC metastasis and recurrence. In this study, heparanase and nm23−H1 protein in 72 HCC cases were tested and analyzed to get insight into the relationship between the expressions of these genes and the clinicopathological parameters and post−operative metastasis and recurrence of HCC, providing the basis for further study and overall evaluation of heparanase and nm23−H1.

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MATERIALS AND METHODS

Clinic materials

All cases were randomly chosen from the hepatectomies performed over a 1~2 year span in the First Affiliated Hospital, Guangxi Medical University, China between May, 2002 and December, 2003. Among them, there were 72 cases of HCC, 62 cases of cirrhosis and 23 cases of normal control from the surrounding liver cavernous haemangioma tissues. Clinicopathologic

variables of sex, age, presurgical serum alpha-fetoprotein (AFP), histologic grades, clinical stages, portal vein tumor embolus, tumor capsular infiltration, tumor nodes and tumor size were checked through medical records, retrospectively. The age of HCC patients was ranged 22~75 years old, with a median of 47.1 years old. Among 72 patients, 63 were male, 9 female, forty-four cases were with presurgical serum AFP positive (≥

Table 1 The expression of heparanase and nm23-H1 in HCC

Factors		No. of patients	heparanase				nm23-H1			
			+	rate(%)	χ^2	p-value	+	rate(%)	χ^2	p-value
Tissues	HCC	72	29	40.28			13	18.11		
	Cirrhosis	62	4	6.45	⋆		23	37.10	⋆⋆	
	Normal liver	23	1	4.35			10	43.48		
Age	≥50	31	10	32.26	1.198	0.231	8	25.81	1.476	0.14
	<50	41	19	46.34			5	12.20		
Sex	Male	68	28	41.18	0.637	0.61	13	19.12	0.959	0.545
	Female	4	1	25			0	0		
Differentiation	Well	6	3	50			3	50		
	Moderately	44	17	38.64	⋆		8	18.2	⋆⋆	
	Poorly	22	9	40.91			2	9.1		
Clinical stage	Ⅰ Ⅱ	37	8	21.62	3.295	0.001	12	32.4	3.238	0.001
	Ⅲ Ⅳ	35	21	60			1	2.9		
Metastasis and recurrence	Yes	31	19	61.29	3.978	0.000	1	3.2	2.738	0.000
	No	33	5	15.15			10	30.3		
Serum AFP (μg/L)	≥400	44	23	52.27	2.583	0.01	3	6.82	3.086	0.02
	<400	28	6	21.43			10	35.71		
Portal vein tumor embolus	Presence	31	20	64.52	3.621	0.000	1	3.23	2.825	0.005
	Absence	41	9	21.95			12	29.27		
Tumor capsular infiltration	No capsular or capsular infiltration	49	21	42.86	0.647	0.518	6	12.24	1.858	0.063
	No capsular infiltration	23	8	34.78			7	30.43		
No. of tumor nodes	Multiple	29	19	65.52	4.048	0.000	3	10.34	1.814	0.07
	Single	43	10	23.26			10	23.26		
Tumor size(cm)	≥5	44	21	47.73	1.604	0.109	7	15.91	0.589	0.556
	<5	28	8	28.57			6	21.43		

⋆heparanase: HCC versus cirrhosis: χ^2=4.424, p=0.000; HCC versus normal liver: χ^2=3.363, *P*=0.001; cirrhosis versus normal liver: χ^2=0.473, *P*=0.636.

well-differentiated versus moderately-differentiated: χ^2=0.528, *P*=0.673; well-differentiated versus poorly-differentiated: χ^2=0.392, *P*=0.764; moderately-differentiated versus poorly-differentiated: χ^2=0.177, *P*=0.86.

⋆⋆nm23-H1: HCC versus cirrhosis: χ^2=2.595, *P*=0.009; HCC versus normal liver: χ^2=3.806, *P*=0.000; cirrhosis versus normal liver: χ^2=1.634, *P*=0.102.

well-differentiated versus moderately-differentiated: χ^2=1.747, *P*=0.222; well-differentiated versus poorly-differentiated: χ^2=2.277, *P*=0.141; moderately-differentiated versus poorly-differentiated: χ^2=0.964, *P*=0.335.

