

RESEARCH ARTICLE

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Characterization and Validation of Hepatocellular Carcinoma (HCC) Xenograft tumor as a Suitable Liver Cancer Model for Preclinical Mesenchymal Stem Cell Studies

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Abstract

Background: Hepatocellular carcinoma (HCC) is the fifth most diagnosed cancer and the third leading cause of cancer-related death. sorafenib is used as a standard therapy to treat HCC. mesenchymal stromal cells (MSCs) have also been used to suppress HCC. Here we investigate the development of a xenograft model of liver cancer to study the homing of hpMSC-GFP cells, tumor kinetics and molecular characterizations of HCC. **Methods:** To create xenograft models of HCC, HepG2 cell lines were inoculated into the flanks of 9 nude mice bilaterally. Animals were then divided into three groups: the first group received hpMSC-GFP systemically, the second received intra-tumoral hpMSC-GFP and the third received PBS. The first two groups were sacrificed after 72 hours of MSCs injection but the third group was followed up for forty days. One tumor from each animal was then transferred to formalin buffer for H&E staining and immunohistochemistry analysis (KI67 and CD34), and the other tumor was used for ex-vivo imaging. Blood samples were taken from all subjects before sacrificing them. **Results:** Histopathological fidelity of heterotopic HePG2 xenograft models to human HCC tumors was demonstrated. Biochemical evaluation suggested the health of the animal's liver and kidneys. Ex-vivo imaging illustrated homing of more hpMSC-GFP cells in tumor tissues derived from the group receiving intra-tumoral hpMSC-GFP. **Conclusion:** A standard method was used to inoculate tumor cells and the intervention was shown to be safe to liver and kidneys. Local injection of MSCs can be used as cell therapy to fight neoplasms.

Keywords: Hepatocellular carcinoma- sorafenib- human placenta Mesenchymal stem cell- animal model

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Introduction

The occurrence of cancer has been increasing recently due to both the aging population, and an increased prevalence of smoking, obesity, and other established risk factors. Globocan estimates that about 14.1 million new cancer cases and 8.2 million deaths occurred in 2012 worldwide. Liver and stomach cancer in males and cervical cancer in females are also accounted as leading causes of cancer death in less developed countries (Torre et al., 2015). Primary liver cancer, which consists predominantly of hepatocellular carcinoma (HCC), is the fifth most common cancer worldwide and the third most common cause of cancer mortality (El-Serag and Rudolph, 2007). Early diagnosis is crucial

for curative treatments such as surgical resection, radiofrequency ablation, and liver transplantation, as opposed to treatments like sorafenib and trans-arterial chemo-embolization which are reserved for more advanced cases (Bellissimo et al., 2015). Before the introduction of sorafenib, cytotoxic agents, hormonal therapies, or their combinations have been the cornerstones of systemic chemotherapy for advanced HCC. However, several randomized controlled trials comparing the effect of doxorubicin monotherapy and placebo have shown no survival advantage for this regimen (Ikeda et al., 2015). Currently, the only systemic molecular therapy available to target HCC is sorafenib (a multi-kinase inhibitor) which can improve the median life expectancy of patients for up to only 1 year (Choi et al., 2015). Another

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therapeutic approach for hepatic regeneration that has been proposed in the last decades is cell therapy with Mesenchymal stem cells (MSCs). Transplantation of bone marrow mesenchymal stem cells (BM-MSCs) has been assessed as an alternative therapy to replace liver transplantation in several trials to treat liver cirrhosis (Huang et al., 2013). MSCs exhibit potent pathotropic migratory properties that make them attractive for use in tumor prevention and treatment. However, little is known about the underlying molecular mechanisms MSCs use to target tumor cells (Hou et al., 2014). MSCs are being widely studied as potential cell therapy agents due to their immune modulatory properties, which have been established by *in vitro* studies and in several clinical trials (Amorin et al., 2014).

Development of novel therapeutic approach requires appropriate research tools. Animal models are one of the most important means of evaluating cancer treatment by cell therapy or novel drug candidates in cancer treatments (Abeni et al., 2017). Numerous experimental models have been developed for describing the pathogenesis of HCC, including chemically induced HCC mice models by administration of a genotoxic compound alone or in combination with another agent. In addition, xenograft HCC models have also been employed by implanting hepatoma cell lines in mice, which are suitable for drug screening. We must however be prudent when extrapolating such data as multiple cell lines have been used. Therefore, development of new animal models is essential for better visualization and understanding the etiology of different malignancies. Over the last several years, a great number of *in-vivo* HCC models have been developed for such purpose and have significantly contributed to unveiling the pathophysiology of liver tumors (Heindryckx et al., 2009). Furthermore, Rats (*Rattus norvegicus*) or mice (*Mus*)-because of their short lifespan, high breeding capacity, and easier handling- have been the most popular models for cancer research, especially in studying the development of HCC (De Minicis et al., 2013).

