Detection of Isolated Disseminated Tumor Cells in Bone Marrow and Blood Samples of Patients With Hepatocellular Carcinoma

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Background: Patients with hepatocellular carcinoma (HCC) often develop recurrences after curative resection or liver transplantation. Therefore, tumor cell dissemination must have occurred preoperatively or intraoperatively. Current staging methods cannot reliably detect micrometastasis. Reverse transcription–polymerase chain reaction (RT-PCR) for α-fetoprotein (AFP) has been used to detect circulating liver cancer cells, but results in blood samples have been contradictory.

Hypothesis: AFP–RT-PCR is a specific and sensitive assay for the detection of disseminated tumor cells in central venous blood and bone marrow samples of patients with HCC and has prognostic relevance.

Design: Prospective consecutive series.

Setting: University hospital.

Patients and Methods: We performed preoperative, intraoperative, and postoperative analyses of central venous blood samples and preoperative analysis of bone marrow samples of patients with HCC and patients without malignant disease, using a modified AFP–RT-PCR method. Preoperative serum AFP levels were measured. Clinical follow-up ranged from 4 to 20 months.

Main Outcome Measures: Sensitivity and specificity of AFP–RT-PCR, correlation of AFP–RT-PCR results to tumor stage and tumor recurrence.

Results: In serial dilution experiments, 50 AFP-expressing HepG2 cells were detected in 10 mL of blood. Peripheral blood samples of 20 healthy volunteers and bone marrow samples of 21 patients with benign diseases consistently tested negative for AFP, whereas 4 of 24 patients with HCC showed AFP expression in bone marrow samples. All these patients had advanced disease; however, correlation of positive RT-PCR results to tumor stage was not significant (P = .07). One of the 4 AFP-positive patients developed an intrahepatic recurrence soon after liver transplantation. Central venous blood of patients with HCC (n = 24) and patients with benign liver diseases (n = 13) equally demonstrated AFP-expressing cells. There was no correlation of RT-PCR results to serum AFP levels.

Conclusions: Perioperative screening for micrometastasis in bone marrow of patients with HCC is sensitive and specific with AFP–RT-PCR and may have prognostic relevance. α-Fetoprotein is not a suitable marker for the detection of tumor cells in central venous blood samples.

PATIENTS AND METHODS

BONE MARROW AND BLOOD SAMPLES

For bone marrow samples, 10 mL of bone marrow of the iliac crest of both sides was aspirated after bone marrow puncture; blood samples (10 mL) were obtained through a central venous catheter positioned in the superior vena cava or right atrium and all samples were diluted with 10 mL of phosphate-buffered saline. After density centrifugation through Ficoll-Paque (Pharmacia Biotech, Freiburg, Germany) for 30 minutes at 400g, mononuclear cells were harvested from the interphase and washed twice in phosphate-buffered saline. The cell pellet was then shock-frozen in liquid nitrogen and stored at −80°C.

CELL LINE AND SERIAL DILUTION EXPERIMENTS

The hepatoblastoma cell line HepG2 was cultured in Dulbecco modified Eagle medium supplemented with 10% fetal calf serum. Cell counting was performed in a hemocytometer. Serial dilutions of HepG2 cells with phosphate-buffered saline were then added to 10 mL of blood from healthy donors (corresponding to approximately 10^8 peripheral mononuclear cells) and Ficoll-Paque density centrifugation was performed as described above.

RNA EXTRACTION

Total RNA from bone marrow, blood, cell lines, and frozen tissue sections of tumor and normal liver tissue was extracted using a commercially available RNA isolation system (TRI- Reagent, Molecular Research Center, Cincinnati, Ohio) in accordance with the recommendations of the manufacturer. To eliminate contaminating DNA within the RNA preparation, 1 µg of RNA was digested with ribonuclease-free deoxyribonuclease I for 15 minutes at 25°C as recommended by the supplier (Life Technologies Inc, Gibco BRL, Karlsruhe, Germany).

