Orthotopic Implantation of Human Hepatocellular Carcinoma in Mice: Analysis of Tumor Progression and Establishment of the BCLC-9 Cell Line

Carolina Armengol, Gemma Tarafa, Loreto Boix, Manel Solé, Rosa Queralt, Dolores Costa, Oriol Bachs, Jordi Bruix, and Gabriel Capella

ABSTRACT

Purpose: To allow the longitudinal investigation of molecular events associated with the progression of human hepatocellular carcinoma (HCC), we sought to develop a murine model by orthotopic implantation of tumor fragments obtained from patients diagnosed at early stage.

Experimental Design: Tumor pieces (2 × 2 mm) were implanted on the liver surface of nude mice. After xenograft growing, subsequent passages were performed to achieve long-term implant viability. Isolation of tumoral hepatocytes was done to establish new cell lines. HCC characteristics, proliferation rate, apoptotic index (terminal deoxynucleotidyl transferase-mediated nick end labeling), and expression of cell-cycle regulators (cyclins E and A, p21Cip1, p27Kip1, p16INK4a, pRb, and p53) were assessed by Western blot and immunohistochemistry, to correlate them with tumor progression.

Results: Five (50%) of the 10 primary HCCs resulted in small slow-growing liver implants. Three of them are viable after 48 months, whereas the remaining two survived for 15 and 13 months. Xenografts throughout passages exhibited a more aggressive phenotype with a poorer degree of differentiation, intense proliferation, moderate apoptosis, cell-cycle deregulation, p53 alterations, microvascular invasion, and dissemination. In one single passage, we observed critical growth delay, which was associated with significant p27kip1 overexpression. We established the anchor-free growing BCLC-9 cell line from one xenograft. This has gains of chromosomes 7, 5p, 6q, and 9q, is hepatitis B virus-DNA positive, does not secrete α-fetoprotein, and has TP53 missense mutations in codons 192 and 242.

Conclusions: The orthotopic implantation of early HCC fragments in nude mice provides a useful model to investigate the mechanisms of human HCC evolution and to establish new cell lines.

INTRODUCTION

Hepatocellular carcinoma (HCC) mostly affects patients with viral cirrhosis (1, 2) and constitutes a worldwide major cause of cancer-related death (3, 4). At initial evolutionary stages, the tumor is well differentiated and the progression to more advanced stages with loss of differentiation and appearance of an invasive phenotype may require months or even years (5, 6). Interestingly, in a minority of patients, HCC can regress or even disappear because of unknown mechanisms (7). Tumor progression is achieved by the sequential accumulation of molecular alterations that bring the cells into a more transformed stage characterized by an increased proliferation. Cell proliferation is tightly regulated by the cell cycle machinery (8) that includes cyclins, cyclin-dependent kinases (CDK), and their inhibitors (CDK inhibitors) as p27kip1, p21Cip1, p16INK4a, pRB, and p53) were assessed by Western blot and immunohistochemistry, to correlate them with tumor progression.

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Notes: C. Armengol and G. Tarafa contributed equally to this study.

Requests for reprints: Jordi Bruix, BCLC Group, Liver Unit, Hospital Clinic, Villarroel 170, 08036 Barcelona, Spain. Fax: 34-932279803; E-mail: jbruix@clinic.ub.es.
To establish new human cell lines, we sought to use pathological specimens for xenografting and characterize their successful liver implants (xenografts). We studied the mechanisms involved in developing an animal model derived from early human HCC and evaluated whether it reproduces the evolutionary characteristics observed in humans. Accordingly, intact specimens of human HCC at initial stages were orthotopically implanted in nude mice, and their progression was assessed pathologically and by registering proliferation activity and apoptotic rate, as well as the expression of cell cycle regulators (cyclin E, cyclin A, p21Cip1, p27Kip1, p16InK4a, pRb, and p53). Finally, on establishing successful liver implants (xenografts), we sought to use them to establish new human cell lines.

