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Hepatocellular carcinoma drug resistance models

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Abstract

Hepatocellular carcinoma (HCC) is the second leading cause of cancer-related death worldwide. Although drug therapy has been well developed and applied, its clinical efficacy is limited due to primary or acquired drug resistance in most HCC patients. Therefore, it is of great clinical significance to elucidate the key molecular mechanisms of resistance and improve the sensitivity of HCC cells to drugs. At present, a variety of HCC drug resistance models have been developed to find out resistance mechanisms, screen biomarkers, and explore strategies to reverse drug resistance, including traditional HCC drug resistance models, HCC patient-derived drug resistance models, three-dimensional drug resistance models, transgenic drug resistance models, and multi-drug resistance models. Here, we searched PubMed, Embase and Web of science for studies related to HCC drug resistance models in recent years, systematically summarized the established methods and characteristics of these models, reviewed their applications and compared their advantages and disadvantages, aiming to provide reference for the selection of appropriate models for HCC drug resistance research.

Keywords HCC, Drug resistance, Cell line, Patient-derived xenograft, Transgenic model

Introduction

Hepatocellular carcinoma (HCC) is the most common primary liver cancer, accounting for about 90% of all cases and the second leading cause of cancer-related deaths worldwide [1, 2]. Although significant progress has been made in the prevention, diagnosis and treatment of HCC, more than 50% of HCC patients are in the middle or advanced stage at the time of diagnosis, and 70% of patients relapse within the first five years of initial treatment due to its insistent and rapid onset [3]. Early HCC may be managed with radical interventions such as surgical resection, while systematic therapy like chemotherapy is the mainstay for improving survival in middle and advanced stages of HCC [4]. Although traditional drug regimens such as cytotoxic chemotherapy and tyrosinase inhibitors, as well as the most popular immunotherapies in recent years such as immune checkpoint inhibitors, can extend progression-free survival and overall survival of HCC patients to some extent, patients will inevitably develop resistance. And about 90% of HCC patients died due to resistance [5]. Therefore, elucidating the mechanism of HCC drug resistance and exploring reversal strategies are key clinical issues that need to be addressed. HCC drug resistance models are reliable tools to solve these problems. In recent years, the use of various HCC drug resistance models has led to the discovery and proposal of molecular mechanisms and reversal strategies. Some of these strategies have shown promising clinical efficacy [6–8].However, with the advent of the new drug era, there are numerous models

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for drug resistance in HCC, each presenting its own set of advantages and disadvantages, making it challenging for researchers to identify the most suitable model from this array. Therefore, we reviewed the established methods (Fig. 1) and characteristics of the main HCC drug resistance models, such as traditional HCC drug resistance model, HCC patient-derived drug resistance model, three-dimensional drug resistance model, transgenic drug resistance model and multi-drug resistance model, discussed their applications and compared their advantages and disadvantages (Table 1), in order to offer guidance in selecting appropriate drug resistance models for related research.

Traditional HCC drug resistance model

Traditional HCC drug resistance models are based on commercial HCC cell lines, which are mainly divided into in vitro models and in vivo models. In in vitro models, commercial HCC cell lines are cultured continuously in increasing concentrations of drug for weeks to months until drug-resistant HCC cell lines are formed. For the establishment of in vivo models, BALB/c nude mice or nu/nu mice are most commonly used. Drug-resistant HCC cell lines can be directly transplanted into mice. Drug-resistant mice can also be established by subcutaneous or orthotopic transplantation of commercial HCC cell lines into mice, followed by continuous administration. At present, there are only about 30 HCC cell lines [9]. HuH7 and Hep-3B are two classic HCC cell lines. Based on the traditional HCC drug resistance models formed by these cell lines, a clear gene-protein-phenotype drug resistance axis is constructed outside clinical trials, providing a solid material basis for preclinical exploration of drug resistance mechanisms and reversal strategies.

The commercial HCC cell lines used in in vitro models retain the characteristics of primary cancer cells, and have the advantages of cell homogeneity, easy gene editing, and reproducible results. In vitro models are mainly used to identify the expression of various genes and proteins, but they also face problems such as genomic instability and lack of specific tumor microenvironment [9, 10]. In vivo models can better simulate the tumor microenvironment. In practice, both models are often used together to further verify the drug resistance pathways and molecular mechanisms, and can also be used for drug screening and efficacy verification to reverse HCC resistance [11]. Doxorubicin, sorafenib and lenvatinib are major research objects, probably because they are the most widely used drugs in clinical practice, and also

