Clinical Observations

HEMATOGENOUS SPREADING OF HEPATOCELLULAR CARCINOMA CELLS: POSSIBLE PREDICTOR OF RECURRENCE OR METASTASIS

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ABSTRACT

Objective: To investigate the status of hematogenous spreading of hepatocellular carcinoma (HCC) before or after surgical treatment or transcatheter arterial chemoembolization (TACE) and to elucidate the significance of peripheral blood Alpha-fetoprotein (AFP) mRNA expression in predicting recurrence or metastasis of HCC. Methods: Peripheral venous bloods were collected from 60 patients with HCC, 20 of whom had received TACE before blood samples were collected, and from 30 subjects as control (10 cases with benign liver disorders, 20 healthy donors). AFP cDNA was amplified from 5 ml whole blood by nested reverse transcription polymerase chain reaction (RT-PCR). Results: Of the 60 patients with HCC, 32 cases (53.3%) had positive AFP mRNA in their peripheral blood. In 33 patients with intra-and/or extrahepatic metastasis, 27 (81.1%) were positive for AFP mRNA. In patients who didn't yet have metastasis when samples were collected, 11 (29.7%) gave positive AFP mRNA, 6 of whom developed tumor recurrence or metastasis after the samples were collected. The presence of AFP mRNA correlated with the stage of HCC and the presence of intrahepatic and/or extrahepatic metastasis, but did not correlate with tumor size and serum AFP level. There was no significant difference in AFP mRNA expression before and after surgical treatment or TACE. Conclusion: Detection of AFP mRNA by PCR provides a sensitive and specific assay of hematogenous dissemination of HCC. TACE can not prevent metastasis of HCC. Systemic chemotherapy or immunotherapy is needed to prevent occult or overt metastasis.

Key words: Liver cancer, AFP, Gene expression, Metastasis

Hepatocellular carcinoma (HCC) is a common cancer in China. Although some advances have been achieved for the diagnosis and treatment of HCC in recent years, the long-term outcome for patients with HCC remains very poor. The major obstacle to the improvement of prognosis for HCC is the high incidence of recurrence or metastasis after routine surgical treatment or thanscatheter arterial chemoembolization (TACE). Therefore, the present study was designed to investigate the value of AFP mRNA as a predictor for the risk of tumor recurrence or metastasis. In addition, the effect of TACE on the hematogenous spreading of hepatocellular carcinoma cells was also investigated.

MATERIALS AND METHODS

Patients and Samples

5 ml peripheral blood from 60 patients with HCC and 30 subjects as control (10 cases with benign liver disease and 20 healthy donors) were collected. 20 cases underwent transcatheter arterial chemoembolization (TACE) before blood samples were collected. After the samplings, 10 cases received surgical treatment, 17 cases underwent TACE.

Separation of Mononuclear Cells

The mononuclear cells were separated by Ficoll-Hypaque centrifugation method and the cells were rinsed twice with PBS.

RNA Preparation

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

Reverse Transcription

Moloney murine leukemia virus reverse transcripts (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

External (EX-sense and EX-antisense) and internal (IN-sense and IN-antisense) primer pairs were administered for the first PCR reaction or the second PCR reaction, respectively. EX-sense primer: 5'-ACTGAATCCAGAACACTGCATAG-3'. EX-antisense primer: 5'-TGCAGTCAATGCATCTTT-CACCA-3'. IN-sense primer: 5'-TGGAATAGCTT-CCATATTGGATTC-3'. IN-antisense primer: 5'-AAGTGGCTTGAACAAACTGG-3'. Primers were synthesized by Shanghai Shengong Biotechnique Company.

PCR reaction

The first PCR mixture contained 3 μl of the synthesized cDNA solution, 2.5 μl of 10 × polymerase reaction buffer, 1.5 mM $MgCl_2$, 200 uM each of dCTP, dATP, dGTP, and dTTP, 16 pmol of each external primer (EX-sense and EX-antisense), and 1 unit of Taq DNA polymerase. The of PCR mixture was subjected to 40 cycles PCR amplification using protocol TOUGHDWN in PTC-100 programmable thermal cycler (MJ Research, USA). β_2 microglobulin mRNA was co-amplified during the RT-PCR test as an internal standard.

