PO Box 2345, Beijing 100023, China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

WJG, 2000 February; 6(1):61-65
World Journal of Gastroenterology
 ISSN 1007-9327

Recurrence or metastasis of HCC: predictors, early detection and experimental antiangiogenic therapy

Jiang YF, Yang ZH and **Hu JQ**

Subject headings carcinoma, hepatocellular; neoplasm metasta sis; angiogenesis; liver neoplasms

Abstract

AIM To investigate the predictors for recurrence or metastas is of HCC, and to evaluate the effect of antiangiogenic therapy on the growth of transplantable human HCC in nude mice.

METHODS RT-PCR was used to measure the expression of matrix metalloproteinase-9 (MMP-9) and vascular endothelial growth factor (VEGF) in 56 pairs of nontumorous liver and tumor samples. Sixty blood samples from human HCC were examined by nested RT-PCR to find out AFP mRNA. Recombinant human endostatin and polyclonal antibody against VEGF were administered to treat human HCC transplanted in nude mice.

RESULTS Thirty of 56 HCC samples showed stronger expression of MMP-9 in tumorous tissues than in nontumorous tissues. Fifteen of the 26 patients with relative expression level of MMP-9 more than 0.34 developed tumor recurrence or metastasis, whereas only 7 of 30 patients with relative expression le velless than 0.34 developed tumor recurrence ($P<0.05$). There was no significant difference in the relative expression level of VEGF between patients with postoperative recurrence or metastasis and those without recurrence. AFP mRNA was detectable in 53.3% of patients with HCC. The sensitivity and specificity of AFP mRNA as a marker to detect hematogenous dissemination of HCC cells was 81.8% and 84.4%, respectively. Recombinant human endostatin and polyclonal an tibody against VEGF inhibited the growth of transplantable HCC in nude mice by 52.2% and 45.7%, respectively.

CONCLUSION MMP-9 expression in HCC correlates with the postoperative recurrence or metastasis of HCC. Patients with high level of MMP-9 expression in HCC are susceptible to metastasis. AFP mRNA could serve as an indicator of hematogenous spreading of HCC cells in circulation and a predictor of recur rence or metastasis of HCC. Antiangiogenesis may be an adjuvant therapy for HCC.

INTRODUCTION

Hepatocellular carcinoma (HCC) is a common cancer in China and in other Asian countries or in south part of Africa. Although some advances have been achieved in the diagnosis and treatment of HCC, the long-term outcome for patients with HCC is still very poor[1]. The prognosis for HCC depends mainly on the clinico-pathological characteristic regarding invasion and metastasis. The major obstacle to the improvement of the prognosis for HCC is the high incidence of recurrence or metastasis after routine surgical treatment or transcatheter arterial chemoembolization (TACE). Therefore, the following prospective study was designed to inves tigate molecules responsible for postoperative recurrence of HCC by focusing on matrix metalloproteinase-9 (MMP-9) and vascular endothelial growth factor (VEGF). Furthermore, we also studied the detection of hematogenous spreading of HCC cells at a relatively early stage, and the experimental anti-angiogenic therapy for HCC in an animal model.

MATERIALS AND METHODS

Materials

Tumorous and nontumorous liver samples were obtained from 56 HCC patients who underwent hepatectomy. Peripheral venous blood samples were collected from 60 patients with HCC and 30 subjects as control (10 patients with liver cirrhosis and 20 healthy donors). Recombinant human endostatin and polyclonal antibody against VEGF were used to treat HCC transplanted in nude mice.

