Impact of Cytomorphological Detection of Circulating Tumor Cells in Patients With Liver Cancer

Giovanna Vona,[1] Laurence Estepa,[2] Christophe Béroud,[2] Diane Damotte,[3] Frédérique Capron,[4] Bertrand Nalpas,[1,5] Alexandra Mineur,[5] Dominique Franco,[6] Bernard Lacour,[2] Stanislas Pol,[5] Christian Bréchot,[1,5] and Patrizia Paterlini-Bréchot[1,2]

The clinical impact of circulating tumor cell (CTC) detection is controversial, mainly due to drawbacks of molecular approaches applied to this field. We sought to determine if the specific identification and counting of circulating tumor cells by cytomorphologic analysis has clinical usefulness. Peripheral blood (6 mL), treated using isolation by size of epithelial tumor cells, was obtained from 44 patients with primary liver cancer (PLC) and without metastases, 30 patients with chronic active hepatitis, 39 with liver cirrhosis, and 38 healthy individuals, and followed up for a mean period of 1 year. We searched for β-catenin mutations in 60 single microdissected CTCs. One patient with liver cancer developed extrahepatic metastases during follow-up. CTCs and microemboli were found in 23 of the 44 patients with liver cancer and in none of the patients with chronic active hepatitis, patients with cirrhosis, or healthy subjects. Their presence was significantly associated with tumor diffusion ($P = .0001$) and portal tumor thrombosis ($P = .006$). Both the presence ($P = .01$) and number ($P = .02$) of CTCs and microemboli were significantly associated with a shorter survival. β-Catenin mutations were found in 3 of 60 CTCs, arguing against their impact on the initial step of tumor cell invasion. In conclusion, the highly sensitive and specific detection of CTCs and microemboli may have clinical implications for cancer staging and outcome prediction. We also show the feasibility of molecular studies of individual circulating tumor cells, aimed at identifying gene mutations involved in tumor invasion. (HEPATOLOGY **2004;39:792–797.**)

The spread of malignant cells in the peripheral blood of patients with cancer plays a major role in the process leading to metastases and/or recurrence after surgery.[1] In the case of patients with primary liver cancer (PLC), circulating tumor cells (CTCs) have been reported; however, their clinical impact on disease outcome is presently unknown.[2] This is mainly because (1) specific tests for identifying and counting CTCs are lacking and (2) extrahepatic metastases occur only rarely in these patients.[3] Furthermore, the significance of CTC and circulating tumor microemboli (CTM) detection in patients who are going to die without developing tumor recurrence or metastases is also unknown.

The major limitation of detection of CTCs is that they are rare (≤1/mL) and thus require a highly sensitive and specific method for their identification and characterization. Molecular methods such as reverse-transcriptase polymerase chain reaction have been used for this purpose in the past[4,5]; however, a number of technical and methodologic drawbacks have limited their interpretation. Isolation by size of epithelial tumor cells (ISET) allows a highly sensitive enrichment, morphological identification, and counting of CTCs and CTM.[6] We have now applied this approach to patients with PLC in order to evaluate its clinical impact.

Recently, mutations and/or deletions clustered in exon 3 of the β-catenin gene[7,8]—which encodes a mutifunc-

Abbreviations: CTC, circulating tumor cell; ISET, isolation by size of epithelial tumor cells; PLC, primary liver cancer; CTM, circulating tumor microemboli; CT, computed tomography; EAC, epithelial atypical cell; PEP, primer extension preamplification; PCR, polymerase chain reaction; HCC, hepatocellular carcinoma.

From [1]Pasteur/INSERM Unit 370, Faculté de Médecine Necker Enfants Malades, Paris, France; the Departments of [2]Biochemistry, [3]Pathology, and [5]Hepatology, Hôpital Necker, Paris, France; and the Departments of [4]Pathology and [6]Surgery, Hôpital A. Beclère, Clamart, France.

Received May 23, 2003; accepted November 24, 2003.

Supported by grants from Institut National de Santé et Recherche Médicale, Ligue National contre le Cancer, Association pour la Recherche contre le Cancer, and Paris Biotech.

