Hematogenous Dissemination of Hepatocytes and Tumor Cells after Surgical Resection of Hepatocellular Carcinoma: A Quantitative Analysis

Ivy H. N. Wong, Wan Y. Lau, Thomas Leung, Winnie Yeo, and Philip J. Johnson

ABSTRACT

The only hope of long-term survival for patients with hepatocellular carcinoma (HCC) is surgical resection or liver transplantation. However, recurrence or metastasis formation is common after surgery. We aim to assess whether surgical resection leads to hematogenous dissemination of malignant and nontumor hepatocytes and determine the quantity and timing of hepatocyte shedding into the circulation. Using semiquantitative reverse transcription-PCR for α-fetoprotein (afp) and albumin (alb) mRNAs, we measured the mass of malignant and nontumor hepatocytes in 53 peripheral blood samples collected preoperatively, intraoperatively, and postoperatively from 13 HCC patients. We compared these data with those in 54 control samples collected from 24 healthy subjects and patients with chronic hepatitis/cirrhosis and 10 hepatocellular adenoma patients who underwent resection. Clinicopathological information of HCC patients was obtained during 3-year follow-up. In 100% (23 of 23) of HCC and adenoma patients, afl mRNA levels increased 10–10^6-fold intraoperatively and then markedly declined within 8 weeks after operation. Levels of afl mRNA increased 5–7600-fold preoperatively in 8% (1 of 13) and postoperatively in 70% (9 of 13) of HCC patients. All five HCC patients with persistently elevated afl mRNA levels died from intrahepatic/extrahepatic metastasis, liver recurrence, or persistent HCC within 1 year after surgery. The absence/clearance of afl mRNA in 75% (six of eight) of survivors was strongly associated with the absence of metastasis/recurrence (P = 0.02). We present evidence that alb-expressing hepatocytes are released intraoperatively into the circulation, and afl-expressing tumor cells are disseminated mostly postoperatively that may potentially be the source of recurrence or metastasis. Sequential quantification of both alb and afl mRNAs may provide insights for risk assessment and prognostic indication.

INTRODUCTION

The only hope of long-term survival for patients with HCC is surgical resection or liver transplantation. However, after apparently curative resection, recurrences are common, and they largely account for poor survival rates of only 68.5% at 1 year and 31.9% at 5 years after resection, and >80% of recurrences are intrahepatic (1–3). Liver recurrences are presumably caused by the incomplete resection of HCC, leaving behind residual tumor at the site of the resected stump, the presence of undetectable regional micrometastasis, and/or the spillage of tumor cells into the bloodstream during surgical manipulation (4, 5). The regenerating liver, after resection, may be a particularly fertile ground for these tumor cells to proliferate.

It has been conceived that one may infer the presence of circulating HCC cells and hence the potential for metastasis if liver-specific alb and afl mRNAs are detected in peripheral blood (6–12). There has been emerging evidence suggesting that afl mRNA detection is strongly associated with the presence of metastasis or liver recurrence, particularly after surgery (8–12). Nonetheless, extremely sensitive nested RT-PCR applied in these studies might possibly give rise to false positivity because nontumor hepatocytes could also express afl mRNA at low levels. In addition, “illegitimate transcription” among normal PBMCs is a potential problem that needs to be addressed (13). To distinguish between the presence of circulating HCC cells/nontumor hepatocytes and normal PBMCs, we have developed a sufficiently sensitive and well-optimized semiquantitative RT-PCR method for measuring levels of afl and alb mRNAs with reference to the equivalent number of HepG2 cells (14).

In the present study, we aimed to assess whether surgical maneuver or resection of HCC could lead to hematogenous dissemination of malignant and nontumor hepatocytes. The quantity and timing of hepatocytes shedding into the circulation of HCC patients were also monitored by semiquantitative RT-PCR before, during, and after surgery. To determine the clinical significance of the findings, we correlated the numbers of circulating HCC cells and nontumor hepatocytes...
cytes detected with the clinical outcomes of the patients studied in 3-year follow-up.