In this study we aimed to firstly validate that the tumors are all composed of HCC cells, secondly to make sure that our treatment would not harm the animals' liver or kidneys (hence checking for urea, creatinine and liver enzymes) and finally to verify if more hpMSC have been implanted in site after 72 hours as compared to the systemic injection.

Materials and Methods

Cell culture

The HepG2 cell line and hpMSC-GFP were obtained from the National Center for Biological and Genetic Resources of Iran (IBRC) and SABZ biomedical (http://www.sabzgroup.com), and cultured in RPMI-1640 (Gibco R0883, USA) media and DMEM (Gibco D5796, USA), supplemented with 10% fetal bovine serum (cat. number 10-091-148, USA), Penicillin-Streptomycin (Gibco: cat. number: P4333, USA) (100 U/ml), in standard condition of incubator at 37°C in a 5% CO₂ atmosphere and 95% humidity.

Animal model

A total of 9 male athymic nude mice (nu/nu; C57BL/6) aged 4 to 6 weeks were obtained from Omid Institute for Advanced Biomodels, and their treatment was according to guidelines outlined by the Institutional Ethical Committee under specific pathogen-free conditions. The mice were housed and maintained under optimized hygienic conditions in an individually ventilated cage system. The average temperature of each cage was 23°C with relative humidity of 65%. Animal feeding was with autoclaved commercial diet and water *ad libitum*, and triple ethical principles of working with animals including reduction, refinement, and replacement were implemented. For HCC tumor implantation, 1×10^7 of human HepG2 cells were suspended in 100 μ l of serum-free medium supplemented with 100 μ l Matrigel (Corning, product number: 354230, USA) and then subcutaneously injected into the right and left flanks of each mouse. Tumors were monitored three times a week and tumor volume was calculated with the aim of Vernier calipers. The volume of tumors was calculated using a standard formula ($\text{length} \times \text{width}^2 \times 0.52$) and growth curves were drawn (Tomayko and Reynolds, 1989).

When the tumor volumes reached to higher than 200 mm³, treatment was initiated and mice were randomized into three groups: one receiving hpMSC-GFP via IV injection, the other receiving hpMSC-GFP through intratumoral injection, and the control group. Injection of human placenta-derived hpMSC (5×10^5 cells) in the first group was via tail veins and in the second group was into the tumor margin, 15 days after HepG2 cells injection.

Ex vivo imaging was performed after 72 h of injection of hpMSC-GFP for the first two groups.

Forty days later, the third group of mice were sacrificed after blood samples were drawn under anesthesia with ketamine/xylazine and the tumors were harvested and fixed in 10% formalin and were analyzed in the pathology laboratory.

Histopathology and Immunohistochemistry

For the pathologic examination for each tumor specimen, one slide was stained with hematoxylin and eosin (H&E) and another slide was evaluated by immunohistochemistry (IHC) analysis and was stained with tumor marker antibodies (Biocare, CA, USA) for assessment of tumor proliferation (Ki-67 cat. number: CRM325 A,B,C) and tumor angiogenesis using microvessels density (MVD) & (CD34 cat. number: CM 084 A,B,C).

Biochemical tests

As for biochemical analysis, blood samples were collected and centrifuged at 3000x rpm. The levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT), BUN and creatinine were then quantified from serum, for safety assessment. All tests were measured with an automated biochemical analyzer (Mindray), after which statistical analysis was performed.

Statically analysis

In this exploratory step, our primary goal was not focused on hypothesis testing and any level of difference

among groups would have suffice our conclusion. Therefore we followed the crude method of “resource equation” which uses E to calculate the sample size:

$E = \text{Total number of test entities} - \text{Total number of groups}$.

Since each xenograft tumor is considered a single entity, and two xenografts were implanted onto each mouse, we had 18 tumors in total that were allocated to 3 groups, E was equal to $18-3=15$ that can be considered as adequate.

In our research we used 18 tumors in 3 groups so $E=(18-3)=15$ lies between 10 and 20 that can be considered as adequate.

