

The properties of tumor-initiating cells from a hepatocellular carcinoma patient's primary and recurrent tumor

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Hepatocellular carcinoma (HCC) is associated with a high morbidity and mortality due to its high rate of recurrence. However, little is known about the biological characteristics of recurrent HCC cells. A single patient's primary and recurrent HCC-derived cell lines, Hep-11 and Hep-12, respectively, were established by primary culture. These two cell lines have the same hepatitis B virus integration site and share many common amplifications and deletions, which suggest that they have the same clonal origin. While Hep-11 cells were non-tumorigenic at 16 weeks following injection of up to 10 000 cells, injection of only 100 Hep-12 cells was sufficient to initiate tumor growth, and all single Hep-12 clones were tumorigenic in immunodeficient mice. Compared with Hep-11, Hep-12 cells expressed the oval cell markers *AFP*, *NCAM/CD56*, *c-kit/CD117*, as well as multiple stem cell markers such as *Nanog*, *OCT4* and *SOX2*. In addition, >90% of Hep-12 cells were aldehyde dehydrogenase positive. They were also less resistant to paclitaxel, but more resistant to doxorubicin, cisplatin and hydroxycamptothecin (HCPT), which had been administered to the patient. Furthermore, Hep-12 cells expressed higher levels of poly (adenosine diphosphate-ribose) polymerase-1 (PARP-1) than Hep-11, and PARP-1 inhibition potentiated the sensitivity to HCPT in Hep-12 cells but not in Hep-11 cells. These results indicate that a large population of the recurrent HCC-derived Hep-12 cells were tumor-initiating cells and that elevated expression of PARP-1 was related to their resistance to HCPT.

Introduction

Hepatocellular carcinoma (HCC) is one of the deadliest cancers, accounting for ~500 000 deaths each year (1). More than 80% of patients are diagnosed with advanced or unresectable HCC, which carries a particularly dismal prognosis (2). All the current routine treatments for HCC, such as surgical resection, percutaneous ethanol injection, arterial embolization, interventional chemotherapy and radiofrequency ablation, are frequently complicated by recurrence and

Abbreviations: ABCG2, ATP-binding cassette superfamily G member 2; ADM, doxorubicin; AFP, α -fetoprotein; ALDH, aldehyde dehydrogenase; CSC, cancer stem cell; CDDP, cisplatin; EpCAM, epithelial cell adhesion molecule; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HCPT, hydroxycamptothecin; IC₅₀, the half maximal inhibitory concentration; MDR1, multidrug resistance gene 1; mRNA, messenger RNA; PARP-1, poly (adenosine diphosphate-ribose) polymerase-1; SCID, severe combined immunodeficiency; TIC, tumor-initiating cell.

metastasis following intervention. The fact that the overall recurrence rate of HCC can exceed 70% underscores the need for novel therapeutic strategies (3). We believe that analysis of the biological characteristics of recurrent HCC cells may facilitate the innovation of such strategies.

The cancer stem cell (CSC) hypothesis postulates that cancer occurrence, recurrence and metastasis are driven by CSCs, which are characterized by unlimited self-renewal and lineage capacity (4,5). CSCs have been successfully identified and characterized in HCC cell lines and biopsies (6–10). They were found to be CD133⁺, CD90⁺ or to be of oval cell origin. However, since these markers are not expressed exclusively in liver CSCs and since there exist a multitude of markers, the identification of CSCs in a given HCC cell population by these markers would not be feasible. Instead, a functional assay, *in vivo* serial transplantation, is considered as the gold standard for CSC identification (11).

It is generally thought that CSCs are resistant to conventional chemotherapy, resulting in tumor recurrence and treatment failure (4,5,12). An enriched CSC population has been identified in breast cancer specimens from patients who received chemotherapy (13) and in glioma xenografts following radiotherapy (14). There have also been multiple reports that CSCs isolated from a variety of human cancer cell lines display multidrug and/or radiotherapy resistance, resulting from intrinsic properties such as quiescence, DNA repair capacity and expression of transporters proteins such as ATP-binding cassette superfamily G member 2 (ABCG2) and multidrug resistance gene 1 (MDR1) (12,15–17). However, the drug resistance profile of CSCs of various origins and the underlying mechanisms still remain largely unknown. In fact, to date no investigations have evaluated the CSC-like properties and chemoresistance profile of recurrent HCC cells.

We have previously described the establishment of a pair of cell lines, Hep-11 and Hep-12, from a single patient's primary and recurrent HCC tissues, respectively. The recurrent HCC-originated Hep-12 cells were initially shown to be persistently tumorigenic and might possibly serve as the targets for immunotherapy (18). Here, we further characterized their properties of tumor initiation and drug resistance. The results of a serial transplantation assay in immunodeficient mice, including population and clonal analysis, suggest that a large population of the recurrent HCC-derived Hep-12 cells are tumor-initiating cells (TICs). We further analyzed the expression of several stem cell markers, as well as the drug resistance profile of these cells. Interestingly, we found that elevated expression of poly (adenosine diphosphate-ribose) polymerase-1 (PARP-1) in these cells increased resistance to hydroxycamptothecin (HCPT). These results are possibly important not only because they describe the biological characteristics of a population of recurrent HCC-derived cells but also because they might provide insight into the development of novel therapies directed against recurrent HCC.

Materials and methods

Patient information and collection of the primary and recurrent HCC tissues

The patient was a 47-year-old man who was found with extremely high levels of serum α -fetoprotein (AFP, 2733 ng/ml; normal range: 0–7 ng/ml) and positive antibodies against Hepatitis B e-antigen and Hepatitis B viral core protein. He underwent HCC resection of his right liver in February 2004. The pathological diagnosis of the primary tumor was as follows: HCC, 7 × 5 cm, with visible intravascular cancer embolus, negative surgical margins and chronic cholecystitis. Five weeks later, he was treated by transarterial chemoembolization twice with an interval of 7 weeks. The combination of pirarubicin (50 mg), carboplatin (300 mg) and HCPT (30 mg) was first administered, and the drugs of cisplatin (CDDP, 80 mg), epirubicin (60 mg) and HCPT (30 mg) were injected during the second transarterial chemoembolization procedure. The patient then received a left lateral hepatic lobectomy following HCC recurrence 7 months

later, and the pathological diagnosis was HCC, grade II–III, 8 × 6 cm, with no intravascular cancer embolus and with negative surgical margins. Primary tumor tissue, recurrent tumor tissue and blood samples were obtained from this patient for research purposes with written informed consent, which was approved by the Ethics Review Committee of Beijing Cancer Hospital.

