Genes & Diseases (2020) 7, 336–350

Available online at www.sciencedirect.com

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REVIEW ARTICLE

Study on the hepatocellular carcinoma model with metastasis

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Received 11 October 2019; received in revised form 7 December 2019; accepted 31 December 2019
Available online 8 January 2020

KEYWORDS
Animal model;
Cell model;
Chemically induced HCC model;
Genetically engineered mouse model;
Hepatocellular carcinoma;
Metastasis;
Patient derived xenograft model

Abstract Hepatocellular carcinoma (HCC) is one of the most common causes of cancer-related death around the world due to advanced clinical stage at diagnosis, high incidence of recurrence and metastasis after surgical treatment. It is in urgent need to create appropriate animal models to explore the mechanism, patterns, risk factors, and therapeutic strategies of HCC metastasis and recurrence. However, most of the established models lack the phenotype of invasion and metastasis in patient, or have unstable phenotype. To establish HCC models with stable metastasis phenotype requires profound understanding in cancer metastasis biology and scientific methodology. Over the past 3 decades, HCC models with stable metastasis have been extensively studied. This paper reviewed the history and development of HCC animal models and cell models, focusing on the screening and maintaining of metastatic potential and phenotype. In-depth studies using these models vastly promote the understanding of cellular and molecular mechanisms and development of therapeutic strategies on HCC metastasis.

Introduction

Hepatocellular carcinoma (HCC) is the second leading cause of cancer death in male, and the sixth leading cause in female around the world. China accounts for about 50% of total cases and death.[1] Metastasis is the most fundamental biological characteristic and main cause of treatment failure and cancer death.[2] Even in small HCC, the metastasis and recurrence rate can reach up to 50%.[3–5] Most of the HCC metastasizes to other organs through blood vessel,

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Peer review under responsibility of Chongqing Medical University.

https://doi.org/10.1016/j.gendis.2019.12.008

with lymphatic metastasis being the second most frequent route. Direct spreading, invasion or implantation is also found in HCC metastasis.

The extrahepatic hematogenous metastasis usually occurs in early stage and involves large extent of organs or tissues. Zhu et al[6] found that extrahepatic organ metastasis through hematogenous route happened in more than 60% of HCC patients, with lung being the most frequently involved organ (approximately 90% in all hematogenous metastasis). Liu et al[7] performed autopsy on 56 primary HCC patients, and revealed 85 organ metastases and 78 lymph node metastases. In an autopsy study of 387 patients with 14 various primary cancers, the metastatic rate of HCC was 51.8%. In another autopsy study of 240 HCC patients in Japan,[8] the intrahepatic metastasis rate was 78.5%, hematogenous metastatic rate 59.5%, portal vein tumor thrombus 57.9%, lymph node metastasis 32.9%, locally direct spreading 21.3%, hepatic vein tumor thrombus 12.1%, biliary tract invasion 4.2%, and perihepatic dissemination 3.8%. Among organs involved, pulmonary metastatic rate was 49.2% (118/240). These data suggested that lung was a more favorable organ of extrahepatic metastasis in HCC. Therefore, one of the vital emphasis on the study of HCC metastasis should be the mechanism of pulmonary metastasis.

Experimental HCC models with metastasis are essential for exploring mechanism of HCC metastasis and elucidating the contributing factors. However, the invasion and metastasis characteristics in clinical patients are not always able to be replicated in animal models or cell lines. The features of both tumor specimens and conditions of host animal for developing metastasis models are fundamental issues to be solved during mechanism studies of cancer metastasis. Therefore, the significance of establishing HCC model with metastasis is more than just constructing a laboratory platform. Instead, it is important to explore the basic laws and mechanism of HCC metastasis, so that HCC model with metastasis can be established by certain scientific approaches rather than by chances. This review is intended to summarize the progress on the construction of HCC animal and cell models with metastasis and/or metastatic potential.

HCC animal model with metastasis

Spontaneous HCC models

Spontaneous tumor model develops naturally in animals without any intentional intervention, reflecting the accumulation of environmental carcinogens, cancer-promoting substances and tumor susceptibility, replicating human HCC in terms of antigenicity, growth pattern, cell kinetics and differentiation.[9] However, there are many uncontrollable factors in spontaneous HCC model. First, the susceptibility varies among different species, with C3H mice and CBA mice being more susceptible to spontaneous HCC.[10] Second, even in the same species, the incidence and the time of tumor development could be largely different between male mice and female mice.[11] Third, the histopathology of tumor is markedly heterogeneous. In a study of 1,000 wild rats, only one cavernous hemangioma, two fibromas and one adenofibroma were found, showing great variance within only four models.[12] In terms of metastasis, pulmonary metastasis was not observed until 24 months, with an incidence rate of only 4.5%.[9] To sum up, spontaneous HCC model is usually characterized by low incidence, long incubation period, great phenotype heterogeneity, and low spontaneous metastasis rate. Therefore, spontaneous HCC model is currently not a favorable option, except for the purpose of providing tumor source for transplantation models.[13]

Chemically induced HCC models

Exposure of chemical, physical, biological, and other carcinogenic factors are critical links in the pathogenesis of HCC due to the essential function of xenobiotic detoxification in liver. Among the carcinogenic risk factors, chemical agents are the most frequently used method to induce HCC model, because it is technically easy to perform and can replicate human HCC in terms of pathogenetic mechanism and clinical-pathological presentation. Dimethylnitrosamine (DMN), diethylnitrosamine (DEN), N-nitrosomorpholine (NMOR), N-butyl-N-(4-hydroxybutyl) nitrosamine, 1,2-dimethylhydrazine, and 2,2′-dihydroxy-di-N-propylnitrosamine are several kinds of frequently used chemical carcinogens for the inducing of HCC models.[14] Metastasis in chemically induced HCC models mostly occurred through blood vessels.

