Isolation and Characterization of Hepatic Cancer Cells with Stem-Like Properties from Hepatocellular Carcinoma

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Abstract

Background & Aims. Major burdens in the treatment of hepatocellular carcinoma (HCC) are the high percentage of recurrence and resistance to chemotherapy. Hepatic cancer stem cells provide a reservoir of cells that can self-renew, maintain the tumor by generating differentiated non-stem cells which make up the bulk of the tumor and are responsable for recurrence after ablative surgery and chemoradiotherapy. The objective of this study was to identify and characterize a self-renewing subpopulation of human liver tumor cells with a distinctive genetic profile that adds the capacity to proliferate despite chemotherapy and promotes cancer recurrence. **Methods**. Stemness properties of tumor cells isolated from a HCC biopsy were established by their capacity to form spheroids and by cell proliferation assays. The cells also showed enhanced chemoresistance to cancer drugs. The up-regulation of stem cell markers is proven by immunocytochemistry stainings and reverse transcription – PCR. **Results**. Cells had a high proliferative potential, even when cultured in medium supplemented with doxorubicin and carboplatin, eliminated Rhodamine 123 immediately in culture and also formed spheroids in suspension. Molecular diagnosis techniques showed that cells expressed the stem cell markers Oct 3/4 and CXCR4. Cells were also positive for CD133 and CD90 cancer stem cell specific markers, with monoclonal antibody staining. **Conclusion**. The unique characteristics identified in cancer stem cells explain self-renewal and could drive metastasis in patients that have received treatment for cancer. The identification and cloning of such cells can aid in developing of better therapeutic approaches for patients with HCC, as chemosensitive pretherapeutic assays or targeted therapies.

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Key words

Cancer stem cells – hepatocellular carcinoma – isolation – characterization – chemotherapy resistance.

Introduction

Hepatocellular carcinoma (HCC) is the third leading cause of cancer-related death and accounts for as many as 600,000 deaths worldwide annually [1]. While HCC is common in Southeast Asia and sub-Saharan Africa, its incidence has continued to increase in the United States and Europe in the past 25 years. Incidence and mortality rates are expected to double in the next two decades [2-4]. Most patients with HCC also suffer from coexisting cirrhosis, which is the major clinical risk factor for hepatic cancer and is correlated to hepatitis B or C virus infection. However, cirrhosis from non-viral causes such as alcohol abuse, hemochromatosis and primary biliary cirrhosis are also associated with an elevated risk of liver cancer [5].

Patients who succumb to advanced stage liver neoplasia inevitably become refractory to chemotherapy, resulting in disease progression and death. Although much is known about the etiological agents of HCC, the cellular and molecular pathogenesis of this type of cancer is not well understood. One emerging model for the development of drug-resistant tumors invokes a pool of self-renewing malignant progenitors known as tumor initiating cells or cancer stem cells (CSC). The relapse that often follows clinical remission is likely due to the failure of eradicating CSC which, despite the tumor bulk shrinkage, can subsequently reproduce the entire malignant phenotype.

It is thought that CSC are able to survive conventional treatments, which usually target fast dividing cells, because they share the same basic molecular and functional characteristics with normal stem cells, such as expression of membrane efflux transporters, enhanced DNA repair and the ability to modulate and balance their own differentiation and self-renewal capacity according to genetic constraints and environmental stimuli [6-8].

Cancer stem cells were originally identified in leukemia and more recently in solid tumors [9-13]. The first isolation

from HCC was reported by Sell et al [14]. In the current study, using a primary human HCC biopsy, we report the first isolation and characterization of cancer cells with stem-like properties in our country. These cells express specific pluripotency and malignancy markers such as CXCR4, Oct3/4, CD 133 and CD 90, having a unique genetic profile that confers the distinctive characteristics in terms of their tumorigeneicity, resistance to chemotherapy and capacity to promote a pro-inflammatory microenvironment. Consequently, their specific detection and targeting could be highly valuable for the therapy of recurrent, chemoradioresistant disease.