Culture of HCC cells

The establishment of cell lines, Hep-11 and Hep-12, from the above patient by primary culture has been described in our previous paper (18). In brief, tumor specimens were mechanically dissociated and layered onto a 75%/100% two-step Ficoll gradient. Tumor cells, which enriched in the upper interface, were cultured in RPMI1640 (Invitrogen, Grand Island, NY) supplemented with 20% autologous serum or 10% fetal bovine serum (once the autologous serum was depleted). Established cell lines were passaged regularly by trypsinization. Cells used in this study were passaged between 5 and 15 times unless noted otherwise.

Southern blotting

Restriction enzyme-digested genomic DNA was electrophoresed, transferred onto nylon membranes (Hybond-N, Amersham Pharmacia Biotech, Little Chalfont, UK) and hybridized with a ³²P-labeled 3.2 kb BamHI fragment of full-length hepatitis B virus (HBV) genomic DNA (subtype: adr) in ExpressHyb solution (Clontech, Palo Alto, CA). Signals were detected by autoradiography with X-ray film.

HBV–human genomic junction identification

Human genomic sequences adjacent to HBV integration sites were identified by using the cassette ligation-mediated-polymerase chain reaction (PCR)-based method, which has been described previously (19). The PCR products were sequenced and blasted using National Center for Biotechnology Information website (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) to identify the human genomic sequences adjacent to the HBV sequence.

Array comparative genomic hybridization analysis

The array comparative genomic hybridization was performed by KangChen Bio-tech (Shanghai, China) by using the Aigent Human Genome 4X44K CGH Microarray that contains 43 000+ coding and non-coding human sequences, according to the manufacturer's instructions. The raw array comparative genomic hybridization profiles extracted from Agilent Feature Extraction 10.5.1.1 were processed to identify statistically significant transitions in copy number using the aberration detection method algorithm found in Agilent DNA Analytics 4.0.

Anchorage-independent growth assay

Cells were suspended in 0.3% agar/culture medium and plated at a density of 150 cells per well in a 24-well plate, which had been previously coated with 0.5% agar. In total, 150 µl of fresh media was added to each well every 3 days. After 3 weeks, colonies were quantified by the investigators without magnification, and images were then recorded with a stereo microscope (Olympus, Tokyo, Japan).

Severe combined immunodeficiency xenograft transplantation

The serial transplantation assay was performed as described previously (6,18). Cells were suspended in 50 µl of a 1:1 suspension of RPMI1640 and Matrigel (BD Biosciences, Bedford, MA) and transplanted subcutaneously into the axilla of severe combined immunodeficiency (SCID) mice (4–6 weeks old, Vitalriver Laboratory Animal, Beijing, China). A subset of sorted positive and negative cells was injected into the opposite flanks (ventral side) of the same non-obese diabetic/SCID mice. Tumor formation was monitored weekly. Once identified, tumors were either fixed and stained with hematoxylin and eosin or cultured for 1 week followed by transplantation of 100 cells into another SCID mouse.

For single-cell clone analysis, Hep-12 cells were resuspended and diluted to a density of one cell per 0.4 ml culture medium. In total, 200 µl of cell suspension was added into each well of 96-well plates. Wells with only one cell were marked, and fresh media was added every 3 days. Single-cell clones were expanded and transplanted into two to three SCID mice with 1000 cells per site at two sites per mouse.

All animal protocols were performed in accordance with the institutional guidelines for the use of laboratory animals.

Flow cytometry

Cells were dissociated with 0.02% ethylenediaminetetraacetic acid/phosphate-buffered saline, then resuspended in phosphate-buffered saline with 2% bovine serum albumin and stained with either fluorescence-conjugated specific antibodies or isotype-matched mouse immunoglobulins at 4°C for 30 min. The detailed antibody information is available upon request.

To quantify the aldehyde dehydrogenase (ALDH)-positive population, the Aldefluor™ kit was purchased from StemCell Technologies (Vancouver, BC, Canada) and used according to the manufacturer's instructions. In brief, cells were resuspended in Aldefluor assay buffer containing ALDH substrate

(BODIPY-aminoacetaldehyde, 0.625 µg/ml per 1 × 10⁶ cells) and incubated at 37°C for 40 min. As negative control (for gate setting), an aliquot of each cell line was treated with a specific ALDH inhibitor, diethylaminobenzaldehyde, at 50 mmol/l.

Labeled samples were analyzed or sorted using a FACSCalibur™ or FACSAria™ flow cytometer (BD Biosciences).

Reverse transcription–PCR analysis

Total RNA was extracted with TRIZOL reagent, and reverse transcription into complementary DNA was performed using the Superscript III kit (Invitrogen). PCR primers used are available upon request. The human stem cell RT² Profiler™ PCR Arrays from SuperArray Bioscience (Frederick, MD) were also used to profile the genes differentially expressed in these cells according to the manufacturer's instructions.

Western blotting

Cell lysis, sample preparation, sodium dodecyl sulfate–polyacrylamide gel electrophoresis separation and electrotransfer onto Immobilon-P blotting membranes (Millipore, Billerica, MA) were performed according to basic laboratory protocols. Immunoblots were stained sequentially with primary antibodies [mouse anti-PARP-1 (Santa Cruz Biotechnology, Santa Cruz, CA) or anti-β-actin (ICN Biomedicals, Costa Mesa, CA)] and then with horseradish peroxidase-labeled goat anti-mouse secondary antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA). Detection was performed using a chemiluminescence detection system (Pierce, Rockford, IL).

