Original Article
Establishment and characterization of six novel hepatocellular carcinoma cell lines from Chinese patient-derived tumor xenografts

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Abstract: The unmet clinical needs in population with hepatocellular carcinoma (HCC) makes it urgent to construct more patient-derived preclinical HCC models, including patient-derived xenografts (PDXs) and patient-derived cell lines (PDCs), to improve the therapy for HCC. The observation that dramatic inter-tumor heterogeneity exists in HCC patients and the development of precision cancer care also needs more preclinical HCC models with distinct genomic signatures to test targeted compounds *in vitro*. In present work, 6 novel Chinese PDX-derived HCC cell lines were established. They displayed significant difference on cell morphology, growth rate, chromosomal number, mRNA levels of some cancer-related genes, and the response to 12 antitumor agents. Among which, CNHCC0106 grows at the fastest rate, harbors the highest expression of *BCL2, RAF1,* and *MET*, and responds well to everolimus but not to sorafenib, which may serve as a useful model for new mechanism of action exploration of some anticancer drugs. Our work enlarges the number of HCC cell lines that can be used for further exploring the molecular mechanism of HCC and anticancer drug screening.

Keywords: Hepatocellular carcinoma, patient-derived xenograft (PDX), antitumor agents

Introduction

Hepatocellular carcinoma (HCC) is the leading cause of cancer deaths with massive patient population in China (466.1 and 422.1 thousands of people are estimated to occur and be dead with this disease, respectively, in 2015) and increasing incidence and mortality in Western countries [1, 2]. The survival rate for HCC patients is dismal: The 1- and 5-year relative survival rates for HCC patients are 43% and 17%, respectively, in the United States (American Cancer Society. Caner Facts & Figures 2015.); while in the United Kingdom, the 1-year relative survival rates for HCC patients is just 20% [3]. The not-well-understood molecular pathogenesis of HCC, the lack of good diagnostic markers and treatment strategies, and clinical heterogeneity makes management of HCC a great challenge [4-6].

With the advances in sequencing technologies in the past decade, the landscape of genomic alterations in HCC patients has been uncov-

ered. Recurrent mutations and copy number variations are occurred in HCC patients in the following pathways: Telomere stability, p53/cell cycle control, Wnt/β-catenin signaling, Chromatin remodeling, Ras/PI3K/mTOR pathway, Oxidative stress, FGF signaling, VEGF signaling, JAK/STAT signaling, PDGFR signaling, and IGF signaling [6-10]. However, most common mutations are undruggable, such as TERT promoter, TP53 and CTNNB1. Furthermore, up to seven large, randomized phase III clinical trials investigating other molecular therapies in HCC have failed to improve on the results observed with sorafenib, the only targeted agent FDA-approved for the treatment of advanced HCC patients. Potential reasons for this include issues with trial design, a lack of predictive biomarkers of response, and intertumour heterogeneity [6, 11], which has been described by several previous studies [10, 12].

Cancer cell lines and patient-derived xenograft (PDX) models have been widely used in preclinical studies and regarded as essential tools for

Table 1. Information of patients

Cell line	Age/sex	Clinical Pathological diagnosis	Site	HBsAg	HCV	HIV
CNHCC0101	49/M	Poorly to moderately differentiated HCC	Right lobe	+	-	-
CNHCC0104	58/M	Poorly differentiated HCC	Right lobe	+	-	-
CNHCC0106	64/M	Poorly differentiated HCC	Left lobe	-	-	-
CNHCC0109	70/M	Poorly differentiated HCC	Middle lobe	+	-	-
CNHCC0111	61/M	Poorly to moderately differentiated HCC	Right lobe	+	-	-
CNHCC0112	66/F	Poorly differentiated HCC	Left lobe	+	-	-

Table 2. Quantification of chromosome aberrations

Cell line	Chromosome quantity
CNHCC0101	42 ± 3
CNHCC0104	75 ± 6
CNHCC0106	50 ± 5
CNHCC0109	57 ± 8
CNHCC0111	58 ± 7
CNHCC0112	88 ± 14

oncology drug development and prediction of the cancer therapy [13]. In 2012, two research groups carried out next-generation sequencing and drug screen for large-scale cancer cell lines [14, 15]. The genomic signatures, including gene mutation, copy number variation, chromosome translocation, and gene expression, in these cell lines were correlated to their response to antitumor agents. By linking drug activity to the functional complexity of cancer genomes, systematic pharmacogenomic profiling in cancer cell lines provides a powerful biomarker discovery platform to guide rational cancer therapeutic strategies [15]. After then, increasing studies used cell line panel of some type of cancer or cell lines harboring some given genomic variation (usually more than 40 cell lines, including commercial and primary cell lines) to uncover biomarkers for some anticancer agents [16-18]. However, there are only 28 liver cancer cell lines in Cancer Cell Line Encyclopedia (CCLE), most of them are derived from Western population. Regarding the massive population of HCC patients in China and the high intertumour heterogeneity of HCC, it is of great importance to construct more Chinese patient-derived HCC cell lines to boost the drug therapy for HCC. In the present study, 6 novel HCC cell lines were established from PDX models. The cell morphology, growth rate, chromosomal number, mRNA levels of some cancer-related genes, and response to 12 antitumor agents of these cell lines were investigated.