G.V., L.E., and C. Béroud contributed equally to this work.

Address reprint requests to: Patrizia Paterlini-Bréchot: Pasteur/INSERM Unit 370, Faculté de Médecine Necker, 156, rue de Vaugirard, 75730 Paris cedex 15, France. E-mail: paterlini@necker.fr; fax: +33-1-40615581.

Published online in Wiley InterScience (www.interscience.wiley.com).

DOI 10.1002/hep.20091

tional protein involved in cell–cell adhesion and Wingless/Wnt signaling[9,10]—have been reported in an average of 20% (18%–41%) of human PLC. β-Catenin exon 3 mutations have been shown to block the protein phosphorylation and ubiquitination. This leads to stabilization of cytoplasmic-free β-catenin and its translocation to the nucleus, where it has been reported to transcriptionally activate target genes like cyclin D1 and matrix metalloproteinase.[7,11] In this work we looked for β-catenin mutations in circulating CTCs in an attempt to assess their impact on the early steps of tumor cell invasion in patients with PLC.

Materials and Methods

We studied 44 patients with PLC and without extrahepatic metastases (32 men and 12 women 50–79 years old), 30 patients with chronic hepatitis (16 men and 14 women 35–56 years old), 39 patients with liver cirrhosis (25 men and 14 women 43–62 years old), and 38 healthy individuals (23 men and 15 women 31–52 years old). Patients with PLC were tested at diagnosis. Clinical, biochemical, and histopathologic analyses were then performed to evaluate the extent of tumor development. Eligibility for surgery was defined according to the following parameters:

- Pugh's score lower than 7
- serum bilirubin concentration below 25 micromoles/L
- absence of ascites
- absence of extrahepatic metastases
- less than four tumor nodules
- presence of a noninvaded, 10-mm-wide or larger liver area around the tumor at computed tomography (CT) analysis, which suggests the absence of tumor diffusion.

Consistently, we will use the term "localized tumor" or "diffuse tumor" according to the absence or presence of signs of tumor invasion around the lesion assessed by CT. Patients with localized tumors underwent liver resection. Extrahepatic invasion was also carefully ruled out preoperatively through abdominal CT and chest X rays. Portal thrombosis was assessed by pathologic and/or imaging analyses. Extrahepatic metastases were investigated using chest X rays, ultrasonography, and CT. Patients were followed up for a mean period of 50 ± 48 weeks. Informed consent was obtained from each patient who participated in the study. The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki as reflected in *a priori* approval by the Necker Hospital review committee.

The ISET method has already been reported.[6] Peripheral blood (6 mL) was collected on buffered ethylendiaminetetracetic acid, diluted 1:10 with the filtration buffer, and filtered using an ISET device (Metagenex.fr, Paris, France). At the end of this step, each spot on the filter contains the large cells derived from 1 mL of blood. Three spots per sample—equivalent to 3 mL of blood—were stained and/or immunostained (anti–α-fetoprotein antibody).

CTCs were identified as large cells (cell diameter >25 μm) with a high nucleus/cytoplasmic ratio, irregular chromatin, and/or nuclear shape. CTM were identified as groups of adherent tumor cells. Cells having intermediate characteristics (*e.g.*, large cells with a normal nucleus/cytoplasmic ratio and/or regular chromatin or nuclear shape) were classified as epithelial atypical cells (EAC). Nuclear shape and cell size were assessed using the Adobe Photoshop software and compared to the size of the filter-calibrated pores (8 μm).

Single-stained or immunostained tumor cells were captured by way of laser capture microdissection (Acturus Pix cell II, Mountain View, CA) using the combination of low-energy solid laser and a simple transfer film on a capsule. DNA was extracted by using lysis buffer (100 mM Tris-HCl pH 8 and 400 μg/mL proteinase K) at 37° C for 16 hours. After proteinase K inactivation at 90° C for 10 minutes, DNA was amplified using the modified primer extension pre-amplification (PEP) protocol as previously described.[6]

The β-catenin exon 3 was amplified by way of a nested PCR protocol using 10 μL of the PEP product and the following β-catenin primers: forward primer 5′-ATTTGATGGAGTTGGACATGGC-3′, reverse primer 5′-ATCAGCTCTTGTTCTTGAGTGA-3′. The first PCR round was performed in 100 μL of PCR buffer containing 2 mM $MgCl_2$, 0.25 mM desoxynucleoside triphosphates (dATP, dTTP, dCTP, and dGTP), 10 pmoles of each β-catenin primer, and 2.5 units of Taq polymerase (PerkinElmer Cetus, Emeryville, CA).