A volume of 8 μl PCR product was added in 2% agarose gel containing 0.5 μg/ml. After electrophoresis, the gel was placed under ultraviolet ray to see whether the specific band of 176 base pairs was observable. If no specific band was observable, 2 μl PCR product was reamplified with internal primers for nested PCR. The final product was electrophoresised on 2% agarose gel for the specific band of 101 base pairs.

RESULTS

AFP mRNA from Clinical Samples

The frequency of AFP mRNA positive cases in 60 patients with HCC was 32 of 60 (53.3%), 21 of which were positive after the first PCR reaction (Figure 1), the remaining 11 cases gave positive AFP mRNA after the second PCR (Figure 2). The frequency of AFP mRNA positivity in patients with liver cirrhosis and in healthy donors was 1 of 10 and one of 20, respectively.

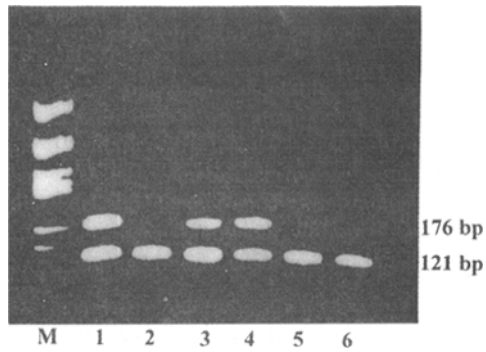

Fig 1. Electrophoresis analysis of the first RT-PCR products amplified from cDNA obtained from peripheral venous blood of patients with HCC. M, PGEM-7ZF(+)/Hae III marker (SABC); Lane 1, lane 3 and lane 4 showed positive bands of AFP (176bp) and positive bands of β_2-MG (121bp); Lane 2, lane 5 and lane 6 showed positive bands of β_2-MG.

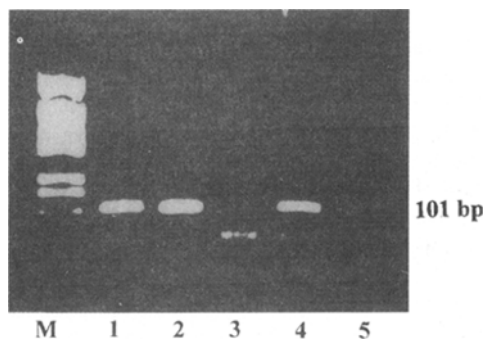

Fig 2. Electrophoresis analysis of nested RT-PCR products. M, PGEM-7ZF (+)/Hae III marker (SABC); Lane 1, lane 2 and lane 4 showed positive bands of AFP (101bp).

The 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. 21 cases suffered from tumor recurrence or metastasis before samples were collected. Of the 11 patients with detectable AFP mRNA in peripheral blood but without metastasis at sample collecting time, 6 cases developed metastasis

or tumor recurrence during the follow-up period observed. 6 of 28 patients without detectable AFP mRNA developed metastasis (Table 1). Patients with positive AFP mRNA in peripheral blood were far more susceptible to metastasis or tumor recurrence than those without detectable AFP mRNA ($P<0.01$), while the serum AFP level didn't correlate with cancer metastasis or recurrence.

Table 1. Relationship between AFP mRNA expression in peripheral blood and recurrence or metastasis of HCC

AFP mRNA	Total number	Intrahepatic metastasis	Extrahepatic metastasis	Portal venous invasion
Positive	32	20	4	3
Negative	28	5	1	0

According to TNM staging, the frequency of AFP mMNA positive cases in each stage was: 1 of 2 in stages I, 4 of 34 (11.7%) in stage II, 7 of 12 (58%) in stage III, 9 of 12 (75%) in stage IV. These results showed strong correlation with clinical severity. Although AFP mRNA positivity was more frequent in patients with large (exceeding 5 cm in diameter) tumors (67.7%) than in those with small (under 5 cm) tumors (37.9%), but without a significant statistical difference ($P>0.05$). There also significant difference in the frequency of AFP mRNA positivity between patients with high serum AFP level (exceeding 200ng/ml) and patients with serum AFP level less than 200 ng/ml ($P>0.05$).