Yang Fu Jiang, Zhi Hua Yang and Jin Qun Hu
Cancer Institute, Chinese Academy of Medical Sciences & Peking Union Medical College, Beijing 100021, China
Dr. Yang Fu Jiang, male, born on 1971-03-28 in Chongqing, graduated from West-China University of Medical Sciences in 1993, got Ph.D. at Peking Union Medical College in 1999. Now postdoctoral fellow at Albert Einstein College of Medicine, U.S.A, having 4 papers published.
Correspondence to: Dr. Zhi Hua Yang, Department of Molecular Biology, Cancer Institute, Chinese Academy of Medical Sciences, Beijing 100021, China
Tel. +86-10-67781331 Ext. 8439, Fax. +86-10-67782259
Email.Zhyang@public.bta.net.cn
Received 1999-07-03 **Accepted** 1999-09-15

Methods

RNA preparation The total tissue RNA was extracted with TRIzol (Life Technologies, Inc. Gaithusburg, USA), precipitated in ethanol and resuspended in sterile RNAase-free water for storage at -70 ℃.

Reverse transcription Moloney murine leukemia virus reverse transcriptase (M-MLV RT) was used to synthesize a complementary DNA strand in the presence of random primer from 6 μg single stranded RNA.

PCR amplification Primer sequences. Sense primer for VEGF: 5'-TTGCTGCTCTACCTCCAC-3'. Antisense primer for VEGF: 5'-AATGCTTTCTC-CGCTCTG-3'. Sense primer for MMP-9: 5'-CG-GAGCAGGAGACGGGTAT-3'. Antisense primer for MMP-9: 5'-TGAAGGGGAAGACG-CACGCACAGC-3'. Sense primer for internal control of β_2-MG: 5'ACCC CCACTGAAAAAGATGA-3'. Antisense primer for β_2-MG: 5'-ATCTTCAAACCTCCATGATG-3'. All primers were synthesized by Shanghai Sangon Biotec hnique Company. PCR reaction: the 25 μL of PCR mixture contained 2 μL of the synthesized cDNA solution, 2.5 μL of 10× polymerase reaction buffer, 1.5 mM $MgCl_2$, 200 μM-each of dCTP, dATP, dGTP, dTTP, 10 pmol of each primer (sense and antisense); and 1 unit of Taq DNA polymerase. The PCR mixture for VEGF was subjected to 40 cycles PCR amplification using protocol TOUCHDOWN in PTC 100 programmable thermal cycler (MJ Research, USA). Cycle conditions for amplifying MMP 9 included a 94 ℃ denaturation (30 s, first cycle 1 min), a 60 ℃ annealing (30 s), and a 72 ℃ extension (60 s). After the final cycle, tubes were placed at the extension temperature for 5 min.

Assay of PCR production A volume of 7 μL PC R products was added in 1.5% agarose gel containing 0.5 μg/mL EB, after electrophoresis, the gel was placed under ultraviolet ray to analyze the results. The density and area of each band were measured using Image Master VDS software (Pharmacia, Sweden). The relative mRNA level of VEGF of MMP-9 gene in tumor or nontumorous tissues was calculated using the house-keeping gene β_2-MG as an internal control.

Nested RT-PCR amplifying human AFP A 5-mL heparinized blood sample from each patient was taken for AFP mRNA determ ination. Cell pellets were obtained from heparinized blood samples. The total RNA was extracted with TRIzol and resuspended in RNAase-free water. Reverse transcription was performed using random primers and PCR using specific AFP primers. β_2-microglobulin mRNA was co-amplified during the RT-PCR test as an internal control. The 25 μL of first PCR mixture containing external sense and antisense primer for AFP were subjected to 40 cycles PCR amplification using protocol TOUCHDOWN in PTC-100 programmable thermal cycler (MJ Research, USA). A volume of 8 μL PCR products was added in 2% agarose gel for electrophoresis. If no specific band of 176 base pairs was observable, 2 μL PCR product was ream plified with internal primers. The final product was electrophoresed on 2% agarose gel for the specific band of 101 base pairs.

Experimental antiangiogenic therapy of HCC Preparation of recombinant human endostatin. The human endostatin cDNA which encoded 184 amino-acids was cloned from human fetal liver. The recombinant human endostatin was expressed in a prokaryotic system. The recombinant endostatin unde rwent denaturation in 8 mol/L urea and was refolded in Sephadex G-100 column.

