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Original Articles

AFP mRNA level in enriched circulating tumor cells from hepatocellular carcinoma patient blood samples is a pivotal predictive marker for metastasis

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ABSTRACT

Circulating tumor cells (CTCs) quantification may be helpful for evaluating cancer dissemination, predicting prognosis and assessing therapeutic effectiveness and safety. In the present study, CTCs from blood samples of 72 patients with hepatocellular carcinoma (HCC) were enriched with anti-EpCAM nanoparticles. AFP mRNA level was detected by nested polymerase chain reaction (PCR) after enrichment of CTCs from HCC blood samples at 0, 3, 6, 9 and 12 months after hepatectomy, respectively. AFP mRNA expression in CTCs was positive in 43 patients (59.7%) and negative in 29 patients (40.3%) before hepatectomy. Among 43 patients with positive AFP mRNA expression in CTCs before hepatectomy, 10 and 11 were diagnosed as intrahepatic/extrahepatic metastasis before and after hepatectomy, respectively. In addition, these 21 patients with metastasis had persisting positive AFP mRNA of CTCs during the whole tested year. Specifically, 3 patients with AFP mRNA negative in CTCs before hepatectomy changed to be positive at 6 and 9 months, and 2 of them were diagnosed as metastasis 12 months after hepatectomy. We conclude that the positive AFP mRNA of CTCs can be a pivotal predictor for HCC metastasis before and after hepatectomy. The release of AFP expression from hepatocellular carcinoma cells into circulation must be a major source of HCC metastasis.

Introduction

Circulating tumor cells

Circulating tumor cells (CTCs) are traveling cells in physiological fluids released from a primary or metastatic tumor. CTCs quantification may be helpful for evaluating cancer dissemination, predicting prognosis and assessing therapeutic effectiveness and safety. During tumor management, one of the important tasks is to predict the metastatic ability of a primary tumor. Detachment of cancer cells from primary tumors is an initial step in the metastatic process. Furthermore, CTCs can be used in subsequent proteomic and functional genetic analyses [1–3]. Isolation of CTCs from peripheral blood may facilitate early diagnosis and timely treatment. As CTCs are extraordinarily rare in the background of millions of normal hematopoietic cells (approximately 1 CTC in the background of millions of normal hematopoietic cells), their identification and characterization require extremely sensitive and specific analytical methods, which are usually a combination of enrichment and detection procedures, posing a great technological challenge to our current knowledge. In fact, liquid biopsy, a method of isolating CTCs from peripheral blood, is expected to complement conventional tissue biopsies in the detection of metastatic tumors. During the detection of CTCs, the specific proteins overexpressed on the surface of their plasma membrane are often determined, and these proteins are often used as targets in CTCs sensing methodologies for different types of cancer cells.

Epithelial cell adhesion molecule

Epithelial cell adhesion molecule (EpCAM), a type I glycosylated membrane protein with a molecular weight of 30–40 kDa, has been found to be involved in tumor invasion and metastasis [4–6]. EpCAM is abundant in most epithelial tissues but absent in blood cells and

Abbreviations: CTCs, circulating tumor cells; HCC, hepatocellular carcinoma; AFP, alpha fetoprotein; HepG2, hepatocellular carcinoma cell lines; EpCAM, epithelial cell adhesion molecule.

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functions as a hemophilic Ca^{2+}-independent cell–cell adhesion molecule. Furthermore, EpCAM is considered a general marker for a large variety of epithelial cells. Thus EpCAM could be applied as a proper target to separate CTCs from the blood. However, many molecular analyses on CTCs demonstrate that CTCs and normal epithelial cells share the same molecule of EpCAM. Method of detecting CTCs by EpCAM is not always specific, which means further verification should be applied to characterize the CTCs captured by EpCAM antibody.

Currently, a novel method that has been very well established for immuno-isolation of CTCs from blood is to conjugate antibodies against EpCAM to magnetic beads, followed by purification of captured cells through a magnetic field, which has been used to enrich CTCs from the blood of patients with breast, prostate, and colon cancers [7–9].