MATERIALS AND METHODS

Human HCC Tissues

Ten fresh pieces of tumor tissue were obtained at the time of surgical resection or liver transplantation, in a series of nine patients diagnosed with early HCC (Table 1). All but two patients had underlying cirrhosis due to hepatitis B (HBV) or hepatitis C viruses (HCV). The two patients with a healthy liver had no serological markers of active viral infection. However, in one of them, HBV-DNA was found in the liver tissue, thus indicating occult HBV infection. In two patients (HC-3 and HC-7), small additional well and moderately differentiated tumors without evidence of vascular invasion were disclosed at pathological examination in explanted liver, probably representing occult HBV infection. In two patients (HC-3 and HC-7a) with a healthy liver, HBV-DNA was found in the liver tissue, thus indicating occult HBV infection. In two patients (HC-3 and HC-7a), small additional well and moderately differentiated tumors without evidence of vascular invasion were disclosed at pathological examination in explanted liver, probably representing occult HBV infection. In two patients (HC-3 and HC-7a), small additional well and moderately differentiated tumors without evidence of vascular invasion were disclosed at pathological examination in explanted liver, probably representing occult HBV infection.

Patient code | Sex/ Age (yr) | Underlying disease | Trb | AFP | Tumor size (cm) | Differ. Degree | Cell pattern | Ki67 LF (%) | No. of HCCs | Vascular invasion |
---|---|---|---|---|---|---|---|---|---|---|
HC-1 | M/63 | HCV liver cirrhosis | R | N | 4 | W | Trabecular-acinar | 8.1 | 1 | No |
HC-2 | M/52 | Healthy liver | R | N | 3.4 | W to Mod | Trabecular-acinar | 0.2 | 1 | No |
HC-3 | F/57 | HCV liver cirrhosis | LT | N | 2 | W to Mod | Trabecular-acinar | 3.3 | 4 | No |
HC-4 | F/65 | HCV liver cirrhosis | R | E | 6 | W to Mod | Trabecular | 11.6 | 1 | No |
HC-5 | F/71 | HBV liver cirrhosis | R | E | 1 | Mod | Trabecular | 21.3 | 1 | No |
HC-6 | F/69 | HCV liver cirrhosis | R | E | 3 | W | Trabecular | 16.7 | 1 | No |
HC-7 | M/60 | HBV liver cirrhosis | LT | N | 5 (A) | Mod | Trabecular-acinar | 0 | 4 | No |
HC-8 | M/56 | HCV liver cirrhosis | LT | N | 5.5 | Mod | Macrotubecular-pseudoacinar | 7.6 | 1 | No |
HC-9 | F/70 | HBV Healthy liver | R | N | 7 | W | Microtubecular-pseudoacinar | 21.6 | 1 | No |

Table 1: Clinical and pathological characteristics of patients with hepatocellular carcinoma (HCC) from which orthotopic implants were derived.

- Defined according to pathology examination.
- Treatment; HCV, hepatitis C virus; HBV, hepatitis B virus; R, surgical resection; LT, liver transplant; AFP, α-fetoprotein; N, normal levels (≤20 ng/mL); E, elevated levels (>20 ng/mL); Differ., differentiation; W, well differentiated; Mod, moderately differentiated; LI, labeling index.
- Number of cells stained/total cells analyzed in Ki67 immunohistochemistry.
- Data of patient HC-7 reflect the characteristics of the two nodules (A and B) used for grafting.

Thus, it has been useful to study the mechanisms involved in a terminal tumor stage, but it offered no insight into the processes that regulate the biological evolution at earlier stages. The present investigation assesses, first of all, the feasibility of developing an animal model derived from early human HCC and evaluates whether it reproduces the evolutionary characteristics observed in humans. Accordingly, intact specimens of human HCC at initial stages were orthotopically implanted in nude mice, and their progression was assessed pathologically and by registering proliferation activity and apoptotic rate, as well as the expression of cell cycle regulators (cyclin E, cyclin A, p21Cip1, p27Kip1, p16InK4a, pRb, and p53). Finally, on establishing successful liver implants (xenografts), we sought to use them to establish new human cell lines.