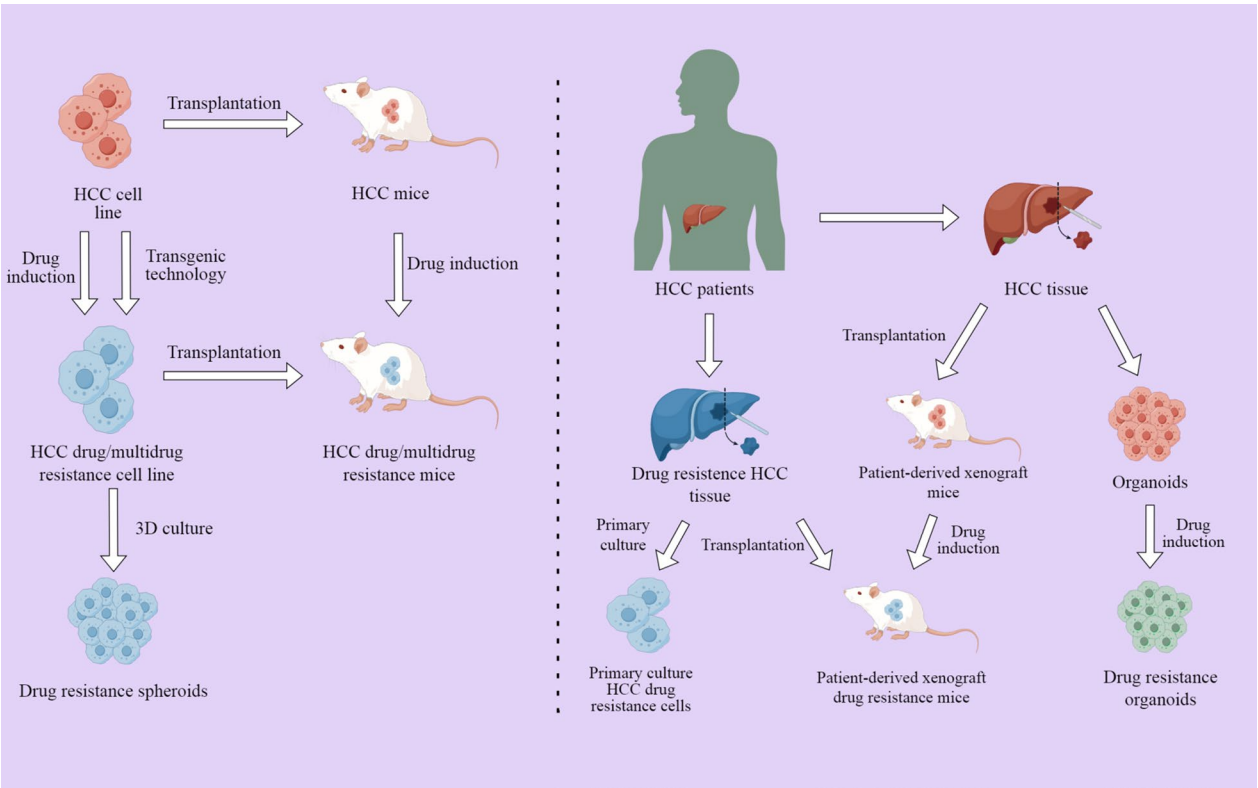

Fig. 1 Establishment process of HCC drug resistance model

Table 1 Establishment methods, advantages and disadvantages and applications of major HCC drug resistance models

Models		Establishment methods	Advantages	Disadvantages	Applications
Traditional HCC drug resistance model	In vitro model [9]	Commercial HCC cell lines are cultured for months at increasing concentrations of the drug	Retain the characteristics of primary cancer cells, have cell homogeneity, easy genetic manipulation and easy reproduction of results	Genomic instability and lack of specific tumor microenvironment	Identify the expression of various resistance related genes and proteins
	In vivo model [11]	HCC resistant cell lines are transplanted into mice, or HCC cell lines were transplanted into mice and then administered repeatedly to form HCC resistant mice	Better simulate the tumor microenvironment	Time-consuming, requires a lot of animals	Drug screening and validation for reversing HCC resistance
HCC patient-derived drug resistance model	HCC patient-derived drug resistance cell line [33]	Derived from cancer tissues from HCC resistant patients treated by primary cell culture and passage	Preserve the gene and drug resistance characteristics of individual HCC cells	The small number of biopsy samples, the small tissue sizes available, and the technical difficulties in isolating and culturing cells	Analyze the individual characteristics of HCC, study the drug resistance mechanism of different HCC subtypes
	HCC PDX drug resistance model [34]	The cancer tissue of drug-resistant HCC patients was transplanted into immunodeficient mice, or the cancer tissue was transplanted into immunodeficient mice and then given a certain dose of the drug through continuous iteration and finally acquired resistance	Better retain the clinical characteristics of tumors, and stronger correlation between drug response and clinical efficacy, provide the body physiological environment for tumor growth	Expensive, low success rate of transplantation, long culture time, and the original human stromal cells in the tumor are gradually replaced by mouse stromal cells with the growth of xenograft	Studies of human-tumor-animal interactions and mechanisms of drug resistance, screening for drugs that reverse resistance, and preclinical testing of individualized treatment strategies
Three-dimensional drug resistance model [43]		3D culture technology	Better preserve the characteristics of the original tumor and can be studied at the genetic level	Expensive and time-consuming, low success rate of modeling, and difficult to reproduce complex TME	Research on potential targets of new drugs and identification of prognostic biomarkers and mechanisms of drug resistance
Transgenic drug resistance model [62]		Gene editing technologies, such as CRISPR/Cas9	Stable, the detailed mechanism of resistance can be studied	Complex operation, time-consuming	Study on single drug resistance mechanism
Multidrug resistance model		Drug or physical induction, transfection of multi-drug resistance genes	The mechanism of multidrug resistance in tumors can be studied	Cross resistance to cytotoxic chemotherapy drugs	Study on multi-drug resistance mechanism and reversal strategy

the drugs with the most reported drug resistance. Table 2 summarizes traditional HCC resistance models induced by other drugs.

