

Nested RT-PCR in detection of blood AFPmRNA in animal model of human hepatocellular carcinoma

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OBJECTIVES: To detect blood AFPmRNA in the nude mice bearing with human hepatocellular carcinoma (HCC) by using nested reverse transcriptase polymerase chain reaction (nested RT-PCR), and assess its significance in HCC distant metastasis.

METHOD: We detected 20 blood samples from the nude mice bearing with human HCC by nested RT-PCR to find out AFPmRNA.

RESULT: AFPmRNA was detected in 6 blood samples from the nude mice bearing with human HCC (30.0%), in which 4 mice developed distant metastasis.

CONCLUSION: AFPmRNA may be used as an efficient and sensitive marker to detect blood spread of HCC cells. It can predict the occurrence of HCC distant metastasis.

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Key words: hepatocellular carcinoma; RT-PCR; AFPmRNA; nude mice

Introduction

Hepatocellular carcinoma (HCC) is common in China, and about 200 000 patients may die of HCC each year. Prognosis of patients with HCC is estimated by several factors such as histological differentiation of tumor cells, tumor size, and extent of lymphatic or hematogenous spread. One of the difficulties in the management of HCC is its extensive involvement, including intrahepatic metastasis, venous invasion, and distant metastasis. Some patients with HCC may develop metastatic nodules shortly after curative resection or chemoembolization. Moreover, orthotopic liver transplantation for unresectable HCC yields disappointing

results because of a high recurrence rate.^[1] It is assumed that HCC cells have been disseminated in the systemic circulation and proliferated in an immunosuppressive environment during or after therapy. To improve the prognosis of HCC, it is important to detect such blood-borne circulation of HCC cells before treatment.

Reverse transcription-polymerase chain reaction (RT-PCR) technique has been used to detect tumor cells in peripheral blood,^[2-4] bone marrow,^[5] and lymph node.^[6] In this study, we detected blood AFPmRNA in the nude mice bearing with HCC by using nested RT-PCR and assessed its significance in blood spreading or distant metastasis.

Methods

Animals

SMMC-7721 cell lines secreting AFP positive, provided by the Pathology Department of this university^[7] were inoculated into the neck and back of nude mice 4 weeks after birth (BALB/C/NU), with approximately 10^7 HCC cells each time. Six

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weeks later, the nude mice bearing with HCC 2–4 cm in diameter could be used experimentally (15–20 g in weight). A total of 20 nude mice bearing with HCC were utilized.

Samples

Both eyes of each nude mouse bearing with HCC were taken out for blood collection (about 1 ml). The tumor was removed for further observation of integrity as well as its relations to other tissues. Later on, the liver, lungs, kidneys and other organs of the mice were resected for pathological examination.

Methods

About one ml of whole blood obtained from each mouse was put into a heparinized tube. AFPmRNA was detected in peripheral blood with nested RT-PCR.

Detection of AFPmRNA

Heparinized whole blood was centrifuged with the removal of the plasma fraction. The cellular fraction was enriched for mononuclear cells or possible tumor cells according to the method described by Komeda.^[2] Total cellular RNA was extracted with the single-step method of RNA isolation.^[8] Reverse transcription reaction was carried out in 20 μ l reaction mixture using a first-strand cDNA synthesis kit (Promega, USA) according to the manufacturer's instructions. Nested PCR was conducted by adding 5 μ l cDNA to 100 μ l reaction mixture containing 10 mmol Tris-HCl (pH 9.0), 50 mmol potassium chloride, 4.5 mmol magnesium chloride, 250 nmol dNTP, 15p mol of each outer primer (EX-sense and EX-antisense), and 2.5 units of Taq DNA polymerase (Promega, USA). The reaction mixtures were subjected to 35 cycles of amplification in a programmable thermal cycler (Perkin-Elmer Cetus, USA) by using the following sequence: 94 °C for 1.5 min, 57 °C for 1.5 min and 72 °C for 2.5 min, plus a final extension step at 72 °C for 10 min. A sample of 10 μ l of the first amplification product was further amplified using an inner pair of primers (IN-sense and IN-antisense). To verify the amplified AFP DNA frag-

ment, samples were digested with the restriction enzyme Pst I and analysed by electrophoresis on a 15% polyacrylamide gel and stained with ethidium bromide for the specific bands of 174bp (first amplification product) and 101bp (second amplification product). Nested PCR was conducted two or three times for samples with conflicting results. The designing of external and inner pair of primers was as follows:

EX-sense 5'-ACTGAATCCACAACACTGCCA-TAG-3'; EX-antisense 5'-TGCACTCAATGCATC-TTCACCA-3'; IN-sense 5'-TGGAAATAGCTTCCA-TATTGGATTC-3'; IN-antisense 5'-AAGTGCCT-TCTTGAACAAACTGG-3'.