Relationship between AFP mRNA Expression and Therapy

Of the 60 patients with HCC, 14 patients underwent surgical treatment, 35 received transcatheter arterial chemoembolization (TACE). 15 of 33 blood samples collected before surgical treatment or TACE showed detectable AFP mRNA in peripheral venous blood. 21 of 35 samples collected after TACE or surgical treatment had detectable AFP mRNA ($P>0.05$). 6 of 15 (40%) samples collected before TACE showed detectable AFP mRNA in peripheral venous blood, 11 of 18 (61%) samples collected after TACE showed detectable AFP mRNA ($P>0.05$).

DISCUSSION

The first choice of treatment for patients with HCC is surgical treatment or intervention therapy. However, the incidence of tumor recurrence was as high as 40%-70% after surgery or transcatheter arterial chemoembolization.[1] One of the major factors contributing to tumor recurrence is incomplete resection or some minimal residual disease, including tumor cells spreading hematogenously before surgical treatment. The major obstacle to the improvement of the long-term outcome for patients with HCC is the high incidence of recurrence or metastasis, even during the course of therapy. If metastasis can be sensitively determined at early stage, it can be expected that good outcome may be achieved with combined therapy administered in time. The preoperative level of serum AFP has not been considered to be a predictive factor for recurrence in previous studies. Nevertheless, according to this study and other reports, AFP and mRNA proved to be useful in the detection of circulating hepatocellular carcinoma cells, even if overt metastasis was not confirmed.[2-4] The detection of tumor cells in peripheral blood by means of RTPCR is a very attractive measure. According to this study, as a tissue specific transcripts, AFP mRNA was present in venous blood of 53.3% of patients with HCC, while serum AFP level exceeded 200ng/ml in only 36.7% of patients with HCC. Of 16 patients whose serum AFP level ranges from 25ng/ml to 200 ng/ml, 11 cases had positive AFP mRNA in peripheral venous blood. In addition, 6 of 22 patients whose serum AFP level was less than 25 ng/ml had positive AFP mRNA in peripheral venous blood. All these data indicate that AFP mRNA is also a valuable marker for the diagnosis of liver cancer. It's very useful to detect AFP mRNA in peripheral blood and AFP in serum.

In addition, AFP mRNA can serve as an indicator of hematogenous spreading of hepatocellular carcinoma cells in the circulation. The presence of AFP mRNA in peripheral blood correlates closely with metastasis or recurrence of HCC. The sensitivity and specificity of AFP mRNA as a marker to detect hematogenous dissemination or as a predictor of recurrence or metastasis of HCC reachs a degree of 81.8% and 84.4%, respectively. 6 of 11 patients without intra- or extra-hepatic metastasis at the time that the samples were collected developed tumor recurrence or metastasis later. These data indicate that the detection of AFP mRNA in peripheral blood by means of RT-PCR is useful in predicting the increased probability of metastasis or recurrence after radical therapy. The result also suggests that tumor cells are present in the peripheral blood of patients with small HCC, which indicats that the status of hematogenous spreading of HCC cells could not be ignored even if the liver tumor is small. This is consistent with the high incidence of tumor recurrence of small HCC.

There was no significant difference in AFP

mRNA expression between the group of blood samples collected before TACE and the group of samples collected after TACE, which indicates that circulating HCC cells could not be eliminated by TACE alone. This results were consistent with the results of some prospective and retrospective studies that showed TACE could not improve the survival or prevent tumor recurrence in patients with HCC.[5-7] These data showed that systemic chemotherapy or immunotherapy is required to decrease the frequency of tumor recurrence or metastases before or after loco-regional therapy including surgical treatment or TACE. The detection of tumor cells in peripheral blood by means of RT-PCR is useful in identifying a subpopulation of patients with HCC cells in peripheral blood and a more vigilant follow-up or more aggressive management is a very important measure to improve the outcome of those patients.

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