Materials and methods

Patients and sample collection

Tissue samples were collected from 15 patients diagnosed with HCC who underwent curative resection without any adjuvant therapy before for the purposes of the study (**Table 1**). All HCC tumor tissues used for PDX model establishment were obtained from Huashan Hospital in accordance with protocols approved by the Institutional Ethics Committee of Huashan Hospital and with written informed consent from each patient.

Generation of PDX models

6-8 week-old female SCID mice (Beijing Vital River, China) were used for human HCC fragments implantation. Mice were maintained under specific-pathogen-free (SPF) conditions. The fresh tumor tissue specimens were rinsed twice with Hank's balanced salt solution (HBSS) containing antibiotic and transported on ice. As described previously [19], tumor tissues were cut into 3×3×3 mm pieces and implanted subcutaneously into the flank of SCID mice. After outgrowth of patient tumor and reaching a size of approximately 500 mm^3, PDX tumors were harvested and passaged, and/or used to establish *in vitro* cultures. Tumors were typically retransplanted three times (i.e. up to p4).

Establishment of HCC cell lines from PDX models

Harvested xenografts were minced, placed in 5% FBS containing DMEM/F-12 1:1 with collagenase IV (0.5 mg/ml, Sigma) in a tube and incubated at 37°C for 60 min with vortexing every 10 min. The dissociated suspension was passed through a 70 μm strainer to obtain single cells and washed with culture medium. Cell aggregates retained on top of the filter

Table 3. Primers for qPCR

Gene	Forward	Reverse
BCL2	catgtgtgtggagagcgtcaa	agtcatccacagggcgatgt
MET	agcgtcaacagagggacct	gcagtgaacctccgactgtatg
CTNNB1	gcagagtgctgaaggtgctat	tctgtcaggtgaagtcctaaagc
JAK2	caggaacaagatgtgaactgtttc	cccatgcagagtctttttcag
RAF1	attgggaaatagaagccagtga	caaaagagcctgacccaatc
ACTB	gcatcccccaaagttcacaa	ggacttcctgtaacaacgcatct
EGFR	ccactgcaaaacactaaagatcca	ttatcctactgtacctgccatgactt
TOP1	ccctgtacttcatcgacaagc	ccacagtgtccgctgtttc

were put in a separate dish. Isolated cells and aggregates were grown in DMEM/F-12 1:1 containing 5% FBS. Purity of the epithelial culture was assessed by cell morphology. For selective trypsinization, cultures were washed twice with PBS, followed by 2-3 min incubation with 0.05% Trypsin/0.02% EDTA solution at 37°C. Detached cells were gently washed away with 5% serum containing medium and selective removal of fibroblast was repeated once cells reached confluence. Cells were subcultured at 70-80% confluence and used for further experiments after at least 30-50 passages.

Growth kinetics in vitro

After establishment, growth kinetics of the tumor cells was obtained by seeding the cells at the density of 10000 cells/well into 12-well plate. The number of cells in each well was counted at a 24 h interval and the average value of duplicates was used to calculate the doubling time and plot their growth curve. The doubling times of the 6 HCC cell lines were calculated with the aid of GraphPad Prism 5.0 software by exponential growth equation.

Chromosome analysis

The chromosome specimens of the established HCC cell lines were valued as previously described [20]. Cells at logarithmic phase were harvested and suspended in 0.075 mol/L KCl hypotonic solution and then fixed in fix solution (methanol: glacial acetic acid = 3:1). Chromosome specimens were stained with Giemsa and Chromosome numbers of M phase cells were counted under a microscope (Olympus IX51). The chromosome frequency of each cell line was analyzed by Origin software.

HBV DNA integration analysis by PCR

Genomic DNA from tumor cells was isolated using QIAamp DNA Mini Kit (Qiagen, USA). 10 ng of genomic DNA was used to amplify the *C, P, S,* and *X* gene in the PCR system, respectively. The primers for each gene were list below: C: forward 5'-TTGCCTTCT-GACTTCTTTCC-3', reverse 5'-TCTGCG-AGGCGAGGGAGTTCT-3'; P: forward 5'-GGGTCACCATATTCTTGGGA-3', reverse 5'-CCCCGCCTGTAACACGAGCA-3'; S: forward 5'-ACTCACCAACCTCTTGTCCT-3', reverse 5'-GACAAACGGGCAACATACCT-3'; X: forward 5'-CCGATCCATACTGCGGAAC-3', reverse 5'-GC-AGAGGTGAAGCGAAGTGCA-3'. The PCR products were electrophoresis on 1.5% agarose. The length of PCR product should be 441, 577, 130, and 340 bp for *C, P, S,* and *X* gene, respectively.