400μg/L), 28 negative. All cases were initial hepatectomies in order to avoid the secondary changes of healing post biopsy. In the group of cirrhosis, 41 were male, 21 female, their ages ranged 15~74 years old, with a median of 43.3 years old. In the group of normal control, 16 were male, 7 female, Their ages ranged 18~72 years old, with a median of 42.3 years old. Written informed consent was obtained from the patients and clinicians to use the samples for research. The histopathologic diagnoses were made according to the WHO international histologic classification of HCC, there were 6 cases with well differentiated, 44 cases moderately differentiated, 22 cases poorly differentiated. According to clinic tumor-node metastasis (TNM) standard, their clinical stages were stage Ⅰ, 5 cases; stage Ⅱ, 32 cases; stage Ⅲ, 13 cases; stage Ⅳ, 22 cases. Clinical information was obtained from the records. Among 72 HCC patients, 64 cases had the follow-up data by medical records, telephone and mail, among which there were 31 cases with metastasis in 20 months, 33 cases without. Histopathological diagnosis and classification were made by the same pathologists.

Immunohistochemical staining

For the immunohistochemical study, the method was employed as described [18] performed with the polyclonal antibody: anti-heparanase (Boster Corp, Wuhan, China) and the monoclonal antibody: anti-nm23-H1 (Maxim Corp, Fuzhou, China). Sections of the gastric carcinoma tissue were used as positive controls for heparanase and normal gastric tissues were the positive controls for nm23-H1. Negative control experiments were carried out by substituting the primary antibody with phosphate buffered saline (PBS). The positive signal for heparanase and nm23-H1 appeared as yellow-brown staining in cytoplasm of the cells. Two independent observers scored 300 cells per slide as positive or negative. The results from these two observers were averaged to obtain the percentages of positive cells per sample [11]. In all tumors diagnosed as positive, more than 25% of cells reacted with the antibody.

Statistical analysis

All collected data were analyzed with a statistical program (SPSS 13.0 for Windows). Chi-square test and Pearson correlation analyses were employed, $P<0.05$ was considered as statistically significant.

RESULTS

Expression of heparanase in HCC and the relationship with the clinic-pathological parameters

As showed in table 1, the positive rate of heparanase expression in HCC tissues was significantly higher than that in the cirrhosis 6.45%($\chi^2=4.424$, $P=0.000$)and normal liver tissues($\chi^2=3.363$, $P=0.001$). The expression of heparanase in HCC tissues in the clinical TNM stage Ⅰ, Ⅱ was obviously lower than that in the stage Ⅲ, Ⅳ ($\chi^2=3.295$, $P=0.001$). Heparanase expression in the cases without metastasis within 20 months was significantly lower than that in the group with metastasis ($\chi^2=3.978$, $P=0.000$). Heparanase expression in the groups of AFP≥400μg/L, portal vein tumor embolus and multiple tumor nodes were significantly higher than that in the groups of AFP<400μg/L($\chi^2=2.583$, $P=0.01$), without tumor embolus($\chi^2=3.621$, $P=0.000$)and single node ($\chi^2=4.048$, $P=0.000$). However it was not correslated with patients´ age, sex, histological classification, cirrhosis, tumor capsular infiltration or tumor diameter.

Expression of nm23-H1 in HCC and the relationship with the clinic-pathological parameters

As also showed in table 1, the positive rate of nm23-H1 in HCC tissues was significantly lower than that in the cirrhosis ($\chi^2=2.595$, $P=0.009$)and in normal liver tissues ($\chi^2=3.806$, $P=0.000$). The expression of nm23-H1 in HCC tissues in the clinical TNM stage Ⅰ, Ⅱ was obviously higher than that in the stage Ⅲ, Ⅳ ($\chi^2=3.238$, $P=0.001$), nm23-H1 expressed in the cases without metastasis within 20 months significantly lower than that in the group with metastasis ($\chi^2=2.738$, $P=0.000$), in the groups of AFP ≥400μg/L and portal vein tumor embolus were significantly higher than in the groups of AFP<400μg/L ($\chi^2=3.086$, $P=0.02$)and without tumor embolus ($\chi^2=2.825$, $P=0.005$), while it was not associated with patients´ age, sex, histological classification, cirrhosis, tumor capsular infiltration, tumor nodes or tumor diameters.