HCC doxorubicin resistance model

Doxorubicin, a natural anthracycline, has broad spectrum anticancer activity and is the standard treatment for advanced liver cancer [12]. In order to clarify the key molecular mechanism of doxorubicin resistance in HCC and enhance the sensitivity of HCC cells to it, a variety of HCC doxorubicin resistance models have been established. Zhuo et al. [13] treated the parental Huh-7 cell line with doxorubicin at an initial concentration of 0.02 μg/ml, gradually increasing the concentration to 4 μg/ml, and formed a doxorubicin Huh-7 resistant cell line after 10 months. Compared with the parental Huh-7 cell line, the doxorubicin-resistant cell line showed significant differences in miRNA expression, with 53 miRNAs up-regulated and 52 miRNAs down-regulated. By the same method, they constructed resistant Huh-7 cell lines of cisplatin, carboplatin, mitomycin C and vincristine respectively, and found that miRNA expression was different among these cell lines. However, miR-27b, miR-181a, miR-146b-5p, miR-181 d and miR-146a were significantly upregulated in all the five drug-resistant cell lines. Using a similar approach, miR-215 [14] and miR-760 [15] have been confirmed as key targets of doxorubicin drug resistance in subsequent studies. Cao et al. [16] obtained doxorubicin-resistant Huh-7 and Hep3B cell lines by the same method, and in vitro experiments found that lncRNA MALAT1 was highly expressed in doxorubicin-resistant Huh-7 and Hep3B cells, and promoted drug resistance of liver cancer cells through the MALAT1/miR-3129-5p/Nova1 axis. Moreover, Li et al. [17] found that circRNA was highly expressed in doxorubicin-resistant Huh7 and HCCLM3 cells. Zhang et al. [18] found that NAT10 was involved in doxorubicin resistance by regulating EMT in a xenograft model based on Huh-7 cell lines. In addition to nucleus-related genetic material, Zhu et al. [19] also found that mitochondrial DNA TFAM was up-regulated in Huh-7 cell lines resistant to doxorubicin and sorafenib, suggesting that TFAM may be related to the proliferation and chemotherapy sensitivity of hepatoma cells.

HCC sorafenib resistance model

Sorafenib, a multikinase inhibitor, was approved by the US FDA in 2007 for the treatment of unresectable HCC patients and remains the first-line treatment for advanced HCC [20]. The common clinical dose of sorafenib is 400 mg bid, which significantly prolongs the median survival of HCC patients [21]. However, resistance often occurs within 6 months of treatment [22]. In order to elucidate the key molecular mechanism of sorafenib resistance in HCC patients, researchers used different methods to establish HCC sorafenib resistance model. Huang et al. [23] exposed HuH7 to 0.5 μM of sorafenib for a long period of time and gradually increased the dose to 10 μM, thereby achieving stable resistance. They found that ABCC5 was significantly upregulated in sorafenib resistant HCC cells and produced acquired resistance via the PI3 K/AKT/NRF2 axis. Lu et al. [24] used 0.625 μM sorafenib as the starting concentration, and gradually increased the concentration to 10 μM (the highest clinically achievable concentration) to establish sorafenib resistant Huh7 and Hep3B cell lines. In in vitro models, they found that CD24 was overexpressed in resistant

Table 2 Traditional HCC resistance models induced by other drugs

Drug	Commercial HCC cell line	Mechanism	In vitro/in vivo	Reversal drug of resistance	Establishment of drug resistance	References
Oxaliplatin	Bel-7402	EMT	In vitro/in vivo		The initial drug concentration of 2 μmol/l was cultured for four cycles, and then the concentration was progressively increased to 4, 6, 12, 24 and 48 μmol/l over 9 months	[63]
Oxaliplatin	SNU-182	PBK/TOPK	In vitro		The initial drug concentration was 0.1 μM for one month and then increased by 0.1 μM every 2 weeks until 1 μM	[64]
Cisplatin	LM3, Huh7	Aerobic glycolysis	In vitro	Canagliflozin	10–25 μM, four months	[65]
Regorafenib	SMMC-7721, Huh7	Pin1	In vitro		Low concentration (0.5 μM) and intermittent gradient induction for 6 months	[66]
5-FU	BEL7402	MRP1, Bclxl, TS	In vitro		1 μmol/l for 10 months	[67]
Bortezomib	HuH7	Proteasome	In vitro		A gradual increase in the concentrations of drug for at least 6 months from 40 to 3000 nM	[68]

cells. Then, they subcutaneously transplanted the resistant cell lines into nu/nu mice, and found that CD24 promoted sorafenib resistance through autophagy regulation. Xu et al. [25] found a new circRNA in LM3 resistant cells cultured for 12 months with gradually increasing the dose of sorafenib. This circRNA can be upregulated in sorafenib resistant HCC cells, and by binding to YBX1, a major oncogenic protein in the cytoplasm, prevents the nuclear interaction between YBX1 and E3 ubiquitin ligase PRP19, thereby blocking PRP19-mediated degradation of YBX1 and promoting drug resistance of HCC cells. Wu et al. [26] placed Huh7 and LM3 cells in an environment of 1% O_2 and gradually increasing sorafenib concentration to construct hypoxia-induced sorafenib resistant HCC cell lines. The resistant cells showed the downregulation of the mitochondrial autophagy core regulatory gene ATAD3 A and regulation of overactivated mitochondrial autophagy by the ATAD3 A-PINK1/PARKIN axis. ATAD3 A may be a functional target of miR-210-5P, and further in vivo experiments suggest that targeting the axis of Mir-210-5p-ATAD3 A may be a novel therapeutic target for sorafenib resistant HCC patients.