For nested PCR, 2 μL of the first reaction product was subjected to further amplification in a microfuge tube containing 100 μL of PCR buffer and 10 pmoles of the two β-catenin internal primers: forward primer 5′-ACATGGCCATGGAACCAGACAGA-3′ and reverse primer 5′-GAGTGAAGGACTGAGAAAATCCCTG-3′. Amplifications were carried out for 40 cycles in a thermocycler (9500, PerkinElmer) (30 seconds each at 94° C, 55° C, and 72° C). Amplification products were analyzed by way of 2% agarose gel electrophoresis and ethidium bromide staining. The exon 3–specific bands were purified by spin column and sequenced on an ABI Prism 310 automated sequencer (Applied Biosystems, Foster City, CA) using a Big Dye terminator cycle sequencing kit and AmpliTaq DNA polymerase. We used the two β-catenin internal primers as sequencing primers. Mutations were confirmed by sequence analysis of both DNA strands obtained from two independent PCR tests. In each test, we introduced as a positive

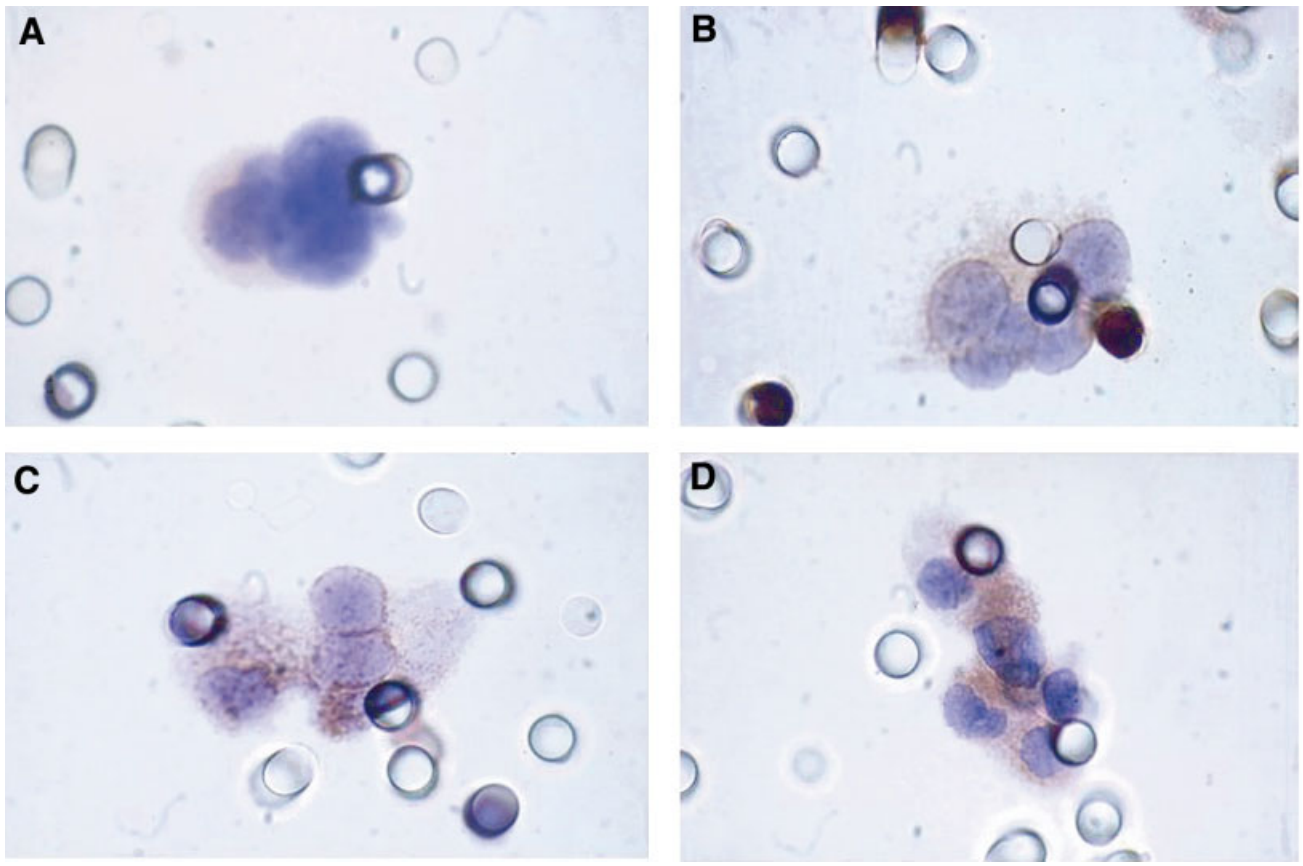

Fig. 1. CTCs and CTM isolated by way of ISET from the peripheral blood of patients with liver cancer. (A) Anti–α-fetoprotein–positive mononuclear CTC characterized by large size (42 μm), a high nucleus/cytoplasmic ratio (0.83) and irregular nuclear shape. (B) Anti–α-fetoprotein–positive plurinucleated CTC characterized by large size (41 μm) and a high nucleus/cytoplasmic ratio (0.7). (C) Anti–α-fetoprotein–positive CTM formed by a monunucleated CTC (**left**) and a trinucleated CTC (**right**). (D) Anti–α-fetoprotein–positive CTM formed by several CTCs. Cell nuclei are counterstained with Mayer's hematoxylin ($\times$100).

control one single microdissected HuH6 cell (known to have a mutation in β-catenin exon 3).

The χ^2 test or Fisher exact test was used for statistical analyses of between-group frequencies. The cumulative overall survival rates were calculated using the Kaplan-Meier method. Survival curves were compared by means of the log-rank test. Univariate and multivariate analyses were made using SPSS-10.0 statistical software (SPSS Inc., Chicago, IL). Multivariate analysis was performed using the Cox model approach and the following variables:

