



***In vitro* three-dimensional culture of hepatocellular carcinoma to measure prognosis and responsiveness to chemotherapeutic agents**

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Hepatocellular carcinoma (HCC) is considered the sixth most common cancer and the third most common cause of cancer-related death worldwide. In addition, HCC is the second most common cancer, second only to lung cancer, in China (1). Over the last 3 decades, incidence rates and mortality rates have gradually increased in the United States (2). Analysis of HCC data from 1990–2014 from the World Health Organization (WHO) and EU indicated that the mortality rates would continue to increase through 2020 in the EU, North America and Latin America. However, in eastern Asia, the rates are predicted to be two to five fold increase compared with those countries (3). Early diagnosis and effective treatment are significant in HCC; however, HCC is typically diagnosed in late stages when surgical resection is not an option. Chemotherapeutic agents, such as sorafenib and doxorubicin, represent a major therapeutic option to alleviate a locally advanced disease and improve survival rates. However, the tumor structure, its invasive nature and metastatic potential and response to HCC chemotherapies are key factors affecting prognosis. During recent decades, two-dimensional culture has been the most common technique used to test the efficacy and safety of drugs before clinical trials, but two-dimensional culture cannot mimic the complex pathophysiological conditions present in HCC. In addition, the lack of specific growth factors and differentiation factors, which are required

to mimic the tumor microenvironment *in vivo*, causes the drugs to fail to achieve satisfactory effects in clinical trials. Three-dimensional cell culture combined with the advantages of animal research and two-dimensional cell culture, which can simulate the tumor microenvironment *in vivo* (e.g., cell-cell communication and cell-extracellular matrix interaction), reflect the growth characteristics of HCC and the effectiveness of chemotherapy drugs more accurately than two-dimensional cell culture. Currently, three-dimensional cell culture has become a suitable model for researching expression, migration, invasion, and apoptosis and performing drug screening in HCC.

HCC is a highly malignant disease with poor prognosis. Tumor morphology, metastatic nature and invasiveness have been used to predict the long-term prognosis of HCC. Cancer cell line spheroid culture was investigated over 40 years ago. Xu *et al.* discovered that when HCC cells were cultured with alginate gel (ALG) beads, HCC cells proliferated to form 3D spheroids and exhibited increased metastatic ability compared with adhesion cells, and these properties are similar to those of liver cancer tissue (4,5). Most tumor cells grew and proliferated slowly in spheroids, whereas an increased proportion of apoptotic cells indicated nutrition deficiencies and anoxia at the spheroid core. This 3D model of HCC mirrored many clinical pathological features of HCC *in vivo* (6). Researchers developed a novel

in vitro tissue-like metastatic HCC spheroid model using a biodegradable poly-lactic acid-co-glycolic acid (PLGA) scaffold within an RWV bioreactor system. In this system, HCC tumor cells exhibited close cell-cell connections, and cells formed tight junctions with the extracellular matrix when HCC spheroids were cultured for 15 days. The morphological traits of cells revealed a tissue-like structure that was distinct from HCC monolayers and mirrored the morphology and tissue ultrastructure of HCC *in vivo*. Moreover, the 3D HCC cells described above were transported into the livers of nude mice and exhibited evidence of intrahepatic and distant metastasis (7). In addition, solid tumor growth and metastasis are resistant to angiogenesis and blood supply. Angiogenesis marks the transformation of a benign tumor into a life threatening disease. In recent years, vasculogenic mimicry (VM) has become a new tumor blood supply system that has been identified in some highly malignant tumors. In this system similar vascular-like channels are formed through the self-deformation of cells and the remodeling of extracellular matrix. In the absence of endothelial cells (ECs), malignant tumor cells could undergo deformation to promote tumor growth, invasion and metastasis to provide blood supply for microcirculation of the pipeline, which represents an important form of blood supply in the growth and metastasis of malignant tumors (8). Researchers have mentioned that 3D co-culture of HepG2 cells and ECs could induce vascular-like network formation to mimic the vasculature of a liver tumor (9).