HCC lenvatinib resistance model

Lenvatinib, like sorafenib, is a multikinase inhibitor that was approved by the U.S. FDA in 2018 as first-line treatment for unresectable HCC [27]. Lenvatinib shows a longer median survival compared to sorafenib [28], but still inevitably develop resistance after 7 months of treatment [29]. The HCC lenvatinib resistance model established by different methods has become an important tool for many researchers to explore the mechanism of lenvatinib resistance. Ao et al. [30] treated parental Huh7 cells with 2 mM of lenvatinib, and then the concentration gradually increased at a rate of 1 mM per week, reaching 10 m M after 2 months, and finally established a lenvatinib resistant Huh7 cell line. Lenvatinib resistant Huh7 cells showed the activation of MAPK/MEK/ERK signaling pathways and upregulation of epithelial mesenchymal transformation markers, with the upregulation of 16 cytokines (such as vascular endothelial growth factor (VEGF), platelet-derived growth factor-aa (PDGF-AA)), and has strong proliferation and invasion ability. Zhao et al. [31] established two lenvatinib resistance in vitro models with different methods. Huh7 cell lines were incubated with lenvatinib below IC50 and the lenvatinib dose was slowly increased at 0.25 μmol/l/time for 6–7 months, thus establishing lenvatinib-resistant Huh7 cell lines. They found that the expression of vascular endothelial growth factor receptor 2 (VEGFR2) and its downstream RAS/MEK/ERK signaling were significantly upregulated in lenvatinib-resistant HCC cells, while the expressions of VEGFR1, VEGFR3, FGFR1-4 and PDGFRα/β were not significantly different. ETS-1 was identified as the cause of VEGFR2-mediated lenvatinib resistance. By establishing three kinds of drug-resistant HCC models, Hu et al. [32] found that HCC cells could enhance their exocytosis to lenvatinib by activating EGFR and stimulating the EGFR-STAT3-ABCB1 axis, thereby developing resistance to lenvatinib. First, they cultured HuH7 and PLC/PRF/5 cells in 3 μmol/L and 20 μmol/L lenvatinib medium, respectively, and changed the cell medium every 48 h until the cells had spread 90% in the dish, and then passed. After the replication and passage of the cells, the concentration was increased by 0.5 μmol/L until the rapid proliferation of HuH7 cells at 30 μmol/L lenvatinib and the rapid proliferation of PLC/PRF/5 cells at 60 μmol/L lenvatinib, which took at least 6 months, and finally formed lenvatinib-resistant HuH7 and PLC/PRF/5 cell lines. In addition, they established a lenvatinib-resistant C57BL/6 mouse, that is, after subcutaneous injection of Hep1-6 cells into mice to form solid tumors, lenvatinib was given at a dose of 10 mg/kg/d for 28 days and repeated for 3 generations, finally forming a stable lenvatinib-resistant mouse model. Combined with in vivo and in vitro results, they found that EGFR and ABCB1 inhibitor erlotinib can effectively inhibit the exocytosis of HCC cells against lenvatinib, reduce drug resistance and increase anti-cancer effects.

HCC patient-derived drug resistance model

Since traditional HCC drug resistance models cannot reflect the complexity of the interaction between human tumor microenvironment and other factors, HCC patient-derived drug resistance cell lines based on primary cell culture technology and HCC patient-derived xenograft (PDX) drug resistance models based on xenotransplantation technology can better overcome those limitations. HCC patient-derived drug resistance cell lines are the primary HCC drug resistance cell lines obtained through a series of processes including resection of HCC drug-resistant patients'cancer tissues, tissue digestion, cell isolation and purification, and primary cell culture. HCC PDX drug resistance models can be established in immunodeficient mice by orthotopic (liver) or ectopic (subcutaneous) transplantation of patient-derived drug-resistant HCC tissues, and can also be iteratively obtained by transplanting non-drug-resistant HCC tissues into immunodeficient mice and then giving them a certain dose of drugs. The primary HCC drug resistance cell lines can retain the genes and drug resistance characteristics of HCC cells, but there are also problems such as a small number of biopsy samples, small available tissue size, and technical difficulties in isolating and culturing these cells [33]. HCC PDX drug resistance models can preserve the proportion of primary tumor

cells and stromal cells, histopathological structure, mutation information, gene profile and tumor microenvironment [34]. Compared with the traditional in vivo drug resistance model, HCC PDX drug resistance model can better retain the clinical characteristics of tumors, and the correlation between drug response and clinical efficacy is also stronger, but there are some problems such as expensive, low transplant success rate, and long culture time [35]. More importantly, as the xenograft grows, the original human stromal cells in the tumor will gradually be replaced by mouse stromal cells [36]. At present, sorafenib is often used to establish HCC patient-derived drug resistance models (Table 3), which are mostly used for screening and validation of combination therapy strategies and mechanism studies on drug resistance and reversal.