- presence versus absence of portal thrombosis
- presence versus absence of CTCs/CTM
- monofocal versus multifocal tumor
- eligibility to surgery versus ineligibility to surgery
- Child-Pugh class A versus Child-Pugh class B or C.

A P value of less than .05 was considered significant.

Results

None of the 38 healthy individuals scored positive for CTCs or CTM. None of the 30 patients with chronic active hepatitis and none of the 39 patients with cirrhosis scored positive for CTCs or CTM. Clinical, biochemical, and histopathologic analyses showed that 22 patients had a localized tumor, which was treated during follow-up by means of liver tumor resection. Twenty-two patients had diffuse, multinodular tumors and were considered ineligible for surgery. Twenty-three patients with PLC scored positive for CTCs (Fig. 1, Table 1), including two patients with CTM, five of whom had a localized tumor.

Table 1. CTCs and EACs in PLC and Control Patients

	Total Number of Patients	Number of Patients With CTCs/CTM	Number of Patients With EACs
PLC	44*	23 (1–10)†	22 (1–9)
Cirrhosis	39	0	6 (1–5)
Chronic hepatitis	30	0	7 (1–6)
Healthy individuals	38	0	0

Range of CTCs/CTM or EACs per 3 mL of blood is shown in parentheses.

*Twenty-two patients with localized PLC (eligible for surgery) and 22 patients with diffuse PLC (ineligible for surgery).

†Includes five patients with localized tumors and 18 with diffuse tumors, two of whom had tumor microemboli.

Table 2. CTCs According to Tumor Characteristics in 44 Patients With PLC

	Total Number of Patients	Number of Patients With CTCs	*P* Value
Eligibility for tumor resection			
Eligible	22	5	$P = .0001$
Ineligible	22	18	
Tumor noduli			
1	16	6	NS
>1	27	16	
Not evaluated	1		
Size (cm) of the largest nodule			
≤3	16	8	NS
>3-<10	14	7	
>10	11	5	
Not evaluated	3		
Portal thrombosis			
Absent	24	8	$P = .006$
Present	20	15	

Abbreviation: NS, not significant.

