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Diversity of Hepatocellular Carcinoma Clones Bearing Hematopoietic Malignancies-Related Chromosomal Translocation

Romain Parent,[1]* Marie-Laure Plissonnier,[1] Brigitte Bancel,[2] Wan-Li Liao,[1] Sylvie Rumin,[1] Remal Asaad,[1] Marianne Till,[3] Damien Sanlaville,[3] Fabien Zoulim,[1] Christian Trépo,[1] and Marie-Jeanne Marion[1]

[1]*DevWeCan Laboratories of Excellence Network (Labex), Université de Lyon, Lyon Cancer Research Center, Inserm U1052–CNRS UMR5286, Lyon, France*

[2]*Laboratoire d'Anatomie Pathologique, Hôpital de la Croix-Rousse, Lyon, France*

[3]*Laboratoire de Cytogénétique, Hôpital Edouard Herriot, Lyon, France*

ABSTRACT

Interpatient heterogeneity of hepatocellular carcinoma has been in-depth addressed. Intrapatient heterogeneity is less known. Four clones were freshly isolated from an Edmondson grade I HCV-associated hepatocellular carcinoma. Biochemical approaches, functional assays and cytogenetics were used. Albumin inducibility was uncoupled from canonical cytokeratin profiles, suggesting pathological combinations of hepatospecific and biliary markers. Poor differentiation and TGFβ's proproliferative effect on all clones were observed. TGFβ, Interferon α and doxorubicin sensitivity levels were found highly heterogeneous. Progenitor and stem cells markers OV6 and EpCAM were mutually exclusively expressed. All clones were CD44+, while none expressed CD90, CD133, or CD117. Three clones displayed a liver progenitor OV6+ phenotype, and were susceptible to hepatocytic differentiation, among which one fibroblastoid clone displayed intrahepatic parenchymal engraftment capability. A fourth clone, the less motile, displayed a cancer stem cell *EpCAM*+ phenotype, was essentially β-catenin negative, and was as expected devoid of hepatocytic differentiation capability, yet the most sensitive to doxorubicin treatment. Cytogenetics evidenced in all clones a t(12;22)(p11;q11) translocation found in several myelodysplastic syndromes. All clones, that probably derive from EpCAM+ tumor cells, display aberrant E-cadherin cytosolic localization. Because of their diverse pathophysiolocal features, these freshly isolated, low population doubling-defined, HCC clones may provide novel opportunities to tackle HCC heterogeneity in a single patient background for therapy improvement purposes, especially regarding recently developed targeted strategies. J. Cell. Biochem. 115: 666–677, 2014.

KEY WORDS: LIVER; HEPATOCELLULAR CARCINOMA; PROGENITOR CELLS

Hepatocellular carcinoma (HCC) represents the third commonest cancer type and is associated with HCV infection. The incidence of HCC continues to increase worldwide [El-Serag, 2012]. HCC pathogenesis comprises a multistep process that involves genetic and epigenetic events occurring during initiation and progression of the disease. According to this theory, external stimuli would induce alterations on mature hepatocytes or stem cells [Thorgeirsson and Grisham, 2002; Farazi and DePinho, 2006; Villanueva et al., 2007] leading to apoptosis, cell proliferation, dysplasia and neoplasia [Thorgeirsson and Grisham, 2002]. During the preneoplastic stage, activation of mitotic signaling pathways may confer growth advantages and induce the selection of dysplastic cell clones. These clones, organized in dysplastic nodules surrounded by connective tissue, could lately acquire a malignant phenotype after exposure to additional genomic insults such as point mutations, chromosomal arm gains or losses or aberrant promoter methylation of key genes [Villanueva et al., 2007]. The prognostic variability of individuals with HCC supports the notion that this disease comprises several biologically distinct subgroups. This variability probably reflects a molecular heterogeneity that the research community has mainly tackled from the interindividual prospective [for example, see Lee et al., 2006; Guichard et al., 2012; Nahon and Zucman-Rossi, 2012]. It

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*Correspondence to: Lyon Cancer Research Center, Inserm U1052–CNRS UMR5286, 151 Cours Albert Thomas, F-69424 Lyon Cedex 03, France. E-mail: romain.parent@inserm.fr
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is well established that a large number of genetic and epigenetic alterations are accumulated during the hepatocarcinogenesis process. For instance, studies indicate aberrant activation of signaling pathways involved in cellular proliferation (e.g., epidermal growth factor and RAS/mitogen-activated protein kinase pathways), survival (e.g., Akt/mechanistic target of rapamycin pathway), differentiation (e.g., Wnt and Hedgehog pathways), and angiogenesis (e.g., vascular endothelial growth factor and platelet-derived growth factor), which is heterogeneously represented in each tumor [Hoshida et al., 2010] and may hence differentially react to treatment and heavily influence disease outcome. Hence, the deleterious clinical heterogeneity of HCC, mainly because of its development on a previously long-lived diseased or even cirrhotic liver and the lack of good diagnostic markers and treatment options, demands further consideration for personalized therapy development [Villanueva and Llovet, 2011]. Yet, genotypic, phenotypic, and functional tumor heterogeneity studies, seldom based on freshly isolated single cell lines, have to our knowledge rarely been studied at the single patient level with several low population doubling clones.

In the present study, we provide data regarding the morphological, histological, functional and cytogenetic features of four clones isolated from a single patient's HCV-associated, differentiated Edmondson grade I HCC, which bear putative liver progenitor cells properties.

MATERIALS AND METHODS

PATIENT AND INITIAL CLONES ISOLATION

Tumor tissue has been obtained in accordance with French law and the National Ethics Committee, from a patient with Hepatitis C undergoing hepatocellular carcinoma (HCC) resection, as described elsewhere [Gripon et al., 2002; Parent et al., 2004]. Four clones subsequently named CLP11, 12, 13, and 21 have successfully propagated. In parallel, tissues were fixed in Bouin's solution and embedded in paraffin. Serial sections were used for Trichrome staining for diagnosis and immunohistochemistry characterization.

CELL CULTURE

Cells were seeded and maintained in Williams'E medium supplemented with 1%DMSO (Sigma) for differentiation purposes as described previously [Parent et al., 2004].

PROLIFERATION ASSAYS

Cells were plated in triplicate in 24-well plates and counted every day for 4 days after seeding by direct counting with Trypan blue exclusion using a Malassez hemocytometer. For TGFβ (10 ng/ml), IFNα (2,000 IU/ml) and doxorubicin (1 μg/ml) treatment at these indicated doses, cells were plated in triplicate in 96-well plates and the cell proliferation assay was performed using Neutral Red uptake assay as described by Repetto et al. [2008].

IMMUNOFLUORESCENCE AND IMMUNOHISTOCHEMISTRY

Immunofluorescence and immunohistochemistry have been performed as described previously [Parent et al., 2004]. For immunochemistry, bound immunoglobulins were detected using an HRP-coupled secondary antibody (Dako) according to the manufacturer's specifications. For immunofluorescence, bound anti-E-cadherin (Transduction Laboratories) and anti-β-catenin (Santa Cruz) immunoglobulins were detected using an Alexa 488-coupled secondary antibody (Life Technologies) according to the manufacturer's specifications. Hoechst 33258 (Sigma) was used for nuclei counterstaining.

