Roles of Tumor Microenvironment in Hepatocellular Carcinoma

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Abstract: Hepatocellular carcinoma (HCC) is one of the most malignant human cancers, with a high mortality rate worldwide despite its early diagnosis in patients and improvement in therapeutic technology. Most cases of liver cancer show a strong resistance to anticancer therapy. Moreover, liver cancer patients generally have poor tolerance to chemotherapy due to liver dysfunction. In these situations, liver-targeting drugs with fewer side effects and a high efficacy are urgently needed during drug discovery for liver cancer. Researchers have aimed to derive target genes and drug candidates for HCC; however, the development of targeted drugs has not yet improved the outcome significantly.

Recently, the role of the tumor microenvironment (TME) in HCC has been probed to combat this deadly disease. A deeper knowledge of the crosstalk between tumor cells and their TME is needed to fully understand tumor development, progression and chemo-resistance in HCC because this cancer develops from chronically damaged tissue that contains large amounts of inflammation and fibrosis.

In this review, we summarize how distinct stromal cells of TME are involved in tumorigenesis and chemoresistance in HCC and the significant challenge to recapitulate tumor complexity and heterogeneity enhancement.

Keywords: Co-culture, drug development, hepatocellular carcinoma (HCC), multicellular tumor spheroid model (MCTS), tumor microenvironment (TME), tumorigenesis.

INTRODUCTION

Hepatocellular carcinoma (HCC) is the most common malignant tumor and the second leading cause of cancer-related deaths worldwide [1]. Chronic infection by hepatitis B and C, cirrhosis, and alcohol use are leading causes, as well as metastases from tumors elsewhere in the body [2]. Over the past decade, advances in treatment, surgical techniques, radiology, and liver transplantation have resulted in considerable improvements in the therapy for HCC. However, the prognosis for this disease is very poor because only 10-20% of liver tumors can be removed surgically [3]. Moreover, the cumulative 3-year recurrence rate after resection with a curative aim is approximately 80%. More importantly, recurrence after resection usually results in a high rate of mortality [4].

Presently, sorafenib, a multikinase inhibitor, is approved for the treatment of patients with advanced HCC. Generally, the medication costs of sorafenib are approximately US $5,400 per month; however, the drug only extends lifespan by an average of 2.8 months with various side effects [5]. Thus, novel therapeutic strategies are needed to improve the liver cancer patient’s quality of life.

For a long time, oncologists have studied the functions of oncogenes and tumor suppressor genes in tumorigenesis. In the recent years, the concept of cancer biology is changing from the genetics of tumor cells alone to studying the complicated interplay between cancer and the tumor microenvironment (TME).

The TME is the cellular environment in which the tumor exists, including the surrounding blood
vessels, immune cells, fibroblasts, other cells, signaling molecules, and the extracellular matrix (ECM) [6-8]. Recent studies have shown that the stromal cells in solid tumors have a dynamic and flexible function in tumor proliferation, invasion and metastasis, and the cells of the TME can regulate the response of cancer cells to chemotherapy [9].

To enhance our understanding of the communication between cancer cells and their microenvironment, we should solve some important questions such as what is the contribution of distinct components in the TME to tumor progression, what type of signals do cancer cells receive from the stromal cells in HCC and how do these signals promote malignant growth.

To solve these questions, many groups have challenged the modeling of tumor complexity and heterogeneity in various ways to mimic the in vivo TME. In this review, we describe the roles of distinct stromal cells of the TME in tumorigenesis and chemo-resistance in HCC and the significant challenge to model of tumor complexity and heterogeneity. A better understanding of interplay between tumor cells and the TME may be useful to devise new therapeutic strategies for HCC.

INFLUENCE OF THE TME COMPONENTS

Cancer-associated Fibroblasts

Fibroblasts are the most abundant cell type in connective tissues that maintain the structural framework of tissues through the secretion of ECM components. They also play a critical role in wound healing to support repair. Unlike normal fibroblasts, cancer-associated fibroblasts (CAFs), which are a specialized group of fibroblasts in cancer, can significantly promote the growth and invasion of tumor cells in various cancers, such as breast, prostate and pancreatic carcinoma [10-13]. CAFs directly affect tumor progression through enhancement of the expression of mitogens, cytokines, MMPs and ECM components that include hepatocyte growth factor (HGF), epidermal growth factor (EGF), vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), basic fibroblast growth factor bFGF, stromal cell-derived factor (SDF-1) and Interleukin-6 (IL-6) [10, 14-17].