The presence of CTCs/CTM was more frequent in patients with diffuse tumors ($P = .0001$) and was associated with portal tumor thrombosis ($P = .006$) but not with the number and size of tumor nodules (Table 2). Only one patient—who had a diffuse tumor—developed extrahepatic metastases during follow-up. Twenty-one patients died during follow-up. The analysis of the relationship between CTCs/CTM in blood and the histologic evidence of microinvasion of cancer cells in tumor vessels was performed in 18 of the 22 patients who underwent liver tumor resection. Pathologic analysis revealed that tumors spread into the venous vessels around the tumorous tissue in 6 out of 18 patients, including the five who had CTCs/CTM in the peripheral blood. This result is consistent with the association we found between portal tumor thrombosis and presence of CTCs/CTM. CTCs/CTM presence was significantly more frequent in patients in Child-Pugh class B or C than in patients in Child-Pugh class A ($P = .001$). The α-fetoprotein value was also more frequently above the normal level in patients with than in patients without CTCs/CTM ($P = .03$). Both the presence of portal tumor thombosis ($P = .03$) and of multifocal tumors ($P = .02$) were significantly associated with Child-Pugh class B or C.

In univariate analysis, patients who were eligible for surgery (with localized tumor) showed a significantly increased survival compared with patients who were ineligible for surgery (with diffuse tumor) (Fig. 2A; $P = .04$). Patients without CTCs/CTM showed a significantly increased survival compared with patients with CTCs/CTM (Fig. 2B; $P = .01$). Patients having at least one CTM or four or more CTCs per 3 mL of blood showed a significantly decreased cumulative survival compared with patients with one to three CTCs and patients without CTCs per 3 mL of blood (Fig. 2C, $P = .02$). However, when CTCs/CTM plus EACs were taken into account, no significant association of their presence with survival was found in the group of nonoperated patients (Fig. 2D). Survival was significantly shorter in patients with more severe cirrhosis (Child-Pugh class B or C) compared with patients in Child-Pugh class A ($P = .02$). Presence of monofocal tumors was associated with a better survival than presence of multifocal tumors (80% versus 20%; $P = .03$). Absence of portal tumor thrombosis was also significantly associated with a longer survival ($P = .01$).

In the multivariate analysis, when all the five criteria that were significantly associated with a better survival in the univariate analysis (eligibility for surgery, absence of CTCs/CTM, Child-Pugh class A, presence of monofocal tumors, and absence of portal thrombosis) were taken into account, none of them was independently associated with survival. Because this result may be related to the high number of the included parameters compared with the number of events and the size of the sample, we repeated the analysis excluding the Child-Pugh class and the eligibility for surgery—which are composite variables—and found that only portal tumor thrombosis was associated with decreased survival as an independent variable ($P = .05$; RR: 3.22), while the presence of CTCs/CTM did not reach significant value ($P = .16$; RR: 2.2).

β-Catenin mutations were assessed in isolated CTCs and CTM (Table 3). One cell from the HCC-derived cell line, HuH6—which carried a β-catenin mutation at codon 34 (Gly to Val)—was inserted as positive control in each series of tested samples. The HuH6 β-catenin mutation was consistently found at codon 34 (G to T) in each test, demonstrating that the PEP and the nested PCR protocols did not modify the amplified sequence. As negative controls, we used single microdissected cells from cell lines HuH7 and LNCaP and 24 nontumorous cells (monocytes) microdissected from the patients under study. None of these cells scored positive for β-catenin mutations. A variable number of CTCs (from 4 to 13 per patient; total: 60) were microdissected from 10 patients—including two CTM from two different patients—and tested for mutations in the exon 3 of β-catenin. A β-catenin mutation was found in two CTCs (codon 42: C to T, Thr to Ile; codon 66: G to T, Trp to Leu) and one CTM (codon 30: A to G, Tyr to Cys) derived from three different patients. These results show that cells carrying a β-catenin gene mutation are not specifically selected in CTCs spread from the primary liver tumor.