Materials and methods

Primary tumour cell culture

The HCC biopsy was provided by the Department of Abdominal Surgery, 3rd Surgical Clinic in Cluj Napoca, Romania. Signed informed consent was obtained from the patient prior to sample acquisition in accordance with all ethical and legal aspects. The primary lesion was diagnosed as Edmondson-Steiner grade II HCC (Fig. 1). The tumour tissue was washed and minced with fine scissors into fragments of 1x1x1 mm^3 before culture in Petri dishes with 1 ml of fetal calf serum (FCS). After three hours, the dish was replenished with 3 ml of DMEM medium, supplemented with FCS, glutamine, antibiotics and non-essential aminoacids (all from Sigma Aldrich, St Louis, MO, USA).

When a monolayer of primary tumour cells was formed, cells were detached using trypsin/ EDTA and resuspended in defined media: DMEM/F12 (1:1) medium, supplemented with 15 ng/ml basic fibroblast growth factor (bFGF), 20 ng/ml epidermal growth factor (EGF), 2mM/l L-glutamine, 4 U/l insuline growth factor (IGF) and B 27 supplement (1:50) (Sigma Aldrich). Trypan blue staining confirmed more than 80% viability after the procedure. Cells were cultured in a humidified amosphere at 37 ˚C and 7% CO_2.

Colony formation assay

Human tumour cells expanded in culture to 70-80% confluency were harvested using trypsin/EDTA. Cells were counted and then diluted in complete culture medium before being plated at about 100 cells per 100-mm tissue culture dish in complex DMEM medium. Cells were incubated for 10 days at 37˚ C in 5% humidified CO_2, afterwards washed with PBS and the visible colonies were counted [15].

Formation and culture of tumor spheres

Tumour cells at different passages were plated at a density of 10^5 cell/ml in serum-free culture media. The formation of tumour-spheres was observed under an Olympus CKX 41 inverted light microscope, at 100X and 200X magnification.

Tumor spheres were cultured alternately in serum-based medium and serum-free stem cell based growth medium

Non-adherent tumor spheres were seeded in DMEM/F12 (1:1) medium, supplemented with FCS and the cells had attached to the bottom of the flask and grown into cell monolayers for one week. The flask was washed with PBS

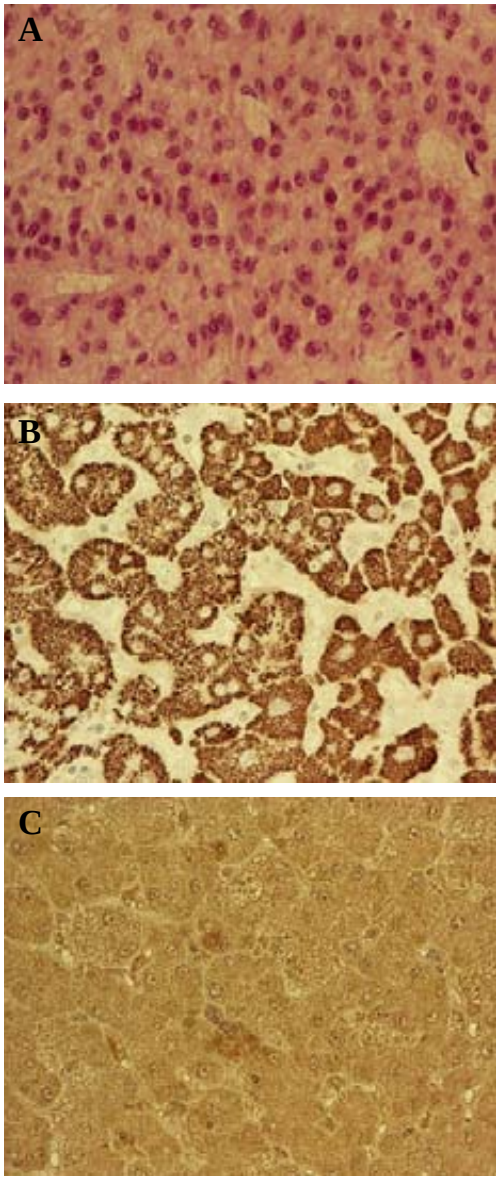

Fig 1. Malignant cellular proliferation, with moderate nuclear polymorphism (A). Hepatocyte-positive immunohistochemical reaction confirms the diagnosis of hepatocellular carcinoma (B). Focal positive reaction for β Human chorionic gonadotrophin (β CGH) (C).

to remove the bovine serum and defined stem cell growth medium was later added. These procedures were repeated thrice and morphological changes in the tumor cells were observed under an inverted light microscope.