CAFs are extremely critical components of the HCC microenvironment because most HCC cases are derived from fibrosis and cirrhosis. However, the crosstalk effects between HCC and fibrosis have not been well studied in the liver cancer microenvironment.

To date, only a few studies have addressed that H-CAFs (hepatocellular carcinoma-associated fibroblast) are an important factor for promoting the growth of HCCs in vitro and in vivo. Chuang et al. demonstrated [18, 19] that co-culture of HCCs and CAFs in vitro can enhance proliferation, migration, and invasion of HCC by changing of gene expression in HCC cell lines. H-CAFs can specifically up-regulate CCL2, CCL26, IL6, and LOXL2 genes which are related to proliferation, migration, invasion and angiogenesis in HCC cells. H-CAFs also regulate the cytokine levels. Qi Zhang et al. showed [20] a similar tendency in H-CAFs to support tumorigenesis by HGF secretion. A positive relationship between the distribution of H-CAFs and tumor growth was detected on the clinical level using 43 cases of HCC patient-derived materials. Furthermore, activated H-CAFs exhibited deactivation of natural killer (NK) cells by the regulation of PGE2 and IDO, thereby fostering tumor growth and progression [21]. This study showed that complicated multiple inter-actions between different types of components in the TME induced tumor development, progression, and chemo-resistance.

In summary, H-CAFs play a critical role in tumorigenesis by various mechanisms in HCC. However, the association between HCCs and CAFs is incompletely understood to treat liver cancer patients by targeting H-CAFs.

Hepatic Stellate Cells

Hepatic stellate cells (HSCs) play critical roles in diverse aspects of liver physiology, including liver organogenesis, regeneration, and HCC. HSCs are found in the space of Disse between the sinusoidal endothelial cells and hepatic epithelial cells [22]. HSCs were first described by Karl von Kupffer as Sternzellen (star cells) because normal stellate cells have a typical star-like morphology [23, 24]. Since then, stellate cells were found in several other organs, including the kidney, pancreas, and lung [25-28].
HSCs are quiescent and accumulate numerous vitamin A lipid droplets in healthy liver [29, 30]. When the liver is wounded by viral infection or hepatic toxins, HSCs undergo phenotypic transformation from quiescent cells to activated myofibroblast-like cells and secrete diverse cytokines, growth factors, and EMC proteins to protect the liver. The hallmarks of HSC activation are a reduced level of intracellular lipid droplets, increased expression of αSMA and production of ECM, and morphological changes [31-33].

HSCs are involved in the process of liver regeneration to appropriately control liver growth by producing antigenic factors and growth factors, changing the expression profile of cytokines and chemokines, and modulating endothelial cell and hepatocyte proliferation. The modulation of the balance between HGF and TGF-β1 can control the initiation and cessation of liver generation [34]. The neurotrophin receptor p75NTR is expressed in HSCs after fibrotic and cirrhotic liver injury in humans. p75NTR may lead to the termination of liver regeneration via the induction of the apoptosis of activated HSCs [35].

In addition to liver development and regeneration, HSCs exhibit biological functions in liver carcinogenesis. HSCs can induce phenotypic changes in cancer cells through the production of growth factors and cytokines such as HGF and IL-6. Reciprocal crosstalk between HCC and HSCs exists. When HSCs are co-cultured with Huh7 or HepG2 cells, HSC activation and migration are detected [36]. Activated HSCs can regulate the migration and proliferation of HCC cells via the modulation of TGF-β and ECM-related proteins. Moreover, the interaction between HCC and activated HSCs formed pro-angiogenic microenvironment by the overexpression of VEGF-α and metalloproteinase-9 (MMP9) [32, 37, 38]. Because active HSCs are involved in tumor onset and progress, targeting HSCs may represent a promising therapeutic strategy.

Vasculature

The vascular endothelium can control the transport of nutrients into tissues and maintain the flow of blood. The structure of the vascular endothelium is composed of endothelial cells, smooth muscle cells and a basement membrane. The endothelial cells form a continuous and uniform mono-layer in normal tissues and express various receptors of angiogenic factors including VEGFRs, Tie-2, EGFR, PDGFR, and CXCRs. Activation of receptors in endothelial cells trigger several signal cascades to regulate survival, proliferation, and invasion.