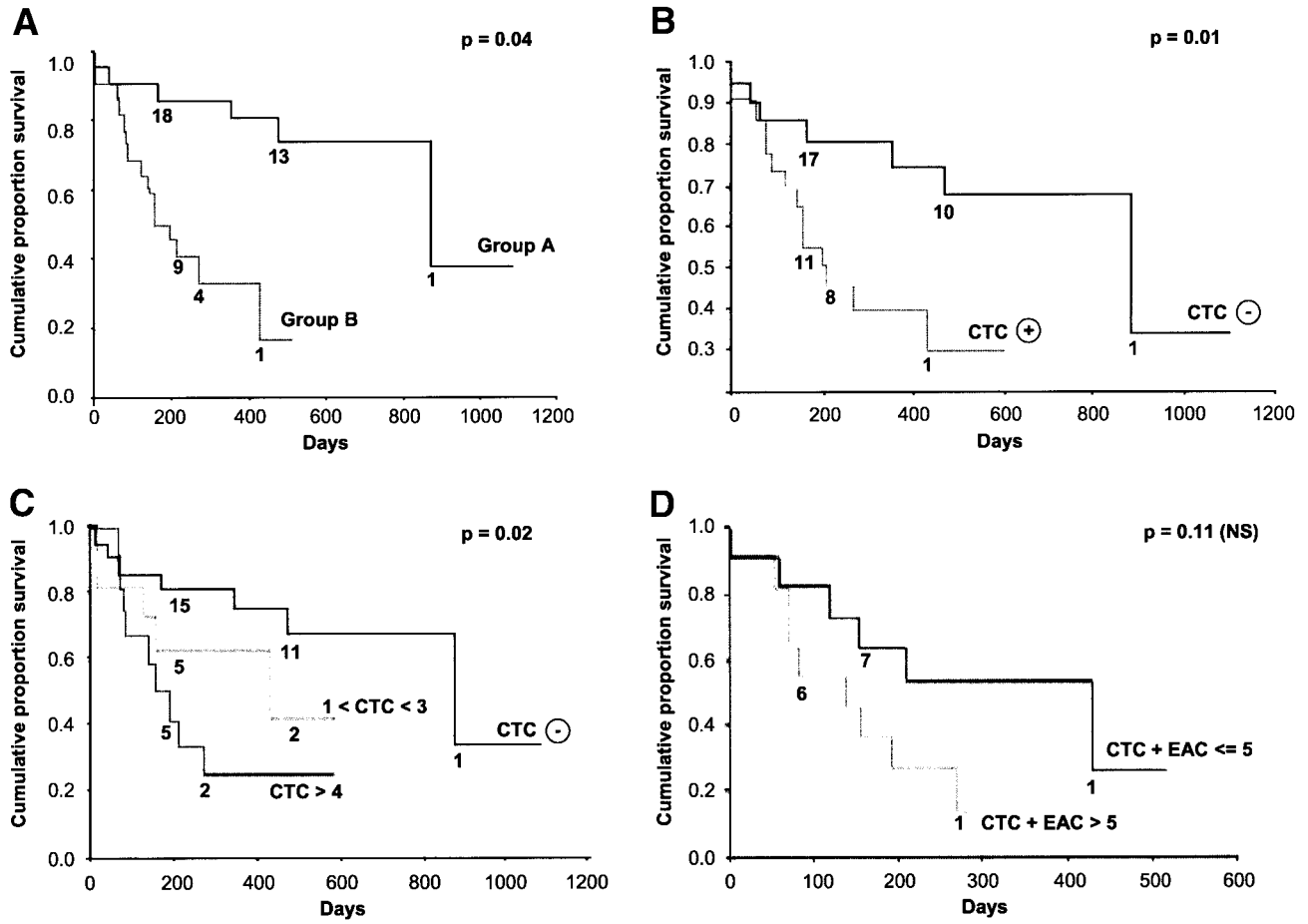

Fig. 2. Kaplan-Meier survival curves of patients with PLC. Patients were classified according to: (A) localized tumor (group A) and diffused tumor (group B); (B) presence (CTC+) and absence (CTC−) of CTCs per 3 mL of blood; (C) number of CTCs: absence (CTC−), one to three CTCs (1<CTC<3), and four or more CTCs (CTC>4) per 3 mL of blood; (D) presence of more (CTC+EAC>5) or less (CTC+EAC≤5) than five CTCs plus EACs per 3 mL of blood (analysis performed in patients with diffused tumors only). Numbers below the curves indicate the number of patients still at risk at the corresponding time. *P* value was calculated using the log rank test.