As early as 1956, Magee et al[15] firstly reported that DMN was a promising chemical agent to induce hepatic tumor accompanied with metastasis in rats (Table 1). Using this method, researchers have been devoting to the construction of HCC model with hematogenous metastasis. Later, based on the three-stage assumption (initiation, promotion, and progression) in liver carcinogenesis, a two-step method (initiation and promotion) were innovated to produce HCC model, mostly composed of the combination of DEN or other chemical compounds as the initiating agents and phenobarbital as the promoter.[16] In 1983, Dennis et al[17] demonstrated that partial hepatectomy was also a promising promoting stimulus in inducing liver cancer. In the late 1980s, it was reported that NMOR could induce HCC oncogenesis and spontaneous pulmonary metastasis in F344 rats by adding it to drinking water.[18]

However, tumor incidence rate in corresponding chemical agents was the major observation target, instead of HCC metastasis. In the late 1990s, Masui et al[14] was the first to demonstrate that pulmonary metastatic rate of DEN could be significantly enhanced to 100% by increasing the dosage of NMOR to 120 ppm. In 1999, Futakuchi et al[19] further demonstrated that combination of DEN and NMOR was the most efficient method to induce HCC with significant pulmonary metastasis. The HCC incidence rate and pulmonary metastatic rate reached 94%—100% and 69%—84% respectively, with histopathology being moderately differentiated HCC and moderately to poorly differentiated carcinoma. One important molecular feature of this model is the progressive decline in cadherin expression during the transformation from adenocarcinoma to HCC, and eventually to metastatic cancer, which mimicked the malignant biological behavior in patient.

Table 1 Synopsis of chemically induced HCC model with metastasis.

Chemical agents	Characteristics	Metastasis	Comments	References (Year)
DMN induced model	Months 6.5—10: primary hepatic tumor development	Pulmonary (7/20) and intra-abdominal (3/20) metastasis	The first to provide methods for chemical agent induced HCC model	Magee et al (1956)[15]
DEN and 2-AAF + PH induced model	Months 8: 68%—71% HCC;	9.1%—16.7% metastatic rate	Starting the method of "two-step" (initiation, promotion) model; DEN alone could not induce carcinogenesis;	Solt et al (1983)[17]
[PH + B(a)P] + [2-AAF + CCl_4] induced model	Months 18: 82% (14/17) HCC	23.5% (4/17) pulmonary metastasis		
[PH + 1,2-DMH] + [2-AAF + CCl_4] induced model	Months 18: 67% (8/12) HCC	16.7% (2/12) pulmonary metastasis		
DEN induced model	Months 11-: 100% HCC	25%—50% metastasis rate in B6C3F_1 mice; 0%—33% metastasis rate in C3AF_1 mice	Revealing heterogeneity between different species; Younger mice have faster HCC development than older mice	Vesselinovitch et al (1984)[20]
NMOR induced model	Months 25: 63% (15/24) HCC (NMOR 100 mg/L); Months 40: 67% (16/24) HCC (NMOR 40 mg/L)	NMOR 100 mg/L: 4.2% metastatic rate; NMOR 40 mg/L: 29.2% metastatic rate	Focusing on the dose response in F344 rats when administered with NMOR	Lijinsky et al (1988)[18]
DEN + PB induced model	Months 10: 30% (6/20) HCC	NA	PB shortened the time to HCC appearance	Klaunig et al (1988)[23]
DEN + NMOR induced model	Months 4: 100% (15/15) HCC	Months 7: 100% (15/15) pulmonary metastasis	Providing an optimal method for the inducing of HCC with significant pulmonary metastasis	Masui et al (1997)[14]
DEN + NMOR induced model	Months 2: 60% (9/15) HCC; Months 4: 100% (13/13) HCC; Months 5.5: 94% (17/18) HCC	Months 2: 0% (0/15) pulmonary metastasis; Months 4: 69% (9/13) pulmonary metastasis; Months 5.5: 84% (16/19) pulmonary metastasis	Established relatively stable HCC model for studies on metastasis; Providing experience for metastatic model construction	Futakuchi et al (1999)[19]
NNM induced model	Months 6.8: 100% (15/15) HCC;	Months 7: 87% (13/15) metastasis rate	WS/Shi was the most sensitive species to NNM compared with SD/gShi, and F344/DuCrj rats	Murai et al (2000)[24]
DEN + PB induced model	Months 5: 67% (6/9) macroscopic hepatic masses; Months 9: 100% (9/9) macroscopic hepatic masses	NA	PB did not influence the incidence of macroscopic hepatic masses	Goldsworthy et al (2002)[25]
DEN + NMOR induced model	Months 3.5: HCC was observed; Months 5: 100% HCC	Months 5.5: first pulmonary metastasis; Months 9: 60%	Modifying the experimental protocol to improve survival and to	Yoshino et al (2005)[22]

Table 1 (*continued*)

Chemical agents	Characteristics	Metastasis	Comments	References (Year)
		pulmonary metastasis; Months 10: 100% pulmonary metastasis	establish a better animal metastasis model	
DEN in GDF-15 deleted mice	Months 6: 80% (16/20) HCC	No metastasis	GDF-15 had no apparent effect on HCC tumor formation rate, growth rate, or invasiveness in DEN-induced HCC	Zimmers et al (2008)[26]
DEN in ATM mutated mice	Months 1.3: development of HCC; Months 12: 100% HCC	Pulmonary metastasis in 50% of $ATM^{+/+}$ and 52% of $ATM^{+/-}$ mice; Months 12: 100% metastasizing HCC in wild type or $ATM^{+/-}$ mice	Hepatocarcinogenesis is abrogated in ATM-deficient mice	Teoh et al (2010)[27]
DEN + PB	Months 8: microscopically and macroscopically detectable tumors; Months 14: HCC	NA	Exploring the tumor genomes of DEN induced HCC for the first time; Beta-catenin mutation and activation of the Wnt/β-catenin pathway were not involved in tumor initiation of this model	Aleksic et al (2011)[28]

Abbreviations: HCC, hepatocellular carcinoma; DMN, dimethylnitrosamine; DEN, diethylnitrosamine; 2-AAF, 2-acetylaminofluorene; PH, partial hepatectomy; B(a)P, benzo(a)pyrene; 1,2-DMH, 1,2-dimethylhydrazine; NMOR, nitrosomorpholine; PB, phenobarbital; NNM, N-nitrosomorpholine; GDF-15, Growth/differentiation factor-15; ATM, ataxia telangiectasia mutated.