**Orthotopic Implantation in Nude Mice**

Five-to-6-week-old male nu/nu Swiss mice, weighing 18–22 g (Iffa-Credo Animaux de Laboratoire, L’Abresle, France), were used for HCC implantation. Two pieces of each tumor were implanted in two different mice. The mice were anesthetized using i.p. injection of Avertin, 2,2,2-Tribromoethanol (SIGMA Chemical Co) at a dose of 0.3 mg/g. After performing a median laparotomy, the tumor fragment was anchored to the anterior hepatic lobe with a Prolene 6-0 suture. The abdominal incision was closed with staples. In the first passages, another tumor piece was placed s.c. after Matrigel soaking. Mice were inspected twice a week. Development of the successful liver implant was examined by an exploratory laparotomy under anesthesia at least every 6 weeks. Successive passages into new mice were done at least 3 months after implantation. At that time, the mice were sacrificed by cervical dislocation and were extensively explored to rule out the presence of hepatic or extrahepatic dissemination. At each passage, small fragments of the xenograft were snap-frozen or formalin fixed, and, if enough tissue was available, we used fragments to isolate tumoral hepatocytes. Blood was obtained by cardiac puncture.

**Characterization of Xenografts**

All of the primary HCC tumors and xenografts collected throughout successive passages were characterized in detail. The human origin of the passaged xenografts was confirmed by checking for microsatellite instability (20) within two markers, analysis. The remaining fragments were fixed in formalin and embedded in paraffin for conventional pathological study and immunohistochemistry.

The Ethics Committee of the Hospital Clinic approved this study. Informed consent was requested from the patients before tissue collection. Animal-use and animal-care protocols were approved by the Ethics Committee of Animal Experimentation of Institut Català d’Oncologia and by the Animal Experimentation Commission of Generalitat de Catalunya according to National and European legal requirements.
BAT26 and D12S95, which show distinct and specific band profiles between human and mouse samples.

**Study of Cell Proliferation and Apoptosis.** Cell proliferation and protein expression of cell cycle regulators was assessed by Ki67 immunohistochemistry and Western blot, respectively, as published elsewhere (21). In one of the tumors (HC-2), we did not obtain enough protein yield. Fifty μg of protein, 25 μg for pRb Western, were separated electrophoretically in SDS-polyacrylamide gels and transferred onto Immobilon-P membranes (Millipore, Bedford, MA). To control protein loading, strips were incubated with 20% Ponceau S solution (SIGMA Chemical Co). Antigens were identified using polyclonal antibodies against cyclin A, p21Cip1, p16Ink4a, pRb (1:100 dilution except 1:200 for pRb; sc-751, sc-397, sc-468, and sc-050, respectively; Santa Cruz Biotechnology, Inc, Santa Cruz, CA), cyclin E (1:2500 dilution, 06-459; Upstate, Lake Placid, NY), and monoclonal antibody anti-p27Kip1 (1:200 dilution, sc-050, respectively; Santa Cruz Biotechnology, Inc). To correct for potential contamination of mouse cells (stromal and blood cells), we assumed a 10% cell contamination. Hence, to properly assess the protein expression by Western blot, we added in each blot a lane containing 20% of mouse liver sample as a reaction control. This did not show any significant band in any Western blots.

Apoptosis was estimated by measuring nuclear DNA fragmentation using the terminal deoxynucleotidyl transferasemediated nick end labeling assay (22). p53 protein was studied by immunohistochemistry using monoclonal antibody to human p53 (1:50 dilution, BP53-12; Novocastra, Newcastle, United Kingdom). Papillary endometrial adenocarcinoma and 0.1% BSA in PBS instead of primary antibody were used for positive and negative control, respectively. In addition, Tp53 gene mutations between exons 4 to 9 were analyzed by PCR using human intronic primers (23) and by direct sequencing.