According to designing of primer pairs, the PCR products of 176bp and 101bp were amplified from AFPcDNA by external (EX-sense and EX-antisense) and internal (IN-sense and IN-antisense) primer pairs, respectively. The locations of primer pairs were as follows: EX-sense in exon 1 (AFPmRNA nucleotides 90–112), EX-antisense in exon 2 (AFPmRNA nucleotides 263–241), IN-sense over exon 1 and exon 2 (AFPmRNA nucleotides 122–145), IN-antisense in exon 3 (AFPmRNA nucleotides 222–200). cDNA sequences were based upon those previously reported.^[9]

Statistical analysis

The relationship between the presence of AFPmRNA in peripheral blood and various clinical parameters was examined by the Chi-square test.

Results

Local infiltration and metastasis were not detected in the 20 mice, in which 6 showed AFPmRNA in blood (30.0%); 4 of the 6 nude mice showed distant metastasis in the lung, liver or kidney (66.7%). The relationship between detection rate of AFPmRNA in blood and distant metastasis is shown in Table 1.

The diameter of tumors in the 6 nude mice with AFPmRNA positive in blood were more than 3 cm; none of them developed distant metastasis in tumors under 3 cm in diameter (Table 2).

Six of 12 nude mice bearing with HCC showed

Table 1. The relationship between detection rate of AFPmRNA and distant metastasis

Group	n	Number of metastasis	Metastasis rate (%)
AFPmRNA (+)	6	4	66.7*
AFPmRNA (-)	14	0	0

* compared with AFPmRNA(-) group, $P < 0.01$.

Table 2. The relationship between tumor diameter and AFPmRNA detection rate

Diameter (cm)	n	Number of AFPmRNA (+)	Positive rate (%)
> 3	9	6	66.7*
< 3	11	0	0

* compared between two groups, $P < 0.01$.

Table 3. The relationship between AFP levels in serum and AFPmRNA positive rate

AFP levels ($\mu\text{g/L}$)	n	Number of AFPmRNA (+)	Positive rate (%)
>4000	12	6	50.0*
<4000	8	0	0

* compared between two groups, $P < 0.01$.

AFPmRNA positive (AFP levels in serum beyond 4000 $\mu\text{g/L}$), and none of the remaining 8 mice showed AFPmRNA positive (AFP levels below 4000 $\mu\text{g/L}$) (Table 3).

The positive products of 174bp and 101bp of the first and the second PCR were not detected in the groups of negative controls.

The first cycles products of PCR were 174bp which can be cut into two pieces of 102bp and 72bp by restriction enzyme Pst I. The second cycles products of PCR were 101bp which can be cut into two pieces of 60bp and 41bp by restriction enzyme Pst I.^[10]

Discussion

Metastasis of carcinoma is one of the most important factors affecting prognosis. The presence of circulating tumor cells is known to be an indicator for hematogenous spread of tumor cells leading to metastasis at other organs. If metastasis in the early stage can be determined sensitively, we should be able to select more beneficial therapeutic methods.

PCR is a very sensitive method that detects minute volumes of DNA copies. Some researchers^[4,11,12] combined PCR with reverse transcription to detect micrometastasis of melanoma, prostatic cancer, and neuroblastoma. Comparison of the sensitivities of RT-PCR and nested PCR revealed that nested PCR provides a 100-fold sensitivity.^[13] Besides, nested PCR has increased the specificity owing to the use of two pairs of specific primers. Therefore, nested PCR is superior to RT-PCR with respect to the specificity and sensitivity in the detection of micrometastatic tumor cells.^[14] Recent studies^[16,17] on HCC have suggested that human AFP gene other than albumin mRNA can be used as a target.^[2,14,15]

The occurrence of tumor metastasis depends on the balance between the selected properties of tumor cells and the reactivity of the host.^[18] In the process of metastasis, tumor cells are scattered from the original site, spreaded hematogenously, arrested at the small vessels, and extravasated from the vessels. During these processes, tumor cells are attacked and destroyed by immunologically responsive cells or by mechanical force. Thus, a moderate number of circulating tumor cells is needed to form foci of micrometastasis. Liotta et al^[18,19] reported that less than 0.01% of circulating tumor cells may successfully establish metastatic clones.

Free mRNA is so fragile under conditions of abundant RNase activity that the specific mRNA in blood will indicate the presence of intact cells producing such proteins just before the extraction of RNA, not circulating free mRNA.^[2]

In this study, AFPmRNA (30.0%) was detected in 6 of the 20 nude mice bearing with human hepatocellular carcinoma, of which 4 showed distant metastasis (66.7%). None of the remaining 14 nude mice with AFPmRNA negative in blood developed distant metastasis ($P < 0.01$), indicating that distant metastasis in the nude mice bearing with HCC may take place, while HCC cells spreading into blood, as Paget's theory "seeds and soils", suggests that the circulating tumor cells or micrometastasis may serve as "seeds", anchoring and arrested by some tissues. If the microenvironments (serve as "soils") of secondary sites are favorable, HCC cells can survive and re-grow to form

a secondary tumor. The detection rate of AFPmRNA is related to AFP level, tumor size, and distant metastasis ($P < 0.01$). In other words, AFPmRNA in blood may be a prerequisite for distant metastasis of HCC.

In conclusion, AFPmRNA as an efficient and sensitive marker can be used to detect blood spread of HCC cells, and predict the distant metastasis of HCC.

Competing interest

The author or authors do not choose to response to the statements listed in Instructions for Authors.

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