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide assay and statistics

A total of 10⁴ cells was added into each well of a 96-well plate. Drugs were added the next day and remained in culture for 48 h. When PARP-1 was inhibited with 200 µM NU1025 (Alexis Biochemicals, San Diego, CA), NU1025 or dimethyl sulfoxide was added. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (20 µl of 5 mg/ml; Amresco, Solon, OH) was added to each well, and the plates were incubated for another 4 h. The supernatant was then discarded, and 150 µl dimethyl sulfoxide was added into each well. After the plates were shaken for 10 min in the dark, the absorbance was measured at 490 nm (referenced to 630 nm) with an ELISA reader (Bio-Rad, Hercules, CA). The mean of three to six replicate wells for each drug concentration was used to calculate inhibition rate. The half maximal inhibitory concentration (IC₅₀) was calculated according to an equation described previously (20): $\log(Fa/Fu) = m \log(D) - m \log(Dm)$.

The data are shown as mean ± SD of three independent experiments. Statistical significance of the results was calculated by Student's *t*-test.

Results

In vitro growth characteristics of Hep-11 and Hep-12 cell lines established from the same patient's primary and recurrent HCC tissues, respectively

By primary cell culture, we have derived two cell lines, Hep-11 and Hep-12, from a single patient's primary and recurrent HCC tissues, respectively (18). The Hep-11 cells were typical epithelial cells with polygonal morphology, whereas the Hep-12 cells were round, adhered loosely to flasks and formed spheres both in serum-containing (Figure 1A, also see ref. 18) and serum-free media. Furthermore, soft agar assay revealed that ~30% of Hep-12 cells were capable of forming visible clones, whereas few colonies were identified in Hep-11 cells (Figure 1B–D). This was in agreement with the tumorigenicity assay data: Hep-12 formed tumors at ~10 days following injection of 2 × 10⁶ cells, whereas Hep-11 produced visible nodules only at 6 months following injection of 5 × 10⁶ cells.

Both Hep-11 and Hep-12 cells have the same clonal origin

HCC recurrence may originate from intrahepatic metastasis or independent multicentric occurrence. In HBV-associated HCC, the origin of recurrence can be determined by analyzing the integration sites of HBV DNA (21). Since wild-type HBV genomic DNA contains only one HindIII restriction enzyme site and no EcoRI site, the bands seen in a Southern blot following digestion by these enzymes are diagnostic of HBV–human genomic DNA junctions unless a new point mutation(s) presented in the HBV genomic DNA has created a new site(s). Southern blotting results indicated that both Hep-11 and Hep-12 cells had similar HBV integration patterns (Figure 1E). Further cloning of the HBV–human genomic junction revealed that, in