**HCV and HBV Detection.** The presence of HCV-RNA and HBV-DNA in serum and tissue was assessed by reverse transcription after PCR (24) and nested PCR (25), respectively.

**Isolation and in Vitro Culture of Human Tumoral Hepatocytes.**

A xenograft piece was minced and then digested with 0.5 mg/ml collagenase type IV (SIGMA Chemical Co) at 37°C. Material was then placed in culture flasks containing supplemented Dulbecco’s modified Eagle’s and Nutrient Mixture F-12 HAM medium with 10% fetal calf serum in a humidified incubator at 37°C with 5% CO₂ in air. Medium was changed 3 times a week. For subculture, 0.25% trypsin-EDTA (Biological industries, Kibbutz Beit Haemek Israel) was used. Cultured cells were frozen and stored in liquid nitrogen in 10% dimethylsulfoxide in fetal calf serum.

**Characterization of Human HCC Cell Line.**

**Determination of Doubling Time.** Cells (5 × 10⁴) were seeded in 60-mm culture dishes. Viable cells determined by trypan blue exclusion were counted with a Neubauer chamber every day during the first 5 days after trypsin treatment. Population doubling time was calculated by plotting the mean cell number of three dishes versus days in culture during the exponential growth phase.

**Cytogenetic Analysis.** The cytogenetic study was carried out on G-banded chromosomes obtained from 24-h non-stimulated cell culture after adding 10 μg/ml colcemid (Life Technologies, Inc., Grand Island, NY). Karyotype was described according to the International System for Human Cytogenetic Nomenclature (26). Comparative genomic hybridization (CGH) and fluorescence in situ hybridization (FISH) analysis were performed with VYSIS material (Downers Grove, IL) according to the manufacturer’s protocols. In CGH analysis, 1 μg of DNA from cell line and normal reference were labeled with different fluorescent dUTP by nick translation. Labeled probes were mixed and hybridized onto healthy male metaphase target slides. Repetitive sequences were blocked by COT-1 DNA. The average ratio profile was calculated based on the analysis of 15 selected metaphase images. Thresholds for gains and losses were set at 1.25 and 0.75, respectively. FISH analysis was done using WCP5, WCP9, and WCP13 probes. FISH and CGH images were captured, processed, and evaluated with a Cytovision ULTRA (Applied Imaging, Pittsburgh, PA).

**Anchorage-Independent Growth Assay.** This was performed in three six-well dishes by suspending 5 × 10⁴ cells in 0.3% agar in complete medium and overlaying the suspension on presolidified 0.5% agar. Cells were incubated at standard conditions for 3 weeks.

**Study of Cell Proliferation and Cell Cycle Regulators.** Ki67 and p53 immunohistochemistry and Western blot of cell cycle regulators were assessed in nonsynchronized nonconfluent cells as described previously. For Western blot analysis, cells were lysed in 80 mM Tris-HCl (pH 6.8) with 2% SDS. Lysates were then sonicated twice for 10 s, and protein content was estimated by Lowry procedure.

**RESULTS**

**Orthotopic Implantation in Nude Mice.**

Successful hepatic grafting with growth of a small tumor nodule with well-defined limits and less than 1 cm in diameter was registered in five implants (Table 2; Fig. 1, A and B). Hepatic grafting coincided with the growth of the s.c. seed. Successful implants were obtained from patients with HBV or HCV infection, and also from tumor developed in a normal liver without evidence of viral infections. Three of the xenografts (HC-3, HC-7A, and HC-9) are still viable throughout 48, 33, and 32 months involving 12, 9, and 7 passages, respectively. The other two xenografts (HC-2 and HC-5) survived for 15 (3 passages) and 13 (3 passages) months, respectively. The successful grafting of the xenografts was not related to any of the clinico pathological or molecular primary tumor characteristics depicted in Table 1.

Local and distant dissemination was observed in two perforated xenografts (Table 2). HC-3 and HC-7A displayed several satellite nodules with evidence of vascular invasion. A later passage of HC-3 showed lymph node metastasis that was orthoimplanted in new mice, resulting in xenograft HC-3Mx.
Characterization of Orthotopic Implanted Tumors

Microsatellite analysis confirmed the human origin of the xenografts and did not disclose instability in any sample (data not shown).