Multidrug resistance assay

To assess the resistance of cells isolated from HCC to chemotherapy, cells were seeded at 3000 cells/well in 96-well plates. The culture media was DMEM/F12 supplemented with growth factors. Cancer stem cells were compared with HFL human lung fibroblasts (European Collection of Cell Cultures, Budapest, Hungary) and with MLS human ovarian tumor cells (courtesy of Dr Yael Schiffenbauer, Medis El Ltd, Yehud, Israel). Six hours later, all cells were treated with 5

μg/ml carboplatin and 5 μg/ml doxorubicin. After 24 and 48 hours, the relative cell number was determined by standard MTT assay [16].

Rhodamine 123 efflux assay

Hepatic cancer stem cells, MLS ovarian tumor cells and HFL human fibroblasts were seeded in specific culture media supplemented with FCS, non-essential amino acids, L-glutamine and antibiotics, at 20 x 10^4 cells/ml. Cells were stained with 10 μM Rhodamine 123 and then incubated for three hours at 37˚C and 7% CO_2. After culture, all cell types were washed three times with PBS before intracellular fluorescence studies, according to Donnenberg et al [17] .

RNA extraction and RT-PCR analysis

Total RNA was isolated from hepatic cancer stem cells and from placental mesenchymal stem cells which are used as a control, being already known to express the genes of interest (unpublished data). RNA isolation was performed from subconfluent monolayers of adherent cells plated on 6-well dishes, using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. 1μg of total RNA was used for reverse transcription with the ImProm Reverse Transcription System (Promega, Madison, WI, USA). Only mRNA was transformed into cDNA by using oligo-dT primers in the reaction mixture, together with: AMV reverse-transcriptase 15 u/μg; buffer solution (10 mM Tris-HCl, pH=9.0; 50 mM KCl; 0,1% Triton X-100); dNTP solution, 1 mM each; MgCl2 5mM; recombinant ribonuclease inhibitor 1 u/μl; ultrapure nuclease-free water. The cDNA was amplified using GoTaq PCR Core System II (Promega). The primers used were designed according to the corresponding human genes: CXCR4 (sense, 5'-ATTCCTTTGCCTCTTTTGCAGATATA-3'; antisense, 5'-ATGGCCAGGTAGCGGTCCAGACTGATGAA-3'), Oct-3/4 (sense, 5'-AGGAGTCCAGGAC-ATCAAAG-3'; antisense, 5'-TCGTTTGGCTGAATACCTTC-3'), and human β2-microglobulin (sense, 5'-ACTCCAAAGATTCAGGTTTACTC-3', antisense, 5'-CATGATGCTGCTTACATGTC) was used as an internal control for PCR. Amplification reactions were performed on a Techne TC3000 thermal cycler (Bibby Scientific Ltd, Staffordshire, United Kingdom) at 95°C for 30 seconds, 52°C for 1 minute (t=56°C for Oct-3/4, 52°C for β2-microglobulin and 61°C for CXCR4), and 72°C for 2 minutes for 40 cycles. The PCR products were then separated by electrophoresis on 2% agarose gell and photographed with a UV transilluminator.

Immunocytochemical staining

Hepatocellular carcinoma derived adherent cells were labeled with anti-human antibodies and fixed with 4% paraformaldehyde for 20 minutes. After blocking with Bovine Serum Albumine 10% (Sigma Aldrich), cells were incubated overnight with two primary antibodies: CD133 Rabbit anti-human monoclonal antibody (Sigma Aldrich) and CD90 Mouse anti-human monoclonal antibody (Becton Dickinson, Franklin Lakes, NJ, USA), diluted 1: 100. For cell staining with secondary antibodies, the protocols used fluorescein isothiocyanate (FITC) Goat anti-mouse IgG (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Cell nuclei were counterstained with 4,6-diamidino-2-phenylindole (DAPI). For permeabilization we used 0.01% Tween 20 or 1% Triton X-100. The fluorescent cells were visualised with an inverted phase Zeiss Axiovert microscope using filtres of 488, 546 and 340/360 nm.

Statistical analysis

Statistical significance values were obtained using a one-way analysis of variance (ANOVA), with a 95% confidence level using GraphPad Prism 5 statistics program. All experiments were performed in triplicate.