As listed in Table 1, the phenotype of pulmonary metastasis was frequently observed in chemically induced HCC model. However, the development of HCC and potential of metastasis vary with the changes of age, gender and species of mice,[20,21] as well as the dosage of chemotoxic agents. Even DEN plus NMOR in F344 rats was a relatively mature method, slight changes in age (6 weeks vs. 6 weeks vs. 5 weeks), DEN (100 mg/kg, 4 weeks vs. 100 mg/kg, single intraperitoneal injection vs. 100 mg/kg, single intraperitoneal injection), and NMOR (120 ppm, 24 weeks vs. 120 ppm, 22 weeks vs. 40 ppm, 14 weeks) vastly influence the time of pulmonary metastasis and incidence rate.[14,19,22] Nevertheless, DEN plus NMOR was still a more effective and stable protocol for chemically induced HCC model with significant pulmonary metastasis when comparing other counterparts in Table 1.

Patient derived xenograft (PDX) HCC model

In 1969, Rygaard proved for the first time that T cell development in mice were obstructed due to thymic aplasia and cellular immunodeficiency caused by congenital gene mutation, which made the grafting of xenogeneic skin tissue and tumor possible.[29,30] PDX model of HCC started in the mid-1970s. In 1976, Shimosato for the first time reported the establishment of murine HCC model.[31] Tumor was grafted subcutaneously in the back of BALB/c nude mice using cuff technique. The tumor volume reached 40 mm × 30 mm × 15 mm 28 days later, accompanied with local lymphatic metastasis. In 1979, Hirohashi et al[32] studied several subcutaneous HCC nude mouse models by consecutive passaging, demonstrating that most of the HCC were able to maintain primary histological features during passaging. This HCC model mimicked patient in terms of protein expression level and correlation among tumor growth speed, histological differentiation and alpha-fetoprotein (AFP) expression level, indicating that PDX model was a promising and reliable platform in the future studies of HCC.

At the same period, studies on PDX models were also carried out at Liver Cancer Institute of Fudan University (former Liver Cancer Institute of Shanghai Medical University). In 1982, the earliest HCC nude mouse model in China, $LTNM_1$ and $LTNM_2$,[33,34] were established by subcutaneous grafting of AFP-positive tumor sample to the neck and back

of nude mice via trocar. Later in 1986, 2 heterotopic subcutaneous nude rat models, $LTNR_1$ and $LTNR_2$, were constructed.[35] Compared with the HCC nude mouse model, the nude rat model had the advantages of more tumor nodules with more adequate blood supply, and was more convenient for experimental operation. But both nude mouse and nude rat subcutaneous models lacked the feature of infiltration or metastasis, failing to fully reflect all the malignant biological behaviors of human HCC. Therefore, subcutaneous model was still a transitional model before optimal model was established.

The advantages and disadvantages of different transplantation methods, including subcutaneous, intraperitoneal, and intrahepatic nude rat models were compared and concluded by Bao et al.[36] Subcutaneous model had the advantages of high successful rate, high growth speed, which was suitable for batch experiment. However, the deficiency on the microenvironment, biological behavior of infiltration and metastasis greatly limits the application of subcutaneous models in HCC study. Intraperitoneal model is inconvenient for the measurement and observation of tumor hiding deep in the peritoneal cavity, but the incidence rate of infiltration, metastasis, and ascites is significantly elevated compared with subcutaneous model. Intrahepatic model possesses the advantages of short latent period and rapid growth. Besides, it replicates human HCC, showing characteristics of infiltration, metastasis, ascites, and tumor loci. Intrahepatic model might bring practical significance for the exploration of interventional treatments, for example, intubation, ligation, and embolism.

By the mid-1990s, owing to the aforementioned well-rounded studies, the theoretical basis and experimental method have been gradually concluded and refined to produce ideal HCC PDX model with significant phenotype of metastasis. Improved experimental protocol has been put into practice, including the following three major aspects. (1) Tumor source: HCC specimen was obtained from the metastatic loci rather than the primary tumor, so as to selectively increase the metastatic potential of original tumor tissue; (2) Implantation method: To provide a more favorable microenvironment for tumor growth and the expression of metastatic feature, tumor samples were grafted orthotopically to murine liver; and (3) Serial selection: Several rounds of *in vivo* selection by continuously grafting tumor into liver was applied to further promote and stabilize the metastatic potential of HCC. With the refined methods, HCC PDX models with high-metastatic potential were gradually constructed and put into HCC studies. HCC PDX models can be further divided into three types based on the features of dominant metastasis behaviors, including HCC model with hematogenous metastasis, HCC model with intrahepatic metastasis, and HCC model with lymphatic metastasis (Table 2).