According to the accepted rule of thumb for ample size in animal study (Charan and Kantharia, 2013), any sample size, which keeps E between 10 and 20 should be considered adequate.

$E = \text{Total number of animals} - \text{Total number of groups}$

Ethical concern

The experimental procedure was approved by the National Committee for ethics in animal research.

Results

After subcutaneous inoculation of a hepatocellular carcinoma cell line (HepG2 cell line) into 9 male mice,

18 tumors were formed (Figure 1A) and tumor cells did not grow in only one inoculation site. The growth curve of tumors is shown in Figure 1B. For measuring tumor take rate, mean doubling time (The time needed for a tumor to double in volume, doubling time (DT) (Esmail, 2010) was calculated. These results verify that the method used has been valid and that the cells have been potent enough to grow the tumor.

After sacrificing the mice, internal organs were dissected to find macrometastases; none were found.

Quantification Figure 2 -B

The software “Image Pro Plus 6.0” (USA) was used to calculate the green surface area which contained GFP. A mean and standard deviation for each group was then calculated; the results are as follows:

Systemic group = 38.5 ± 5.8 ; local group = 78.4 ± 4.9 ; control group = 12.1 ± 3.4 .

Similarly, the density of green color in control group was strikingly less than the other two groups and the local group yielded the most color density among the three.

H&E stained tumors showed highly proliferative hyperchromatic cells with anisokaryosis and anisocytosis. Numerous mitotic figures and multinucleated bizarre cells were also observed (Figure 3, A and B). Numerous necrotic areas were illustrated as well (Figure. 3, C). Due to the high grade of malignancy, the tumor was diagnosed

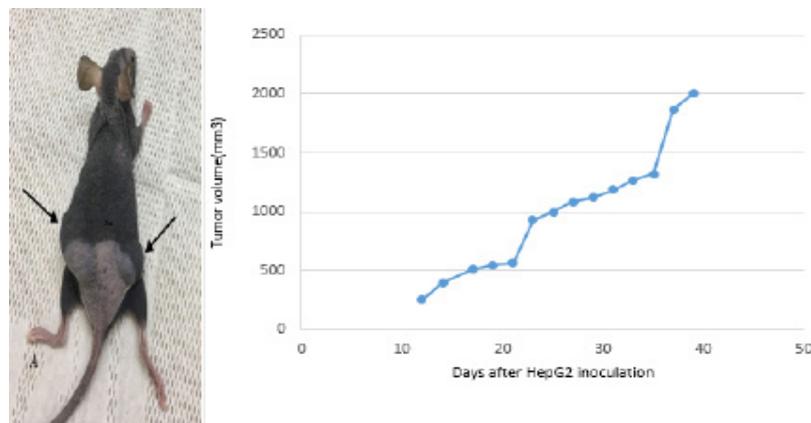


Figure 1. A) Heterotopic HepG2 Xenograft Liver Cancer: 1×10^6 B) Growth Curve of HCC Tumors During 30 Days after Inoculation of HepG2 cells. Error Bars Represent Standard Deviation

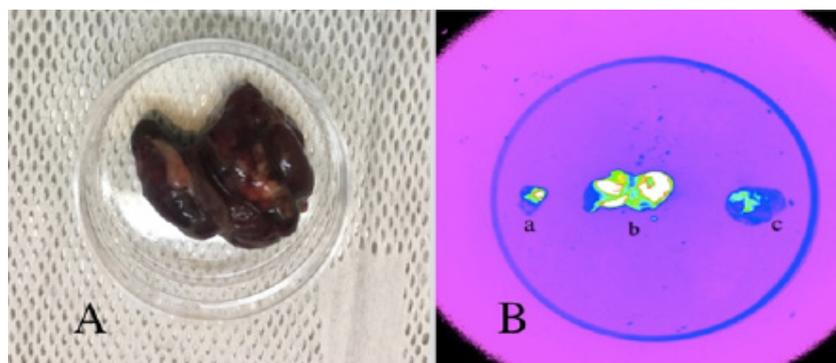


Figure 2. A. Note Tumor Size as Compared with the Plate Size. B. Ex-vivo Imaging of Tumor Tissues were Derived from Three Groups of HCC Animal Models: a: Systemic Injection of hpMSC-GFP. b: Local Injection of hpMSC-GFP. c: PBS Injection