PATIENTS AND METHODS

Patients. With informed consent and ethics approval from the Ethics Committee of the Chinese University of Hong Kong, 83 peripheral blood samples were collected during preoperative, intraoperative, and postoperative periods from 23 patients with HCC or hepatocellular adenoma who underwent surgical resection with curative intent. Preoperative blood samples were collected from the patients studied at least 1 h before surgery. Intraoperative blood samples were collected immediately after tumor resection. Postoperative blood samples were collected at 1–7 months after surgery. The diagnosis of HCC was confirmed histologically on each resected specimen where the tumor size and the mass of hepatectomy were measured. The 10 patients with hepatocellular adenoma served as control subjects undergoing surgical resection. As negative controls for semiquantitative RT-PCR, 24 peripheral blood samples were collected from healthy subjects and patients with chronic hepatitis or cirrhosis. HCC patients were regularly assessed for tumor recurrence by the measurement of serum AFP, ultrasound examination with computed tomography, and hepatic angiography as appropriate. Clinical and histopathological features of the HCC patients studied were obtained during 3-year follow-up to evaluate the clinical significance of the data (Table 1).

PBMC Isolation and RNA Extraction. PBMCs were isolated by Ficoll-Paque (Pharmacia, Biotech, Uppsala, Sweden) from 20 ml of citrated blood collected from the studied patients and controls. After washing in 30 ml of PBS and centrifugation at 100 × g for 10 min, the cell pellet was resuspended in 1 ml of PBS, and the number of PBMCs was counted in a hemocytometer. After centrifugation, the cell pellet was resuspended in 0.5 ml of guanidinium thiocyanate solution, and total RNA was extracted by a single-step method (15).

Cell Culture. The hepatoblastoma cell line HepG2 (American Type Culture Collection, Rockville, MD) was used to establish calibration curves for assessing the amounts of Alb and afp mRNAs. The cell line was cultivated in RPMI 1640 added with penicillin, streptomycin, and 10% fetal bovine serum (Life Technologies, Inc., Gaithersburg, MD). The medium was changed every 3 days, and the cells were harvested when the growth was subconfluent. The total number of cells was then counted in a hemocytometer.

Spiking Experiment Using HepG2 Cells. To simulate the presence of HCC cells in the circulation of HCC patients, total RNA was first extracted from 10⁷ PBMCs from a healthy subject and 10⁶ HepG2 cells. Aliquots of total RNA from 10⁶ PBMCs were mixed with HepG2 total RNA, corresponding to 1, 10, 10², 10³, 10⁴, and 10⁵ HepG2 cells (based on the calculation of the average amount of HepG2 total RNA extracted per cell). The RNA mixtures were then subject to semiquantitative RT-PCR for constructing the calibration curves (14).

Semiquantitative RT-PCR. Total RNA (1–2 μg) was denatured at 65°C for 2 min and annealed with 1 μg of random primer. Denatured at 65°C for 2 min and annealed with 1 μg of random primer. The RNA mixtures were then subject to semiquantitative RT-PCR for constructing the calibration curves (14).

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Hepatocellular carcinoma. HBsAg, hepatitis B surface antigen. W, well-differentiated; m, moderately differentiated; P, poorly differentiated; U, unclear differentiation stage; S, major hepatic resection; H, minor hepatic resection. N, the reference range for negative controls including healthy subjects and patients with chronic hepatitis or cirrhosis.

States whether the resection margin was free of tumor (f) or close to tumor (T) and whether the tumor was encapsulated (C) or not (NC).

Peripheral blood samples were not collected afterwards.
primers at 37°C for 10 min (14, 16). Reverse transcriptase reaction was carried out in 1× reverse transcriptase buffer [50 mM Tris-HCl (pH 8.3), 75 mM KCl, and 3 mM MgCl₂] with 10 mM DTT, 0.5 mM deoxynucleotide triphosphates, and 0.5 μl of RNase block (Stratagene, La Jolla, CA). cDNAs were synthesized at 37°C for 1 h using 200 units of Moloney murine leukemia virus reverse transcriptase (Life Technologies, Inc.), and the reaction was stopped at 70°C for 7 min (14, 16).