HCC model with hematogenous metastasis

In clinical conditions, the most frequent site of distant metastasis is the lung, due to dissemination of tumor cells via the blood stream, hemodynamic characteristics of the liver and the intrinsic biological features of the tumor, for example, increased proliferation, invasion, and motility (Fig. 1).[2,37]

In 1996, high metastatic LCI-D20 model of HCC was constructed by Liver Cancer Institute of Fudan University (Table 2).[38–40] Surgical specimens from 30 patients were used for transplantation, among which one case from a 39-year old man showed pulmonary metastasis. LCI-D20 was constructed via *in vivo* clonal selection by repeated "lung foci to liver".[40,41] LCI-D20 mimicked biological behaviors of human HCC, including local-regional growth and invasion, and spontaneous metastasis to lung, liver, and lymph node. This model manifested 100% transplant-ability and the high metastatic ability was maintained to more than 120 passages, with short passage duration of about 20 days. Both LCI-D20 and the patient showed Edmonson Grade-II HCC microscopically, accompanying with the expression of AFP and hepatitis virus-B surface antigen (HBsAg). At the same period, an orthotopic HCC model with low metastatic potential, LCI-D35, was established for comparative studies.[38,39] To this point, HCC PDX models in accordance with clinical patient and more relevant to clinical setting were successfully established. LCI-D20 was now widely applied in the study of HCC recurrence and metastasis, for example, the studies of antisense oligonucleotides and gene transfection,[42] extracellular matrix,[43] intercellular cell adhesion molecule (ICAM),[44] anti-angiogenesis, and inducers for cellular differentiation.[45]

HCC model with intrahepatic metastasis

HCC metastasis mostly started with intrahepatic spreading. Therefore, it's important to establish HCC model with intrahepatic metastasis. In 1993, Aruga et al[46] firstly introduced a subcutaneous HCC model with spontaneous liver metastasis. However, the established subcutaneous model might not fully reflect the process of intrahepatic metastasis. Kuriyama et al[47] grafted carbocyanine dye (DiI)-labeled HCC cells under the capsule of liver tissue. Fluorescence microscopy and laser confocal microscopy revealed that cancer cells invaded portal vein nearby, but did not invade central vein. However, cancer cells with DiI label did not show the sign of migration. The author repeated the experiment above after inducing cirrhosis using intraperitoneal injection of thioacetamide. The speed cancer cells spread to the portal vein area was significantly elevated in mice with cirrhosis than in normal mice,[48] which indicated that HCC cells were prone to invade portal vein, especially under the circumstances of liver cirrhosis. Kuriyama again proved that cirrhosis is an important contributing factor for HCC development and metastasis. With the successful experiment, HCC model with intrahepatic metastasis were widely applied in the interventional experiments, for example heat treatment and radiofrequency ablation.[49,50]

HCC model with lymphatic metastasis

The ascitic type HCC model H22 possesses stable tumorgenicity, but not fully presenting the characteristics of metastasis. In 1984, tumor cells from H22 model were injected subcutaneously into the foot pad of the inbred 615

Table 2 Synopsis of patient-derived xenograft HCC model with metastasis.

High metastatic HCC Model	Characteristics	Metastasis	Comments	References (Year)
Hematogenous metastasis model				
LCI-D20	Days 10 ± 1.5: 100% transplant-ability (96/96) for 18 passages	Weeks 6—8: all transplanted mice died of serious metastasis; Spontaneous metastasis to lung (78/78), liver (78/78), and lymph node (78/78)	Having significant and stable phenotype of hematogenous metastasis; Played a vital role in the exploration of HCC metastasis and recurrence	Sun et al (1996)[40]
LCI-D35	Low metastatic ability	No metastasis in the liver, lung and lymph node	Used as a control compared to LCI-D20; The biological features remained unchanged until 59 passages	Sun et al (1995, 1996, 2001)[38,39,41]
Intrahepatic metastasis model				
Subcutaneous liver metastatic HCC model	Weeks 2: latent period; Weeks 4: 1.0 cm in average diameter	Weeks 4: significant liver metastasis in 100% mice (5/5); 100% liver metastasis after the third generation and even after 18 generations	Non-orthotopic intrahepatic metastasis model	Aruga et al (1993)[46]
HCC cell lines inoculated HCC model	Li7 and KYN-2 cell lines: multiple small liver tumors	Li7 and KYN-2 cell lines: vascular tumor thrombi and intrahepatic metastasis;	Rho/p160ROCK signaling pathway is critical in intrahepatic metastasis of human HCC.	Genda et al (1999)[62]
Mouse HCC tumor implanted model	100% transplant-ability	Weeks 4: 100% (7/7) intrahepatic metastasis; and 28.6% (2/7) pulmonary metastasis	Reflecting the steps of HCC metastasis; Intrahepatic tumor presented time-dependent growth pattern;	Sawada et al (2002)[63]
Lymphatic metastasis model				
Lymphatic metastasis model with H22 tumor cells	Constructed by injecting cells obtained by repeated lymphatic system screen; Cell subclones with different metastatic potential was isolated from lymphatic metastatic models	100% (10/10) lymph node metastasis	First established HCC model with significant lymphatic metastasis, and heterogeneity inside this model was further illustrated	Ling et al (1984, 1990)[51,55]
Lymphatic metastasis model with Hca-F cells	No metastasis to other organs except for lymph node	High metastatic rate during the 17 generations (80%—100%)	Lymphatic metastatic models with stable and high metastatic rates	Li et al (1998)[58]
HCC model with lymph node-specific metastasis	Using lymph node metastasis from HCCLM3 as the tumor source; Using fluorescent stereomicroscopy to detect metastasis loci	Weeks 6: 100% lymph node metastasis	Applied convenient method to detect tiny metastasis loci	Tao et al (2011)[59]

(*continued on next page*)

Table 2 (*continued*)

High metastatic HCC Model	Characteristics	Metastasis	Comments	References (Year)
Annexin A7 down-regulated lymphatic metastasis model	Downregulation of the Annexin A7 gene significantly inhibit lymph node metastasis	100% lymph node metastasis rate	Illustrating the potential of lymph node metastasis in terms of gene expression	Jin et al (2013)[60]

Abbreviations: HCC, hepatocellular carcinoma.

mice, and extensive lymphatic metastasis occurred 21 days later, including ipsilateral axillary lymph nodes, bilateral inguinal lymph nodes, bilateral axillary lymph nodes, lumbar and renal hilar lymph node.[51] After 20 rounds of repeated *in vivo* lymphatic system selection, the lymphatic metastasis rate was elevated and even involved paratracheal lymph nodes.[52] Using *in vivo* screening for 25 rounds, the ascites of H22—F25 was cultured *in vitro* to establish a mouse HCC cell line H22—F25/L.[53,54]

In 1990, Ling further isolated five cell clones ($16A_3$, $16C_3$, $22C_5$, H_7, and A_2) from H22—F25/L cell line,[55] including one high metastatic cell line ($16A_3$) and one low metastatic cell line (A_2). The subclone of $16A_3$ and A_2 were able to preserve the metastasis potential within 20 passages. However, the high-metastatic potential of $16A_3$ was relatively unstable and could be preserved within 20 consecutive *in vitro* passages, but might change significantly following further passages.[56] To get cell lines with stable metastasis potential, Ling suggested to freeze primary cultured cells as much as possible or to further separate subclones with high metastasis potential.[57] By applying limited dilution method, 8 subclones were isolated, among which HCa-F25/CL16A3-F (F) and HCa—F25/CL16A3-D (D) had the highest metastasis rate.