RT-PCR ANALYSIS

Sequences for oligonucleotide primers in the AFP complementary DNA (cDNA) sequence were identified through the Heidelberg Unix Sequence Analysis Software Program (German Cancer Research Center, Heidelberg). A nested PCR protocol was developed to increase sensitivity and specificity of the method. Primers were located on different exons to allow distinguishing between amplified spliced transcripts and contaminating DNA on the basis of their different product sizes (Figure 1). The annealing temperature of the first PCR was chosen below that of the nested PCR to minimize binding of the outer primers to amplification products in the nested PCR. Various annealing temperatures were systematically tested to achieve maximal sensitivity and specificity. First-strand cDNA was synthesized from 1 µg of RNA in a total volume of 20 µL with a reverse transcription kit (Life Technologies Inc) according to the recommendations of the manufacturer and using the outer 3’ PCR-primer 1245rev as the specific primer.

First PCR was performed using primers 775for (TGA GTC AGA AGT TTA CCA AAG) and 1245rev (CTT CTC CTT TAT CCT TGC). Five microliters of RT reaction mixture was used to amplify AFP cDNAs in a total reaction volume of 100 µL containing the above primers, 25 mmol/L each; deoxynucleotide triphosphate, 0.2 mmol/L; magnesium chloride, 1.5 mmol/L; Taq DNA polymerase, 2.5 U; and PCR buffer consisting of Tris-hydrochloride, 20 mmol/L, and potassium chloride, 50 mmol/L (PCR Kit; Life Technologies Inc). Thirty-five cycles of amplification were done at 30-second intervals at temperatures of 93°C, 62°C, and 72°C, with a final extension step of 10 minutes at 72°C. A total of 20 µL of this reaction mixture was then transferred to the nested PCR, which was also performed in a total volume of 100 µL with primers 954for (TGC AAA CTG ACC ACG CTG CAA C) and 1228rev (TGG CAT TCA AGA GGG TTT TCA GTC). Forty cycles of amplification were done at 30-second intervals at temperatures of 93°C, 66°C, and 72°C, with a final extension step of 10 minutes. The PCR products were subjected to electrophoresis in 2% agarose gels and visualized.

healthy controls, therefore rendering it unsuitable as a marker for tumor dissemination. Results for detecting α-fetoprotein (AFP)-expressing cells in peripheral blood samples have been more promising, although here too specificity remains a problem. Patients with acute and chronic hepatitis and liver cirrhosis without malignant disease have also tested positive for AFP mRNA in peripheral blood samples of 24 patients with HCC and 21 patients without malignant disease for expression of AFP mRNA.

SENSITIVITY AND SPECIFICITY OF AFP–RT-PCR

In cell spiking experiments, a 275–base pair PCR product was amplified from samples enriched with HepG2 cells in a dilution of up to 50 cells in an overall volume of 10 mL, thereby amounting to a sensitivity of about 5 cells in 1 mL of blood (approximately corresponding to 5 cells in 1 × 10^7 peripheral mononuclear cells) (Figure 2). The above PCR fragments were shown by Southern blotting to hybridize to an oligonucleotide probe specific for AFP, thus confirming it as corresponding to an AFP cDNA fragment.

All bone marrow samples from 21 patients without malignant disease and all peripheral blood samples of the 20 healthy volunteers consistently tested negative for AFP..
expression. Tumor tissue from patients with HCC all tested positive for AFP expression, as expectedly did all normal liver tissue samples. A considerable number of control patients with benign liver diseases, however, showed positive RT-PCR results for AFP mRNA in central venous blood samples (Table 1).