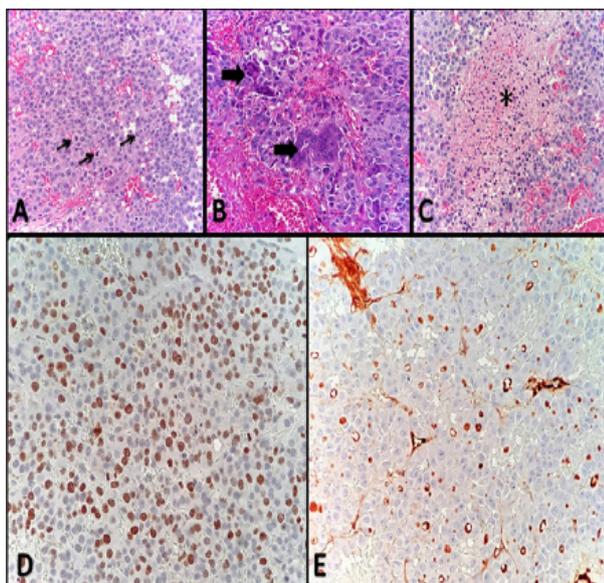


Figure 3. Photomicrographs of Xenograft Hepatocellular Carcinoma. A, B and C) H&E Staining of Tumor Cells. A) Highly Malignant Cells with Anisocytosis and Anisokaryosis (pleomorphism) are Observable, thin Arrows Show Mitotic Figures. B) Multinucleated bizarre cells (thick arrows), C) Necrotic areas (asteroid). D and E) IHC staining of tumoral cells. D) using Ki-67 marker. Immunoreactive nuclei indicate high proliferation in the tumor xenograft model. The brown color indicates positive staining for Ki-67 marker. E) IHC staining using CD34 marker. The brown lumens show immunoreactive endothelial cells and neo-vascularization

as undifferentiated carcinoma. IHC examination showed that more than 70% of cells were Ki-67-positive. Moreover, CD34 expression (Figure 3, E) indicated the intensity of angiogenesis in tumors (neovascularization).

The results suggest that the tumors were indeed composed of hepatocellular carcinoma cell.

Analysis of biochemical factors

The average levels of AST, ALT, BUN, and creatinine were measured. Statistical analysis that was performed on these results did not show any significant differences between the samples indicating the adequate safety of these therapeutic approaches on the animals' liver and kidneys.

Discussion

This study aimed to characterize comprehensively the xenograft hepatocellular carcinoma model of the HepG2 cell line. Pathological and kinetic growth properties were also demonstrated. The HepG2 cell line was derived from a 15-year-old male caucasian liver cancer patient (2018). The latent period after inoculation of the cultured cell suspension was relatively short and lasted approximately 10 days after injection. The present study showed the take rate 100%. whereas is caused by inoculation of the smaller number of cells in our study.

Microscopic data from this study was indicative of the validity of this model. Given the short doubling time of HepG2 xenograft models, all nuclear features of

a highly malignant cell can be observed. Active nuclei and high proliferation index (Ki-67) indicate a high level of metabolism in malignant cells. Ki-67 expression in tumors is associated with prognosis of HCC patients and is an independent prognostic indicator after resection that could be an important factor in the decision-making for adjuvant therapy (King et al., 1998).

HCC is one of the most vascular solid tumors and angiogenesis plays an important role in its development, progression, and metastasis (Qin and Tang, 2002). We have reported the high rates of tumor angiogenesis and the newly formed micro vessels in this study by CD34 labeling and demonstrating a high MVD. High metabolic activity in tumor cells induces hypoxia, which in turn enhances the HIF-1 alpha gene expression and thus begins the phase of angiogenesis with new blood vessels forming. Anti-angiogenic therapy can be considered as a tool to prevent the progression of HCC and other chronic liver diseases (Coulon et al., 2011).

In conclusion, this study represents a comprehensive model of the HepG2 xenograft for in vivo and ex vivo imaging studies that enable researchers to assess new therapeutical approaches for the treatment of liver cancer with hpMSC. Further studies are pending completion that will assess the therapeutic effects of mesenchymal stem cells on HCC.

Tumor optimization would have been a reasonable choice before conducting this study in order to control the quality of cells and the method used.

This study represents a comprehensive model of HepG2 xenograft for in vivo and ex vivo imaging studies that enable researchers to assess new therapeutical approaches for treatment of liver cancer with hpMSC. Further studies are pending completion that will assess the therapeutic effects of mesenchymal stem cells on HCC.

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