In the following 2 decades, several lymphatic metastatic models have been established, using similar method to Ling et al (Table 2).[58,59] Using gene knock up and knock down technique, Jin et al[60] demonstrated that knock down of Annexin A7 expression could significantly induce lymphatic metastasis in HCC model. In the treatment study, Liu et al[61]

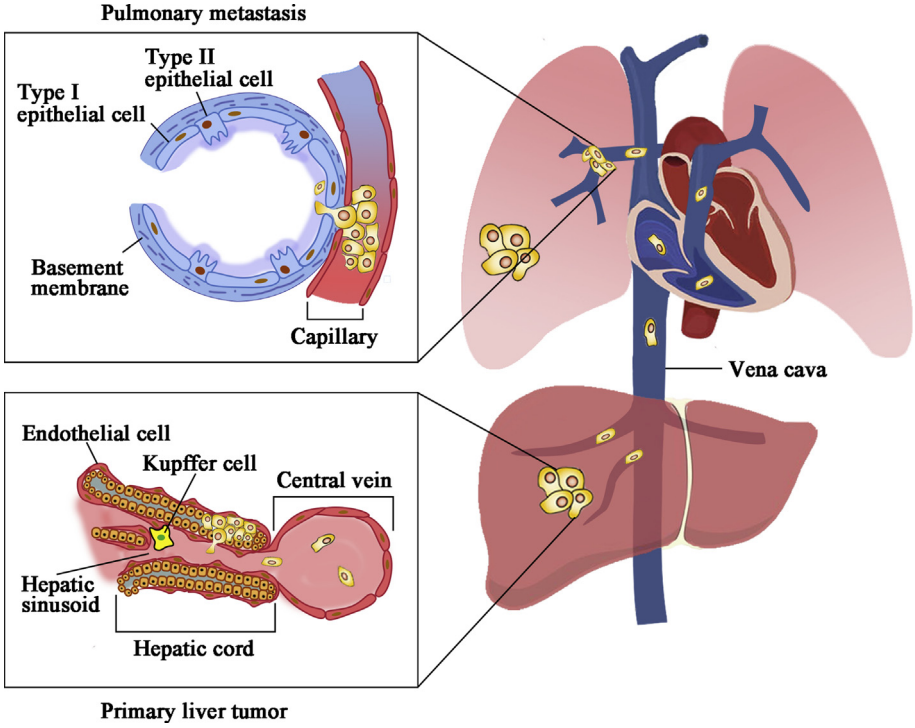

Figure 1 Primary HCC tumor metastasizes to the lung via bloodstream in clinical settings. Experimental models of HCC established by orthotopic implantation of intact tumor tissue into nude mice optimally mimic human primary HCC, as the tumor microenvironment is also transplanted, creating similar liver and lung microenvironments favorable for the growth of metastatic cells.

showed that oxyresveratrol could prevent tumor growth and lymphatic metastasis by inhibiting tumor angiogenesis and lymph-angiogenesis.

The current status and development of HCC animal models

Chemically induced and PDX models are currently not meeting the demand of the research on hepatocarcinogenesis mechanism in specific liver disease context. On the one hand, the histopathological process and mutation profile vary in different liver disease, requiring HCC model induced by corresponding liver diseases or gene mutation. On the other hand, PDX models using immunocompromised mice lack one or more major human immune cells, and was not able to replicate full human immune response. Therefore, humanized mouse models with minimal differences between human and mouse immune system are developed to provide an ideal model for reliable translational studies of immunotherapy in HCC.

Since the first genetically engineered mouse (GEM) model was established by Chisari[64] in 1989, the techniques in GEM model construction have been gradually improved. Nowadays, there are mainly four kinds of methods, including mouse embryo manipulation, Cre-Lox recombination, hydrodynamic injection and clustered regularly interspaced short palindromic repeats (CRISPR)-CRISPR associated protein 9 (Cas9).[65] GEM model related studies have been focusing on the role of specific gene and the interaction of different genes in hepatocarcinogenesis. Through the knock in or knock out of HCC-associated genes, the process of tumor development including hepatitis, hepatocyte hyperplasia, hepatic adenoma and HCC can be highly replicated from clinical HCC (Synopsis of genetically engineered HCC model has been listed in Supplementary Table 1). Therefore, GEM models are ideal and important tools in the exploration of the mechanism of hepatocarcinogenesis. However, the metastatic potential of GEM HCC models is not clear yet, with only few reports mentioning metastasis status, but lacking detailed illustration (Supplementary Table 1).[66–71] In the future development of GEM HCC models, the knock in of metastasis regulator genes, such as MET,[72] could be a promising way to facilitate the study on the metastasis mechanism of HCC. Apart from GEM models, several liver disease-associated HCC models have been successfully established, including HBV/HCV models,[64,73–79] alcohol models[80–82] and non-alcoholic fatty liver disease or non-alcoholic steatohepatitis model (methionine and choline-deficient diet model[83–86]; choline-deficient L-amino-defined diet model[87–89]; high fat diet model[89–92]; fast food diet model[93,94]).