WOUND REPAIR AND TRANSMEMBRANE MIGRATION ASSAY

Wound was performed with a 20 μl pipet tip. Cells were then rinsed with warm fresh medium, and new medium was added for the indicated times. For migration assays, cells were plated in Boyden chambers equipped with 8 μm inserts (BD Biosciences) in 24-well plates according to commercial instructions. To minimize bias due to post-translocation or post-scratching proliferation, 3 μg/ml mitomycin (Sigma) was used.

IMMUNOBLOTTING

Immunoblotting has been performed as described previously [Parent et al., 2004], followed by chemiluminescence-based quantification of the bound secondary antibodies' signal using the Bio-Rad Chemidoc apparatus and quantification software. Signals shown have been normalized to β-actin signals.

QUANTITATIVE RT-PCR

Total RNAs were extracted using Extract-all (Eurobio). One microgram of RNA of each sample was DNAse I-digested (Promega) then reverse transcribed using the MML-V enzyme. Real-time quantitative RT-PCR was performed on a LightCycler 480 device using the LC480 SYBR GREEN I Master mix (Roche). Normalization was done using *GUS1* signals. Oligonucleotide sequences are available on request.

CYTOGENETICS

Chromosome analysis was performed using exponentially growing cells by R-Giemsa banding. The modal number was determined from the analysis of six metaphases for each CLP cell line. Description of the karyotypes was done according to the International system for Human Cytogenetics Nomenclature (ISCN, 1995) with 350 band stage. FISH experiments were performed using whole chromosome 12 and 22 painting probes (WPC12 and 22). Probes and slide preparation as well as hybridization experiments were performed as previously described [Romana et al., 1994].

TUMORIGENICITY ASSAYS

CLP cells expanded in complete medium were harvested, spun down, and resuspended in Williams E medium + glutamine without any additive. Aliquots of 150 μl were injected subcutaneously in the upper back of 6-week old female athymic nude mice. Five million cells of each cell line were injected into two mice. Two mice were injected with control medium. Animals were housed and fed ad libitum according to French regulations, examined twice a week, and euthanized 17 weeks after injection. Autopsies were carried out on organs susceptible to engraftment (lung, brain, spleen, kidney, liver, pancreas) and tissues were either snap frozen in liquid nitrogen or formalin-fixed for histological examination after HES staining. Analysis was carried out blindly by a pathologist (BB). Mice were housed and treated according to French regulations.

RESULTS

HISTOPATHOLOGICAL ANALYSIS OF THE INITIAL HUMAN TUMOR

The tumor from the HCV-infected, cirrhotic, 70-year-old female caucasian patient who gave rise to the cell lines described herein was first analyzed. The cirrhotic parenchyma showed a whitish 18-mm tumor nodule with focal extra-nodular growth. Histologically, the tumor consisted of a well-differentiated Edmondson grade I HCC, displaying a "nodule-in-nodule" structure (Fig. 1A). Architectural pattern was trabecular with rare and empty glandular structures and some round nuclei observed were adenoma-like, exhibiting homogeneous chromatin and inconspicuous nucleoli (Fig. 1B). Large nuclei with small nucleoli were also identified (Fig. 1C). A millimetric focus of small cells with increased nuclear density was observed, associated with a high nuclear-to-cytoplasmic ratio, hyperchromatic nuclei and scant, basophilic or clear cytoplasm (Fig. 1D). No oval shaped cells, biliary differentiation, or fibrous stroma have been detected. Cytokeratin 7 stained few small tumor cells at the periphery of some nodular foci (Fig. 1E,F). HepPar1 signal was found positive throughout the tumor. Neither pCEA nor CD10 nor the mesenchymal/epitheliomesenchymal transition markers Vimentin, Twist-1 or Snail-1 stainings yielded any signal in validated clinical grade assays using a positive controls. Stem cells markers such as EpCAM, CD133 and CD56 were found negative as well in the same procedures