HCC patient-derived drug resistance cell line

Lim et al. [37] isolated primary drug resistance HCC cells (YUMC-R-H1, YUMC-R-H2, YUMC-RH3, YUMC-R-H4 and YUMC-R-H5) from 5 sorafenib resistant HCC patients, and cultured them in RPMI-1640 medium containing 15% fetal bovine serum. Compared with sensitive HCC cells, these resistant HCC cells exhibited significantly upregulated stem cell markers (e.g. CD13, CD24, CD44, CD90, and Oct4), significantly increased levels of calcium-mediated and survival-related target genes (e.g. ATP2 A subtypes and B-cell lymphoma 2 (Bcl-2)), and high enrichment of Notch, calcium, and cancer stem cell signaling pathways. In addition, SERCA, a key regulatory protein involved in calcium homeostasis and anti-apoptosis, was significantly expressed in drug-resistant HCC cells, providing a material basis for exploring whether SERCA inhibitors are candidate drugs to reverse sorafenib resistance in HCC. Hashiba et al. [38] obtained sorafenib resistant HCC cell lines (HCC-SR) from HCC patients who received sorafenib treatment in STORM trial and were eventually resistant to sorafenib.

HCC PDX drug resistance model

Lim et al. [37] established a HCC PDX drug resistance model by subcutaneous injection of 4.4×10^6 primary drug-resistant HCC cells into female NOG mice, and found that the combination of SERCA inhibitors and sorafenib could significantly reduce the tumor size. Liao et al. [39] transplanted tumor tissue from HCC patients into the axilla of 5–6 week-old mice with severe NSG immunodeficiency to construct a PDX model. When the tumor size reached 100 mm^3, sorafenib was given intragastric administration of 80 mg/kg/day. And when the tumor size reached 400–500 mm^3, the tumor tissue was stripped and inoculated

Table 3 HCC patient-derived drug resistance model

Drug	Source of cell line	Mechanism	In vitro/in vivo	Reversal drug of resistance	Establishment of drug resistance	Reference
Sorafenib	Cancer tissue from HCC patients	SCD1	In vivo	SCD1 inhibitor	Mice orally administered 10 mg/kg/day for 26 days	[69]
Sorafenib	Cancer tissue from HCC patients	EPHB2	In vivo		The patient was given 100 mg/kg orally to form resistance, and then the PDX model was established	[70]
Sorafenib mainly	Cancer tissue from HCC patients	SERCA	In vitro/in vivo	SERCA inhibitor	Drug resistant patient tumor tissue	[37]
Sorafenib	Cancer tissue from HCC patients	HIF1α, HSP90α	In vivo	17-AAG	Mice were orally cultured at 80 mg/kg/day for four generations	[39]
Sorafenib	Cancer tissue from HCC patients	CIC	In vitro/in vivo	Regorafenib	Drug resistant patient tumor tissue	[38]
Sorafenib	Cancer tissue from HCC patients	miR-378a-3p	In vivo	GW3965	Mice orally administered 30 mg/kg/day for 21 days	[40]
Sorafenib	Patient-derived HCC 26-0808 A cell line	Wnt/β-catenin	In vivo	Refametinib	Mice orally administered 10 mg/kg/day for 80 to 90 days	[41]
Sorafenib	Cancer tissue from HCC patients	FGF	In vitro/in vivo		Mice orally received 40 mg/kg/day for 100 days	[42]
Sorafenib, Lenvatinib	Cancer tissue from HCC patients	CASP3	In vivo		Sorafenib: Mice 100 mg/kg/day orally for 31 days; Lenvatinib: Mice 30 mg/kg/day for 21 days	[71]
Anti-PD1 therapy	Cancer tissue from HCC patients	circTMEM181	In vivo		Drug resistant patient tumor tissue	[72]

into other immunodeficient mice. After four generations of culture, sorafenib-resistant HCC PDX mice were established. In this model, the expression of HIF1α in internal hypoxic tumor tissues was much higher than that in external hypoxic tumor tissues. In addition, HSP90α directly binds to MLKL to promote chaperon-mediated autophagy degradation under hypoxia, leading to the blocking of necroptosis and eventually acquired resistance. And In vivo studies showed that 17-AAG, an HSP90α inhibitor, can inhibit HSP90α and reverse sorafenib resistance. Hashiba et al. [38] inoculated patient-derived sorafenib resistant cell lines subcutaneously into NOD/SCID mice to establish HCC PDX sorafenib resistant mice. They found CIC mutations and their low expression in HCC-SR cells. Capicua is a general sensor of tyrosine kinase receptor signals encoded by CIC. In vivo experiments showed that regorafenib can inhibit the growth of sorafenib resistant and Capicua inactivated HCC cells, as well as inhibit extracellular signal-regulated kinase phosphorylation. Lin et al. [40] found that the combination of sorafenib with GW3965, an agonist of liver X receptor α (LXRα), can increase the expression of miR-378a-3p and inhibit tumor proliferation, thereby resensitizing resistant cells to sorafenib. Wnt/β-catenin pathway plays an important role in the development of HCC. In order to evaluate whether inhibitors of this pathway can inhibit tumor growth, Huynh et al. [41] established a HCC PDX drug resistance model. They transplanted HCC PDX xenograft cell line HCC26-0808 A into male C.B-17 SCID mice aged 9–10 weeks and administered sorafenib 10 mg/kg/day for 80 to 90 days. When the tumor reached 1500 mm^3, the tumor was isolated and transplanted into other mice until the effect of sorafenib on tumor growth was minimal to form sorafenib resistant PDX mice. It was found that Wnt/β-catenin pathway was activated in drug resistant mice, and sorafenib combined with refametinib significantly inhibited the activation of this pathway and inhibited tumor growth. Hu et al. [42] subcutaneously implanted primary HCC tumor fragments from a 39-year-old female patient into the right side of SCID mice and treated them with sorafenib (40 mg/kg/day, orally, for about 100 days). After passing through Nu/Nu mice, a sorafenib resistant PDX model was established and the resistant cell line LIXC-004SR was isolated. It was found that tumors developed by LIXC-004SR were still resistant to sorafenib and showed significant increase in angiogenesis, which may be due to the activation of FGF pathway to promote angiogenesis. In addition, the LIXC-004SR cell line also exhibited multidrug resistance and genetic instability.