Although the development of liver disease-associated HCC model has mimicked different disease settings to the most extent, most of them failed to replicate full immune response, as well as the feature of metastasis, which is still not sufficient for translational studies, especially preclinical immunotherapy. Up to now, several techniques have been developed to mimic tumor microenvironment, one of which is to transplant human hematopoietic stem cells and precursor cells into the marrow of sub-lethally irradiated mice. And several models for immunotherapy had been successfully established.[95,96] However, an ideal model replicating patient tumor microenvironment and immune response have not been established, requiring further technical advancement. The application of GEM models, liver disease-associated HCC models and humanized HCC models could devote to the construction of HCC animal models which might replicate human HCC to the most extent. However, further emphasis is need on the phenotype selection of HCC metastasis in the above three models.

Cell models

High metastatic human HCC cell lines: MHCC97

Before the 2000s, there were already many kinds of human HCC cell lines, for example, BEL-7402,[97] PLC/PRF/5[98] and SMMC-7721.[99] But most of them lack the phenotype of metastasis or haven't been demonstrated. The metastatic ability, organ affinity, and stability of unexamined cell lines were often indeterminate, though they had some metastatic potential. Therefore, it is inaccurate to name all human HCC cell lines metastatic cell lines. And common HCC cell lines were usually not suitable for experimental studies of HCC metastasis.

It is a complex task to develop metastatic HCC cell lines. However, it is also a compulsory requirement for the studies in HCC metastasis, for example, the procedure of metastasis, metastatic mechanism, the interaction between cancer cells and host animals, the relationship between metastasis, adhesion molecular, oncogenes and their products, and the interventional studies in HCC metastasis. Thus, metastatic HCC cell lines are fundamental experimental materials for the studies of metastatic HCC. The Liver Cancer Institute of Fudan University has been devoted to the development of metastatic HCC cell lines, and Dr. Tian was the first to create the groundbreaking work in this field, establishing human HCC cell line MHCC97 with spontaneously high metastatic potential.[100,101]

High and low metastatic potential HCC cell lines with similar genetic background

The successful establishment of MHCC97 cell line had significantly promoted the studies in the biological features of human HCC. All kinds of cancers, including HCC, develop the diversity of differentiation and phenotype caused by cellular mutations under the influence of genetic instability of cancer cells plus the selective pressure from host and other environmental factors. Heterogeneity pertaining to solid tumor tissue or cell lines refers to the fact that they are composed of a variety of cancer cell clones. It is valuable to screen cell strains of different biological features from a heterogeneous cell line, so that the cell strains can be used for comparative experiments, drug test, and differentiation-inducing therapy. Not all cancer cells in the primary tumor tissues possess the abilities of invasion and metastasis, but some specific subclones do. Even in such subclone groups, the variation in the metastatic potential may still exist. If considering the influences from the host animal and environment, the differences of metastatic

phenotype between different cell subclones may be even more significant. MHCC97 cell line isolated from metastatic LCI-D20 had greater metastatic potential, but it was still a heterogeneous cell group consisted of a variety of cell subclones, including cancer cells with high metastatic ability, low metastatic ability, and even no metastatic ability. Isolating and comparing the difference of cell subgroups with different metastatic phenotype would be helpful for the studies of the mechanism and the finding of molecular marker of HCC metastasis.

Our research group performed *in vitro* monoclonal cell culture on MHCC97. Cell strains from single cells were performed initial screening in nude mice to further identify targeted cell strains. We eventually established cell clone with high metastatic potential (MHCC97-H) and low metastatic potential (MHCC97-L).[102] Comparing these 2 cell clones, MHCC97-H had smaller cell size and faster *in vitro* and *in vivo* growth rate. The result of Boyden chamber *in vitro* invasion assay showed that the number of penetrating cells through the artificial basement membrane in MHCC97-H was significantly higher than MHCC97-L.

At the same period, based on the constructed model,[19] Ogawa et al[103] increased the dosages of DEN to 200 mg/kg and NMOR to 120 ppm. HCC model with pulmonary metastasis was successfully induced on week 24. Four monoclonal cell lines (C1, C2, C5F, and C6) were isolated and established from one murine tumor nodule. Another two cell lines, N1 and L2 were isolated from another primary HCC and pulmonary metastasis respectively. These six cell lines showed similar histopathology after subcutaneous inoculation, but varied vastly in metastatic phenotype when transplanted in different ways. When inoculating cells to KSN nude mice subcutaneously at the dosage of 5×10^6/0.2 ml, C5F monoclonal cell line had the highest pulmonary metastatic rate (89%, 8/9) 5–7 weeks after inoculation. When inoculating cells through tail vein, the N1 and L2 cell line had the highest rates (100%, 12/12 and 3/3 respectively) of pulmonary metastasis, while C5F had the lowest metastatic rate (0%, 0/8). When inoculated by intraperitoneal injection, C1, C6, N1, and L2 caused obvious hemorrhagic ascites and intraperitoneal dissemination, with infiltration in hepatic capsule in N1 cell line and pulmonary metastasis in C6 and C5F. Lower expression level of *KAI-1* and heparinase genes in C5F were observed compared with other cell lines, indicating heterogeneity in metastatic model.

HCC cell models selected by different rounds of pulmonary metastasis in nude mice

On the basis of the work finished above, our team inoculated MHCC97-H to nude mice to further screen by three, six, and nine rounds of pulmonary metastasis selection. Pulmonary metastasis from the third, sixth, and ninth round screens were harvested to establish cell strains, named HCCLM3, HCCLM6, and HCCLM9 (Fig. 2), with unique characteristics of high pulmonary metastatic rate.[104] All the three cell strains presented as polygonal epithelial cancer cells, with similar diameter for around 50 μm, rich cytoplasm, large and round nucleus with homogeneous light red staining, clear nuclear membrane and two to seven large and clear nucleoli. Cells were arranged with single layer