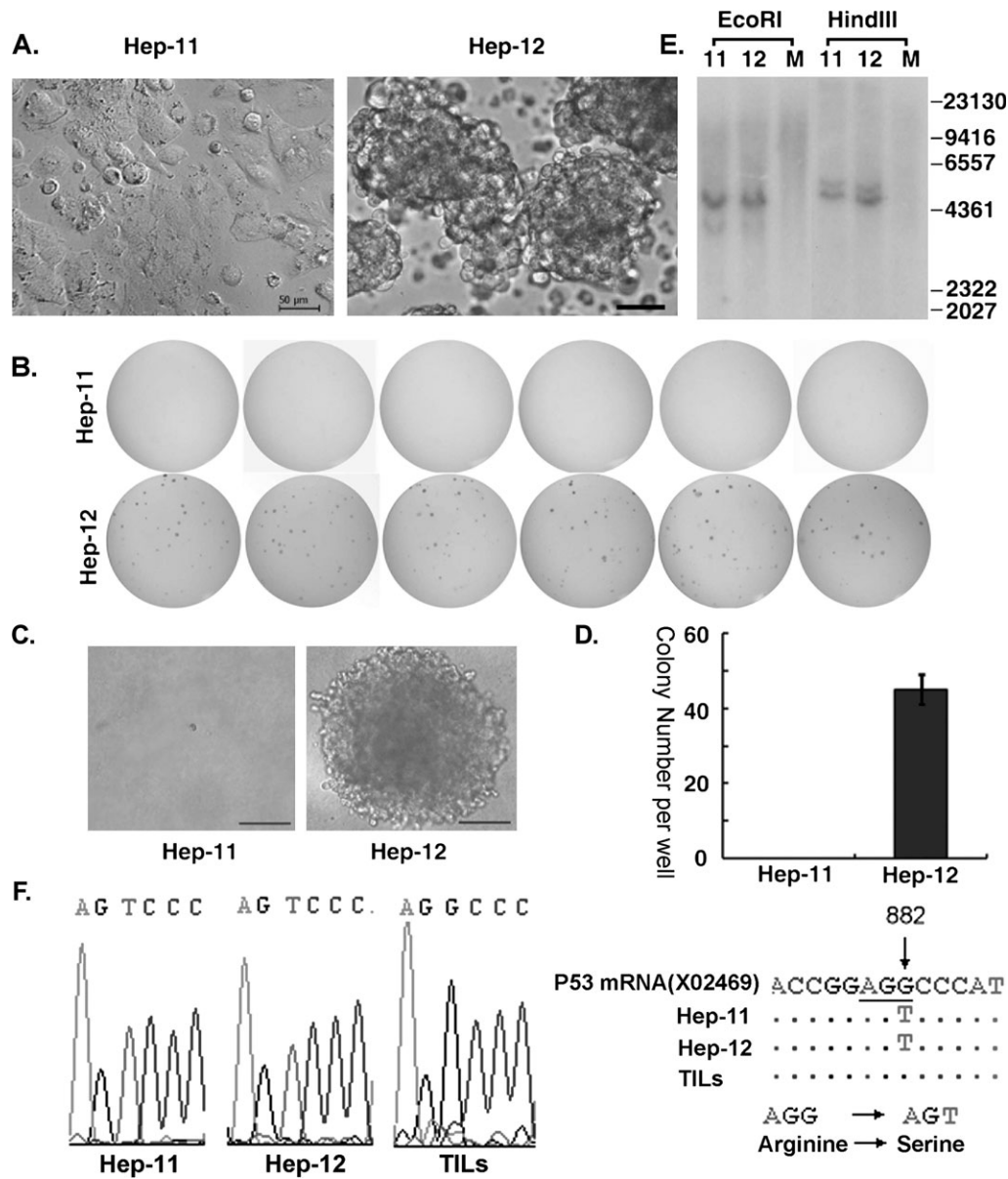


Fig. 1. Characterization of two HCC cell lines from a patient's primary and recurrent tumors. (A) Morphology under a differential interference contrast microscope. Hep-11 cells (Passage 15) are classic epithelial-like cells, whereas Hep-12 cells (Passages 12) form spheres. Bar = 50 μ m. (B–D) Soft agar assay results. (B) Colonies of Hep-11 (Passages 10) and Hep-12 (Passages 7) cells visualized under a stereo microscope. (C) Colony of Hep-11 and Hep-12 cells under high power. Bar = 100 μ m. (D) Clone numbers per well. Data are reported as mean \pm SD. (E) Southern blot analysis of HBV integration patterns; 11, 12 and M represented Hep-11, Hep-12 and negative control MCF-7 cells, respectively. (F) Sequencing chromatograms for *p53* mutation analysis. Genomic DNAs from Hep-11, Hep-12 and tumor-infiltrating lymphocytes (TILs) (18) were PCR amplified and sequenced to detect mutations in exon 5–9 of *p53* (primers available upon request). An AGG (Arg) to AGT (Ser) mutation in exon 7 at codon 249 was found in both Hep-11 and Hep-12 but not in the tumor-infiltrating lymphocytes from the same patient.

both cell lines, HBV sequence was followed by the same human genomic sequence, which was identified as part of gene LINGO2 (*Homo sapiens* leucine-rich repeat and Ig domain-containing 2) in chromosome 9 by the Blast search method (supplementary Figure 1 is available at *Carcinogenesis* Online). These data confirm that both Hep-11 and Hep-12 have the same HBV integration site, suggesting they may have the same clonal origin.

Furthermore, we have employed array-based comparative genomic hybridization analysis as an independent method to determine the relationship between these two cell lines. As shown in supplementary Table 1 (available at *Carcinogenesis* Online), they share many common amplifications in chromosome 1, 3, 5, 6, 7, 8, 11, 13, 15, 16 and 17 and deletions in chromosome 3, 4, 5, 6, 8, 9, 10, 11, 12, 15, 16, 18 and Y, although each cell line did possess additional unique amplifications and deletions to a lesser degree. In addition, both cell lines

contained the same *p53* mutation at base pair 882, which is a known hot spot mutation in *p53* that leads to a change in amino acid from arginine to serine (Figure 1F) of exon 7 at codon 249. These data further support the hypothesis that Hep-11 and Hep-12 arise from the same progenitor cell.

The expression of putative stem cell surface markers in Hep-11 and Hep-12

In an initial attempt to find possible HCC stem cell markers and to determine if the recurrent HCC-derived Hep-12 cells were enriched for CSCs, we first analyzed the expression of hepatic putative normal and CSC markers (6–10,22) CD34, CD56, CD117, CD133, CD90, epithelial cell adhesion molecule (EpCAM), SSEA-4 and CD44 in these cells by flow cytometry. As shown in Figure 2A, none of the Hep-11 or Hep-12 cells expressed CD34. Almost every cell in each

cell line expressed EpCAM. The expression of CD44 and SSEA-4 was detected in >90% of Hep-11 and ~60% of Hep-12 cells. Greater than 85% of Hep-12 but only 3% of Hep-11 cells were CD56 positive. Only a minor population (~1%) of Hep-12 cells and no Hep-11 cells were CD117 positive. Surprisingly, the previously reported HCC stem cell populations of CD133⁺ (90.1% in Hep-11 versus 69.5% in

Hep-12) and CD90⁺ (32.2% in Hep-11 versus 3.2% in Hep-12) were not enriched in Hep-12 cells at all.

We then sorted some CD90⁺, CD117⁺ and CD56⁺ cells from their negative counterparts and transplanted them into non-obese diabetic/SCID mice (Table I). It seems that none of these molecules alone is sufficient to define CSCs in HCC because no positive cells from