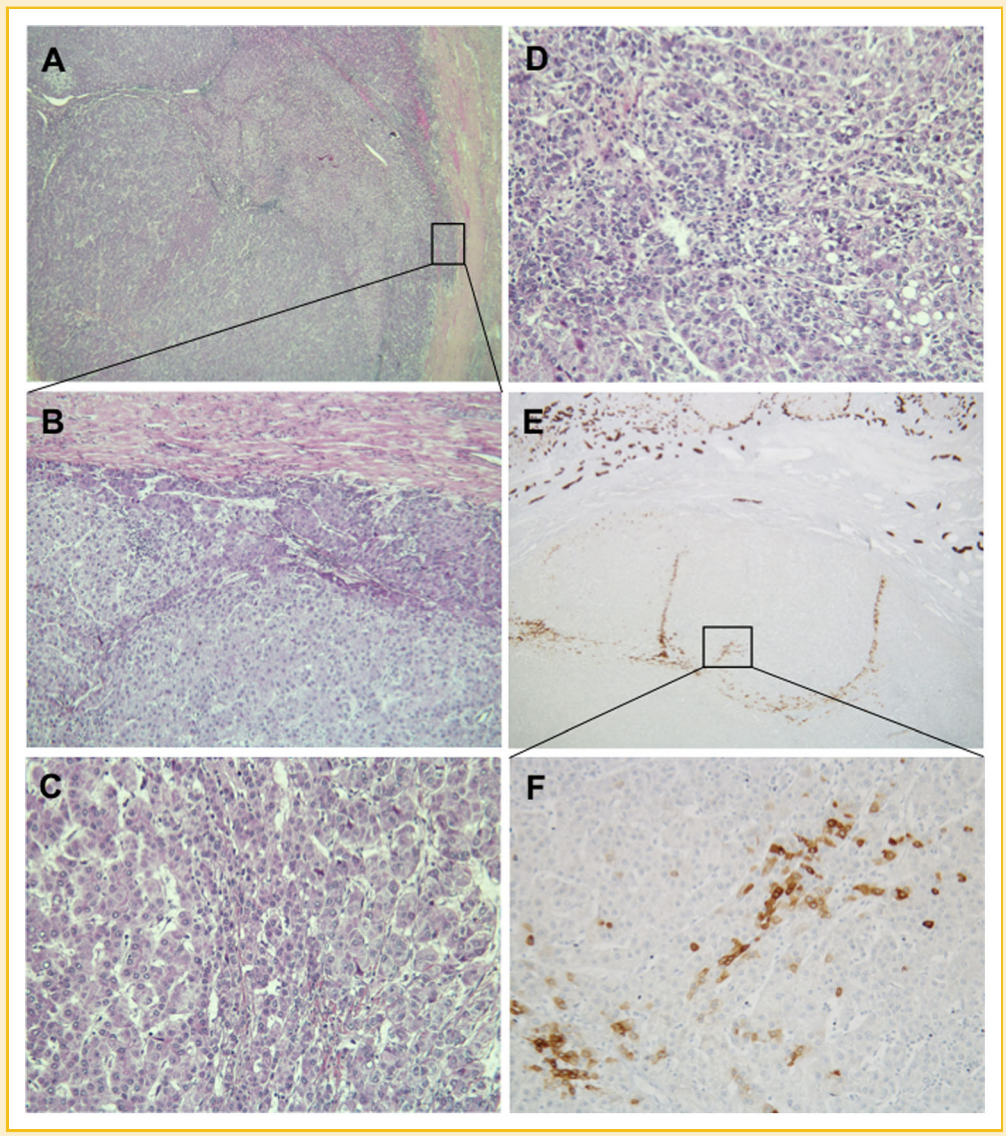

Fig. 1. Histological and immunohistochemistry analysis of the human initial tumor. A: Low magnification picture showing the hepatocellular carcinoma from which CLP cells originate exhibiting a "nodule-in-nodule" pattern by Masson's trichrome staining (10× magnification). B: Magnification (20×) of (A). Edmondson grade 1 hepatocellular carcinoma (bottom of the picture) showing thick trabeculae and minimal nuclear alterations. Note a small-cell dysplastic area at the margin of the nodule (top of the picture). C: Magnification (40×) of the tumor bulk area of (B), showing a representative well-differentiated area. Note the presence of small hepatocytes in the center, at the periphery of a nodular focus. D: Microscopic foci within the tumor with small cell alterations and increased nuclear density (40× magnification). E: Positive cytokeratin 7 immunostaining of small hepatocytes while the tumor bulk remains negative (10× magnification). F: Magnification (40×) of (E).

(not shown). Altogether, these results suggest that CLP cells arose from a well-differentiated HCC hosting infiltrates of cytokeratin 7 positive cells, and composed of several histological foci that may explain CLP cells heterogeneity.

IN VITRO MORPHOLOGY OF CLP CELLS

About 25 clones of CLP cells were clonally isolated, expanded and subsequently frozen from the initial patient's liver. Among these, only four clones remained capable of sustained proliferation. Cell cycle analyses were performed by FACS after PI staining, and demonstrated that all clones displayed similar proliferation rates in in vitro culture (not shown). More than 100 population doublings after their initial isolation, population doubling times were close to 1.5 days for all cell lines. While none of the cell lines displayed terminal hepatocytic differentiation-related features, two of them did evolve towards two distinct cell types, reminiscent of the bipotent liver progenitor HepaRG cells [Parent et al., 2004]. We then sought to observe their morphological features prior to, and at the end, of a hepatocytic, DMSO-based, differentiation process. CLP cells were photographed at the proliferative and differentiated stages. At the proliferative stage, all cells displayed features characteristic of a dedifferentiated epithelial phenotype (Fig. 2A, first lane, left panel). At the differentiated stage, even though induction of features demonstrative of terminally differentiated cells such as bile canaliculi and polygonal shape did not occur, more specific data emerged: CLP11 cells evolved towards an heterogeneous population consisting of poorly polarized areas composed of large and flat cells often surrounded by cords of smaller and thicker cells. Dark cytosol possibly loaded with hepatospecific glycogen granules is yet present (Fig. 2A, first lane, right panel). In contrast, DMSO-treated CLP12 cells exhibited a homogenous monolayer in which densely arranged cells also loaded with dark cytosol are present (Fig. 2A, second lane, right panel). CLP13 cells, as CLP11 cells, evolved towards two distinct populations as well upon differentiation, although in a much less organized fashion than CLP11 cells. Moderate but constant cell detachment and death was visible in the culture of CLP13 cells. Dark cytosolic inclusions were also present in this cell line (Fig. 2A, third lane, right panel). Finally, upon differentiation, CLP21 cells evolved towards a densely packed, fibroblastoid, phenotype, a pattern suggesting strong resistance to differentiation, even though, again, dark cytosol reminiscent of the possible presence of hepatospecific glycogen granules was visible in these cells (Fig. 1B, last lane, right panel). Bile canaliculi represent one of the terminal differentiation features that, when present, recapitulate satisfactory differentiation along the hepatocytic lineage [Lazaro et al., 2003]. They were not expressed by any of the clones upon analysis by a fluorescein accumulation assay (not shown). In summary, while none of the cell lines displayed terminal differentiation-related features, two of them did evolve towards two distinct cell types, suggesting a potential implication of such bipotent progenitors in HCV-related carcinogenesis.

CANONICAL HISTOLOGICAL FEATURES OF CLP CELLS

We then sought to gain insight on CLP cells' phenotype. To this aim, canonically recognized histological markers in hepatology such as the hepatospecific albumin marker, and the biliary epithelium cytokeratin 7, 18, and 19 markers were studied by immunoblotting in proliferative and DMSO-differentiated CLP cultures. No cell was found positive for vimentin, α-SMA or von Willebrand factor whatever the CLP clone studied, suggesting absence of contamination by mesenchymal cells such as endothelial or fibroblastic cells, but also the absence of epitheliomesenchymal transition in all cell lines (not shown), despite the fibroblastoid shape acquired by DMSO-treated CLP21 cells. CLP11 cells showed DMSO-independent albumin induction susceptibility, and prominent cytokeratin 7 expression. Cytokeratin 18 and 19 were found present and robustly expressed whatever the differentiation stage considered (Fig. 2B, first couple of lanes of each panel). CLP12 cells displayed also albumin induction upon differentiation, albeit in a DMSO-dependent fashion. No cytokeratin 7 expression could be evidenced in none of the differentiation conditions tested. Cytokeratin 18 and 19 were found both expressed by CLP12 cells. Interestingly, differentiation did increase albumin and decrease cytokeratin 19 levels of CLP12 cells (Fig. 2B, second couple of lanes of each panel). CLP13 cells were found albumin negative whatever the differentiation step and the DMSO presence considered. Cytokeratin 7 positivity was found in both culture conditions, even though this marker's expression decreased upon differentiation. Cytokeratin 18 and 19 were again co-expressed and found substantially constant whatever the culture conditions (Fig. 2B, third couple of lanes of each panel). As for CLP21 cells, albumin was found induced by the differentiation protocol applied to the cells, in a DMSO-unrelated manner. Cytokeratin 7 was found expressed at similar levels in proliferative and differentiated cells. Again, cytokeratin 18 and 19 coexpression occurred, and remained unchanged by the differentiation protocol (Fig. 2B, fourth couple of lanes of each panel). Taken together, these data suggest important phenotypical heterogeneity amongst CLP clones, and at the proliferative stage, strong dedifferentiation because of albumin negativity for all clones. Although all other cell clones were sensitive to the differentiation protocol applied in terms of albumin induction, CLP13 cells remained negative for this protein throughout differentiation. CLP12 cells appear to be the cells that have retained the most hepatocytic features such as albumin inducibility, hepatocytic-like morphology, cytokeratin 7 negativity, and only weak cytokeratin 19 positivity. Altogether, these data suggest the frequent appearance of aberrant histological markers combinations in HCC-related hepatocytic clones, such as cytokeratin 7, 18, and albumin co-expression.