Discussion

In this article we have used ISET to specifically identify CTCs and CTM by way of cytomorphologic analysis. Our results show that the spontaneous circulation of CTCs in peripheral blood is a sign of tumor progression and tumor spread in patients with PLC, a tumor which is known to develop a low rate of extrahepatic metastases. (In fact, only one patient in our study developed extrahepatic metastases.) We found that the presence of CTCs is significantly associated with the presence of a diffuse tumor (defined as a tumor surrounded by a presumably invaded area at the CT analysis), portal tumor thrombosis (detected by way of his-

Table 3. β-Catenin Mutations (Exon 3) in Individually Microdissected Cells

Type of Microdissected Cells (Number)	Number of Patients	Number of Cells With Mutation (Number of Patients)	Type of β-Catenin Mutation
CTCs (60)*	10	3 (3)†	Codon 42: Thr > Ile Codon 66: Trp > Leu Codon 30: Tyr > Cys
Circulating nontumor cells (24)	10	0	
HuH6 (5)		5	Codon 34: Gly > Val‡
HuH7 (3)		0	
LNCaP (3)		0	

*Includes two CTM.

†Includes one CTM.

‡The same mutation was found in all the tested HuH6.

topathologic analysis and/or CT), and advanced liver disease (Child-Pugh class B or C). Therefore, our results are consistent with the idea that in patients with PLC the presence of CTCs may be an interesting marker of tumor spread. We also found through Kaplan-Meier analysis that the presence and number of CTCs were significantly associated with shorter survival. However, the multivariate analysis, performed using the five variables associated with survival in the univariate analysis, showed that none of them was significantly associated survival as an independent variable. Taking into account that this study has been performed on a limited number of patients, our results encourage assessment of the clinical impact of CTC detection by using a cytomorphologic approach on a larger series of patients with PLC. Our preliminary data suggest that noninvasive identification and counting of CTCs in patients with liver cancer could have a clinical impact on staging and a prognostic value independent of the development of metastases. The assessment of the impact of this analysis in patients with solid tumors who develop a higher rate of metastases is ongoing. Compared with expensive and cumbersome molecular techniques (*e.g.*, reverse-transcriptase polymerase chain reaction), ISET is simple, inexpensive, and allows applying the cytopathological diagnosis of tumor cells, already widely used in clinical oncology, to peripheral blood samples. It also affords to specifically identify and count circulating tumor microemboli (CTM), and to perform immunocytological and molecular studies.[6]

A major issue in molecular oncology is the possibility of identifying gene mutations in spontaneous CTCs, which potentially play a role in the first steps of tumor invasion. β-Catenin exon 3 mutations and the nuclear protein accumulation have been reported to be related to increased proliferative activity,[12] reduced cell adhesion and dedifferentiation,[13] tumor progression,[14,15] and increased metastatic potential.[16] On the one hand, expression of mutant nuclear β-catenin has been shown to correlate with noninvasive HCC, absence of portal vein thrombosis, and good prognosis[17]; on the other hand, it has been recently reported that β-catenin is transported along microtubules and accumulates in the tips of membrane protrusion so that it may regulate cell migration.[18] We found genetic mutations of β-catenin exon 3 only rarely (3 cells out of 60) in individual, spontaneously circulating, liver-derived tumor cells, showing that this mutation does not play an important role in the early steps of invasion. However, our repeated analyses of single HuH6 cells, which carry a point mutation at codon 34, and of nontumorous cells, which do not carry mutations of β-catenin exon 3, clearly established that the pre-amplification of single-cell DNA does not modify the target DNA sequence; therefore, this kind of genetic investigation is now feasible for CTCs.