PCR amplification of \( \text{alb} \) and \( \text{afp} \) cDNAs was conducted using gene-specific primers that lie within different exons to give PCR products of 157 and 215 bp, respectively (14). For \( \text{alb} \) cDNA amplification, sense and antisense primers were 5’-TGC TTG AAT GTG CTG ATG ACA GGG-3’ and 5’-AAG GCA AGT CAG CAG GCA TCT CAT C-3’. For \( \text{afp} \) cDNA amplification, sense and antisense primers were 5’-TGC AGC CAA AGT GAA GAG GGA AGA-3’ and 5’-CAT AGC GAG CAG CCC AAA GAA GAA-3’. \( \beta_2 \)-microglobulin mRNA served as an internal control to ensure that an exact amount of high integrity total RNA was reverse-transcribed to produce cDNAs in each assay (14, 16).

PCR was conducted in 1× PCR buffer [20 mM Tris-HCl (pH 8.4), 50 mM KCl, and 2.5 mM MgCl₂] with 0.2 mM deoxynucleotide triphosphates, and 3 μl of cDNAs as templates. Thermocycling was performed as follows: an initial 5-min denaturation at 94°C, followed by 30 cycles of 94°C for 1 min, 61°C for 1 min, and a final extension at 72°C for 10 min (14). Aerosol-resistant pipette tips and separate areas were used for pre-PCR, PCR, and post-PCR procedures. Each sample was analyzed in duplicate. HepG2 RNA standards and multiple water blanks were analyzed in parallel with blood samples in each set of PCR. PCR products were loaded onto 2% agarose gels and stained with ethidium bromide.

The gene identity of the PCR product was verified by nonradioactive Southern blot analysis using an oligonucleotide labeled at the 3’ end with digoxigenin (14, 16). The \( \text{alb} \) probe was 5’-CAC AGC ATT CCT TCA GTT TAC TGG AGA TCG-3’. The \( \text{afp} \) probe was 5’-CAG CAT CGA TCC CAC TTT TCC AAG TTC CAG-3’. Chemiluminescent detection was conducted using CSPD (Boehringer Mannheim, Mannheim, Germany; Refs. 14 and 16). By imaging densitometry (Bio-Rad, Hercules, CA), the PCR signals for blood samples were quantified on the same Southern blots as the PCR products generated for establishing the HepG2 calibration curves (14).

**RESULTS**

**Preoperative, Intraoperative, and Postoperative Levels of \( \text{alb} \) and \( \text{afp} \) mRNAs in Control Subjects.** Two calibration curves were constructed by measuring levels of \( \text{alb} \) and \( \text{afp} \) mRNAs in 1–10⁶ HepG2 cells using semiquantitative RT-PCR (14). According to the HepG2 standard curves, levels of \( \text{alb} \) and \( \text{afp} \) mRNAs were quantified in 83 peripheral blood samples collected preoperatively, intraoperatively, and postoperatively from 23 patients with HCC or hepatocellular adenoma who underwent resection (Fig. 1) and 24 blood samples from healthy subjects and patients with chronic hepatitis/cirrhosis. Changes in the levels of \( \text{alb} \) and \( \text{afp} \) mRNAs were followed and correlated with the clinical outcomes of the HCC patients studied. Among all 10 adenoma patients studied, the \( \text{alb} \) mRNA levels detected before and after surgery were within the “reference range” determined for the 24 negative control subjects. Notably, the \( \text{alb} \)

![Fig. 1 Southern blot analysis and quantification of \( \text{alb} \) and \( \text{afp} \) mRNA signals in peripheral blood samples collected preoperatively (Lane A), intraoperatively (Lane B), and 1–7 months postoperatively (Lanes C, D, and E) from HCC patients P1, P2, and P3.](clincancerres.aacrjournals.org)
mRNA levels increased $10^{-10^4}$-fold during surgery in all of the adenoma patients studied. Conversely, the AFP mRNA levels were within the reference range before, during, and after surgery in all 10 adenoma patients.