The polyclonal antibody against human VEGF was prepared in our lab. Briefly, the recombinant human VEGF165 was expressed in a prokaryotic system, which was administered as antigen to stimulate the production of antibody against VEGF in rabbits. The antiserum was purified using affinity chromatography column of Sepharose CL-4B-VEGF.

RESULTS

Postoperative recurrence or metastasis of HCC patients

In the total of 56 HCC patients, 22 (39.3%) had relapsed within 20 months after operation. Fifty percent of the recurrence occurred within 6 months after operation. Tumor recurrence had no significant correlation with tumor size or degree of pathological differentiation (P>0.05, Table 1).

Table 1 Relationship between clinicopathological characteristics a nd recurrence or metastasis of HCC

Parameter	*n*	Recurrence of metastasis	
		Yes	No
Tumor size (cm)			
≤5 cm	29	10	19
>5 cm	27	12	15
Liver cirrhosis			
Absent	8	3	5
Present	48	19	29
Tumor differentiation			
Well	12	4	8
Moderate	23	8	15
Poor	9	5	4
Not determined	12	5	7
Clinical staging			
Stage I	2	1	1
Stage II	33	10	23
Stage III	18	8	10
Stage IV	3	3	0

MMP-9 expression in HCC correlated with tumor recurrence

In 35 of the 56 HCC samples, transcripts of MMP-9 were detected, 27 of 35 these samples showed significant elevation of MMP-9 expression (> 2 fold) compared with nontumorous liver tissues. Among the 30 patients with a relative expression level of MMP-9 in HCC less than 0.34, only 7 cases developed tumor recurrence, whereas 15 of the 26 patients with relative expression level of MMP-9 more than 0.34 had developed tumor recurrence or metastasis. Moreover, among the 22 HCC patients who suffered from postoperative recurrence or metastasis, 16(88.8 %) tumors (T) had significantly elevated level of MMP-9 expression compared with nontumorous liver tissues (N) (T/N> 2), while only 11(32.3%) of 34 HCC patients who had not yet relapsed had significantly increased level of MMP-9 expression in HCC. Patients with high level of MMP-9 expression in HCC were sus ceptible to tumor recurrence or metastasis.

VEGF expression in HCC not correlated with tumor recurrence

Transcripts for VEGF were detected in 48 of 56 HCC samples and in 36 of 53 nontumorous liver tissues. The level of VEGF expression was elevated significantly in 52% HCC samples compared to nontumorous liver tissues (Figure 1). T he level of VEGF expression in HCC was not correlated with tumor size or the degree of pathological differentiation. Among the 25 patients with significantly elevated level of VEGF expression in HCC, 12 cases (48%) had tumor recurrence, whereas 10 of 31 patients with similar or decreased level of VEGF expressi on had tumor recurrence.

AFP mRNA in peripheral venous blood from clinical samples

The frequency of positive cases in 60 patients with HCC was 53.3% (32/60) (Figure 2). The frequency of AFP mRNA positivity in patients with liver cirrhosis and in healthy donors was 10% and 5%, respectively.

Relationship between AFP mRNA expression and intra or extra-hepatic metastasis

In 32 patients with detectable AFP mRNA in peripheral blood, 27 patients (84.4%) were accompanied with metastasis. Of the 11 patients with detectable AFP mRNA in peripheral blood but without metastasis at collected samples, 6 cases developed metastasis or tumor recurrence later. Six or 28 patients without detectable AFP mRNA developed metastasis. Serum AFP level was not correlated with cancer me tastasis or recurrence.