STEM/PROGENITOR CELLS CHARACTERISTICS OF CLP CELL LINES

Most CLP cells display, albeit to various extents, coexistence of albumin inducibility and biliary markers, which is in favor of a liver progenitor cell status. Progenitor cells signatures are frequent in HCV-associated HCC [Lee et al., 2006]. In this context, we sought to gain insight into the classically used liver progenitor cells marker OV6 [Ji and Wang, 2012]. The possibly bipotent CLP11 cell line was found strongly positive for this marker, as well as for CLP12 and CLP21 cells. In contrast, CLP13 cells were found negative for the OV6 marker (Fig. 3A). EpCAM is a cancer stem cell marker [Mishra et al., 2009; Ji and Wang, 2012]. In this context, we sought to analyze EpCAM expression in CLP cells. Because of the lack of appropriate antibodies in our hands, we quantified *EpCAM* mRNA expression levels in CLP cells (Fig. 3B). *EpCAM* was detected in CLP13 cells only, suggesting that this clone could correspond to a tumor initiating cell. Detection

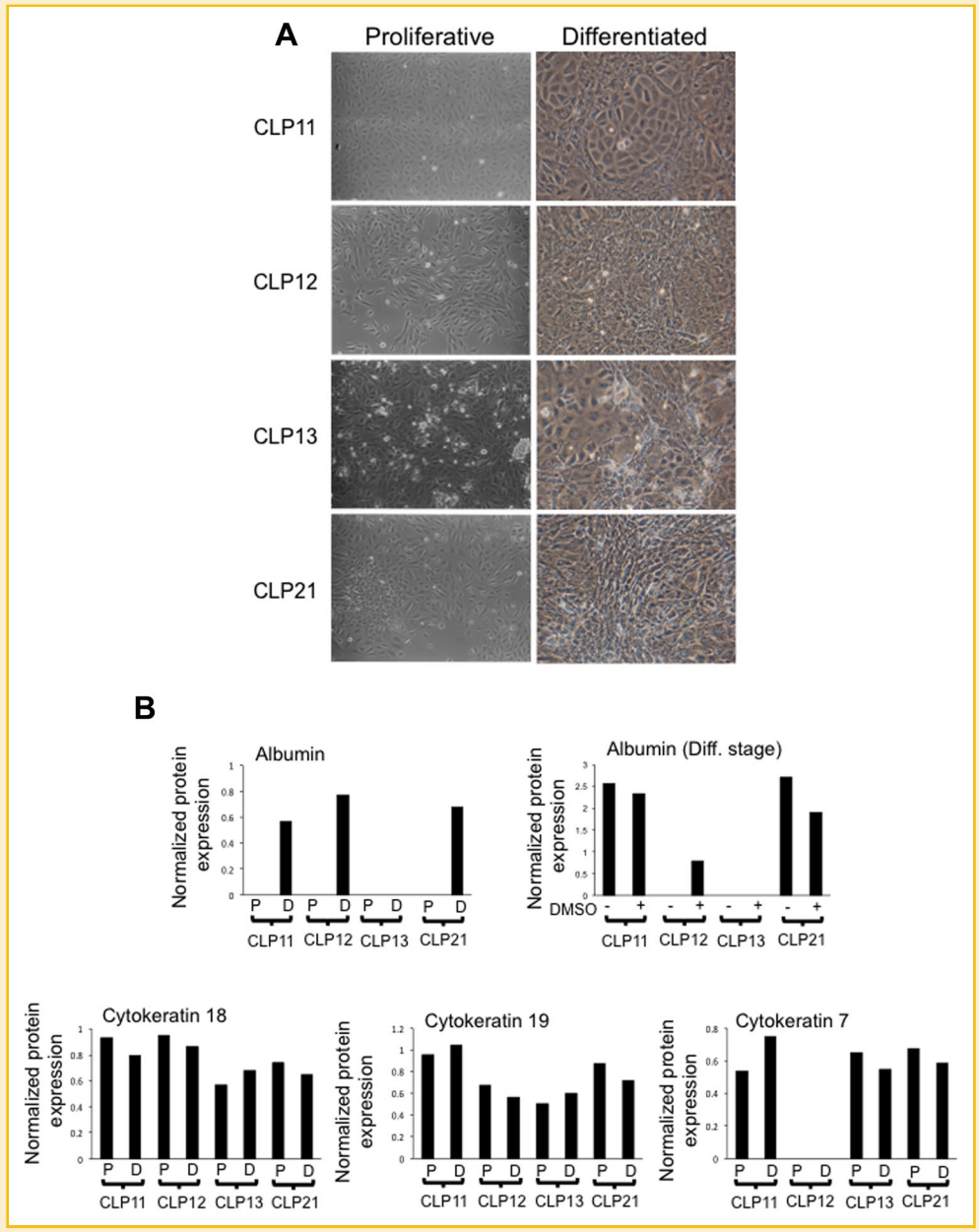

Fig. 2. Morphology and histological markers expression profiles of CLP cells. A: Morphological features of CLP cells. For differentiation, CLP cells were cultured 14 days without and then 14 days with 1% DMSO. Phase contrast micrographs were taken at days 2 and 28 post-seeding (40× magnification). Representative of three independent differentiation processes. B: Histological markers profile obtained by immunoblotting followed by chemiluminescence-based signal quantification normalized to β-actin expression. Representative of three independent differentiation processes. P, proliferative stage; D, differentiated stage.

attempts on other liver stem cell/progenitor cell markers [Mishra et al., 2009; Ji and Wang, 2012] ended up in the detection of similar levels of expression for CD44 whatever the cell line and the differentiation stage considered, while CD90, *CD117* and *CD133* were found not expressed (not shown). E-cadherin and β-catenin are frequently affected function-wise in HCC [Zucman-Rossi, 2010], events being associated with distinct prognostics. These proteins' physiological functionality can be assessed primarily by determining their localization. Hence, E-cadherin and β-catenin were studied by an immunofluorescence approach. E-cadherin was expressed diffusely in the cytoplasm of all cell lines, suggesting the presence of a mutation impacting subcellular localization of the protein (Fig. 3C). β-Catenin mutations are frequent in HCC, leading to its nuclear accumulation at the expense of its plasma membrane, epithelium polarization-related, routing. All clones displayed wild-type β-catenin expression pattern at the plasma membrane except for CLP13 cells that appeared essentially β-catenin-negative whatever the subcellular compartment considered. In summary, all CLP cells