In conclusion, this preliminary work suggests that the specific identification and counting of CTCs/CTM is potentially an interesting test for tumor staging and outcome prediction and could have potential implications for therapeutic choices. A large multicenter study is required to precisely assess the predictive value of the ISET approach. It also shows that the ISET approach allows addressing molecular studies to CTCs, which were previously not available for genetic analysis.

References

1. Vogel I, Kalthoff H. Disseminated tumour cells. Their detection and significance for prognosis of gastrointestinal and pancreatic carcinomas. Virchows Arch 2001;439:109–117.
2. Paterlini-Bréchot P, Vona G, Brechot C. Circulating tumorous cells in patients with hepatocellular carcinoma. Clinical impact and future directions. Semin Cancer Biol 2000;10:241–249.
3. Romeo R, Colombo M. The natural history of hepatocellular carcinoma. Toxicology 2002;181-182:39–42.
4. Ghossein R, Bhattacharya S. Molecular detection and characterisation of circulating tumour cells and micrometastases in solid tumours. Eur J Cancer 2000;36:1681–1694.
5. Ghossein R, Bhattacharya S. Molecular detection and characterization of circulating tumor cells and micrometastases in prostatic, urothelial, and renal cell carcinomas. Semin Surg Oncol 2001;20:304–311.
6. Vona G, Sabile A, Louha M, Sitruk V, Romana S, Schütze K, Capron F, et al. Isolation by size of epithelial tumor cells: a new method for the immunomorphological and molecular characterization of circulating tumor cells. Am J Pathol 2000;156:57–63.
7. Wong C, Fan S, Ng I. beta-Catenin mutation and overexpression in hepatocellular carcinoma: clinicopathologic and prognostic significance. Cancer 2001;92:136–145.
8. Morin P. beta-catenin signaling and cancer. Bioessays 1999;21:1021–1030.
9. Polakis P. The oncogenic activation of beta-catenin. Curr Opin Genet Dev 1999;9:15–21.
10. Polakis P. Wnt signaling and cancer. Genes Dev 2000;14:1837–1851.
11. Buendia M. Genetics of hepatocellular carcinoma. Semin Cancer Biol 2000;10:185–200.
12. Nhieu J, Renard C, Wei Y, Cherqui D, Zafrani E, Buendia M. Nuclear accumulation of mutated beta-catenin in hepatocellular carcinoma is associated with increased cell proliferation. Am J Pathol 1999;155:703–710.
13. An F, Matsuda M, Fujii H, Tang R, Amemiya H, Dai Y, Matsumoto Y. Tumor heterogeneity in small hepatocellular carcinoma: analysis of tumor cell proliferation, expression and mutation of p53 and beta-catenin. Int J Cancer 2001;93:468–474.
14. Calvisi D, Factor V, Loi R, Thorgeirsson S. Activation of beta-catenin during hepatocarcinogenesis in transgenic mouse models: relationship to phenotype and tumor grade. Cancer Res 2001;61:2085–2091.
15. Inagawa S, Itabashi M, Adachi S, Kawamoto T, Hori M, Shimazaki J, Yoshimi F, et al. Expression and prognostic roles of beta-catenin in hepatocellular carcinoma: correlation with tumor progression and postoperative survival. Clin Cancer Res 2002;8:450–456.
16. Ogawa K, Nakanishi H, Takeshita F, Futakuchi M, Asamoto M, Imaida K, Tatematsu M, et al. Establishment of rat hepatocellular carcinoma cell lines with differing metastatic potential in nude mice. Int J Cancer 2001; 91:797–802.
17. Mao T, Chu J, Jeng Y, Lai P, Hsu H. Expression of mutant nuclear beta-catenin correlates with non-invasive hepatocellular carcinoma, absence of portal vein spread, and good prognosis. J Pathol 2001;193:95–101.
18. Jimbo T, Kawasaki Y, Koyama R, Sato R, Takada S, Haraguchi K, Akiyama T. Identification of a link between the tumour suppressor APC and the kinesin superfamily. Nat Cell Biol 2002;4:323–327.