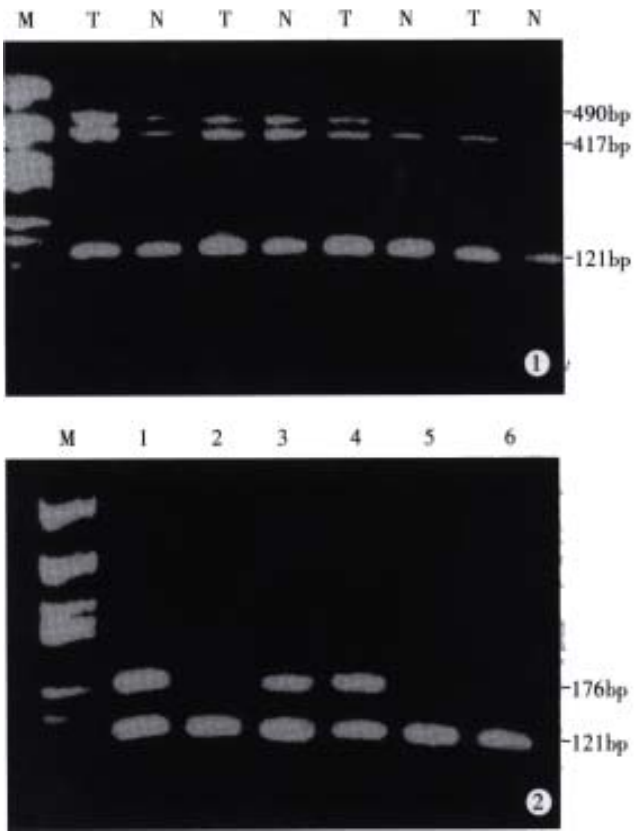

Figure 1 Electrophoresis analysis of RT-PCR product samplified from cDNA obtained from HCC and nontumorous liver tissues. M, DNA m arker (PGEM-7ZF(+) / *Hae*-III); T, tumor; N, nontumorous liver tissues. The positive bands of 417bp, 490 bp, and 12 1bp represent VEGF165, VEGF189, and β_2- MG, respectively.

Figure 2 Electrophoresis analysis of RT-PCR product s from cDNA obtained from peripheral venous blood of patients with HCC. M, DNA marker (PGEM-7ZF(+) / *Hae*-III). Lane 1, lane 3, and lane 4 showed positive bands for AFP (176bp). All lanes showed positive bands for β_2-MG (121bp).

Relationship between AFP mRNA expression and therapy

Of 60 patients with HCC, 14 cases underwent surgical treatment, 35 received transcatheter arterial chemoembolization (TACE). Fifteen of 33 samples collected before operation or TACE showed detectable AFP mRNA in peripheral venous blood. Twenty-one of 35 samples collected after therapy had detectable AFP mRNA (P>0.05).

Recombinant human endostatin and polyclonal antibody against VEGF inhibited the growth of HCC

We used *E. coli*-derived human endostatin to study the effect of endostatin therapy on primary liver cancers. BEL-7402 hepatocellular carcinoma was implant ed into nude mice. The refolded protein of recombinant endostatin was administered to the

mice via peritoneal injection once daily. The growth of primary tumors was inhibited by 52.2% at a dose of 7.5 mg/kg as compared with control mice treated with saline alone. The effect of polyclonal antibody against VEGF on the growth of primary liver cancers was also studied. The polyclonal antibody against VEGF was administered to the mice via peritoneal cavity injection at a dose of 10 mg/kg once daily. The growth of primary tumors was inhibited by 45.7%.

DISCUSSION

The long-term outcome of patients with HCC is still very poor. The major obstacle to the improvement of prognosis for HCC patients is the high incidence of pos toperative recurrence or metastasis. The five year recurrence rate in liver can cers is as high as 40%-70%[2]. Therefore, it is very important to investigate the molecular changes that correlate with recurrence or metastasis of HCC, which is useful to screen HCC patients with high risk of recurrence. Furthermore, the early detection of hematogenous spreading of HCC cells or recurrent lesions and the effective management of recurrent lesions are also important steps to improve the therapeutic effects. We performed a series of study on the recurrence or metastasis of HCC by focusing on some key steps mentioned above.