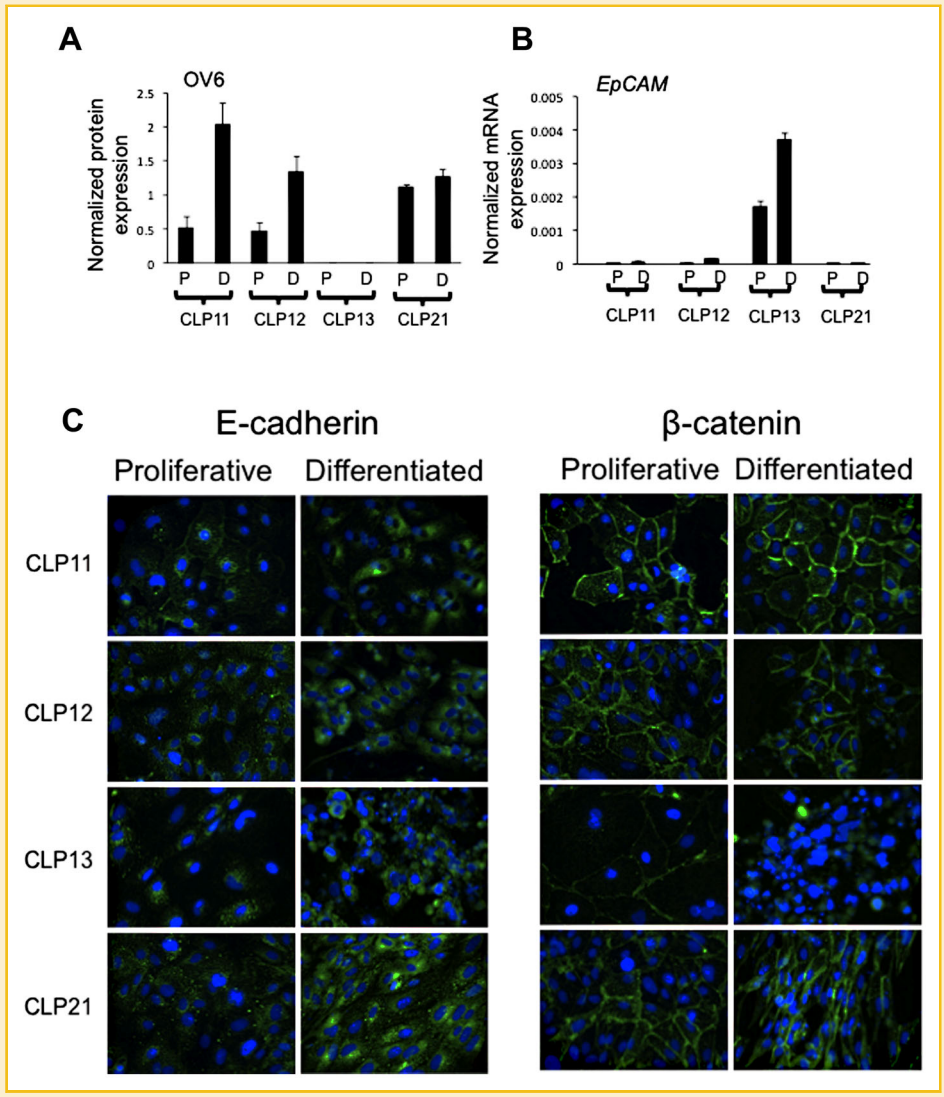

Fig. 3. Liver progenitor/stem cells markers and tight junction proteins expression profile. A: OV6 antigen expression markers profile obtained by immunoblotting followed by chemiluminescence-based signal quantification normalized to β-actin expression. Mean ± SD of three independent experiments. B: *EpCAM* mRNA expression profile quantified by RT-qPCR. Normalization was done using *GUS1* expression levels. Mean ± SD of three independent experiments. C: Tight junction proteins expression patterns of CLP cells. E-cadherin and β-catenin localization patterns (green) were studied by immunofluorescence as described in the Materials and Methods Section. Nuclei counterstaining (blue) was done using Hoechst 33348. Representative of three independent experiments.

displayed mislocalized E-cadherin expression patterns, yet combined with a physiological pericellular accumulation of β-catenin. Through immunoblotting, E-cadherin expression levels were found similar in all cell lines. β-Catenin was found essentially null in CLP13 cells, while also similar in other cell lines (not shown). These data suggest that CLP cells express mutated E-cadherin concomitantly with wild-type β-catenin sequences, and that CLP13 cells may represent the most immature, cancer stem cell-related, clone of the study.

PROLIFERATION PROPERTIES OF CLP CELLS

Transforming growth factor β (TGFβ) has dual effects in hepatocarcinogenesis. While behaving as a tumor suppressor effector in early steps of cell immortalization, it behaves as a tumor enhancer at later stages [Majumdar et al., 2012]. HCC ubiquitously display type I interferon pathway overactivation in vivo [Calvisi et al., 2006], suggesting loss of sensitivity towards this class of cytokines. Doxorubicin is used for HCC cells′ drug sensitivity studies [Barraud et al., 2005]. In order to characterize CLP clones in these regards, cells were followed kinetically in the presence of these three hepatocytic proliferation modulators: TGFβ, IFNα, and doxorubicin (Fig. 4). All TGFβ-treated CLP cells displayed increase rates of proliferation as compared to control cells, suggesting that all cells are substantially advanced in the HCC process. Yet, important heterogeneity was visible since CLP12 cells appeared to be 2.5–10 times more sensitive to TGFβ′s pro-proliferative effect than other cells, while CLP11 cells were

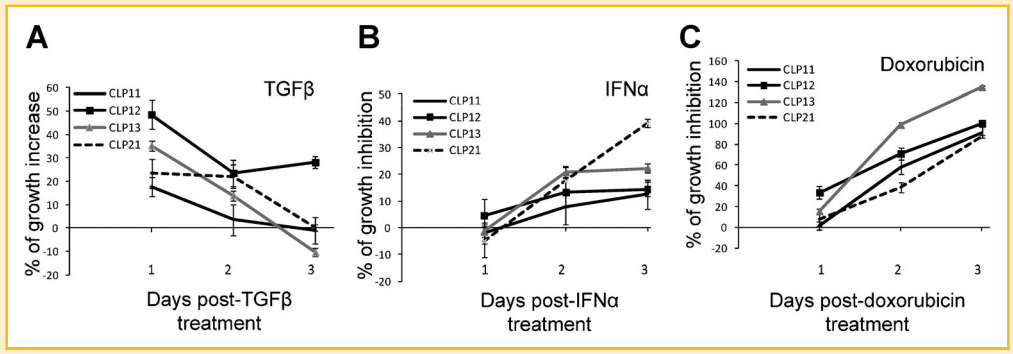

Fig. 4. Heterogeneity of CLP cells sensitivity to TGFβ, IFNα, and doxorubicin. Proliferative CLP cells were treated with TGFβ (A), IFNα (B), or doxorubicin (C) at doses indicated in the Materials and Methods Section. Proliferation was quantified by the Neutral Red assay during 3 days after treatment (shown as Day 0). Mean ± SD of three independent experiments carried out in three technical replicates.

practically insensitive. IFNα-treatment exerted a 40% antiproliferative effect on fibroblastoid CLP21 cells, while CLP11 cells' proliferation rate was practically left unchanged. All CLP clones were, as expected, sensitive to doxorubicin. Yet, CLP13 cells appeared to be the most affected proliferation-wise. It is of note that doxorubicin results have been generated with doses 5–10 times lower than the ones known to be active on Huh7 cells for instance [Barraud et al., 2005] (results not shown). This strengthens the usefulness of these low-passaged HCC clones in terms of their ability to reflect more physiopathologically relevant responses to chemotherapeutic drugs than their highly passaged counterparts that may have been subcloned many years after tumor isolation for their current usage. Taken together, these data show that CLP clones exert different responses to proliferation modulators suggesting important heterogeneity in terms of susceptibility to chemotherapy and cytokines widely known to modulate HCC growth.