Tumor blood vessels are abnormal morphologically. Chaotic networks of tortuous endothelium are defective in tumor blood vessels [39]. The endothelial cells in cancer tissues have an irregular shape and size and are called tumor endothelial cells (TECs). TECs may create leaky vessels or gaps in the vasculature. This process could allow tumor cells to enter the circulation and distribute to other sites. The abnormal function of TECs in cancer tissues is to induce high concentrations of VEGF, which is a main stimulator of angiogenesis and tumor progression [40].

HCC is one of the most vascular types of solid tumor. The growth of liver cancer requires the formation of new blood vessels, and VEGF is critical factor in angiogenesis. VEGF expression is up-regulated in most cases of human HCC [41]. Some studies have shown that VEGF expression is regulated by various factors such as hormones, cytokines, signal molecules, and hypoxia [42-45]. VEGF affects endothelial cells to promote neovascularization in HCC, and the VEGF/VEGFR network may stimulate the growth of liver tumor cells [46].

Presently, multiple agents targeting the VEGF/VEGFR signal cascade are in clinical trials for HCC therapy. Thus far, sorafenib, a typical anti-VEGF agent, is approved for the treatment of patients with advanced HCC. In fact, sorafenib improved the survival in liver cancer patients. Researchers have developed other anti-VEGF agents like sorafenib, but there was no improvement. The molecular pathway in liver angiogenesis remains incompletely elucidated. Therefore, innovative strategies are needed for anti-angiogenesis treatment development by combining research into blocking VEGF signaling with studies of the tumor micro vessel environments in liver cancer.

Cancer Stem Cells

Cancer stem cells (CSCs) have been identified by experiments in which tumor cells were fractionated, characterized by cell surface markers,
and injected at limiting dilutions in mice. Those populations that led to tumor growth in the animal, and that led to tumor growth when that tumor was transplanted into a second animal, are considered CSCs [47, 48]. CSCs are considered the ‘Achilles heel’ due to their strong resistance to chemotherapy and radiotherapy. Thus, the recent advancements in the use of HCC stem cells to develop efficient and organized means to an antitumor agent is quickly gaining recognition as a novel goal. However, chemical screening to identify agents that preferentially kill CSCs is limited by the difficulty of culturing CSCs from solid tumors in vitro—CSC enrichment is rapidly lost in culture [49]. Moreover, CSC existence and roles remain controversial. Commonly, CSCs have been characterized by various cell surface markers, including ABCG2, ALDH1, CD44, CD133, and CD90, although their usefulness in certain tumor types remains debatable [50-52].

Recently, HCC progression has been thought to be derived by CSCs. Many studies have shown that CSC-related surface markers and pathways could modulate tumor development and suppression in liver cancer. Thus far, CSCs in HCC were identified by several cell surface antigens such as CD133, EpCAM, and CD44. Among them, CD133 and EpCAM have attracted considerable attention as representative liver CSC markers. CD133/Prominin-1 is well known as stem cell markers in various types of cancers. Liver cancer patients with high expression of CD133 had a shorter overall survival and higher recurrence rates than patients with low expression of CD133 (Table 1). As it seems to support clinical significance, CD133+ liver CSCs can induce an aberrant signaling pathway rather than CD133- cells [47, 53].

To confer chemo-resistance, CD133+ liver CSCs can modulate the activity of the Akt/PKB pathway, JNK, mTOR, ERK, and β-catenin [54, 55]. Aldehyde dehydrogenase (ALDH) and ATP-binding cassette (ABC) superfamily transporters such as ABCG2 are also elevated in CD133+ liver CSCs [56]. CD133+ liver CSCs can promote angiogenesis via the regulation of the production of IL-8, VEGF, and MMP-2. Current studies have indicated that CD133 is expected as a novel target to overcome chemo-resistance in HCC.