PATIENT STUDY

RT-PCR Results and Serum AFP Levels

Four (17%) of the 24 patients with HCC demonstrated AFP-expressing cells in their bone marrow (Figure 3). All 4 of these patients had advanced disease (TNM stage 3 or 4) (Table 2). 2 patients had surgical explorations without resection as the tumor was macroscopically too advanced, 1 patient already had a recurrence of a primarily resected HCC and had to undergo transplantation, and 1 patient had a tumor larger than 2 cm with vascular infiltration. However, not all patients with advanced disease showed positive RT-PCR results and correlation of advanced tumor stage (stage 3 or higher) to AFP expression in bone marrow samples did not reach significance (P = .07). In central venous blood samples, AFP-expressing cells were found in 7 patients preoperatively (29%), in 11 patients intraoperatively (46%), and in 10 patients 24 hours postoperatively (43%) (Table 1). There was no statistically significant difference between the detection of AFP mRNA in central venous blood samples in patients with HCC or in patients with benign liver diseases nor was there any statistically significant association between the detection of AFP mRNA in bone marrow or blood samples, although all patients with AFP-expressing cells in their bone marrow at least had 1 positive blood sample. Serum AFP levels were elevated in all patients with detection of AFP mRNA in their bone marrow; however, many patients with elevated serum AFP levels did not show AFP-expressing cells in bone marrow, therefore no statistically significant correlation could be found between elevated serum AFP levels and positive AFP–RT-PCR results in bone marrow samples. Two of the 4 patients with positive bone marrow results had been suspected preoperatively; however, postoperative histological assessment ruled out malignancy. Six of the control patients had benign liver tumors, and the other 15 had other benign tumors of the gastrointestinal tract or chronic inflammations previously suspected to be malignant. Central venous blood samples were also obtained from 13 patients with benign liver diseases as described above for the patients with HCC. Peripheral blood samples were additionally taken from 20 healthy volunteers.

STATISTICAL ANALYSIS

Statistical computations were done using the software packages S-PLUS version 3.4 (MathSoft, Inc, Cambridge, Mass) and StatXact3 for Windows (Sytel Software Corp, Cambridge, Mass). Results were defined as statistically significant when the respective test was P<.05.

Stage vs RT-PCR Results

The Fisher exact test and Kendall tau test were used to analyze the correlation between tumor stage and detection of AFP mRNA by RT-PCR in bone marrow and blood samples.

RT-PCR Results in Blood Samples vs in Bone Marrow Samples

Odds ratios were calculated and Mantel-Haenszel and Fisher exact tests were performed to analyze the correlation between detection of AFP mRNA in bone marrow and blood samples.

Serum AFP in Peripheral Blood vs RT-PCR Results

Wilcoxon Mann-Whitney, Fisher exact, and Kendall tau tests were used to analyze the correlation between elevated serum AFP levels in peripheral blood and detection of AFP gene products by RT-PCR in bone marrow or blood samples.

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had antibodies against hepatitis B or C, and the other 2 patients did not show any evidence for viral hepatitis (Table 2).

**FOLLOW-UP**

Patient 3, who had undergone transplantation because of a recurrence of HCC after R0-resection (microscopically tumor-free resection margins), developed a recurrence in the transplanted liver 6 months later and had to undergo liver resection again. One of the other 2 patients with AFP-expressing cells in their bone marrow samples who did not have resection died of his metastatic tumor (patient 17), and the other is still alive with hepatic progression of the malignant disease (patient 20). Patient 21 is clinically still without recurrence; however, the follow-up is only 4 months.

Two of the 20 patients with HCC and negative RT-PCR results in their bone marrow died perioperatively (both due to septic complications after liver transplantation), and 4 patients died of progression of their malignant disease. Excluding the 2 patients who died perioperatively, the median follow-up is 11 months (range, 4-20 months).

**COMMENT**

Patients with HCC often develop recurrences, which are probably caused by preoperatively already existing micrometastases. Therefore, preoperative screening for occult tumor cells is important to adequately select patients for therapeutic regimens. Previous studies have indicated a prognostic relevance for the detection of micrometastasis in bone marrow samples in patients with colorectal and other gastrointestinal cancers. Equivocal results have questioned whether this also applies to tumor cell detection in peripheral blood samples of affected cancer patients. Especially in HCC, conflicting results regarding specificity of RT-PCR–based detection systems in blood samples have been published. The major problem is finding an adequate specific marker, which should obviously not be expressed in normal peripheral blood cells. False-positive results may originate from “illegitimate” transcription, as shown for the albumin gene, or from pseudogenes, as shown for cytokeratin 18 and 19, or may simply be caused by contamination due to the high sensitivity of the method.

We have developed a sensitive and specific RT-PCR method for the detection of AFP-expressing cells

| Table 1. Detection Rates of AFP mRNA in Patients With Hepatocellular Carcinoma and Benign Liver Diseases* |
|---|---|---|
| Venous Blood Sample | Patients With Hepatocellular Carcinoma | Patients With Benign Liver Diseases |
| Preoperative | 7/24 (29) | 3/8 (38) |
| Intraoperative | 11/24 (46) | 10/13 (77) |
| Postoperative | 10/23 (43) | 2/6 (33) |

*AFP indicates α-fetoprotein; mRNA, messenger RNA.