Results

Histopathology and phenotype of primary tumour cells

The tumour biopsy used for primary cell culture had typical histologic features of Edmondson-Steiner grade II HCC. Two weeks after plating the tumour explants and using a method for anchorage dependent stem cell-selective culturing protocol, adherent cells were observed near the tumor explants. After another week, a monolayer of primary tumor cells was formed before being detached using trypsin/EDTA (Fig. 2). In serum-free culture media 70 to 90% of the tumour cells became adherent, with a minority of floating cells forming spheres composed of 3-5 cells (Fig. 3A). After an additional 48 hours, the floating spheres were expanded to contain 15-20 tumour cells, with bright appearance and sharp edge (Fig. 3B).

Hepatic tumour cells express stem cell genes

After the demonstration of tumour-derived cells to organize self-renewing spheroids, the expression of genes specific to tissue or embryonic stem cells was examined. RNA from cancer adherent cells was analyzed by reverse transcription-polymerase chain reaction (RT-PCR) for CXCR4, Oct-3/4 and β2-microglobulin in comparison with mesenchymal stem cells found in the placental chorion (Fig. 4). The cells also expressed the "universal cancer stem cell specific markers" CD 133 and CD90, after immunocytochemical stainings were performed (Fig. 5).

Sphere-forming tumour cells are resistant to conventional chemotherapy

Because Rhodamine 123 uses the same pathways to pass through the membrane as conventional drugs used in oncologic treatments, by measuring the optical density (OD) of this fluorescent substance, we were able to determine indirectly whether the cell population isolated from HCC expressed the proteins responsable for multidrug resistance. Hepatic cancer stem cells were compared with both the HFL human fibroblast cell line and to MLS ovarian tumour cell line (Fig. 6).

To examine whether hepatic tumour cells possessed a hypothesized cancer stem cell chemoresistant phenotype, we also assessed the sensitivity of the cells to carboplatin and doxorubicin under stem cell conditions. Compared with HFL

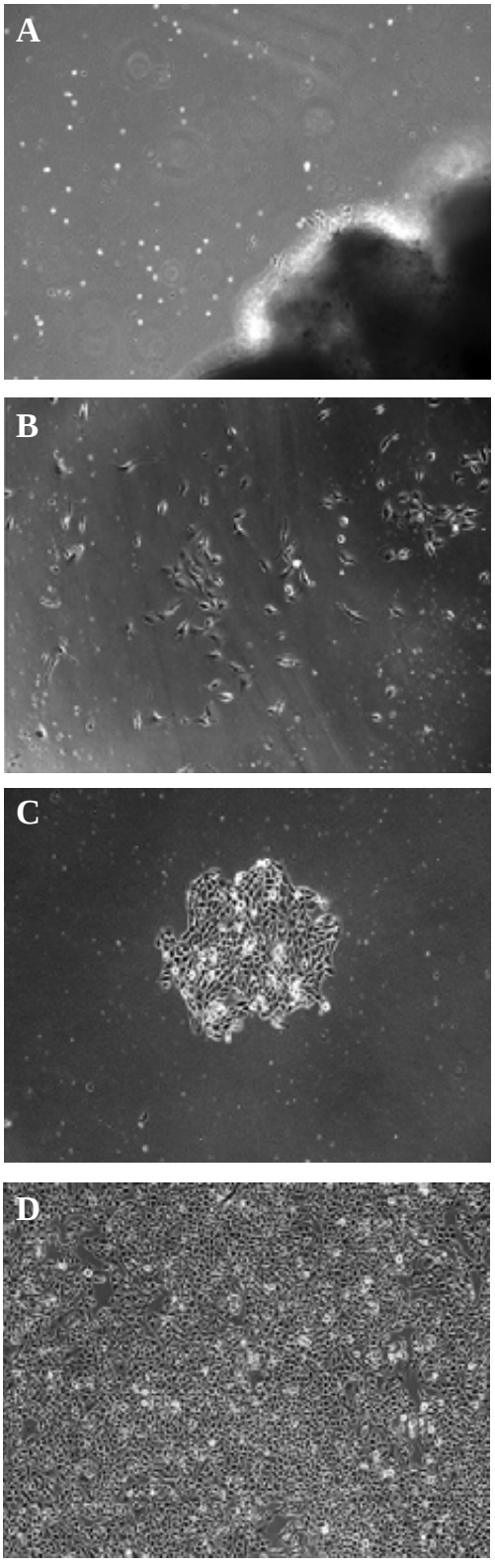

Fig. 2. After 12-14 days, a population of tumor cells was visible near the biopsy fragments (A). After 24 days stem-like cells were isolated (B), having the characteristics of clonal expansion (C) and a very high proliferative potential (D).