Alpha-fetoprotein

Alpha-fetoprotein (AFP) is a glycoprotein comprising of 591 amino acids with a half-life of 5–7 days. Normally, AFP is produced by fetal yolk sac, liver and intestine. Elevated AFP protein level is often used as a serological marker for HCC [10,11] as studies have demonstrated that AFP is overexpressed in liver carcinoma cells [12,13] and strongly correlated with the invasiveness of cancer cells. AFP monitoring in HCC patients has been recommended in many academic organizations including the European Association for the Study of the Liver [14], the Asian Pacific Association for the Study of the Liver [15]. Notably, increase of serum AFP protein level is not only present in HCC patients but also in patients with chronic hepatitis or cirrhosis [16].

Although many studies have elucidated that AFP mRNA in the peripheral blood might be a predictor for HCC recurrence after liver transplantation [17], this technique remains controversial. Furthermore, some research mentions that a larger volume of blood is required (15–20 mL) [16–18]. However, the value of AFP mRNA level in CTCs in predicting HCC recurrence or metastasis before or after hepatectomy has not been well elucidated. In some studies, the positive rates of AFP mRNA were 20%–50% before hepatic resection and transplantation [19–22]. This variability can be attributed to the differences in laboratory techniques, primer selections, intervals between sample collection and processing, and various populations.

In the current study, we have established a method to monitor the AFP expression using nested PCR technology in CTCs enriched by EpCAM antibody coated on the surface of magnetic beads. We try to provide a simple and rapid way to isolate and identify the circulating tumor cells from blood samples. In addition, we attempt to assess the value of AFP mRNA expression of CTCs enriched by anti-EpCAM nanoparticles in predicting HCC recurrence or metastasis before and after hepatectomy.

Materials and methods

Information of HCC patients

Ethics approval was obtained from the Ethics Committee of Beijing Hospital. All the patients enrolled in this study signed the informed consents. The diagnosis of HCC was confirmed histologically. Peripheral blood samples from 72 HCC patients were collected in citrated sodium anticoagulant tubes after hepatectomy at 0, 3, 6, 9 and 12 months, respectively. There were 10 patients diagnosed as metastasis before surgical resection. The number of male (n = 46) was larger than female (n = 26) in HCC patients. Most HCC patients were infected with HBV (n = 48) and some of them were infected with HCV (n = 10). 9 patients were dead within 12 months. These results were consistent with other studies about HCC in China [23,24]. The information of patients was browsed in Table 1. Peripheral blood samples were collected from 20 healthy subjects as negative controls.

Table 1
Different features of HCC patients and AFP mRNA nested PCR results of CTCs captured from blood samples by anti-EpCAM nanoparticles protocol before hepatectomy.

	Total no.	AFP nested PCR results*	
		Negative	Positive
HCC	72	29	43
Female	26	9	17
Male	46	20	26
Metastasis	10	0	10
Death	9[a]	1	8
HBV	48	17	31
HCV	10	1	9

	Positive percent (%)
HCC	59.7
Female	65.4
Male	56.5
Metastasis	100.0
Death	88.9
HBV	64.6
HCV	90.0

* The results of CTCs AFP mRNA nested PCR were tested at the time before hepatectomy.

[a] It is the total dead patients before and after hepatectomy.

Cell culture

Human hepatoblastoma cell line HepG2 was obtained from the Concord Cell Center (Peking Union Medical College, Beijing, China) and applied as a positive control in this protocol. Cells were cultured in Eagle minimum essential medium (MEM, Invitrogen) supplemented with 10% fetal bovine serum (Hyclone, American) and incubated in a humidified condition at a temperature of 37 °C with 5% CO_2. The medium was replaced every 3 days. Cells were harvested when they were subconfluent. The total amount of cells was calculated by a hemocytometer.