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**Figure 1. Schematic illustration of the α-fetoprotein reverse transcription–polymerase chain reaction (AFP–RT-PCR). Arrows indicate the position of primer sets used for PCR (1.PCR) and nested PCR (2.PCR). bp indicates base pair; hybrid oligo, oligonucleotide used for hybridization.**

**Figure 2. Amplification products of α-fetoprotein–expressing HepG2 cells in serial dilutions (0, 5, 50, 500, 5000 cells) in an overall volume of 10 mL of blood (approximately 10⁸ mononuclear cells) of healthy donors. M indicates molecular weight marker; W, water (negative) control.**

**Figure 3. Reverse transcription–polymerase chain reaction (RT-PCR) with Southern blot hybridization of PCR products with an α-fetoprotein (AFP)–specific oligonucleotide. M indicates molecular weight marker; W, water (negative) control; lane 1, negative control cytokeratin-20 amplification product, 290 base pairs (bp) (HT-29 cells); lanes 2-8, AFP amplification products, 275 bp. Lanes 2 to 6 correspond to bone marrow samples of patients 3, 17, 20, 21, and 24; lanes 7 and 8 are positive controls (HepG2 cells).**

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in bone marrow samples. When testing preoperative, intraoperative, or postoperative central venous blood samples from patients operated on for benign liver disease and HCC we found positive RT-PCR results in a comparable percentage in both groups. This is in accordance with Lemoine and colleagues,\textsuperscript{19} who also investigated AFP expression by RT-PCR in intraoperative blood samples and showed comparable detection rates in intraoperative blood samples of patients with HCC and patients without malignant disease (predominantly patients with liver cirrhosis). The high detection rate in patients with benign and malignant diseases in our study may be explained by the position of the central line, which is routinely placed in or just proximal to the right atrium where blood that has just passed through the liver and drained into the inferior vena cava merges with blood from the upper body. Normal liver cells expressing AFP are continuously shed into the hepatic veins and either undergo apoptosis or are filtered out of the blood system when passing capillary beds. Cell shedding may be enhanced by surgical manipulation and inflammatory conditions within the hepatic parenchyma. Central venous blood in our study was drawn before it passed through any capillary bed only a short distance after leaving the liver, therefore possibly accounting for the high rate of detection. This may also be the reason for conflicting results of detection of AFP-expressing cells in blood samples in the literature as the conditions of blood sampling may not have been comparable. Unfortunately, the exact method of obtaining blood is not specified in most articles. It is necessary to further investigate whether the detection of AFP mRNA in peripheral blood samples that have passed through at least one capillary bed is significantly different from the detection in central venous blood samples. In a subgroup of 8 patients, who had all tested positive for AFP mRNA in intraoperative central venous blood samples, only 6 patients showed AFP expression in intraoperative arterial blood samples, thereby suggesting a difference in the detection rate before and after passage through a capillary bed. Preoperative and postoperative blood samples in our study equally revealed positive RT-PCR results in both groups, which also supports the notion that these AFP-expressing cells in the central venous blood are hepatocytes that are later filtered out of the blood or undergo apoptosis. The former concept is further substantiated by the fact that all 20 healthy controls consistently tested negative for AFP expression in peripheral blood samples, thereby making “illegitimate transcription” as cause for the detection of AFP-expressing cells in central venous blood samples in patients with benign diseases very unlikely. In our study, the detection of AFP mRNA was even higher in intraoperative central venous blood samples of patients with benign liver diseases compared with patients with HCC; however, this is easily explicable by a dilution effect. The latter group lost considerably more blood, with HCC; however, this is easily explicable by a dilution of AFP-expressing cells in central venous blood samples. When testing preoperative, intraoperative, or postoperative central venous blood samples from patients operated on for benign liver disease and HCC we found positive RT-PCR results in a comparable percentage in both groups. This is in accordance with Lemoine and colleagues,\textsuperscript{19} who also investigated AFP expression by RT-PCR in intraoperative blood samples and showed comparable detection rates in intraoperative blood samples of patients with HCC and patients without malignant disease (predominantly patients with liver cirrhosis). 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Similar results have been reported from other studies analyzing perioperative tumor cell dissemination where a blood loss of more than 1 L was statistically calculated as cutoff point for reliable intraoperative tumor cell detection.\textsuperscript{31}