EpCAM is expressed in many human cancers with an epithelium origin. Currently, several EpCAM-targeting antibodies and RNAi significantly reduced the tumorigenicity and invasive capacity [57] of various cancers. EpCAM+ HCC cells display liver CSC-like traits, including the abilities to self-renew and differentiate. EpCAM expression is regulated via the activation of Wnt/β-catenin signaling [58]. Because crosstalk between EpCAM and Wnt signaling is associated with a regeneration capacity in liver CSCs, it has important value as a novel target for drug discovery. CSC-related research is an enchanting area to overcome the strong chemo-resistance in HCC. Again, because the roles and existence of CSCs remain controversial, prudent approaches are needed to treat cancer patients by targeting CSCs.

**Immune Cells**

Generally, the functions of the immune system are recognized as the protection of tissues from infection and damage. However, immune systems have also been implicated in promoting and preventing tumor growth. Immune responses in the liver are regulated by a complex interplay of antigen-presenting cells, T cells, and myeloid cell populations. The role of the immune system is complex and can be both pro- and anti-tumorigenic [59] in the development and progression of HCC. The immunosuppressive cell populations in the liver, which include CD4 T cells (Treg), myeloid-derived suppressor cells (MDSCs), tumor-associated macrophages (TAMs), and Th17 cells, affect tumor progression in HCC. Treg and MDSCs have clearly been shown to promote HCC progression. TAMs can also affect HCC tumor progression through NF-kB, STAT-3, and HIF-1 signaling [60]. NKT cell populations can either promote or impair liver cancer spontaneously [61]. The reduction of NK cells in cirrhotic livers is associated with HCC progression. Abnormalities in the B-cell phenotype and function occur in the progress of cirrhosis [63] and HCC; however, B cells can also induce de novo carcinogenesis by chronic inflammation [63]. Today, further studies are needed to elucidate the role of immune cell subsets in HCC for the enhancement of immunotherapeutic strategies and development of cancer vaccines.

**Challenges with the Recapitulation of the TME**

Various types of cancer cell lines have contributed to the improvement of cancer cell biology. However, the mono-culture system has limitations because it cannot estimate the crosstalk between
Table 1. Roles of CD133+ HCC cells in HCC tumorigenesis.

| Cell Type (Cell Line/Patient) | Changed Expression by CD133 | Note | Ref. |
|-------------------------------|-----------------------------|------|------|
| Huh7                          | AFP, GS, CYP3A4             | CD133+ HCC cells possessed higher proliferative and tumorigenic potential and expressed a lower level of mature hepatocyte markers than CD133- HCC cells. | [77] |
| Huh7, PLC8024                 | β-catenin, OCT3/4, Bmi1, SMO, Notch | CD133+ HCC cells exhibited a strong colony-forming ability and proliferative property. They showed progenitor cell-like properties in terms of stemness-related genes, self-renewal ability, and differentiation ability. | [53] |
| SMMC-7221                     |                             | CD133+ HCC cells showed higher survivability to chemotherapy (Doxorubicin, 5-FU) and expression of survival proteins related to the Akt/PKB and Bcl-2 pathways. | [78] |
| PLC/PRF/5, Huh7, Patient (5)  | MMP2, ADAM9                | CD133+ HCC cells displayed a strong invasion capacity and resistance to natural killer cells (NKs) and could produce high levels of VEGF. | [79] |
| PLC8024, Huh7, Patient (12)   | IL-8, ABCC1, Nanog, Notch1, CXCL1 | Neurotensin/IL-8/CXCL1 signaling through MAPK in CD133+ HCC cells played a functional role in the angiogenic, tumorigenic, and stem-like properties of these cells. | [80] |
| Huh7, PLC/PRF/5, Hep3B        | Bmi1, Oct3/4, Nanog, Sox2, Klf4 | Inhibition of mTOR with rapamycin increased CD133+ HCC cells via inhibiting its differentiation potential, retaining stemness properties. | [81] |
| SMMC-7221, Huh7, Hep3B, Patient (5) | | miR-150 down-regulated c-Myb in CD133+ HCC cells, and it affected the self-renewal capacity in these cells. They demonstrated that the high percentage of the G1 phase and apoptosis cell population resulted from the overexpression of miR-150. | [82] |
| Hep3B, SNU475, PLC/PRF/5, SMMC-7221, MHCC-97L, HCC-Ly5 (patient) | GPR87 | The overexpression of GPR87 could regulate CD133 expression positively, and played critical roles in tumorigenesis and stemness. | [83] |
| Patient-derived samples       | | Cytoplasmic CD133 was the main cause of the shorter overall survival of patients with HCC. | |
|                               | β-catenin, Nanog, Oct3/4, Sox2, Nestin, SMO, Bmi1, Notch, ABCG2, ABCB1 | miR-130b could regulate stemness and tumorigenesis via silencing TP53INP1 in CD133+ HCC cells. | [84-87] |
|                               | | For the carcinogenic process, hypomethylation of Line-1 was the most common disorder. Overexpression of CD133 was related to the demethylation of Line-1 in HCC. | |
|                               | | CD133 expression and JNK activation in HCC showed a positive correlation. Resistance to sorafenib was related to the activation of JNK and overexpression level of CD133. | |