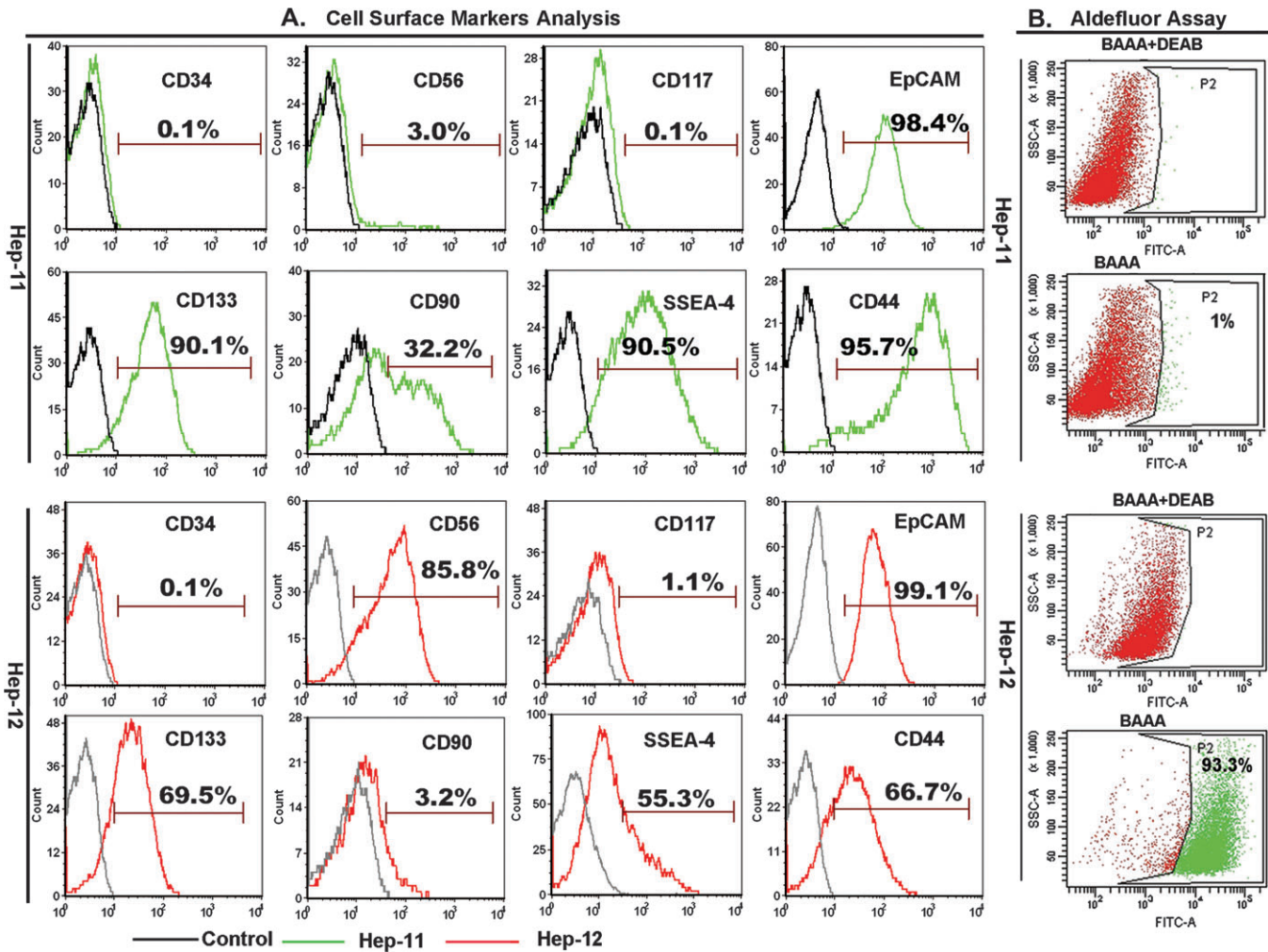


Fig. 2. Flow cytometry analysis of putative stem cell markers in Hep-11 and Hep-12 cells. (A) The expression of cell surface molecules CD34, CD56, CD117, CD90, CD133, EpCAM, SSEA-4 and CD44 was compared between Hep-11 (Passages 8–13) and Hep-12 (Passages 6–10). Cells stained with isotype antibodies served as negative controls. (B) The populations with high ALDH activity measured with the Aldefluor assay in both cell lines. Cells incubated with Aldefluor substrate [BODIPY-aminoacetaldehyde (BAAA)] and the ALDH-specific inhibitor diethylaminobenzaldehyde (DEAB) were used to establish the gate with baseline fluorescence and to define the Aldefluor-positive region (P2).

Table I. Tumorigenicity of Hep-11 and Hep-12 cells in SCID mice

Cells injected	Hep-11			Hep-12		
	10 ⁴	10 ³	10 ²	10 ⁴	10 ³	10 ²
Unsorted	0/5 (16 w) ^d	0/5 (16 w) ^d	0/5 (16 w) ^d	ND	5/5 (5 w) ^b	5/5 (7 w) ^b
CD56 ⁺ ^a	ND	0/5 (5 m) ^d	0/5 (5 m) ^d	ND	5/5 (8 w) ^b	4/5 (16 w) ^b
CD56 ⁻ ^{a,b}	ND	0/5 (5 m) ^d	0/5 (5 m) ^d	ND	3/5 (10 w) ^d	1/5 (5 m) ^d
CD90 ⁺ ^a	ND	0/5 (19 w) ^d	0/5 (19 w) ^d	ND	3/5 (10 w) ^b	ND
CD90 ⁻ ^a	ND	0/5 (19 w) ^d	0/5 (19 w) ^d	5/5 (8 w) ^b	ND	ND
CD117 ⁺	ND	ND	ND	ND	5/5 (7 w) ^b	7/8 (8 w) ^{b,c}
CD117 ⁻	ND	ND	ND	ND	6/6 (7 w) ^b	3/5 (8 w) ^{b,c}

ND, not done; w, weeks; m, months.

^aSorted positive and negative cells were injected subcutaneously in opposite flanks (ventral side) in the same non-obese diabetic/SCID mice.

^bThe time in parentheses refer to the minimum periods to reach the results.

^cTwo hundred cells were injected per mouse.

^dThe time in these parentheses refer to the maximal periods observed.

Hep-11 formed tumors. However, CD56⁺ and CD117⁺ cells sorted from the Hep-12 population do have higher tumorigenicity than their negative counterparts.

A large population of Hep-12 cells was TICs

There are reports that CSCs can account for almost 100% of cultured cells, even in serum-containing medium (23,24), and given the facts that Hep-12 cells grew as spheres and that 200 cells of either CD117⁺ or CD117⁻ could initiate tumor growth at high frequency (7/8 and 3/5, respectively) by 8 weeks after transplantation (Table I), we postulated that most Hep-12 cells were HCC stem cell-like cells. However, since the formation of spheroids is not sufficient to define the presence of stem cells, we performed the serial transplantation assay in SCID mice with population and clonal analysis, as described previously for glioma cells (23), to test this hypothesis.

In the population analysis, various numbers of Hep-11 and Hep-12 cells were injected subcutaneously into SCID mice. As few as 100 Hep-12 cells initiated primary tumor growth in five of five mice (100%) by 5–7 weeks (Table I). Hematoxylin and eosin staining indicated that the tumors formed by Hep-12 cells in SCID mice did not completely resemble the histopathology of either the patient's primary or the recurrent HCC tissue; however, they were closer in histopathology to the recurrent tissue in that they remained poorly differentiated (Figure 3). Furthermore, 100 cells isolated from these primary mouse tumors could induce secondary tumor formation in seven of eight newly injected mice by 8 weeks following the second transplantation. On the contrary, no tumor was detected even after primary transplantation of 10 000 Hep-11 cells after 16 weeks.