Metastasis is the spread of cancer from a primary tumor to distant sites of the body and is a defining feature of cancer. Escape of cells from the primary tumor, intravasation and extravasation are some necessary steps in the process of cancer metastasis[3]. There are a series of collagen containing structural barriers that cancer cells must pass in all steps mentioned above. MMPs are a family of secreted or transmembrane proteins that are capable of digesting extra cellular matrix and basement membrane. MMP-9 is believed to be capable of degra ding type IV collagen, which is a major constituent of basement membrane[4]. Highly invasive tumor cells then, would be expected to secrete large amounts of proteolytic enzyme. The results presented here indicated that MMP-9 mRNA was expressed more frequently in HCC tissues than in corresponding nontumorous ones, and that the degree of MMP-9 mRNA expression in tumors was elevated in 30 of 38 samples with transcripts for MMP-9 compared with corresponding nontumorous tissues. With regard to the correlation of MMP-9 mRNA and tumor recurrence or metastasis, the tumors with high level of MMP-9 expression were more susceptible to relapse or metastasis than those with low level of MMP-9 expression. These results revealed that MMP-9 is an important molecule which participates in the invasion of HCC. MMP-9 expression in HCC is of prognostic significance. MMP-9 can also serve as a potential target for prevention and treatment of tumorr ecurrence or metastasis.

Angiogenesis, the recruitment of new blood vessels, is required for the primary and metastatic tumors to grow beyond minimal size[5]. Vascular endothelial growth factor (VEGF) is an important factor which can promote the proliferation of endothelial cells and the development of new blood vessels[6]. In the present study, we investigated the expression of VEGF mRNA in HCC, we also examined the correlation between VEGF mRNA expression and the recurrence or metastasis of HCC. We found that VEGF mRNA was expressed in most of HCC specimens and nontumorous liver tissues (87.3% *vs* 67.9%). Fifty-two percent HCC specimens exhibited stronger expression of VEGF mRNA in tumorous tissues than in nontumorous ones. No apparent correlation was observed between VEGF expression and tumor size or the grading of tumor differentiation. There was also no significant correlation between VEGF mRNA expression level and tumor metastasis. In addition, the growth of HCC could be inhibited by antibody against VEGF. All these data indicated that VEGF may be involved in the growth of HCC, however, the level of VEGF mRNA expression cannot reflect the potential metastasis of HCC, namely, even if VEGF expression is not strong in HCC, tumor recurrence or metastasis may also occur.

Escape of cells from primary tumor into blood circulation is an indispensable step in the process of blood borne metastasis. The detection of tumor cells in per ipheral blood by means of RT-PCR is a very attractive hypothesis[7]. The oretically, the test could be useful in assessment of prognosis and in predicting the increased probability of metastases. We choose the RT-PCR mRNA AFP as an indicator of liver cells to determine the clinical relevance of the test. Thirty -two of sixty HCC patients involved in this study has positive AFP mRNA test re sult. One tenth patients with liver cirrhosis and 1/20 healthy donors had positive AFP mRNA in peripheral blood. These data suggested that AFP mRNA in peripheral blood is a sensitive marker of presence of HCC cells in circulation, although false positive results may appear occasionally. The frequency of positive cases in patients with metastases was significantly higher than that in patients without occult metastases at collected samples (93.9% *vs* 29.7%), which indicated that the presence of AFP mRNA in peripheral blood correlated with metastasis of HCC. Six of 11 patients who were AFP mRNA-positive and metastasis-free at collected samples had clinically evident recurrence or metasta sis later. Among 26 negative AFP mRNA, metastasis-free patients, 22

patients re mained recurrence-free during the period observed. These data suggest that the detection of AFP mRNA in peripheral blood by means of RT-PCR was useful in predicting the increased probability of metastases, and in identifying a subpopulation of patients with HCC who are at high risk of recurrence. With no significant difference in AFP mRNA expression before and after surgical treatment or TACE, local treatment is insufficient to prevent tumor recurrence. For patients with tumor cells in circulation, more vigilant follow-up or more aggressive management, such as immunotherapy or systemic chemotherapy, should be made.