Pathological Study. This analysis revealed that the xenografts at initial passages did not retain the architectural disposition of corresponding primary HCCs and showed a more undifferentiated cell pattern (Fig. 1C) with some necrotic areas. These malignant hepatocytes exhibited an immature appearance. Throughout successive passages, xenografts maintained a solid and acinar cell pattern (Table 2) with increased mitotic index.

Study of Cell Proliferation and Apoptosis. Ki67 labeling index of the primary tumors was heterogeneous ranging from 0 to 21.6% (Table 1) and relatively low because only two cases exceeded the accepted 20% cutoff used to classify tumors as highly proliferating. Proliferation activity of xenografts (Table 2) was increased with respect to their primary counterparts (Table 1) at first passages and reached almost 100% in the last passages. The increased proliferation in xenografts was associated with an altered expression of cell cycle regulators with respect to their nontumor counterparts, suggesting a cell cycle deregulation (Fig. 2). The expression pattern of positive regulators (cyclin E and cyclin A) in most of perpetuated xenografts increased throughout successive passages. Although the expression of negative regulators (p21Cip1, p27Kip1, p16INK4a) was reduced in several passages, in others their expression was increased. In this sense, in HC-9 at passage 3, we detected an overexpression of p27Kip1 and an induction of p21Cip1 expression with respect to the previous passage, this coinciding with a less aggressive tumor progression; only one of the two grafted mice resulted in a very tiny viable xenograft, which had a very slow growth curve that was maintained in ulterior passages. These expressed CDK inhibitors were functional and able to inhibit CDK2, because we observed a simultaneous decrease of CDK2 total kinase activity (18-fold less).

Table 2  Yield of implantation, pathological characteristics, proliferating activity, and dissemination patterns of human hepatocellular carcinoma (HCC) xenografts in nude mice

<table>
<thead>
<tr>
<th>Xenograft code</th>
<th>Short-term yield</th>
<th>Long-term yield</th>
<th>Viability (mo)</th>
<th>No. of passages</th>
<th>Tumor size (cm)</th>
<th>Differentiation Degreea</th>
<th>Cell patterna</th>
<th>Ki67 Labelling Index (%a,c)</th>
<th>Vascular invasiona</th>
<th>Dissemination pattern</th>
</tr>
</thead>
<tbody>
<tr>
<td>HC-1</td>
<td>No</td>
<td>No</td>
<td>15</td>
<td>3</td>
<td>0.40/0.36</td>
<td>M/ND</td>
<td>Acinar/ND</td>
<td>65.3/ND</td>
<td>No/ND</td>
<td>Intrahepatic</td>
</tr>
<tr>
<td>HC-2</td>
<td>Yes</td>
<td>No</td>
<td>48</td>
<td>12</td>
<td>0.45/0.47</td>
<td>M to P/P</td>
<td>Acinar-solid/solid/ND</td>
<td>29.5/98.6</td>
<td>Yes/Yes</td>
<td>Lymph node</td>
</tr>
<tr>
<td>HC-3</td>
<td>Yes</td>
<td>Yes</td>
<td>16</td>
<td>4</td>
<td>0.41/0.38</td>
<td>M/ND</td>
<td>Solid/ND</td>
<td>85.1/ND</td>
<td>Yes/No</td>
<td></td>
</tr>
<tr>
<td>HC-4</td>
<td>Yes</td>
<td>No</td>
<td>13</td>
<td>3</td>
<td>0.35/0.30</td>
<td>M/ND</td>
<td>Solid/ND</td>
<td>48.1/ND</td>
<td>No/ND</td>
<td></td>
</tr>
<tr>
<td>HC-5</td>
<td>Yes</td>
<td>No</td>
<td>33</td>
<td>9</td>
<td>0.31/0.36</td>
<td>M/M</td>
<td>Acinar-solid/acidinar-solid</td>
<td>96.9/94.8</td>
<td>Yes/Yes</td>
<td>Intrahepatic</td>
</tr>
<tr>
<td>HC-6</td>
<td>No</td>
<td>No</td>
<td>32</td>
<td>7</td>
<td>0.43/0.45</td>
<td>M/M to P</td>
<td>Trabecular/trabecular-solid</td>
<td>96.3/98.2</td>
<td>No/No</td>
<td></td>
</tr>
<tr>
<td>HC-7A</td>
<td>Yes</td>
<td>Yes</td>
<td>16</td>
<td>4</td>
<td>0.41/0.38</td>
<td>M/ND</td>
<td>Acinar-solid/acidinar-solid</td>
<td>96.9/94.8</td>
<td>Yes/Yes</td>
<td>Intrahepatic</td>
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<tr>
<td>HC-7B</td>
<td>No</td>
<td>No</td>
<td>32</td>
<td>6</td>
<td>0.35/0.30</td>
<td>M/M</td>
<td>Solid/ND</td>
<td>48.1/ND</td>
<td>Yes/No</td>
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<tr>
<td>HC-8</td>
<td>No</td>
<td>No</td>
<td>32</td>
<td>7</td>
<td>0.43/0.45</td>
<td>M/M to P</td>
<td>Trabecular/trabecular-solid</td>
<td>96.3/98.2</td>
<td>No/No</td>
<td></td>
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<tr>
<td>HC-9</td>
<td>Yes</td>
<td>Yes</td>
<td>32</td>
<td>7</td>
<td>0.43/0.45</td>
<td>M/M to P</td>
<td>Trabecular/trabecular-solid</td>
<td>96.3/98.2</td>
<td>No/No</td>
<td></td>
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a At early passages (passage 1 except for HC-5 case, which is passage 2)/at late passages (HC-3, passage 6; HC-7A, passage 8; HC-9, passage 4).