**Preoperative, Intraoperative, and Postoperative Levels of \textit{alb} mRNA in HCC Patients.** In all 13 HCC patients studied, the \textit{alb} mRNA levels detected preoperatively were within the reference range that might be attributed to "illegitimate transcription" (14) equivalent to 0–727 HepG2 cells per 20 ml of blood (Fig. 2). Levels of \textit{alb} mRNA were then elevated $50-10^6$-fold intraoperatively in all of the HCC patients studied (Fig. 2), which were significantly higher than the reference range ($P < 0.00001$, Mann-Whitney test). The number of \textit{alb} mRNA-expressing cells detected ranged from 870 to $6.1 \times 10^5$ HepG2 cell equivalents per 20 ml of blood. The intraoperative \textit{alb} mRNA level positively correlated with the mass of hepatectomy, regardless of tumor capsule formation ($r = 0.50; P = 0.06$) but did not correlate with the tumor size ($r = 0.01; P = 0.96$) or the resection margin ($r = -0.02; P = 0.95$; Spearman’s correlation; Table 1). Within 8 weeks after operation, the \textit{alb} mRNA levels declined to the reference range in all of the HCC patients studied (Fig. 2).

**Preoperative, Intraoperative, and Postoperative Levels of \textit{afp} mRNA in HCC Patients.** In all preoperative blood samples (except patient P7’s) and all intraoperative samples analyzed, \textit{afp} mRNA levels were found to be indistinguishable from the reference range ($P = 0.44$ and 0.14 for preoperative and intraoperative samples, respectively, Mann-Whitney test; Fig. 3). In P7, the preoperative \textit{afp} mRNA level was nearly 50-fold higher than the reference range (275 HepG2 cell equivalents per 20 ml of blood) but fell to the reference range during surgery (Fig. 3A).

In postoperative blood samples from 9 of the 13 HCC patients studied (P1, P2, P3, P4, P7, P9, P11, P12, and P13), \textit{afp} mRNA levels were significantly higher (5–7600-fold) than the reference range ($P < 0.00001$, Mann-Whitney test; Fig. 3). Four of these nine patients (P1, P7, P11, and P12) harbored large HCCs $>5$ cm in diameter (Table 1). The number of \textit{afp}-expressing cells detected ranged from 32 to 46,000 HepG2 cell equivalents per 20 ml of blood. The \textit{afp} mRNA levels were persistently elevated in five patients (P1, P2, P7, P11, and P12; Fig. 3A) but fell to the reference range 2–4 weeks later in P3 and P4 (Fig. 3B). In four patients (P5, P6, P8, and P10), levels of \textit{afp} mRNA detected postoperatively were all within the reference range (Fig. 3B). Among the HCC patients studied, the maximal \textit{afp} mRNA level detected over the times did not correlate directly with the tumor size ($r = 0.35; P = 0.23$) or the resection margin ($r = -0.14; P = 0.64$; Spearman’s correlation; Table 1).

**Correlation between Levels of \textit{afp} mRNA and \textit{alb} mRNA/Serum AFP.** Consistent with our previous findings (14), levels of \textit{afp} mRNA did not correlate with levels of \textit{alb} mRNA in 53 peripheral blood samples from the HCC patients studied during the preoperative, intraoperative, and postoperative periods ($r = 0.08; P = 0.55; n = 53$; Spearman’s correlation). In addition, levels of \textit{afp} mRNA detected at different times did not correlate directly with serum AFP levels in the same HCC patients ($r = 0.25; P = 0.08; n = 53$; Spearman’s correlation). Instead, the maximal \textit{afp} mRNA levels detected over the times (postoperatively in 11 of the 13 HCC patients; Table 1) strongly correlated with the amounts of serum AFP monitored at the same times ($r = 0.77; P = 0.002; n = 13$; Spearman’s correlation).

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**Fig. 2** Levels of \textit{alb} mRNA (on logarithmic scale) were measured in peripheral blood samples collected preoperatively, intraoperatively, and postoperatively from 13 HCC patients, with reference to the HepG2 cell line. For patients who showed the same level of \textit{alb} mRNA, a single symbol is illustrated for simplicity. Otherwise, different symbols are used for different HCC patients.
Clinical Relevance of Raised afp mRNA Levels. All of the four patients (P5, P6, P8, and P10) who showed afp mRNA levels within the reference range during the periods monitored remain alive and well with no evidence of tumor recurrence at 3 years (Table 1). Two additional patients (P3 and P4) had evidence of circulating malignant hepatocytes within the first 2 postoperative months, but these cells were cleared within 2–4 weeks. They also remain alive and well with no evidence of recurrence at 3 years (Table 1). Of the other seven patients with postoperatively raised afp mRNA levels, five patients with persistently elevated levels (including P11 and P12 with the highest recorded levels) developed lung metastasis (P11), liver recurrence (P2, P7, and P12), or had persistent HCC with intrahepatic metastasis (P1; P = 0.02, Fisher’s exact test). They all died from the recurrences or metastases (Table 1). The other two patients with raised afp mRNA levels, P9 and P13, were...
Tumor Dissemination After Surgical Resection