In clonal analysis, we obtained 40 Hep-12 single-cell clones by limited dilution in a single 96-well plate. Twenty-two clones were randomly chosen, and each was transplanted into SCID mice at 1000 cells per mouse, and 100% of them were tumorigenic, which demonstrates that a single Hep-12 cell possesses self-renewal capacity, and can initiate HCC tumor growth. Since we diluted the cells as one cell per 400 μ l medium and plated 200 μ l per well, this equals about one cell per two wells. Each of 48 wells of a 96-well plate may have a single cell by chance, hence at least 80% (40/48) of Hep-12 cells are TICs with self-renewal capacity.

The stem cell gene expression profile in Hep-12 cells

To confirm that Hep-12 cells do represent cells with stem cell-like properties, we compared the gene expression profiles of Hep-11 and Hep-12 cells using the human stem cell RT² Profiler PCR array from SuperArray. Hep-12 cells demonstrated upregulation, by at least a 3-fold margin, of multiple genes associated with stem cells, including those regulating cell cycle/division (*EP300*, *PARD6A*, *DHH*); the self-renewal marker *SOX2*; the growth factor *IGF1*; metabolic markers *ABCG2* and *ALDH1A1*; embryonic cell lineage markers *FOXA2*, *IPF1* and *ISL1*; the neural cell lineage marker *NCAM1/CD56* and the notch pathway molecules *DLL3* and *EP300* (supplementary Table 2 is available at *Carcinogenesis* Online). Additional reverse transcription-PCR results also revealed that *AFP*, *CD117*, *NCAM1/CD56*, *Oct-4* and *nanog* were upregulated in Hep-12 cells (Figure 4A).

ALDH is a cytosolic enzyme that is responsible for the oxidation of intracellular aldehydes. Elevated levels of ALDH have been linked to some normal progenitor cells and malignant CSCs (25–27). We therefore employed the Aldefluor assay to assess the size of the ALDH⁺ population in this cell line. Consistent with high level of *ALDH1A1* messenger RNA (mRNA) identified in Hep-12 cells, >90% of Hep-12 cells are ALDH positive, whereas the positive signal in Hep-11 cells was marginally above background (Figure 2B).

Chemoresistance profile of Hep-11 and Hep-12 cells

To further characterize these HCC cells, we analyzed their chemoresistance to four common chemotherapeutic agents, doxorubicin (ADM) (a derivative of pirarubicin and epirubicin), CDDP, HCPT and paclitaxel, all of which except paclitaxel were administered to the patient following the first surgery. Compared with Hep-11 cells, the

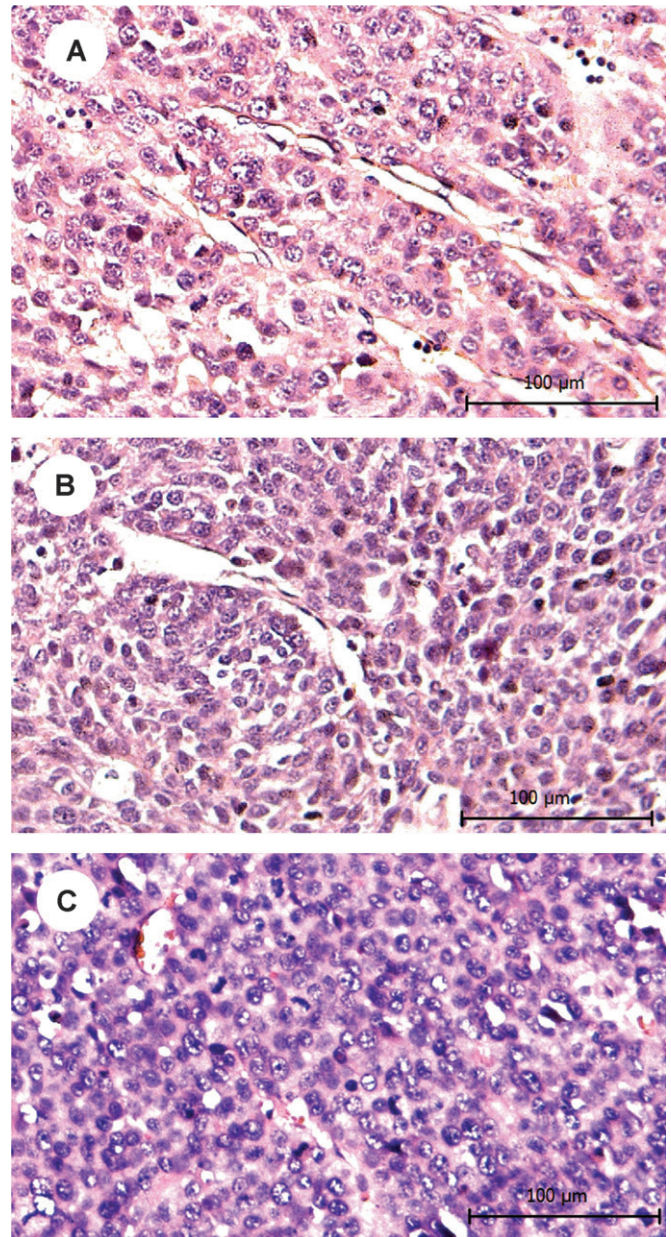


Fig. 3. Representative hematoxylin and eosin staining showing that the tumors formed by Hep-12 cells in SCID mice are poorly differentiated, and somewhat resemble the recurrent HCC tissue from the patient. (A) Hematoxylin and eosin staining of the patient's primary tumor. (B) Hematoxylin and eosin staining of the same patient's recurrent tumor. (C) Hematoxylin and eosin staining of the tumor generated from Hep-12 cells in SCID mice. Bars = 100 μ m.

TIC-enriched Hep-12 cells displayed a selective drug resistance pattern: they were more resistant to ADM, CDDP and HCPT, but relatively sensitive to paclitaxel (Table II). The IC₅₀ values at 48 h for these drugs in Hep-12 cells were about 2-fold (ADM and CDDP), 3-fold (HCPT), and 15% (paclitaxel) of those observed for Hep-11 cells.