In recent years, some studies have suggested that 3D spheroids could reflect gene expression and metabolic profiles that were similar to hepatic tissue; however, 2D cultured cells may lose many important functional characteristics that are expressed *in vivo* (10,11). Compared with 2D monolayer cells, glucose consumption was reduced in HCC spheroids, whereas LDH production increased. Increased levels of albumin, AFP and γ -GT were noted compared with monolayer cells, and these features are consistent with the biochemistry of solid HCC tumors. The expression of CD44, CD29, and MMP9 is increased in metastatic HCC tumors. Tumor cells with increased expression of these proteins in the 3D state could be indicative of intrahepatic metastasis and distant metastasis (7,12). When HepaRG and/or HepG2 cells were cultured in 3D conditions, albumin levels and apoB secretion in per viable cell were increased compared with those in 2D cultured cell. In contrast, the expression of CYP enzymes in 3D HepaRG spheroids was also increased after treatment

with CYP inducers (11,13).

Thus, 3D cell culture may represent a better method than animal research and traditional cell culture to test the effectiveness and safety of drugs as a precursor to clinical trials. This model exhibits advantages of strong repeatability, reduced research time and low cost. Previous research has demonstrated that 2D and 3D cultures exhibited differential sensitivities to targeted agents. The drug-resistant phenotypes of sublines were not expressed when cells were grown as monolayer cultures but were fully recapitulated when cells were grown in three-dimensional conditions, namely, as multicellular tumor spheroids. In addition, the sensitivity to chemotherapeutic drugs in the three-dimensional tumor model is similar to that observed *in vivo* (14). HepG2 in 3D recellularized scaffolds maintained increased oncogenicity (c-MYC, c-Jun, c-Fos, and RAB3B) and multidrug resistance compared with cells maintained in a 2D monolayer or spheroids. The secretion of albumin secretion and α -FP expression decreased in response to a low concentration of methotrexate when HepG2 cells were cultured in 2D the system. In contrast, the same concentration of methotrexate had no significant effect on albumin secretion or α -FP expression when cells were grown in a 3D recellularized scaffold system (15). Given the vascular distribution of HCC, the drug penetration rate far away from blood vessels is lower than that of vascular proximal cells. Thus, in 2D cell cultures, direct exposure of drugs in the prediction of treatment programs is limited, whereas 3D cell culture could effectively imitate the drug reactions that occur in tumor tissues. Thus, 3D cell culture represents a highly efficient platform for chemotherapy drugs screening. Studies have cultured of HCC specimens obtained from 17 patients diagnosed with HCC in a 3D system with 5 fluorouracil (5FU), doxorubicin, docetaxel, irinotecan, and taxol. Interestingly, tumor migration was inhibited by irinotecan, paclitaxel and docetaxel, whereas the effect was variable with 5FU and doxorubicin, which represent the mainstay of HCC treatment (16). In the 3D co-culture system, the result demonstrate that 20 mM sorafenib reduced 66% of networks, 100 nM sunitinib reduced 47% of networks and 1 mM axitinib reduced 53% of networks. These doses were similar to plasma effective concentrations of drugs used *in vivo* (7). In addition, numerous studies have demonstrated that tumor cells in three-dimensional growth can be resistant to chemotherapy and radiotherapy, and cellular resistance characteristics in these culture systems are similar to solid tumors *in vivo*. Thus, 3D cell culture will play an

increasing role in drug screening in the future (17,18).

With the continuous development of HCC and the continuous discovery of anticancer drugs, it is important to establish effective and stable drug screening models that can physiologically reflect liver tumor tissue *in vivo*. The 3D culture of HCC cells is more similar to the actual state of the tumor compared with 2D cultured cells and more accurately reflects cell growth, metastasis and metabolism as well as other states. Thus, *in vitro* 3D cell models are ideal for HCC studies, anticancer drug screening, and the establishment of animal models (19).

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