CTCs isolation by anti-EpCAM nanoparticles

In our present experiment, CTCs were captured immediately by anti-EpCAM nanoparticles and AFP mRNA levels were detected at 0, 3, 6, 9 and 12 months, respectively. Cell lysis buffer (Blood isolation kit, Biochain) was applied to remove the red blood cells. Peripheral blood nucleated cells (PBNCs), including CTCs, were isolated from 3 mL of blood samples (citrated sodium anticoagulant) from each patient. After being washed in phosphate-buffered saline (PBS) and centrifuged at 1500 rpm for 5 min, cell pellets were resuspended in 1 mL of PBS and then 20 μL human anti-EpCAM nanoparticals (MagVigen, Nvigen) were added to incubate for 60 min at room temperature. After incubation, the nanoparticles (with binding cells) were separated from the solution by a magnet.

RNA extraction and nested PCR

Total RNA of CTCs enriched by anti-EpCAM nanoparticles was extracted by TRIzol (Invitrogen) based on the method of acid guanidinium thiocyanate-phenol-chloroform extraction. Total RNA of each sample was denatured at 70 °C for 3 min and annealed with 1 μg of random primer at 37 °C for 2 min. Reverse transcriptase reaction was carried out in 1× reverse transcriptase buffer [50 mM Tris-HCl (pH 8.3), 75 mM KCl, and 3 mM $MgCl_2$] with 10 mM DTT, 1 μL of deoxynucleoside triphosphate (dNTP) mixture (250 mM each dNTP), and 200 units of moloney murine leukemia virus reverse transcriptase (Superscript, Invitrogen), respectively. The cDNAs were synthesized in a total volume of 20 μL at 42 °C for 90 min and the reaction was stopped at 70 °C for 10 min.

In order to increase the sensitivity of this method, we designed nested PCR to amplify AFP mRNA. This step was conducted with gene-specific primers that lie within different exons to avoid nonspecific bindings and could generate outer and inner PCR products of 353 bp and 198 bp, respectively. Sequences of AFP primers used in the experiment were as follows: the sense primers of the outer product was 5′ CAA TTC TTC TTT GGG CTG CTC GCT ATG AC 3′ (AFP 1) and the inner one was 5′ ATG CAG TTG AAT GCT TCC AA 3′ (AFP 3), the antisense primer of the outer product was 5′ AGT GTC TTG TTG AGA ACA TAT GTA GGA CAT G 3′ (AFP 2) and the inner one was 5′ CCA CAT CCA GGA CTA GTT TCT 3′ (AFP 4), respectively. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was served as an internal control to ensure the same amount of total RNA being reverse-transcribed to cDNAs in each assay. For GAPDH RT-PCR amplification, the sense primer was 5′ ACC ACA GTC CAT GCC ATC AC 3′ and the antisense primer was 5′ TCC ACC ACC CTG TTG CTG TA 3′ (451 bp). PCR was performed in a total volume of 25 μL with 1 × PCR buffer [20 mM Tris-HCl (pH 8.4), 50 mM KCl, and 2.5 mM $MgCl_2$], 1 μL of dNTPs mixture (250 mM each dNTP), 10 pmol of sense and antisense primers, 2.5 units of Taq DNA polymerase, 18 μL of cDNA for AFP outer PCR and 2 μL for GAPDH PCR, respectively. The inner AFP PCR amplification was conducted by application of 1 μL of outer AFP PCR products as templates. PCR conditions were as follows: denatured at 94 °C for 5 min, followed by 35 cycles of 94 °C for 30 sec, 60 °C for 30 sec, and 72 °C for 30 sec, and a final extension at 72 °C for 10 min. Then, 5 μL PCR products were electrophoresed in 1.5% (wt/vol) agarose gel stained with ethidium bromide and visualized under a UV light.

The sensitivity of CTCs isolation by anti-EpCAM nanoparticles

In order to test the sensitivity of CTCs enrichment by anti-EpCAM nanoparticles, different concentrations of HepG2 cells were mixed with normal blood samples, and the enrichment of HepG2 by anti-EpCAM nanoparticles was performed afterwards. Captured CTCs were determined from the mixture of HepG2 with different numbers (0, 50, 500, 5000, 50,000) in several 3 mL of normal blood samples. Then, the RNA extraction and nested PCR were performed to evaluate the sensitivity of CTCs isolation by anti-EpCAM nanoparticles.