carcinomas and their TMEs. It is true that heterologous cell types within tumors can actively influence the therapeutic response and shape resistance. To understand the functional role of stromal cells in the TME in tumorigenesis, modeling TME was challenged in various ways. To elucidate the crosstalk between tumor and stromal cells, researchers have challenged direct or indirect co-culture systems. In indirect co-culture systems, conditioned media that contain secreted proteins were utilized to determine the function of the TME because secreted proteins are key intercellular messengers in the tumorigenesis process. Direct co-culture systems, which comprise more than two types of cells in one culture dish or well, has the advantage of evaluating cell-cell interactions in the
TME. Actually, many studies have revealed that the co-culture of hepatocellular carcinoma and stromal cells enhanced the progression of cancers through the activation of specific signal pathways and changes in the cytokine expression profile (Table 2) [62-64].

Recently, a research trend is changing from two-dimensional (2D) to 3D cell culture systems. Actually, attempts to mimic cancer tissues via the creation of 3D culture systems in vitro are not new in cancer biology. Although 2D cell culture systems have taken the lead in cancer research and drug discovery, they are limited in their ability to predict in vivo situations. Because culturing cells in 2D are grown on flat dishes and form unnatural cell attachments, simplifying the assay system in 2D cannot provide the data that would be utilized in translational research.

Globally, the subsequent very well-recognized international laboratory published work clearly highlights the need for complicated 3D cell culture systems as a new methodology to screen for therapeutics on oncology. Because culturing cells in 3D attached to other cells form natural cell-to-cell attachments, cells in 3D culture systems have displayed a spectacular influence on cell polarity, differentiation, signaling cascades, and gene expression relative to cells in 2D culture systems [65, 66]. Therefore, 3D cell culture in vitro has been used in cancer research as an intermediate model between an in vitro cancer cell line culture system and an in vivo tumor. Particularly, liver cells performed more liver cell functions in 3D versus 2D [67-69]. Currently, the importance of the drug development of specific liver cancer through the construction of a 3D tumor microenvironment has been well-recognized. However, it has not been successful worldwide due to the complicated process of establishing a 3D microenvironment.

The tumor microenvironment plays important physiological roles in cell differentiation, tumorigenesis, metastasis, and therapeutic efficiency. Therefore, the multicellular tumor spheroid model (MCTS) has emerged as a powerful method to mirror tumor complexity and heterogeneity enhancement for anticancer research. Reciprocal action between different types of cells in a spheroid produces a critical effect on the sensitivity to chemotherapy and behavior of tumors. Various types of MCTSs have been applied to observe the crosstalk between tumor cells and their stromal cells.

MCTSs that comprise endothelial cells and tumor cells are routinely used to evaluate the antiangiogenic capacity. Under 3D conditions, co-culture of melanoma and vascular endothelial cells (HUVECs) enhance tumor metastasis compared with 2D co-culture [70]. Co-culture of HUVECs and hepatocytes on heterocellular 3D architecture was utilized to monitor cancer angiogenesis [71, 72]. Co-culture of prostate cancer epithelial and stromal cells under 3D conditions influences the secretion of E-cadherin [73]. Co-culture of CAFs

Table 2. Functional studies of stromal cells in HCC using the co-culture system.