CYTOGENETICS

Given the high phenotypical heterogeneity of CLP cells using morphological, functional, and histological antigen probing, we sought to explore CLP cells cytogenetics by R-Giemsa banding. CLP12 and CLP21 cells were diploid while CLP11 cells exhibited tetraploid features. CLP13 cells' karyotype was tetraploid as well (Fig. 5A). All CLP cells displayed the same t(12;22)(p11;q11) translocation with a loss of the short arm of chromosome 12 (red arrow). This t(12;22)(p11;q11) translocation was the sole intrachromosomal aberration observed for CLP11, 12, and 21 cells (Fig. 5B). To investigate the possibility of a fusion protein production because of this translocation, centromeric probes were used in FISH approaches. The der(12) region was dicentric since both centromeric 12 and 22 probes hybridized on der(12), suggesting the absence of a fusion protein (white arrow, Fig. 5C). FISH approaches as well confirmed the deletion of the short arm of one chromosome 12 (not shown). Representative karyotype characteristics are shown in Table I. Taken together, cytogenetic data suggest chromosomal instability in this HCC case, as well as the preexisting status of the t(12;22)(p11;q11) translocation shared by all CLP cells before their likely genotypic and phenotypic divergence.

Cell motility is a widely accepted criterion for cell invasiveness, and a condition for HCC progression. Using the classically used scratch assay, we evaluated the ability of all CLP clones to repair wound 24 and 48 h after scratching using mitomycin in order to prevent the interference of cell proliferation in the results. CLP12 and CLP13 cells proved the less prone to migration. Only CLP21 cells, in accordance with their partly fibroblastoid phenotype, appeared to consistently refill the wound at 48 h post-scratching (Fig. 6A).

We then addressed the invasion/chemotaxis potential of CLP clones. While other cell clones display translocation rates higher than 20%, CLP13 cells are the less prone to invasion/chemotaxis (<10% of translocated and alive cells), perhaps in accordance with their weakly motile, cancer stem cell-like, phenotype (Fig. 6B, higher panel). Interestingly, comparisons of the differences of translocation, percentage-wise, in the presence and the absence of the drug allowed to evaluate the relative ability of all clones to proliferate after translocation. CLP12 displayed the strongest proliferation rate post-translocation (Fig. 6B, lower panel), suggesting this clone may be the most prone to generate active metastasis foci distantly from the primary tumor site.

Altogether, motility experiments suggest that most of these clones acquired substantial motility capabilities, except CLP13 cells which exhibit lower liver-specific cytokeratins levels, null albumin expression, *EpCAM* positivity and the highest doxorubicin sensitivity level in comparison with other CLP clones.

CELLULAR TRANSFORMATION AND LIVER ENGRAFTMENT STUDIES

At this stage, we sought to decipher which of the features previously unraveled could consistently correlate with cellular transformation. To do this, we performed proliferation assays in serum-free medium, soft agar assays, and assessed CLP cells' liver engraftment capability after subcutaneous injection into nude mice. CLP cells were first submitted to proliferation assays in serum-free medium in a 4 days-long trypan blue-based kinetical approach. While CLP11 and 13 cells did not tolerate growth factors deprivation, CLP12 and 21 proliferated at a rate of about +0.5 population doublings per day in average from hours 0 to 96 post-seeding (Fig. 6C). Soft agar assays were repeatedly negative for all clones, using Huh7 cells as a positive control (not

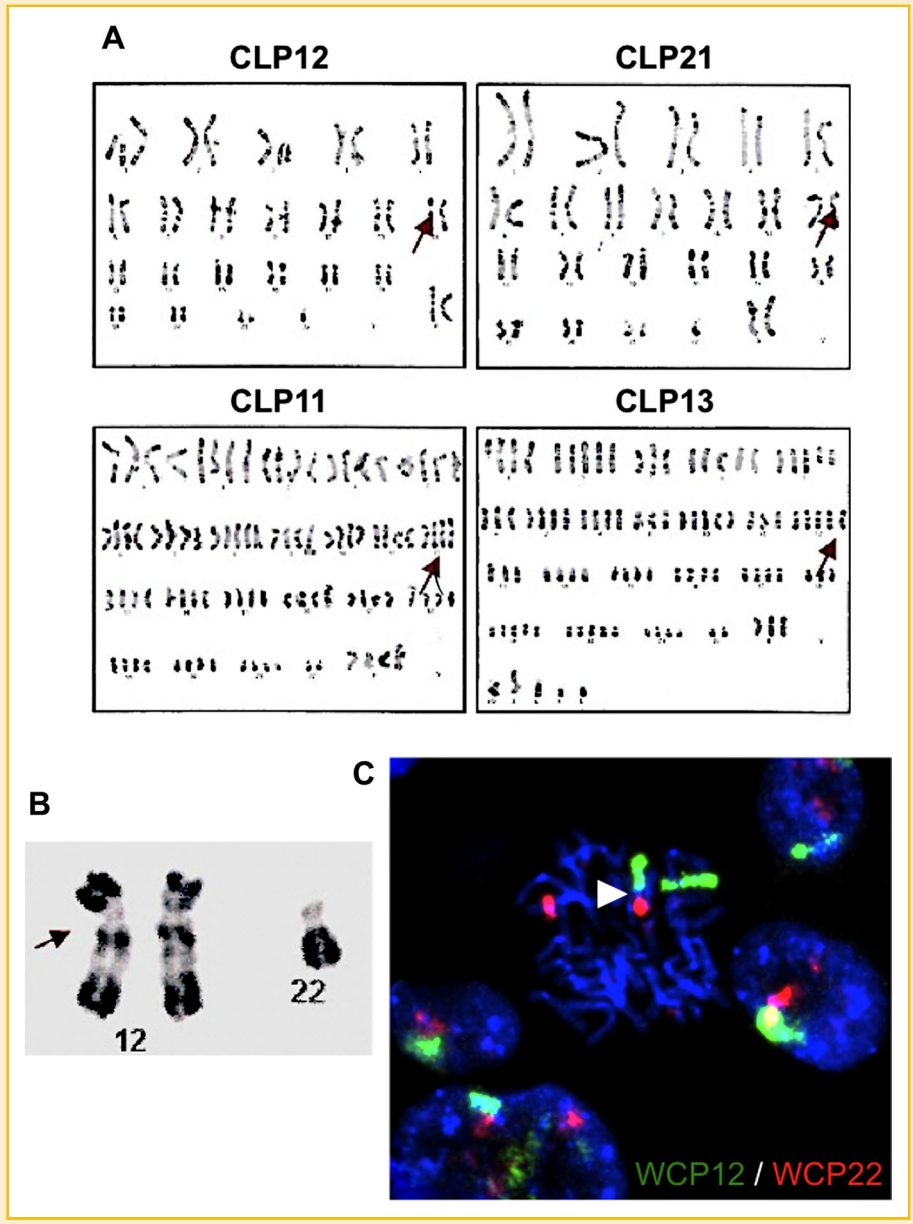

Fig. 5. CLP cells carry the same t(12;22)(p11;q11) translocation, which does not lead to any fusion protein. A: Representative R-banded karyotypes of CLP cells. All CLP cells carried the same t(12;22)(p11;q11) as pointed out by the red arrow shown, and the same loss of one short arm of chromosome 12. B: Zoom-in on the t(12;22)(p11;q11) translocation-bearing chromosome 12/22 pair. Six metaphases were considered for each cell line. C: FISH analysis using whole chromosome painting probe (WCP)12 probe in green, and WCP22 probe in red showing absence of any fusion protein resulting from this translocation (white arrow). Twenty cells were analyzed for each metaphase spread, using chromosome-specific or single locus fluorescent probes.