Three-dimensional drug resistance model

In vitro two-dimensional (2D) drug-resistant cell models lack tumor tissue structure and complexity, and cannot accurately reflect the real biological processes and interactions of tumor cells, stroma and tumor microenvironment. Drug-resistant mice have problems such as long experiment period and poor repeatability. Therefore, there is an urgent need for a readily accessible model that can simulate the real HCC microenvironment in humans for drug resistance studies. Three-dimensional (3D) drug resistance models can reproduce the cellular heterogeneity, structure and function of original HCC tissues, and have become a powerful tool to bridge the gap between 2D drug resistance cell models and animal models, including spheres and organoids [43]. Although both spheres and organoids are cells cultured in vitro by 3D culture technology, they differ in the origin of tumor cells, culture regimen and construction time [43]. Most importantly, organoids can better preserve the original tumor characteristics and can be studied at the genetic level [44]. At present, 3D drug resistance models are limited, mainly because they are more expensive and time-consuming and most of them are difficult to reproduce complex TME processes, and the success rate of modeling is low[45, 46].

In two studies, sorafenib was used to establish a commercial cell line drug resistant HCC sphere model and a patient-derived drug resistant HCC organoid model, respectively. Sariyar et al. [47] mixed 700 sorafenib resistant Huh7 cells and 300 LX2 liver stellate cells in 30 μl culture medium, and established a sorafenib resistant HCC sphere model using the hanging drop method. Sorafenib resistant HCC spheres demonstrated resistance to regorafenib and had a higher CD24 and EPCAM-positive cancer stem cell population compared to parental tumor spheres. In addition, several carcinogenic kinases were upregulated, and importantly, combined inhibition of EGFR and Lyn kinases in this resistant sphere was effective in inducing cell death. Xian et al. [48] successfully constructed 38 HCC organoids from 133 HCC patients' tumors, and randomly selected 4 cases (HCC-10, HCC-25, HCC-52, and HCC-118) to culture with gradually increasing sorafenib concentrations of 1.2–10 μM for 3–5 months. Finally, 4 sorafenib resistant HCC organoids were established. In these organoids, 37 genes (e.g. MCM6, RRS1) were up-regulated, and 207 genes (e.g. TP53INP2, MYH14) were down-regulated. Cancer stem cell-related genes, including Myc- and EGFR-related genes, and EMT-related genes were enriched. Furthermore, there was significant heterogeneity among the four sorafenib resistant HCC organoids. For example, sorafenib resistant HCC-25, HCC-52,

and HCC-10 were associated with up-regulation of stem cell-related genes, while sorafenib resistant HCC-118 was associated with down-regulation of stem cell-related genes. Moreover, sorafenib resistant HCC-118 and HCC-10 was associated with up-regulation of EMT-related genes, while sorafenib resistant HCC-52 and HCC-25 was associated with down-regulation of EMT-related genes. The expression of tumor stem cell markers (e.g. CD133, CK19, and β-catenin) and epithelial markers (e.g. E-cadherin, ZO-1) also showed significant heterogeneity among different drug-resistant organoids [48].