h Differentiation; M, moderately differentiated; P, poorly differentiated; LI, labeling index; ND, not done (material not available).

c Number of cells stained/total cells analyzed in Ki67 immunohistochemistry.

d HC-3Mx xenograft was obtained from the orthotopic implantation of the HC-3 lymph node metastasis.

Fig. 1  Successful orthotopic implant in the mouse liver (A). The xenograft is recognized as a solitary nodule protruding from the liver surface (B). Microscopic picture of HC-3 xenograft, which exhibits a lesser differentiation degree with immature cells and high mitotic activity compared with its primary tumor, H&E; original magnification, ×200 (C).
with respect to the previous passage without p27\textsuperscript{Kip1} overexpression and in the absence of p21\textsuperscript{Cip1} (data not shown).

Primary tumors showed 2–5% of terminal deoxynucleotidyl transferase-mediated nick end labeling-positive cells, whereas this increased to 7–8% in xenografts. p53 nuclear expression was always ≤5% in primary tumors and was considered negative. In contrast, in the different xenografts analyzed (early and late passages) a positive nuclear staining was detected in ≥30% of tumor cells. None of the primaries and xenografts exhibited abnormalities in the \textit{Tp53} gene except for HC-9 xenograft. This showed two previously reported mutations (27) in exon 6 (codon 192, Gln-Arg) and exon 7 (codon 242, Cys-Tyr) in both early and late passages.

\textbf{HBV and HCV Detection.} Sera of mice were constantly negative for both HBV-DNA and HCV-RNA. Whereas HBV-DNA was detected in all surviving xenografts of HBV-related patients, HCV-RNA was not maintained in the xenografts.