| Cell Type | Culture Type | Factors | Pathway Activated | Ref. |
|-----------|--------------|---------|------------------|------|
| HUVEC     | 3D, Direct   | -       | C-Met/AKT, JAK2/STAT3 | [71, 72] |
| Hepatic stellate cell | 2D, Indirect | HGF | FAK-MM9 | [89] |
| Cancer-associated fibroblast | 2D, Direct and Indirect | - | CCL2, CCL26, IL6, LOXL2 | [18] |
| Cancer-associated fibroblast | 2D, Indirect | HGF | - | [20] |
| Human embryo fibroblast | 2D, Direct | Hab18G/CD147 | MMPs | [90] |
| HCC-associated mesenchymal stem cells | 3D, Direct / 2D, Indirect | miR-155 | MMP9 | [91] |
| Tumor-associated macrophages | 2D and 3D, Indirect | IL6 | STAT3 | [92] |
| Tumor-associated macrophages | 2D, Indirect | TGF-β1 | EMT | [93] |

*2D: two-dimensional, 3D: three-dimensional, Indirect: indirect co-culture, Direct: direct co-culture
and salivary gland adenoid cystic carcinoma (ACC) cells under 3D conditions promoted tumor spheroid invasion [74]. The co-culture system of 3D clone cancer cells and stromal fibroblasts also showed a strong invasive phenotype than 3D clone cancer cells alone [75]. Dynamic analysis of hepatocellular carcinoma MCTS formation has shown the fundamental role of E-cadherin and β1-integrin in cell aggregation and multicellular tumor spheroid compaction [76].

These results suggested that the MCTS can remarkably recapitulate the 3D cellular environment and has pathophysiological relevance like in vivo tumors, unlike classical monolayer-based models. Currently, the importance of the drug development of specific liver cancer through the 3D tumor microenvironment has been recognized, but it remains unsuccessful worldwide due to the complicated process of establishing a 3D microenvironment. Institut Pasteur Korea (IPK) has developed MCTSs for high content screening to identify HCC-specific compounds. To configure multicellular tumor spheroids, human hepatocellular carcinoma cells (Huh7) were grown together with human fibroblasts (WI38), human HSCs (LX2), and human umbilical endothelial cells (HUVEC) (Fig. 1). Through the comparison study of the sensitivity to conventional anticancer drugs using our MCTSs and HCC spheroids, MCTSs displayed strong chemo-resistance relative to Huh7-alone spheroids (data not shown). Presently, we expect that the models of multicellular tumor spheroids will contribute to the elimination of false-positive drug candidates during the process of drug discovery and the elucidation of the functional roles of each stromal cell type on tumorigenesis and chemo-resistance.

Recently, tissue-derived tumor spheroids generated from tissues are of growing interest in personalized therapeutic strategies. The current treatment for HCC is surgical resection with limited chemotherapy because of the lack of response in many liver cancer patients. This method will be a clinically available model to monitor chemotherapy drug efficacies in a more natural, clinically relevant environment.

Although the 3D co-culture system is still too immature to mirror the in vivo TME, it is a highly applicable method to elucidate the roles of the TME on tumorigenesis. Furthermore, 3D tumor microenvironment systems will offer a new paradigm for high-throughput drug screening and will significantly improve the efficiency of identifying new drugs for liver cancer treatment.

**CONCLUSION**

Recently, Megan Scudellari mentioned that drug companies have been fighting a losing battle against advanced liver cancer, and this sentiment is presently true [5]. HCCs strongly enhance resistance to chemotherapy together with their TME, because the various components of the TME contribute to many aspects of carcinogenesis, cancer progression, and HCC behavior. Thus, targeting components of the HCC microenvironment might be a useful approach to overcome liver cancer.

To characterize the causes of drug resistance related to TME, the development of sophisticated...
methodologies is essentially needed to reflect the TME. However, these strategies are by no means easy. Because TMEs are different according to various stages of tumor development, drug treatment, and patient properties, it is unclear how to recapitulate the TME like that in vivo. To do this, continuous research on the dynamic changes of TME within patient tissues is essential through the collaboration of basic researchers and clinical pathologists to configure the TME in vitro with physiological relevance. Further studies of the interplay between HCC and the TME should ultimately offer biomarkers that have diagnostic and prognostic value, an opportunity for novel anticancer drug development, and the most appropriate treatment for each patient.

CONFLICT OF INTEREST

The author confirms that this article content has no conflict of interest.

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Roles of Tumor Microenvironment in Hepatocellular Carcinoma

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