A small proportion of the circulating tumor mass could potentially be responsible for the formation of a secondary deposit (22). By lodgment and extravasation, some of the circulating HCC cells detected were possibly arrested by target organs from which they could be released into the circulation again (23), as shown in the five patients who developed metastases or recurrences. It is noteworthy that 80% (four of five) of relapse took place in the liver, a particularly fertile ground for HCC cells to proliferate. In accordance with Paget’s “seed and soil” hypothesis, the disseminating capacity of tumor cells rather than the local outgrowth is controlled by the “soil” characteristic (24). In P3 and P4, who showed no evidence of recurrence or metastasis, almost all afp-expressing circulating cells detected were cleared within 2–4 weeks. The selective growth of highly metastatic tumor cells is definitely required, which are endowed with properties allowing them to complete the metastatic cascade successfully.

The high-risk patients P11 and P12, who showed the largest circulating tumor mass, demonstrated evidence of lung metastasis or liver recurrence after the elevation of afp mRNA levels. A proportion of the HCC cells detected appeared to implant and form clinically detectable metastasis or recurrence in the fertile “soil.” The frequency of relapse/death seems to correlate with the circulating tumor mass, as has been shown by immunohistochemistry in lung cancer patients undergoing surgery (18). Sequential quantification of afp mRNA in peripheral blood would thus shed light on the persistent existence or removal of circulating HCC cells. Conversely, P7, who developed liver recurrence, showed a larger circulating tumor mass preoperatively as compared with that detected postoperatively. It is possible that micrometastasis could be established while still in an intravascular stage, at which some preexisting circulating HCC cells were sequestered and hence undetected postoperatively (25).

It is well documented that gene expression in tumor cells is heterogeneous. Levels of alb mRNA did not correlate directly with levels of afp mRNA in the 53 peripheral blood samples analyzed. The circulating HCC cells detected postoperatively in all of the nine patients expressed the afp gene more strongly than the alb gene. This is in concordance with the considerable heterogeneity in cellular distribution and expression of alb and afp mRNAs in moderately and poorly differentiated HCCs (26). These circulating tumor cells disseminated postoperatively, regardless of the tumor size or the resection margin, and were possibly released from undetectable micrometastases or minimal residual tumor in the liver as a consequence of surgical maneuver. Apparently, they were not liberated from primary HCCs into the circulation during surgical intervention, as implicated by the negligible afp mRNA levels detected intraoperatively in all of the HCC patients studied. It is likely that they were biologically triggered to disseminate postoperatively by cytokines and growth factors released from the regenerating liver (27–30).

A disease-free survival rate of 62% (8 of 13) at 3 years was observed among our patients. Four survivors showed afp mRNA...
levels within the reference range during the periods monitored. The other two showed the clearance of the alpha fetoprotein (AFP) mRNA signal from peripheral blood after its initial detection after resection. Overall, the absence or clearance of AFP mRNA in peripheral blood was strongly associated with the absence of local recurrence or distant metastasis in 75% (six of eight) of survivors. In striking contrast, all of the patients who died from intrahepatic/extrahepatic metastasis, liver recurrence, or persistent HCC had shown persistently raised AFP mRNA levels in the peripheral blood. Our findings could potentially open up prognostic implications that would be consolidated by our current quantitative analysis of a larger cohort of patients together with long-term follow-up.

We present evidence that alb-expressing hepatocytes are released intraoperatively into the circulation, and alb-expressing HCC cells are disseminated mostly postoperatively that may implicitly be the source of recurrence or metastasis in HCC patients undergoing surgical resection. In the near future, this quantitative information may provide an additional guideline for patients undergoing surgical resection. Sequential quantification of circulating HCC cells by our rapid and reliable method may prove valuable for assessing the potential for metastasis and monitoring patients’ response to therapies.

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