fibroblasts and MLS tumour ovarian cells, both carboplatin and doxorubicin IC50 values were greater ($p<0.05$). (Fig. 7). These results support a role for these stem-like cells in hepatic cancer chemoresistance (i.e. failure to eradicate progenitors resulting in tumor regrowth).

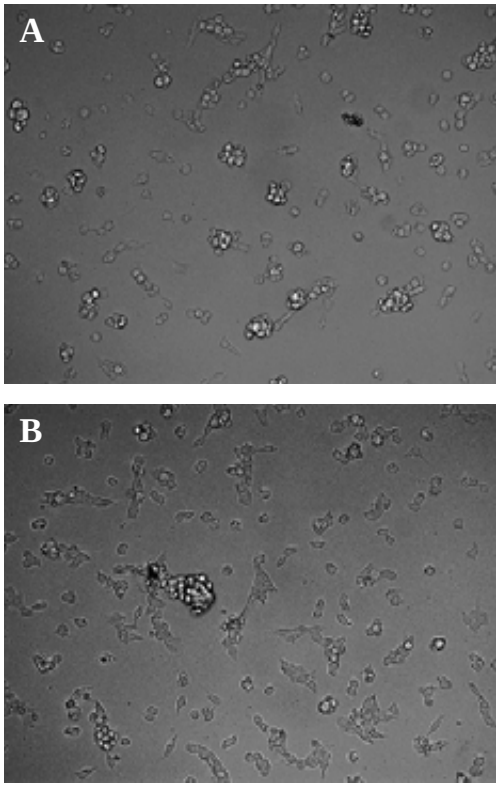

Fig 3. Formation of tumour spheres (A, B).

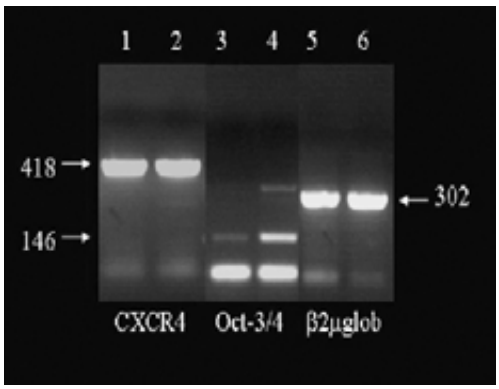

Fig 4. Expression levels of CXCR4, Oct-3/4 and β2-microglobulin as determined by RT-PCR in stem-like cells from hepatocarcinoma (lanes 1, 3 and 5) compared to placenta mesenchymal stem cells (lanes 2, 4 and 6). The numbers near the arrows represent the size in bp of the PCR amplification products corresponding to the three studied genes.

Discussion

Stem cells can be found in many different adult tissues and are important participants in their physiology. Pluripotent cells have three distinctive properties: self-renewal, the ability to differentiate into multiple lineages and the potential to develop extensively. These three properties make a stem