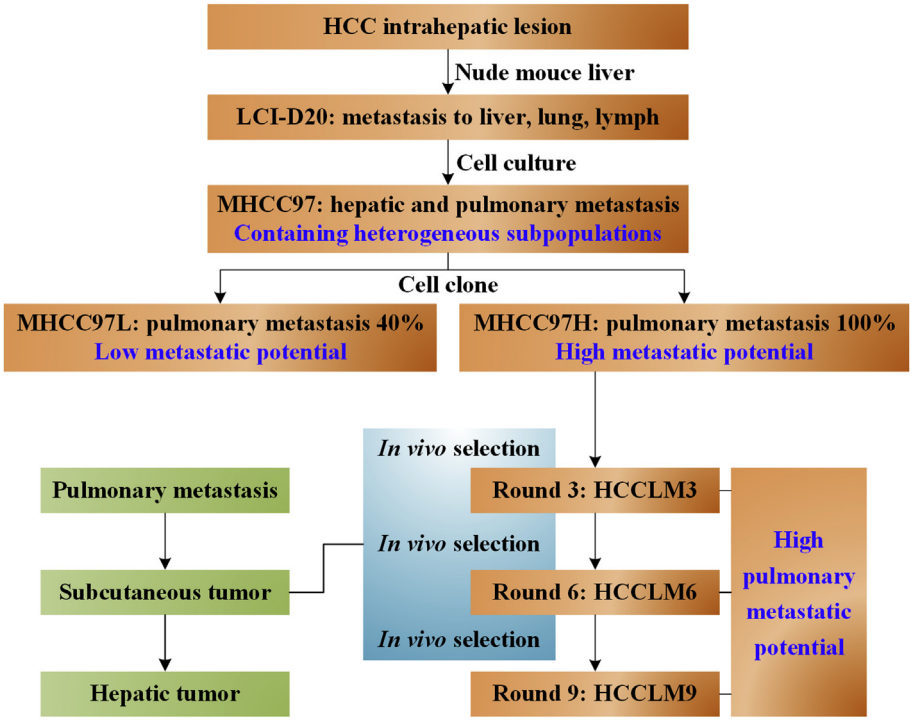

Figure 2 The establishment procedure of MHCC97L, MHCC97H and three high pulmonary metastatic potential cell clones by sequential *in vivo* selection. MHCC97H cell clones isolated from LCI-D20 metastasis was inoculated to BALB/c nude mice, and the pulmonary metastasis were re-inoculated into nude mice for three, six and nine rounds of in *vivo* pulmonary metastasis selection.

and mosaic way, and cytoplasmic bulge were observed between cells. Immunohistochemistry showed positive for AFP, albumin, cytokeratin 8, P16, and negative for P53, nm23, HBsAg. Genetic examination showed HBV DNA integration in cellular genome. Sub-triploid karyotype were found in both cell strains, with the number of chromosomes ranged from 55 to 58 for HCCLM3, and 54 to 57 for HCCLM6.

The application of human HCC cell lines with metastatic potential

HCC metastatic cell lines have been widely applied since they were established, mainly in the field of mechanism exploration and prediction of HCC metastasis.

In the mechanism studies, HCC metastatic cell lines were mainly applied to explore the roles of gene expression, pathways, and epithelial mesenchymal transition (EMT) in HCC metastasis (Fig. 3).

(1) Gene expression and pathways: *KAI1* gene down-regulation was associated with many cancerous invasion and metastasis. Yang et al[105] transfected sense and antisense *KAI1* expression plasmid to the MHCC97-H cell lines, discovering that *KAI1* gene played a role in inhibiting metastasis of HCC cells. Yin et al[106] had proved that co-expression of stemness factors Oct 4 and Nanog were related to metastatic potential of HCC cells. In the following study, Yin further demonstrated that co-expression of genes *Oct4* and *Nanog* contribute to EMT change to promote tumor migration, invasion/metastasis through Stat 3/Snail signaling.[107]

In the pathway studies, Zhang et al[108] proved that cancer-associated fibroblasts (CAFs) can transfer miRNA to HCC cells, by which way miR-320a was able to inhibit MHCC97-H cell proliferation, migration, and metastasis by binding to the direct downstream target PBX3. Thus, miR-320a-PBX3 is an antitumor pathway by inhibiting the activation of MAPK pathway. The loss of exosomal miR-320a contributes to the malignant characteristics of HCC. Zhao et al[109] knockdown ST6Gal-I in MHCC97-H cell lines, and found that proliferation, migration, invasion, and tumor volume in PDX model decreased. *In vitro* study revealed that ST6Gal-I may enhance HCC tumor-genesis and metastasis via the modulation of Wnt/β-catenin signaling pathways.

(2) EMT: EMT was reported to be facilitated by many regulators, and had been shown to be an important mechanism in HCC metastasis and invasion.[107,108,110,111] Xu et al[110] demonstrated that the depletion of SIN1 in MHCC97-H and HCCLM3 cell lines inhibited Akt phosphorylation, leading to down-regulation of Snail, Vimentin, MMP9, and N-cadherin and up-regulation of E-cadherin. And the restoration of Snail expression promoted invasion and migration of HCC cells, demonstrating the promoting role of EMT in HCC cell metastasis. Besides, EMT might also be regulated by Oct 4, Nanog through Stat 3/Snail signaling,[107] CAFs through miR-320a-PBX3 pathway,[107] and ACA11 through PI3K/AKT pathway.[111]

(3) Quantum dots (QD): It's hard to predict or make early diagnosis on HCC metastasis due to the lack of specific biomarker probe. The establishment of HCC