TABLE I. Representative Karyotypes of CLP Cells

	Karyotype description
CLP11	45, XX,-22,der(12)t(12,22)(p11;q11) near diploid 92, X XX X,-22, der(12)t(12,22)(p11;q11) near tetraploid
CLP12	45, XX,-22,der(12)t(12,22)(p11;q11)
CLP13	92, XX XX,-22,der(12)t(12,22)(p11;q11) add(3pter), del(5q),+2. Tetraploid
CLP21	46, XX,+der(7),-22,der(12)t(12,22)(p11;q11)

Six metaphases were considered for each cell line. Abnormalities listed were consistently observed in all metaphases spreads.

shown). Proliferative CLP cells cultured in complete medium were injected into nude mice subcutaneously. Yet, some microscopic lesions such as microvesicular steatosis and congestive sinusoids were found at the hepatic levels of all CLP-injected mice but not in mock-injected mice (not shown). More importantly, staining with a specific anti-human cytokeratin 19 antibody did not yield any signal in CLP11, 12, and 13-injected mice (representative mock-injected slide shown Fig. 6D, panel a). Yet, anti-human cytokeratin 19 antibody allowed localization of isolated cytokeratin 19 positive CLP21 cells, intrahepatically engrafted in close vicinity with

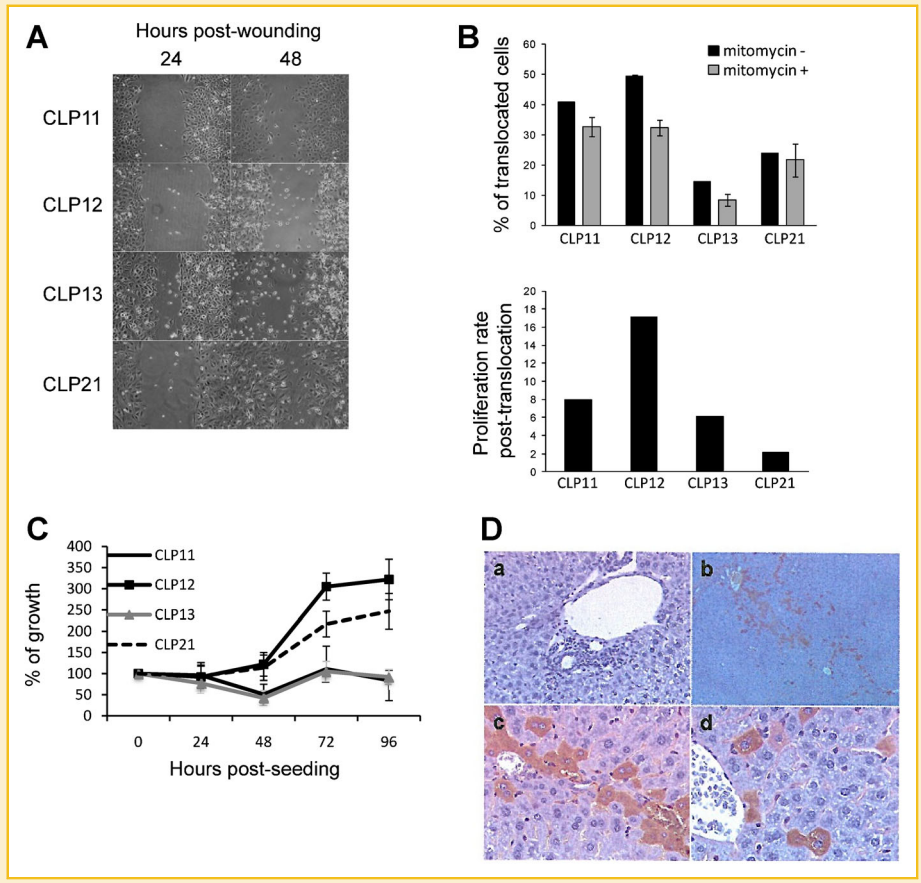

Fig. 6. CLP cells, motility and transformation. A: Wound-healing assay. CLP cells were submitted to wounding with a 20 μl pipet tip. Micrographs were taken 24 and 48 h post-wounding. Representative micrographs of three independent experiments are shown. B: Boyden chamber assay. Cells were seeded in serum-free medium and allowed to translocate in 10% serum-containing medium for 48 h supplemented or not with mitomycin. Mean $\pm$ SD of percentages of translocated cells versus total cells from three independent experiments done in triplicate for each cell line are shown. Comparisons of the clones' differences in lower chamber numbers, percentage-wise, in the presence and the absence of the drug allowed to evaluate the relative ability of all clones to proliferate downstream translocation. C: Proliferation assay in serum-free medium. Cells were seeded and counted every 24 h by trypan blue exclusion assay. Mean $\pm$ SD values from two independent experiments done in triplicate for each cell line are shown. D: Immunohistochemistry analysis of liver tissue sections of CLP21-injected mice. Experiment has been done using two mice for each cell line. (a) liver section from mock-injected mice; (b,c,d) liver sections of mice subcutaneously injected with CLP21 cells. Original magnifications: (a) 20×, (b) 40×, (c,d) 60×.

TABLE II. Summary of CLP Clones Characteristics

	CLP11	CLP12	CLP13	CLP21
Albumin inducibility	Yes	Yes	No	Yes
Cytokeratin 7	Yes	No	Yes	Yes
EpCAM	No	No	Yes	No
CD44	Yes	Yes	Yes	Yes
CD90, CD117, CD133	No	No	No	No
OV6	Yes	Yes	No	Yes
E-cadherin	Mislocalized	Mislocalized	Mislocalized	Mislocalized
B-catenin	Plasma membrane	Plasma membrane	Poorly expressed	Plasma membrane
TGFβ	Weakly Mitogenic	Mitogenic	Mitogenic	Mitogenic
IFNα	Antiproliferative	Antiproliferative	Strongly Antiproliferative	Antiproliferative
Doxorubicin	Sensitive	Sensitive	Most sensitive	Less sensitive
Motility	Yes	Yes	Weak	Yes
Serum deprivation	Sensitive	Resistant	Sensitive	Resistant
Liver engraftment	No	No	No	Yes

central and portal vessels as well as in between (Fig. 6D, panels b, c, and d). No human cells were found whatever the clone injected, neither in any other organ nor at the subcutaneous site of injection upon comprehensive autopsy (not shown). These data suggest that CLP21 cells bearing the most weakened tight junction apparatus, are the most prone to engraftment in an heterologous microenvironment.