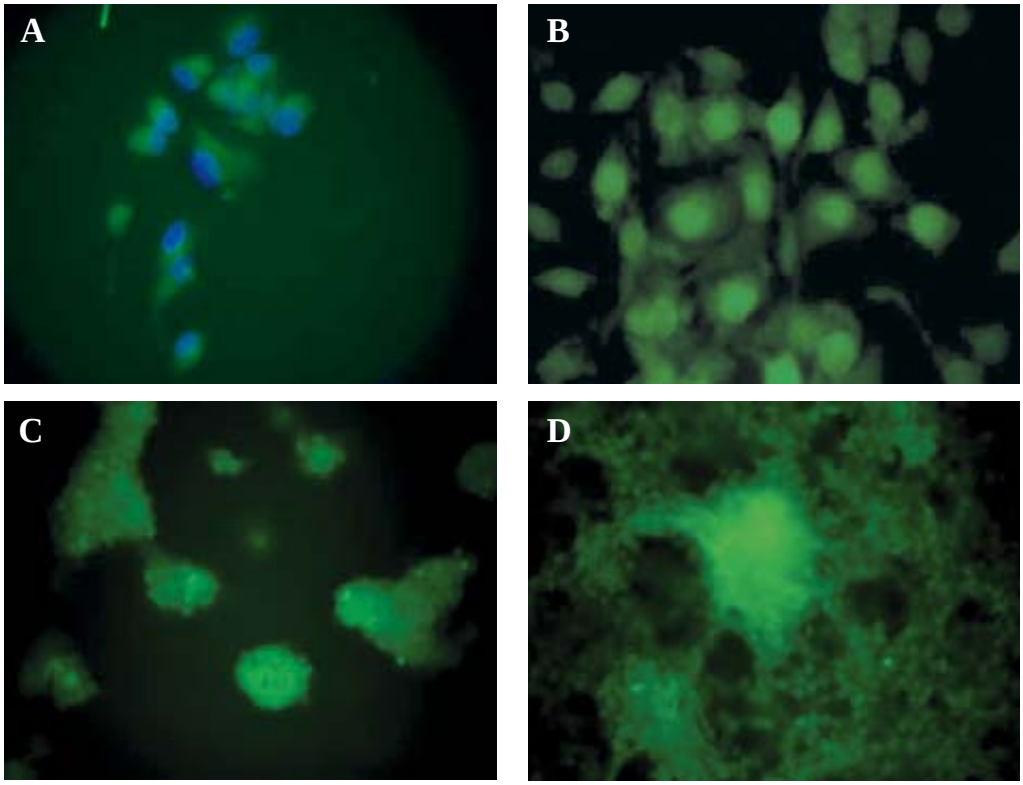

Fig 5. Adherent hepatic cancer stem cells were shown to express the stem cell markers CD133 (A) and CD90 (B). Also, the cancer cells that formed tumour spheres in serum-free culture media, expressed the same specific markers, CD133 (C) and CD90 (D).

cell unique, but the attribute of self-renewal is especially important because its subversion is highly relevant to oncogenesis and malignancy.

Based on the stem cell theory, the human liver has a remarkable capability to restore its functional capacity as a response to the loss of parenchyma. Partial hepatectomy induces regeneration by compensatory hyperplasia, a process in which cells in the remnant liver expand to replace the lost liver function. Hepatic stem cells reside in the most peripheral branches of the biliary tree (the canals of Hering) and have also been proven to proliferate in small cell dysplastic foci and cirrhotic liver tissue [18]. Liver regeneration driven by the replication of hepatoblasts is controlled by multiple pathways organized into different networks involving cytokines, growth factors and other metabolic processes. During the past few years, important factors in these networks have been identified and studied, in particular, the tumour necrosis factor (TNF), interleukin-6 (IL-6), epidermal and hepatocyte growth factors (EGF, HGF) and their corresponding receptors. These transcription factors have also been proven to control key pathways in morphology and embryology, such as TGF-β, Notch2 or Smad [19, 20].

Carcinogenesis, as described by Vogelstein et al in a colorectal carcinoma model, occurs as a multistep evolutionary process involving the disruption of a relatively small number of cellular pathways [21]. Modern genomic analyses of human tumours link the mechanisms of malignant progression to epithelial-mesenchymal transition and the transient expression of a variety of transcription factors [22]. In general, malignant growth requires: self-sufficiency in growth signals, insensitivity to growth inhibitors, evasion from apoptosis, limitless replicative potential, sustained angiogenesis, tissue invasion and metastasis. Among all these characteristics, it is clear that the main event necessary for carcinogenesis is unrestricted proliferative potential, given by cell immortality.

In liver cancer, malignant transformation of hepatocytes occurs mostly through a pathway of hepatocyte turnover and chronic liver injury. Maradpour and Blum have proven that certain hepatotropic viruses, especially hepatitis B virus (HBV), play an additional direct role in the induction of HCC through insertional mutagenesis and transactivation of cellular genes by the HBV X protein. The molecular pathogenesis of HCC in not well understood, despite several studies that have shown chromosomal abnormalities, particularly heterozygosity loss [23]. Heterozygosity loss is characterized by a hemizygous genotype in specific chromozomal regions due to deletions or other mutational events. Because of these mutations only one allele is present. The risk of carcinogenesis increases if the remaining allele is inactivated by a point mutation and contains regulatory elements for the cell cycle, such as a tumour suppressor gene.