Transgenic drug resistance model

Many studies have shown that HCC drug resistance is closely related to abnormal expression of relevant signaling pathways. Therefore, the key molecules of the relevant signaling pathways can be modified by transgenic technology to change the sensitivity of HCC to drugs. Transgenic drug resistance models have become an important tool for the study of the mechanisms of HCC drug resistance. Clustered regularly interspaced short palindromic repeat/CRISPR-associated nuclease 9 (CRISPR/Cas9) is an immune sequence found in bacteria and archaea that consists of a Cas9 protein and single guide RNA (sgRNA) and has been widely used as a gene editing tool [49]. At present, transgenic drug resistance models constructed through the CRISPR/Cas9 system mainly focus on two drugs, sorafenib and lenvatinib. The establishment of transgenic drug resistance models first requires genome-wide CRISPR/Cas9 knockout library screening to identify key sensitive/resistant genes, followed by the construction of transgenic drug resistant cells by knocking out sensitive genes or inserting resistance genes. Lu et al. [50] developed lenvatinib resistant HCC cells with neurofibromin 1 (NF1) and dual specificity phosphatase 9 (DUSP9) knockdown by CRISPR/Cas9 technology. Huang et al. [51] constructed DUSP4-knockout HCC cells, showing that DUSP4 deficiency promoted lenvatinib resistance and restored cell proliferation, survival, and migration. Chen et al. [52] adopted a dual sgRNA CRISPR/Cas9-mediated gene deletion strategy to improve the efficiency of PSTK knockout by integrating two PSTK-specific sgRNA sequences into a single vector. HCC cell lines expressing cas9 were transduced by lectin particles encoding PSTK-sgRNA1-sgRNA2 constructs for 48 h, and then cultured in a culture medium containing puromycin to select successful transduced cells. Finally, PSTK-eliminated drug-resistant HCC cells were established. These cells were then implanted into mice to create an in vivo transgenic drug resistance model.

Multidrug resistance model

Multidrug resistance (MDR) refers to the simultaneous development of resistance to one drug and other drugs with diverse structures and mechanisms, which has become an important factor in the limited efficacy of drug therapy for HCC [53]. To explore the molecular mechanism of MDR related to HCC and its reversal strategy is a key problem that needs to be solved in clinic, and to establish a reliable MDR model for HCC is the key to address this issue. Studies on HCC MDR mainly focused on 5-FU, cisplatin and doxorubicin. Bel-7402 was the most used cell lines to establish MDR models. Due to the complex mechanism of HCC resistance and the differences in cell specificity, induction methods and drugs used, the drug-resistant phenotypes of MDR-cells are not uniform. At present, the establishment methods of MDR models mainly include the induction of MDR cell lines in vitro, the transfection of MDR genes and the induction of MDR in nude mice.

In vitro induced multidrug resistance model

In vitro induced MDR model includes physical induced MDR model and drug induced MDR model. Physically induced MDR model is to simulate the microenvironment of HCC growth in vivo to induce drug resistance, such as hypoxia, low glucose and ionizing radiation [54, 55]. The modeling time of this model is short, usually ranging from several hours to several days, but the resistance index and stability are low. Wen et al. [54] established two physically induced MDR models based on hypoxia and ionizing radiation. They established a hypoxia-induced MDR model by cultivating HCC cell lines (Huh7) in 1% O2, 5% CO2, and 94% N2 incubators for 6 h. The ionizing radiation-induced MDR model is a transient treatment of cells with 10 Gy (X-ray) ionizing radiation of 0.5 Gy/min. The cells showed high resistance to doxorubicin, 5-FU, and cisplatin, and the formation of MDR was found to depend on the calcium-mediated TRPC6/calcium/STAT3 signaling pathway. The expression of MDR related genes in HCC may be another potential mechanism [55].

Drug induced MDR model is established by the method of gradient increase of drug concentration or intermittent induction of high drug concentration, which has high resistance index and stability. The increasing concentration gradient method is to acquire MDR by infiltrating HCC cell lines into increasing drug concentration. This method has low cell proliferation rate, long induction time and is easy to produce pollutants in the process [56]. Zhong et al. [56] established Bel-7402/ADMV cell subline by using adriamycin (ADM) increasing concentration gradient method. Bel-7402 cells with a concentration of 5×10^5/ml were cultured for 24 h, and then cultured in

low concentration (0.01 μg/ml) ADM medium for 24 h. The cells were then digested with 0.25% trypsin and centrifuged at 1000 rpm for 3 min. The cells were collected and re-inoculated in a 25 ml culture vial with a concentration of 1×10^5/ml solution without ADM. When the cell growth was in the logarithmic phase, the drug concentration was increased. The experiment was repeated for about 6 months until the cells grew and proliferated stably in a medium containing 0.5 μg/ml ADM. It was found that Bel-7402/ADM cell sublines were cross-resistant to ADM and CDDP [56]. High concentration intermittent drug induction is to treat HCC cell lines with a single high concentration drug to acquire drug resistance. This method has the advantages of short modeling time, and similar administration to clinical treatment. However, because of the high concentration of the drug, cells may be difficult to tolerate due to sudden changes in the external environment [57].

In vitro transgenic multidrug resistance model

HCC MDR cell line established by drug induction can well simulate the clinical MDR, while the induction time is relatively long, and the biological characteristics and sensitivity of the cells are significantly changed after induction. In addition, when placed in culture without inducing drugs, its stability is poor and resistance is easily lost [58]. Due to the complex mechanism of HCC resistance, this model is not conducive to the study of single mechanism and reversal strategy in HCC MDR phenomenon. Transgenic MDR models are developed by using transgenic technology to transfect MDR genes into HCC cells to form MDR. The HCC MDR cell lines established by transgenic method has the same biological characteristics and sensitivity, and the MDR mechanism is simple and stable, which can better overcome the shortcomings of the above drug-induced cell models [58]. But this method is relatively complicated to operate. Currently, CRISPR/Cas9 is often used to silence or insert MDR related genes in tumor suppressor genes (TSGs) of HCC cell lines. Sanchez-Martin et al. [59] knocked out the tumor suppressor gene ARID1 A in PLC/PRF/5 cells, thereby inducing increased sensitivity to cisplatin, doxorubicin, and cabozantinib without affecting other tumor features.