All CLP cells characteristics investigated in this study are summarized in Table II.

DISCUSSION

HCC heterogeneity represents one of the current therapeutic challenges pertaining to this illness. In order to shed light on this important question in a single genetic background, we isolated numerous proliferating clones from an HCC case, of which only four clones remained compatible with further studies because of their capacity to proliferate. Preliminary morphological studies on emerging clones have allowed objectivation of HCC heterogeneity since possibly bipotent epithelial, homogenous epithelial, and fibroblastoid patterns were seen across these clones. Distinct levels of CLP cells′ sensitivity to DMSO suggest that liver-specific proteins induction may be disrupted in reversible as well as irreversible manners upon hepatocytic carcinogenesis. To what extent this result is linked to other cellular pathogenic events remains to be investigated. Combined with the fact that no hepatocytic organization could be induced by DMSO treatment, unlike HepaRG [Parent et al., 2004] or Huh7.5 cells that do organize [Sainz and Chisari, 2006] used as a control (not shown), we believe that these freshly isolated cells exhibit intrinsically dedifferentiated features that prevent proper differentiation, even in a chemically forced setting.

Cytokeratin profiles are deemed canonical markers for histology and tumor studies [Moll et al., 1982]. Primary hepatocytes are cytokeratin 18 positive, while cytokeratin 7 and 19 negative. Cholangiocytes are cytokeratin 7, 18, and 19 positive [Moll et al., 1982]. Here, we show that while all clones but CLP13 cells are capable of at least weak albumin induction upon DMSO-based differentiation, CLP11, 12, and 21 clones co-express, albeit at various levels and ratios, all intrahepatic cytokeratins, regardless of their hepatocytic or cholangiocytic partial commitment. This persistence of coexpression of hepatocytic and biliary markers combined with CLP cells′ intrinsic lack of complete differentiation capability is to our opinion in favor of their liver progenitor cell status. The CD44 positivity of all clones, the OV6 positivity of CLP11, 12, and 21 clones, the EpCAM positivity of CLP13 cells and the liver progenitor cells signature frequently observed in HCV-related tumors [Lee et al., 2006] strengthen this hypothesis.

Tight junction disorganization [Orban et al., 2008] is a hallmark of HCC development, and E-cadherin down-regulations have been found in HCC [Matsumura et al., 2001; Wei et al., 2002]. All clones probably bear E-cadherin-mislocalizing mutations, while most of them except CLP13 cells express readily detectable levels of plasma membrane, that is, probably wild-type, β-catenin, which could be of interest for studies aiming at uncoupling E-cadherin and β-catenin-related functions in HCC trabecular disorganization. Upon injection into nude mice, no cell clone induced tumors, while only the fibroblastoid CLP21 cells displayed resistance to serum deprivation and exhibited intraparenchymal liver engraftment in a heterologous environment. These data suggest that CLP21 cells have bypassed most of the checkpoints that could constrain their survival and growth in vivo, and that these cells may represent the most aggressive fraction, yet perhaps initially minoritary in the patient′s tissue, of the tumor.

CLP13 cells represent an atypical population of cells in this study and probably also in liver pathophysiology. While OV6 negative, albumin negative, and displaying weak motility, CLP13 retained EpCAM positivity, a feature not seen in the patient′s tumor. These data suggest that CLP13 cells may represent a hepatocytic lineage-uncommitted, minoritary cancer stem cell compartment, of the tumor. Hence, while other CLP cells express liver progenitor cells markers in cell culture, CLP13 cells do not express such features and corresponded either to a more mature phenotype, which is unlikely because of their albumin inducibility resistance, or to a more immature phenotype, hence also devoid of any hepatocytic differentiation capability. CLP13 cells are the most sensitive clone to doxorubicin. This result might be explained by the fact that hepatocyte-related drug efflux and detoxification functions are absent in this cell line that has not engaged into the hepatocytic lineage.

Cytogenetics findings are of interest in terms of liver/hematopoiesis interplay in hepatic disease, and tumor evolution. All clones share a common t(12;22)(p11;q11) chromosomal translocation, suggesting their monoclonal origin. Only CLP13 cells exhibit tetraploidy. Tetraploidy is one of the terminal hepatocytic differentiation markers [Saeter et al., 1988a,b], but is also a prerequisite for cancer stem cell emergence [Nguyen and Ravid, 2006]. The fact that CLP13 cells might have reached tetraploidy through partial redifferentiation appears unlikely since, indeed, they do not display any mature hepatocytic feature. Their EpCAM positivity and OV6 negativity are themselves consistent with the possible cancer stem cell, undifferentiated, status of this clone. The t(12;22)(p11;q11) translocation is common to all CLP clones. Browsing through the Cancer Genome Anatomy Project of the US NCI allowed one to unravel diseases in which this genetic aberration is present. They were all and only myelodysplastic syndromes: lymphoblastic leukemia [Prigogina et al., 1988; Amor et al., 1998], acute anemia with excess of blasts [Yunis et al., 1986] and granulocytic sarcoma [Heimann et al., 1994]. Since hepatic recruitment and transdifferentiation of hematopoietic stem cells may occur [Petersen et al., 1999; Lagasse et al., 2000; Fiegel et al., 2003; Wang et al., 2003; Jang et al., 2004; Wu et al., 2005; Gehling et al., 2010], as is the case in other cancer types [Houghton et al., 2004; Cogle et al., 2007], the possibility that CLPs arose from such an extrahepatic recruitment exists.

We have globally observed strong uncoupling between widely accepted physiological cytokeratins and albumin profiles associations in the literature on the one hand, and our data on the other hand. Two interpretations can be proposed to this observation. Either (i) the stem cell status of these clones accounts for such apparent histological markers aberrations, since cancer stem cells often exhibit in downstream generated tissues co-expression of large markers spectra in such a setting, or (ii) independently emerged clones have made their way in the tissue till they became full-blown HCC components. Since the latter hypothesis is much less likely as it would

necessitate concomitant immortalization of clones derived from different tissues (i.e., biliary and parenchymal) as well as the appearance of the t(12;22)(p11;q11) translocation, one believes that CLP cells may instead derive from a pre-existing or de novo re-emerged cancer stem cell perhaps from which the CLP13 clone is a representative.

In summary, this study allowed the isolation of four untransformed HCC clones displaying strongly distinct sensitivity levels to TGFβ, IFNα, and doxorubicin. Three of which display progenitor properties together with, for each of them, individual features. One of them displays cancer stem cell properties. In this context, CLP clones may constitute useful tools for basic and therapeutic HCC research as well as hepatic pharmacokinetic and toxicokinetic research programs.

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