The TIC-enriched Hep-12 cells express high levels of PARP-1

In order to examine the molecular mechanisms underlying the differential chemoresistance observed in Hep-11 and Hep-12 cells, the expression of drug efflux transporters (*ABCG2* and *MDR1*) (12), the gemcitabine drug resistance gene *CEACAM6* (28) and the DNA repair enzyme *PARP-1* (29) was analyzed by reverse transcription-PCR.

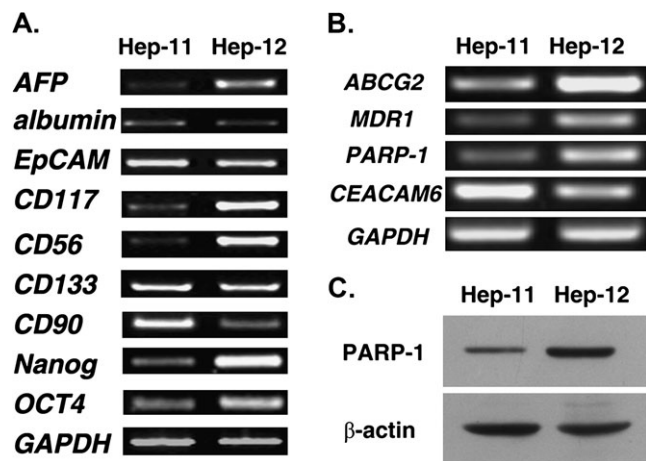


Fig. 4. Genes related to hepatic progenitors and drug resistance are differentially expressed in Hep-11 and Hep-12 cells. (A) Reverse transcription-PCR analysis of the expression of putative hepatic stem cell markers. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control. (B) Reverse transcription-PCR analysis of the expression of drug resistance-related genes. GAPDH served as an internal control. (C) Western blot analysis of PARP-1. β -Actin served as an internal control. The blot shows that PARP-1 is highly expressed in Hep-12 cells compared with Hep-11 cells.

Table II. Chemoresistance properties of Hep-11 and Hep-12 cells

IC ₅₀ ^a (μ M)	IC ₅₀ (μ M)			
	ADM ^b	CDDP ^b	HCPT ^b	Paclitaxel ^c
Hep-11	1.45 \pm 0.36	7.11 \pm 0.30	0.56 \pm 0.13	5.21 \pm 0.60
Hep-12	2.87 \pm 0.20	14.74 \pm 1.63	1.64 \pm 0.18	0.08 \pm 0.00
<i>P</i> value	0.002*	0.001*	0.001*	0.00006*
Hep-12 + DMSO	3.45 \pm 0.34	14.74 \pm 0.19	1.64 \pm 0.26	ND
Hep-12 + NU1025	4.08 \pm 0.70	19.47 \pm 1.19	0.88 \pm 0.11	ND
PF ₅₀ ^d	No potentiation	No potentiation	1.90 \pm 0.52	
<i>P</i> value			0.01**	
Hep-11 + DMSO	ND	ND	0.44 \pm 0.04	ND
Hep-11 + NU1025	ND	ND	0.40 \pm 0.02	ND
PF ₅₀ ^d			1.10 \pm 0.04	
<i>P</i> value			0.36	

DMSO, dimethyl sulfoxide.

^aMean \pm SD for three independent experiments with at least three replicates for each condition.

^bConcentrations were doubled in each increment.

^cConcentrations were increased 5-fold in each increment.

^dThe potentiation factor (PF₅₀) was calculated by the ratio of IC₅₀ (+DMSO) to IC₅₀ (+NU1025).

*Significant difference, Hep-12 versus Hep-11.

**Significant difference, Hep-12 + NU1025 versus Hep-12 + DMSO.

As shown in Figure 4B, in a manner similar to other TICs (16), Hep-12 cells expressed higher levels of *ABCG2* (in agreement with our SuperArray data found in the supplementary Table 2, available at *Carcinogenesis* Online) and *MDR1* mRNA than Hep-11 cells. Interestingly, *PARP-1* mRNA was upregulated in Hep-12 cells. Unexpectedly, the expression of *CEACAM6*, which was found to be upregulated in the side population of liver cancer HuH7 cell line (16), was much lower in Hep-12 cells than in Hep-11 cells.

ADM, CDDP and HCPT are all DNA damage-inducing drugs, and PARP-1 plays an important role in DNA repair (30,31). The above

data suggest that DNA repair capacity may be an important predictor of the chemoresistance of recurrent HCC cells and that this mechanism may be independent from a high drug efflux capacity mediated by ATP-binding cassette transporters. We therefore concentrated on the role of PARP-1 in the drug resistance of these recurrent HCC cells in the following experiments. In agreement with the higher levels of mRNA expression of *PARP-1* in Hep-12 cells, the expression of PARP-1 protein was also detected at higher levels in Hep-12 cells compared with Hep-11 cells by western blot (Figure 4C).

PARP-1 inhibition increases HCPT sensitivity in Hep-12 cells

To evaluate whether elevated PARP-1 levels in Hep-12 cells augment their resistance to DNA damage-inducing drugs, we treated both Hep-11 and Hep-12 cells with a PARP-1 inhibitor, NU1025 (32), prior to analyzing drug resistance. The results indicate that after NU1025 treatment, the IC₅₀ of Hep-12 cells to HCPT decreased from 1.64 \pm 0.26 to 0.88 \pm 0.11 μ M (P = 0.01). However, NU1025 treatment did not increase the sensitivity of Hep-12 cells to either ADM or CDDP. In addition, after NU1025 treatment, the IC₅₀ of Hep-11 cells to HCPT decreased insignificantly from 0.44 \pm 0.04 to 0.40 \pm 0.02 μ M (P = 0.36) (Table II). Hence, elevated PARP-1 levels contribute to the resistance of Hep-12 to HCPT.