\textbf{Establishment and Characterization of Human HCC Cell Line BCLC-9}\n
A long-term viable cell line, named BCLC-9, has been established from xenograft HC-9 and is still maintained 24 months after its obtaining. Plated cells present polygonal and epithelial-like shape under phase-contrast microscope (Fig. 3). They grow as a monolayer or as colonies. Anchorage-independent growth was confirmed when these cells, plated in soft agar, were able to form macroscopic colonies in 2 weeks. Doubling time of BCLC-9 cells at passage 17 was 27.7 h. Karyotype analysis confirmed the human origin, and the hepatocyte lineage was confirmed by cytokeratin profile. The BCLC-9 cell line is VHB-DNA positive, and does not secrete \textit{\textsuperscript{-}fetoprotein}. In addition, BCLC-9 cells expressed E-cadherin, vascular endothelial growth factor, inducible-nitric oxide synthase, and showed an overexpression of Skp2 (data not shown).

\textbf{Cytogenetic Analysis.} The 13 analyzed metaphases of BCLC-9 cells at passage 7 showed a complex karyotype with trisomy of chromosome 7, loss of chromosome 13, and two marker chromosomes, mar1 and mar2 (Fig. 4A). In addition, chromosome 6 was longer in 60% of the metaphases, whereas tetrasomy of chromosome 7 was observed in only 15%.

CGH and FISH analysis were performed to characterize the two marker chromosomes. CGH showed a partial gain of chromosome 5 (p11-pter), chromosome 6 (q24-pter), and chromosome 9 (q22.3-q34), and a whole gain of chromosome 7, confirming the trisomy and tetrasomy observed previously (Fig. 4B). FISH findings disclosed that mar1 was composed of the missing chromosome 13 and of the amplified fragment of chromosome 9, whereas mar2 was the result of the short-arm amplification of chromosome 5 (data not shown). These chromosome alterations were maintained at passage 36 after 16 months of \textit{in vitro} culture.

\textbf{Tumorigenicity Assay.} Tumoral nodules measuring 0.36 ± 0.11 cm developed in 3 of 4 s.c. injection sites 1 month after BCLC-9 cell injection. Pathological analysis showed a human HCC-like pattern mimicking xenograft HC-9.

\textbf{Study of Cell Proliferation and Cell Cycle Regulators.}\n
The Ki67 labeling index in BCLC-9 cells was 84.6%, indicating a high level of proliferating activity that coincided with the analysis of the cell cycle regulators by Western blot analysis. BCLC-9 cells showed a loss of p16\textsuperscript{Ink4a} and an expression profile of cell cycle regulators characteristic of proliferating cells: high levels of cyclins E and A and low levels of p27\textsuperscript{Kip1} and p21\textsuperscript{Cip1} (Fig. 2). Moreover, cells express p53 protein by immunocytochemistry and presented the same \textit{Tp53} gene mutations as reported in the HC-9 xenograft from which they were derived.

![Phase-contrast microscopy image of BCLC-9 cells. ×200.](https://example.com/fig3.jpg)
DISCUSSION

This study indicates that orthotopic implantation of histologically intact tissue of early HCC obtained by surgical resection constitutes not only a useful experimental model to study events associated with tumor progression but also a help in the establishment of new cell lines. Previous investigations in other tumors have shown this potential, but the only data in HCC reported thus far have been obtained from samples derived from patients diagnosed at a very advanced tumor stage (19). The 50% initial take rate compares with the data in other neoplasms and the 30% long-term viability should be considered acceptable because of the early nature of the tumors used. Given the fact that early HCCs are usually well differentiated (5), they will keep several functions of normal hepatic cells, such as apoptotic death due to the stress associated with changes in tumor microenvironment. In that sense, several authors have shown that surgical maneuvers may sharply change the activation of several signal transduction pathways (28–31). This reaction to tissue handling cannot be accurately controlled or prevented and, ultimately, may partially explain the failure to identify any specific pathological or molecular characteristics associated with initial and long-term success. Thus, successful grafting has not been related to the viral etiology of the underlying liver disease, which, in HBV patients, would be potentially mediated by the genetic changes related to the integration of HBV-DNA into the genome of the hepatocytes (1).