\begin{table}[h]
\centering
\begin{tabular}{|c|c|c|c|c|c|c|c|c|}
\hline
Patient No. & TNM Stage (Tumor) & Viral Hepatitis Type & Serum AFP, pg/L & AFP mRNA (Bone Marrow) & \\
\hline
1 & 1 & C & 52 & – & \\
2 & 4 & – & 1658 & – & \\
3 & 4 & – & 1265 & + & \\
4 & 3 & – & 18 & – & \\
5 & 2 & B & 90 300 & – & \\
6 & 4 & – & 972 & – & \\
7 & 2 & C & 16.8 & – & \\
8 & 2 & B & 23 & – & \\
9 & 2 & – & 3.3 & – & \\
10 & 2 & – & 5.5 & – & \\
11 & 3 & – & 5.1 & – & \\
12 & 4 & – & 2.7 & – & \\
13 & 4 & C & 541 & – & \\
14 & 2 & – & 1.7 & – & \\
15 & 3 & – & 5 & – & \\
16 & 2 & – & 65 & – & \\
17 & 4 & – & 14.1 & + & \\
18 & 2 & – & 4.1 & – & \\
19 & 2 & – & 4.6 & – & \\
20 & 3 & – & 1798 & + & \\
21 & 4 & C & 32.8 & + & \\
22 & 3 & C & 795 & – & \\
23 & 4 & – & 67.3 & – & \\
24 & 2 & C & 38.5 & – & \\
\hline
\end{tabular}
\caption{Clinical Characteristics and AFP mRNA Detection Results in Patients With Hepatocellular Carcinoma\textsuperscript{*}}
\end{table}

\textsuperscript{*}AFP indicates \( \alpha \)-fetoprotein; mRNA, messenger RNA; minus sign, absent; and plus sign, present. Boldface items are those patients whose bone marrow samples expressed AFP.

On the other hand, we did not detect AFP-expressing cells in bone marrow samples of 21 patients with benign diseases, but did in 4 of 24 patients with HCC. Correlation of AFP expression in bone marrow samples to tumor stage was not statistically significant, probably due to the limited number of tested patients. The prognostic relevance of these data has to be further evaluated by clinical follow-up. Interestingly, 1 of the 4 AFP-positive patients soon developed a cancer recurrence in the transplanted new liver. The other 3 patients all had advanced disease, and no patient below tumor stage 3 showed AFP-expressing cells in bone marrow. It is certainly premature to derive any therapeutic consequences from these results. However, if detection of tumor cells in bone marrow samples is associated with an increase in the recurrence rate, preoperative screening before liver transplantation for HCC seems warranted. We could also not demonstrate any correlation between elevated AFP serum levels and micrometastasis in bone marrow or blood samples. This is in contrast to the results of Louha et al,\textsuperscript{15} who found a strong correlation between the presence of circulating AFP-positive cells in peripheral blood samples of patients with HCC and serum AFP concentrations. Interestingly, we did not detect any bone marrow micrometastasis in patients with normal AFP levels. The results of Lemoine et al,\textsuperscript{19} however, are in accordance with ours as they too were not able to show any correlation between micrometastatic disease in blood samples and elevated AFP serum levels.

In conclusion, we have developed a sensitive, specific test to detect AFP-expressing cells in bone marrow.
samples. Bone marrow micrometastases were found in 4 of 24 patients with HCC, whereas none of the 21 controls showed positive PCR signals. Potential implications for therapeutic decisions have to be evaluated in further studies and in the clinical follow-up of the tested patients. We believe this assay should be considered in the preoperative workup before liver transplantation to adequately select suitable patients. The AFP–RT-PCR method, however, cannot be recommended for the detection of tumor cell dissemination in blood samples obtained through a central venous catheter from patients with HCC since a large number of control patients with benign liver diseases equally demonstrated AFP-expressing cells. These cells are probably hepatocytes that are regularly shed into the central venous blood and then undergo apoptosis or are filtered out of the blood circulation.

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