The relationship between the expression of heparanase and nm23-H1

Linear correlation analysis showed that there was a linearity negative correlation between the expression of heparanase and nm23-H1 in HCC ($r=-0.271$, $P=0.001$).

DISCUSSION

Heparanase produced by tumor cells degrades heparan sulfate chains and thereby destroys cell-cell and cell-matrix attachments, thus, by degrading the extracellular matrix, the tumor cells can readily penetrate the extracellular matrix or the basement membrane. Heparanase can also potentiate angiogenesis through the release of heparin-binding growth factors and heparan sulfate degradation fragments that promote binding and activation of these heparin-binding growth factors and their receptors[12]. In the present study, the expression of heparanase was significantly higher in HCC than that in the cirrhosis and normal liver tissues. Further more, the expression of heparanase in HCC tissues in the clinical TNM stage Ⅰ, Ⅱ, without metastasis, AFP<400μg/L, without tumor embolus and single node was obviously lower than that in the corresponding groups. The results suggested that the expression of heparanase was positively correlated to poorer prognosis, especially the higher tendency of tumor progression and post-operative metastasis and recurrence.

The nm23 was the first identified metastasis suppressor gene. In 1988, Steeg *et al.* [13] discovered murine nm23 cDNA by using differential colony hybridization between murine K-1735 melanoma cell lines that varied in metastatic potential in vivo. They found that the nm23 mRNA levels of two low metastatic potential cell lines were quantitatively higher than that of five related but highly metastatic cell lines. Later, the examination of protein levels exhibited a similar pattern [14].This discovery aroused much interest in the role of nm23 in progression of carcinomas. Up to now, eight members of the human nm23 family have been reported and are found in multiple subcellular compartments. Two highly homologous genes have been described so far, both located at the long arm of chromosome 17q21.3, coding for the 18.5 and 17 kD proteins nm23-H1 and nm23-H2, respectively. The nm23-H1 and nm23-H2 gene products have been shown to be identical to human nucleoside diphosphate (NDP) kinases A and B [14~16]. Our data showed that the expression level of nm23-H1 was significantly lower in cases of HCC than that in the cirrhosis and normal liver tissues, which indicated that there might exist loss expression of nm23-H1 gene in the HCC. In addition our study also demonstrated that nm23-H1 had relationship with metastasis, clinical stages, AFP level and portal vein tumor embolus, which implied that nm23-H1 had some effects of inhibiting metastasis of HCC and defect of nm23-H1 can also predict HCC metastasis and recurrence.

Cancer recurrence in the remnant liver is considered to be the most important prognostic factor after hepatic resection in patients with HCC[17]. Intrahepatic metastasis and portal vein tumor thrombus are closely correlated with cancer recurrence in the remnant liver[18]. In the process of the HCC metastasis and recurrence, various kinds of genes with different functions effect co-operatingly. Heparance and nm23-H1 have absolutely different biochemical mechanism in HCC. Heparance impels tumor to progress and metastasize by degrading HSPG and releasing angiogenesis factors. As for the mechanism of action of nm23-H1, many scholars have inferred that nm23-H1, by interacting with GTPase-activating protein (GAP) protein, participates in cellular signal transduction, cell differentiation and metastasis. In the present study, the expression of heparanse was negatively related to the expression of nm23-H1, which suggested there may be some relationship between the two genes, but it needs further study. However, it may be valuable to test heparanse and nm23-H1 expression in HCC to predict the clinical metastasis and recurrence.

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