All of the primary tumors that we used belonged to an early evolutionary stage; this was evident because they retained hepatic differentiation, showed no evidence of vascular invasion, had a low-to-moderate proliferation rate, and did not exhibit p53

Fig. 4  BCLC-9 cell shows a complex G-banded karyotype with trisomy of chromosome 7, loss of chromosome 13 and two marker chromosomes, mar1 and mar2 (A). Comparative genomic hybridization findings of BCLC-9 cell line (B) show a partial gain in chromosome 5 (p11-pter), chromosome 6 (q24-qter), and chromosome 9 (q22.3-q34), and a whole gain of chromosome 7.
abnormalities, which in the Western world are mostly detected at an advanced stage (32–34). The evolution of xenografts reproduces the biological progression of human HCC at early stage. They need long periods to reach significant size, have low dissemination potential (in early passages, only one of the five initial successful grafts resulted in intrahepatic dissemination), and, in some cases, the progression might be halted by unknown mechanisms, occasionally leading to tumor regression (as in cases HC-2 and HC-5). However, it has to be noted that xenografts at first passages lose their differentiation degree, show a high level of proliferating activity, and express p53, which in one case was associated with two Tp53 gene mutations. These characteristics reflect mostly an advanced tumor stage and suggest that this model probably selects the more undifferentiated clones that have growth and survival advantages and that can exist even in tumors classified as early stage (35, 36). If not resected, these undetectable and undifferentiated clones would progressively evolve into overt cancer, finally leading to a typical advanced stage. These more aggressive cells would be able to resist extreme conditions such as loss of stimulation by cytokines and growth factors, and low oxygen concentration, this last capacity being at least partially related to p53 loss of function (10, 37).

Our data clearly show that the expression of cell cycle regulators is modified throughout successive passages. Thus, as expected, tumor growth and increased proliferation were associated clearly with an increase of positive cell cycle regulators. Accordingly, the high levels of cyclins E and A that were observed in xenografts unequivocally reflect a proliferating status, because they would not be expressed when cell cycle is arrested. The CDK inhibitor levels in xenografts did not show a consistent pattern and varied from decreases to evident elevations, which could be the result of the activation of growth-inhibitory pathways at early evolutionary stages of HCC. In this sense, the most striking event has been observed in the case HC-9 at passage 3, which presented a sharp increase of p27kip1 expression and a lesser increase of p21Cip1. This coincided with a clear-cut reduction of its progression rate that we attributed mainly to the effects of p27kip1. This is supported by the results of CDK2 activity, which demonstrate that this overexpressed CDK inhibitor was functional and inhibited CDK2-cyclin E and CDK2-cyclin A complexes. We and others (21, 38–40) have shown that HCC with high p27kip1 expression have a better prognosis, and, thus, all these data strongly indicate that this protein is involved in an effective inhibitory pathway that may abrogate aggressive tumor progression at early stages. Two additional xenografts showed significant increases in p27kip1 expression. HC-5 was not perpetuated, suggesting the possibility that p27kip1 levels blocked xenograft viability. By contrast, HC-7A showed high p27kip1 levels and resulted in a viable xenograft with high proliferative index. This observation might coincide with the findings in the few cell lines of European origin: HBGs and HepaRG (42, 43), indicating similarities among cell lines with the same geographic area. Furthermore, the reported gains of BCLC-9 cells have been less frequently observed, and those that have been more frequently described (44, 45) have not been detected. This represents an advantage of the new cell BCLC-9 cell line. Thus, this new cell line and, presumably, the cell lines that will be derived from the other xenografts will enlarge the currently reduced number of European human HCC cell lines, which surely represent a specific genetic background as compared with those derived from Asian patients.

In summary, we have developed an orthotopic model of human HCC in nude mice that reproduces the biological evolution of human HCC. This model constitutes a useful tool to study the molecular basis of the transition from early to advanced carcinomas and represents excellent source material from which to derive cell lines. Both tools should be valuable in the study of HCC progression as well as in the identification and testing of new therapeutic targets both in vitro using cell lines and, thereafter, in vivo using the murine model.

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REFERENCES


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Carolina Armengol, Gemma Tarafa, Loreto Boix, et al.


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