Repeated cyles of cell death and regeneration, as occur in chronic liver disease are important in the genesis of liver cancer. Preneoplastic changes such as hepatocyte dysplasia can result from point mutations in selected

genes, heterozygosity loss, DNA methylation changes and constitutive expression of HGF, TGF-β, Smad or ELF signalling pathways. These factors, together with the cummulative effect of some viral proteins, act to further stimulate the replication of liver stem cells. Influenced by all these pro-mitotic signals, the normal cell cycle is altered and the progenitor cells that have acquired mutations and should normally undergo apoptosis, continue to replicate. This is the moment when the cancer stem cell is born and carcinogenesis is initiated.

Oncogenic mutations allow the mentioned transit amplifying cells to continue to proliferate without entering a postmitotic differentiated state, thereby creating a pool of self-renewing cells in which further mutations can accumulate. This concept has direct relevance to understanding the complex process of cancer progression. For example, homing receptors found on normal hematopoietic stem cells such as the chemokine receptor CXCR4 have been shown to play an important role in promoting the metastasis. Cancer stem cell subpopulations are also naturally quiescent, remaining in G0 phase and thus surviving serum deprivation [24-25]. The CXCR4+ cells we isolated from the HCC biopsy survived the alternate culture in serum-based media and serum-free media and also formed tumour spheres, proving the capability to leave the primary tumour bulk and invade the surrounding tissues. The expression of CXCR4 mRNA has also been shown to influence hepatic cancer cell cycle variation, with important consequences in metastasis and survival, not only for HCC, but also for colon, esophageal or gastric cancer [26-27].

By virtue of their fundamental importance in organogenesis, normal stem cells have evolved mechanisms that promote their survival and resistance to apoptosis, observed in therapy in patients that are given non-myeloablative doses of cytotoxic chemotherapy. Such patients experience a transient decrease in white blood cells, caused by apoptosis of differentiated neutrophils and myeloid precursors, but the stem cells in the bone marrow are not ablated and are able to regenerate a normal hematopoetic system within weeks. Just as normal stem cells may be resistant to the induction of programmed cell death, CSCs may display increased resistance to various drugs or radiation, compared with more differentiated cells that comprise the bulk of the tumour [28]. Our experiments confirm this hypothesis. In comparison to both more differentiated tumour cells and normal human fibroblasts, the liver tumor cells were two-fold or even three-fold more resistant to carboplatin and doxorubicin, drugs that interact with the tumor cell metabolism using completely different pathways.

Resistance to natural hydrofobic drugs generally results from the expression of ATP-dependent efflux pumps with broad drug specificity. Resistance occurs because increased drug efflux lowers intracellular drug concentrations. According to Gottesman et al, drugs that are affected by classical multidrug resistance include the Vinca alkaloids (vinblastine and vincristine), the anthracyclines (doxorubicin and daunorubicin), the RNA transcription inhibitor actinomycin-D and the microtubule-stabilizing drug paclitaxel [29]. In addition, resistance can also be mediated by reduced drug uptake and examples include methotrexate, nucleotide analogues (5-fluorouracil or 8-azaguanine) or platinum-based compunds (cisplatin or carboplatin). Our cells showed little or no sensitivity to both types of cell cyle inteference pathways, confirming the mutidrug resistance hypothesis, a unique characteristic related to the transmembrane proteic carrier ABCG2.

Conclusion

The isolation and culture of hepatic cancer stem cells with stem cell properties (CXCR4+, Oct3/4+, CD133+ and CD 90+) have an important implication for understanding the molecular mechanisms of liver tumorigenesis, as current molecular pathology analysis of global tumour cell populations, such as tumour microarray experiments, may not be sufficient to determine the key molecular alterations in the relatively rare tumour stem cells. Experimenting with hepatic cancer stem cells also has important applications in understanding cancer dissemination and systemic metastasis. The functional analysis provides new means for the testing of novel treatment strategies that focus on the complete eradication of cancer.

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Conflicts of interest

The authors declare no potential conflicts of interest.

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