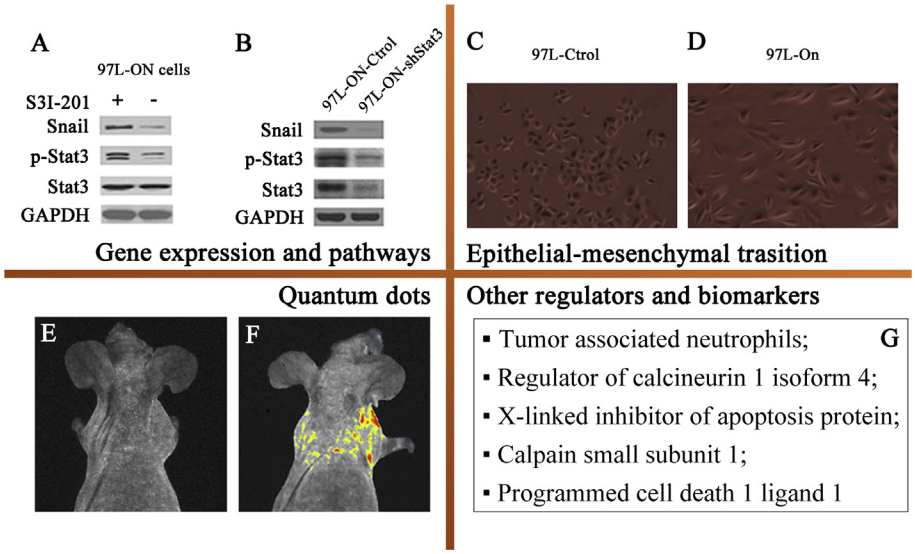

Figure 3 The application of human HCC cell lines with metastatic potential. A: Oct 4/Nanog induced Snail expression was significantly inhibited by a specific Stat 3 inhibitor S3I-201; B: Knockdown of Stat3 reversed Oct-4/Nanog-induced overexpression of Snail in 97 L-ON cells, indicating that co-expression of genes *Oct4* and *Nanog* might modulate metastatic potential by Stat 3/Snail signaling pathway. Picture A and B were cited from Yin et al[79] C and D: MHCC97 L cells (C) underwent dramatic morphologic changes into mesenchymal, fibroblast-like phenotype (D) after treatment with ectopic Oct4 and Nanog expressions. Picture C and D were cited from Yin et al[79] E and F: Comparing to the control group (E), the whole-body imaging showed that the quantum dots fluorescence was localized in the pulmonary metastasis of the model (F), showing the promising function of quantum dots for predicting HCC metastasis. Picture E and F were cited from Chen et al[85] G: Other regulators and biomarkers.

metastatic cell lines made it possible to develop specific and sensitive biomarker probe. QD is one kind of organic dye characterized by broad excitation spectra, size-tunable fluorescence, high photostability, and long fluorescence lifetime.[112] And it is proved to be stable, specific, and biocompatible for ultrasensitive fluorescence imaging of molecular targets in established HCC model system.[113] In the further study, Wang et al[114] labeled HCCLM9 and MHCC97-L cell lines with 6 potential aptamers, and found that LY-1 was a promising molecular probe for the prediction or early diagnosis of HCC.

(4) Other regulators and biomarkers: Zhou et al[115] reported that HCCLM3 formed larger tumors when co-injected with tumor associated neutrophils (TANs). And the mechanism study demonstrated that TANs might promote tumor growth, progression, and resistance to sorafenib through recruiting macrophages and Treg cells to HCC. Jin et al[116] proved that regulator of calcineurin 1 isoform 4 (RCAN1.4) significantly reduced proliferation, migration, and invasive activity of HCCLM3. The downregulation of RCAN1.4 may promote the metastasis of HCC. Besides, including X-linked inhibitor of apoptosis protein,[117] calpain small subunit 1 (Capn4),[118] programmed cell death 1 ligand 1 (PD-L1),[119] CD24,[120] CD151[121], and β-catenin[122] were also possible molecular biomarkers in the diagnosis and therapeutic target of HCC.[2]

Summary

As presented above, there are few models applicable in the studies of HCC metastasis. However, the construction of HCC model with metastasis is far more difficult than routine tumor models. Because, the establishing of HCC model with metastasis is equal to establishing an effective, reliable, and stable experimental system combining *in vivo* and *in vitro* studies, which provides macroscopic observation system replicating the process of metastasis from primary organ to distant organ. Among the limited models, HCC model with spontaneous metastasis, represented by LCI-D20, has several specific advantages. First, the tumor sample used for grafting originated form a patient, with biological behaviors closest to clinical conditions. Second, the model had initially formed a relatively complete system. Human HCC cell line MHCC97 was isolated from LCI-D20, and was successfully isolated for the establishing of MHCC97-H and MHCC-97 L cell strains with different metastatic potential. Furthermore, HCCLM3 and HCCLM6 cell strains from the metastasis of MHCC97-H were isolated and cultured, whose metastatic ability was stronger and biological behavior was closer to clinical course of HCC. This system provided a good foundation for the mechanism studies of HCC metastasis. Third, the studies using this system had produced some encouraging findings, such as the relationship between angiogenesis and metastasis, the hypothesis of multiple genes and multiple stages during metastasis, and the basic and clinical studies on the anti-metastasis of interferon. Throughout the history of the development of the HCC model system with metastasis, it can be seen that every progress in model improvement will greatly promote the understanding of the mechanisms of HCC metastasis and the exploration of new prevention and treatment strategies. The direction of future efforts should be focused on the design of scientific and effective experimental protocols. For example, high-throughput analysis techniques can be applied to find HCC metastasis-related genes and proteins and explore the metastatic rules of HCC models with metastasis. And the final destination is the development of a panel of gene-based biomarkers to predict HCC metastasis and design molecular targeted therapies. Besides, GEM models, liver disease-associated HCC models, and humanized models are models more identical with human HCC and HCC metastasis regarding to the liver disease context.

Funding

This work was supported by Beijing Municipal Administration of Hospitals' Ascent Plan [grant number DFL20180701]; Special Fund for the Capital Characteristic Clinical Medicine Development Project [grant number Z161100000516077]; Beijing Municipal Grant for Medical Talents Group on Peritoneal Surface Oncology [grant number 2017400003235J007]; Beijing Health and Science Technology Achievement and Appropriate Technology Promotion Project [grant number 2018-TG-27] Beijing Natural Science Foundation [grant number 7172108].

Conflict of interests

The authors declare no conflict of interests.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.gendis.2019.12.008.

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