In vivo induced multidrug resistance model

In vivo induced MDR model is established by transplanting HCC cell lines into nude mice through subcutaneous, liver or abdominal injection, and then gradually administrating drugs to induce MDR. This model has the advantages of low reproductive activity, short induction time (generally about 8 weeks), little contamination possibility, and relatively simple operation. In addition, unlike the significant differences among the resistant cells induced in vitro, the morphology of the resistant cells induced in vivo was similar to that of the parents. Zhong et al. [56] established two different human HCC MDR cell lines Bel7402/ADM, that is, nude mouse liver implantation and subcutaneous implantation MDR cell models. 0.2 ml Bel-7402 cell suspension (1×10^8/ml) was injected into the right hepatic parenchyma of nude mice, and another 10 nude mice were subcutaneously injected 0.2 ml Bel-7402 cell suspension (1×10^8/ml) into the right anterior axillary pit. ADM was injected peritoneally at a dose of 1.5 mg/kg per week for 8 weeks. Then, the cells were extracted for primary culture, and purified by multiple passage to obtain the subline Bel-7402/ADML (liver implantation induction) and subline Bel-7402/ADMS (subcutaneous implantation induction). It was found that there were no significant differences in cell morphology, drug resistance multiple, drug flow and efflux, P-gp, MRP and GSH/GST among different tumor implantation methods. The advantages of subcutaneous implantation MDR models are simple to operate and easy to observe, however, because the tumor growth environment is different from that of human HCC, the tumor growth is relatively local and only limited distant metastasis is generated. In contrast, liver implantation MDR model has obvious advantages, especially its tumor growth environment, location and biological behavior are very similar to human HCC, and the proportion of tumor metastasis, invasion and ascites is higher. Therefore, in vivo induced MDR model established by liver implantation in nude mice can better simulate human HCC MDR.

Conclusions

Establishing a stable drug resistance model is the cornerstone of research on HCC drug resistance. In terms of the establishment method of drug resistance models, the development of new model or the study of resistance mechanisms of new drugs can first establish drug-resistant HCC cells through gene editing technology or drug induction, and then further build in vivo drug resistance models. Resistance can also be induced by drug in HCC animal models. Although rodents have made significant contributions to human understanding of HCC resistance mechanisms, their lack of clinical relevance, small size, and low success rate in modeling also limit our more precise understanding of HCC resistance mechanisms. Rabbit VX2 model is a commonly used interventional radiological tumor model, which has the advantages of rapid tumor formation (within a few weeks), high success rate of modeling (95%), large size, and convenient operation [60]. In addition, the rabbit VX2 HCC model has a highly similar anatomical structure to human liver cancer and exhibits high levels of acetaldehyde dehydrogenase

1, which is associated with tumor treatment resistance [61]. Therefore, the rabbit model of HCC drug resistance is expected to be a potential choice for HCC drug resistance research. Compared with traditional commercial HCC cell lines, patient-derived HCC cell lines can better reflect the specific mechanisms of drug resistance during clinical use and better retain individual drug resistance characteristics, providing the possibility of accurately individualized treatment screening. The 3D drug resistance model based on 3D culture technology can more accurately reflect the biological and morphological characteristics of the original tumor, and provide a better choice for the study of drug efficacy and resistance. Transgenic drug resistance models enable individual validation of specific genes or pathways. Compared with the above drug resistance models, HCC PDX drug resistance model can not only retain the clinical characteristics of HCC, but also provide an in vivo physiological environment for tumor growth, which may be the most ideal experimental model for HCC drug resistance related studies. Although the current drug resistance models have certain advantages in HCC resistance related studies and have greatly promoted people's understanding of the potential molecular mechanism of drug resistance, a single HCC drug resistance model has certain defects. Therefore, it may be necessary to combine multiple drug resistance models to conduct targeted research on current issues to bridge the gap between preclinical research and clinical trials, so as to ultimately achieve clinical conversion and application.

Abbreviations

Bcl-2	B-cell lymphoma 2
CRISPR/Cas9	Clustered regularly interspaced short palindromic repeat/CRISPR-associated nuclease 9
Cx32	Connexin 32
DUSP9	Dual specificity phosphatase 9
HCC	Hepatocellular carcinoma
LXRα	Liver X receptor α
MET	Mesenchymal–epithelial transition
NF1	Neurofibromin 1
PDGF-AA	Platelet-derived growth factor-aa
PDX	Patient-derived xenograft
SgRNA	Single guide RNA
2D	Two-dimensional
3D	Three-dimensional
VEGF	Vascular endothelial growth factor
VEGFR2	Vascular endothelial growth factor receptor 2

Author contributions

Conceptualization: Yaling Li; data accumulation, writing original draft preparation: Xiaolu Xie; writing, review and editing: Xiaolu Xie, Yaling Li and Jun Li; figure and table preparation: Yaomin Wang, Ziyi Wang and Lei Zhang; funding acquisition: Yaling Li. All authors have read and approved the submitted version of the manuscript.

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Availability of data and materials

No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate

Not applicable.

Competing interests

The authors declare no competing interests.

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