The effect of conventional chemotherapy on preventing cancer metastasis is not satisfactory mainly due to the multi-drug resistance[8]. Therefore, it is highly necessary to search for new modalities in treatment of cancer metastasis. It is well known that the development of a tumor requires oxygen and nutrients, which are supplied through neovascularization. Therefore, antiangiogen esis and the suppression of the development of neovascularization may offer a novel strategy in overcoming the development and the metastasis of solid tumors[9]. VEGF can promote the development of neovasculization, the antibody against VEGF may inhibit angiogenesis and consequently suppress the growth of tumor. We found that polyclonal antibody can inhibit the growth of human HCC transplanted in nude mice by 45.7%, but not inhibit the growth of tumor thoroughly possibly due to the fact that VEGF was not the only growth factor in the development of neovascularization. Endostatin is an endothelial-specific negative regulator of angiogenesis, previous report suggested that it inhibit the growth of some types of tumor in animal model[10]. We found that the recombinant human endostatin inhibited the growth of human HCC by 52.2%. In addition, both endostatin and antibody against VEGF could inhibit the lung metastasis of murine breast cancer dramatically in an animal model by 68.9% and 71.4%, respectively. It was also reported that antibody against VEGF could inhibit the experimental liver metastasis of human colon cancer in a mouse model[11]. These data suggest that endostatin and antibody against VEGF are useful in prevention or treatment of tumor recurrence or metastasis of HCC. It warrants further research to investigate whether antiangiogenic therapy combined with routine chemotherapy or radiotherapy can improve the effect of tre atment dramatically.

REFERENCES

1 Wang YH, Liu YX, Feng YQ, Zhou NX, Gu WQ, Huang ZQ, Zhao HL, Ji XL. Multivariate analysis of prognostic factors after hepatectomy for primary liver cancer.*Zhonghua Waike Zazhi*,1999; 37:18-21

2 Wu MC. Clinical research advances in primary liver cancer. *WJG*, 1998;4:471-474

3 Aznavoorian S, Murphy AN, Stetler-stevenson WG, Liotta LA. Molecular aspects of tumor cell invasion and metastasis. *Cancer*, 1993;71:1368-1383

4 Powell WC, Matrisian LM. Complex roles of matrix metalloproteinases in tumor progression.*Curr Topics Microbiol Immunol*, 1996;213:1-21

5 Folkman J. Tumor angiogenesis: therapeutic implications. *N Engl J Med*, 1971;285:1182-1186

6 Claffey KP, Robinson GS. Regulation of VEGF/VPF expression in tumor cells: consequences for tumor growth and metastasis. *Cancer Metastasis Rev*, 1996;15:165-176

7 Barbu V, Bonnand AM, Hillaire S, Coste T, Chazouilleres O, Gugenheim J, Boucher E, Poupon R, Poupon RE. Circulating albumin messenger RNA in hepatocellular carcinoma: results of a multicenter prospective study. *Hepatology*,1997;26:1171-1175

8 Goldstein LJ, Galski H, Fojo A, Willingham M, Lai SL, Gazdar A, Pirker R, Green A, Crist W,Brodeur GM, Lieber M, Cossman J, Gottesman MM, Pastan I. Expression of a multidrug resistance gene in human cancers. *J Natl Cancer Inst*,1989;81:116-124

9 Fidler IJ, Ellis LM. The implications of angiogenesis for the biology and therapy of cancer metastasis. *Cell*, 1994;79:185-188

10 O'Reilly MS, Boehm T, Shing Y, Fukai N, Vasios G, Lane WS, Flynn E, Birkhead R, Olsen BR, Folkman J. Endostatin: an endogenous inhibitor of angiogenesis and tumor growth. *Cell*, 1997;88: 277-285

11 Warren RS, Yuan H, Matli MR, Gillett NA, Ferrara N. Regulation by vascular endothelial growth factor of human colon cancer tumorigenesis in a mouse model of experimental liver metastasis. *J Clin Invest*, 1995;95:1789-1794

Edited by Wu XN
Proofread by Miao QH