Discussion

Here, we have characterized two cell lines, Hep-11 and Hep-12, established from the same patient's primary and recurrent HCC tissues, respectively. They have the same HBV integration site and share common genomic aberrations, suggesting that they originated from the same clone. The recurrent Hep-12 cells are characterized by high-level mRNA expression of the oval cell markers (22,33–37) *AFP*, *NCAM/CD56*, *c-kit/CD117*, and many stem cell markers such as *Nanog*, *OCT4* and *SOX2* (38). Interestingly, two genes expressed in pancreatic progenitor cells, *IPF1* and *ISL1*, were found to be upregulated 800- to 1000-fold in Hep-12 cells (supplementary Table 2 is available at *Carcinogenesis* Online). Moreover, most of these Hep-12 cells were observed to be TICs with stem cell-like properties through populational and clonal analysis [in agreement with data from Zheng *et al.* (23)], whereas the majority of Hep-11 cells were observed to be a less tumorigenic cell population. To our knowledge, this is the first pair of primary and recurrent HCC cell lines identified with the same genetic background. These two cell lines could serve as a model system for comparative studies investigating HCC recurrence and TICs.

Although CD56 can be used to differentiate highly tumorigenic cells from those with less tumorigenicity in Hep-12 cells, no single marker has been identified yet that is capable of defining TICs in both cell lines consistently. This finding suggests that TICs should not be defined solely by certain markers since none of the reported markers is specifically restricted to expression in TICs (11).

So far, we do not know the actual frequency of TICs in Hep12 cells and cannot exclude the possibility that Hep-11 cells are immunogenic even in SCID mice or that the Hep-11 to Hep-12 transition is in part due to reduced xenograft rejection in SCID mice as seen in melanoma cells (39). However, the predominance of CD56⁺ cells, which displayed higher tumorigenicity, is ~85% in Hep-12 cells. In addition, ~90% of Hep-12 cell are ALDH positive, which also represents a highly tumorigenic population (25–27). Based on these data, we would estimate that 85–90% of Hep-12 cells are TICs. The clonal analysis data also support this estimation.

Compared with Hep-11, Hep-12 cells exhibited enhanced resistance to chemotherapeutic drugs previously administered to the patient, including ADM, CDDP and HCPT, but displayed poor resistance to a previously unused drug, paclitaxel. These results suggest that (i) primary and recurrent tumor cells might have a different drug resistance profile, and clinical chemotherapy schemes should be designed to administer drugs with different cytotoxic mechanisms to prevent tumor recurrence more effectively; (ii) some TICs, such as Hep-12, may not necessarily exhibit resistance to multiple types of

drugs, even in the presence of expression of the ABCG family member genes and (iii) Hep-12 cells might originate from primary cancer cells selected by chemotherapy. These cells may have survived chemotherapy and driven tumor recurrence in this patient, as has been reported in many other cancers (40,41). Although we cannot exclude the possibility that these Hep-12 cells actually represent a population with high levels of genetic and epigenetic changes that resulted in higher tumorigenicity and drug resistance rather than being CSCs or TICs *per se*, they are indeed the cells responsible for recurrence and should be targeted for therapy regardless of their origin.

The PARP-1-mediated poly adenosine diphosphate-ribosylation reaction results in a unique post-translational modification involved in various cellular processes, including DNA repair, transcriptional control, genomic stability, cell death and transformation (30,31,42). PARP inhibitors can be used to suppress DNA repair and to promote apoptosis in cells that are treated with certain anticancer agents (31,32,43). Here, we have found that the expression of PARP-1 is elevated in HCC tumor initiating cells and that the PARP-1 inhibitor NU1025 enhances HCPT, but not ADM and CDDP, induced cytotoxicity in Hep-12 cells. All three of these drugs are capable of inducing DNA damage: HCPT is a topoisomerase I inhibitor, whereas ADM is a topoisomerase II inhibitor, and it has been reported that NU1025 increases DNA strand breakage mediated by the topoisomerase I inhibitor camptothecin but not by the topoisomerase II inhibitor etoposide in L1210 cells (44). Our results demonstrate that PARP-1 expression does not potentiate the resistance against all types of DNA damage drugs. Hep-12 cells also expressed more *ABCG2* and *MDR1* than Hep-11, and these chemoresistance genes may account for the resistance against ADM and CDDP observed in Hep-12 cells. Our data show that elevated expression of PARP-1 increases the resistance to HCPT in TIC-enriched Hep-12 cells (Table II). Given all the TICs or CSCs found so far are poorly differentiated populations, and Shimizu *et al.* (45) have reported that PARP-1 was highly expressed in poorly differentiated HCC clinical samples, it might be possible that high expression of PARP-1 in HCC TICs is a common feature for many HCC patients. If so, a PARP-1 inhibitor would enhance the treatment efficacy of HCPT and other DNA topoisomerase I antagonists to help to eliminate TICs, and thereby prevent HCC recurrence.

It is not surprising that the tumors formed by Hep-12 cells in immunodeficient mice are not exactly the same as the HCC patient recurrent tissue, although both are poorly differentiated. Since heterogeneity is a common phenotype for almost all kinds of tumors, Hep-12 cells might only represent a subpopulation that survived and expanded through cell culture pressure due to their intrinsic properties or just by chance. It is also possible that they acquired new mutations following growth in culture that rendered them tumorigenic and altered their drug resistance/sensitivity properties. Hence, the data presented here may not necessarily reflect the nature of the cells in the patient. Further work to validate these findings in clinical samples is under way.

In conclusion, our data support the hypothesis that TICs are enriched in a recurrent HCC-derived cell line; these TIC populations are more resistant to chemotherapy agents, which had been previously administered to the patient, and these cells express high levels of PARP-1, which accounts for their resistance to HCPT. These findings may have significant implications for clinical treatment of recurrent HCC. First, post-chemotherapy recurrent HCC may exhibit TIC enrichment, which could explain the difficulty in eliminating recurrent HCC with traditional radiotherapy and chemotherapy. Second, PARP-1 might serve as a molecular therapy target in recurrent HCC. Finally, novel therapeutic strategies for recurrent HCC should be implemented, such as the use of a drug cocktail composed of chemotherapeutic agents with different cytotoxic mechanisms or the use of chemotherapy in conjunction with inhibitors of molecules conferring drug resistance.

Supplementary material

Supplementary Figure 1 and Tables 1 and 2 can be found at <http://carcin.oxfordjournals.org/>

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