Quantitative real-time PCR (qPCR)

Total RNA from the 6 HCC cell lines was respectively isolated with Trizol (Invitrogen, Life Technologies) and synthesized to cDNA for RT-PCR, using PrimeScript RT reagent kit (Takara, RR-074A) and Random Primer (9 mer). The primers used for qPCR validation were list in **Table 3**. Real-time qPCR was performed on CFX-96 (Bio-lab), with endogenous control *hActb*. Gene expression was calculated relative to expression of *hActb* endogenous control and adjusted relative to expression in CNHCC0101 cells.

In vitro anti-proliferation assay

The cell populations were further characterized by analyzing their response to 12 compounds purchased from MedChemExpress (China), including 6 chemotherapeutic agents (Cisplatin, Docetaxel, Doxorubicin, Gemcitabine, Oxaliplatin, and Vinblastine) and 6 targeted compounds (Sorafenib, Everolimus, MK-2206, Pictilisib, Ruxolitinib, and Tideglusib). Cells (500-1000/each well) were grown in 100 μl of DMEM/F-12 1:1 medium containing serum per well in a 96-well plate. After 24 h, the cells were treated with each of the 12 compounds or a solvent control with 0.5% of the final DMSO concentration in medium. Every treatment was triplicate in the same experiment. Then 20 μl of MTS (CellTiter 96 AQueous One Solution Reagent; Promega) was added to each well for 1 to
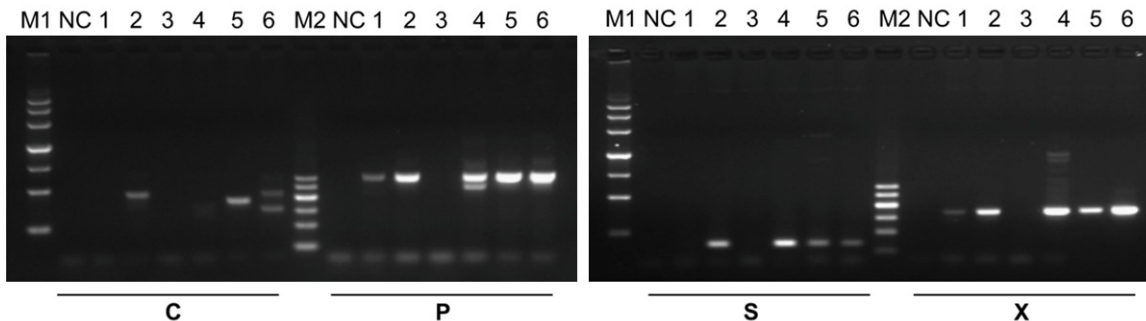
Figure 1. HBV DNA integration analysis by PCR. C, P, S, and X gene were amplified with 10 ng of genomic DNA from each cell line, the PCR products were electrophoresis on 1.5% agarose. M1, DNA marker containing 200, 500, 800, 1200, 2000, 3000, and 4500 bp bands; NC, negative control, containing no genomic DNA but ddH$_2$0 as PCR template; 1-6, containing 10 ng of genomic DNA from CNHCC0101, CNHCC0104, CNHCC0106, CNHCC0109, CNHCC0111 and CNHCC0112, respectively; M2, 100 bp ladder DNA marker containing 100, 200, 300, 400, 500, and 600 bp bands.

4 h at 37°C. After incubation, the absorbance was read at a wavelength of 490 nm according to the manufacturer's protocol. The cell viability was calculated relative to the untreated cells, respectively. The IC50 was determined by GraphPad Prism 5.0 software via nonlinear regression.

Statistical analysis

All data are shown as means ± SD. Statistical analysis was performed with two-tailed Student's t test. Significance was established for *P* values of <0.05.

Results

Establishment of HCC cell lines

Tumor tissues from surgical resection of Chinese patients of HCC were grafted in SCID mice to generate PDX models, and then primary culture of tumor cells was performed for cell lines establishment using the tissues derived from the PDX models. 6 HCC PDX models were successfully constructed out of 15 tumor tissues from different patients, and the HCC cell lines were designated as CNHCC0101, CNH-CC0104, CNHCC0106, CNHCC0109, CNHCC-0111 and CNHCC0112. The information of the patients was listed in **Table 1**. The ages ranged from 49 to 70, and only one patient is female. The HBV DNA integration was examined by amplification of C, P, S, and X gene of virus (**Figure 1**). The results showed that none of the four genes could be detected in CNHCC0106, implicating that there was no HBV replication
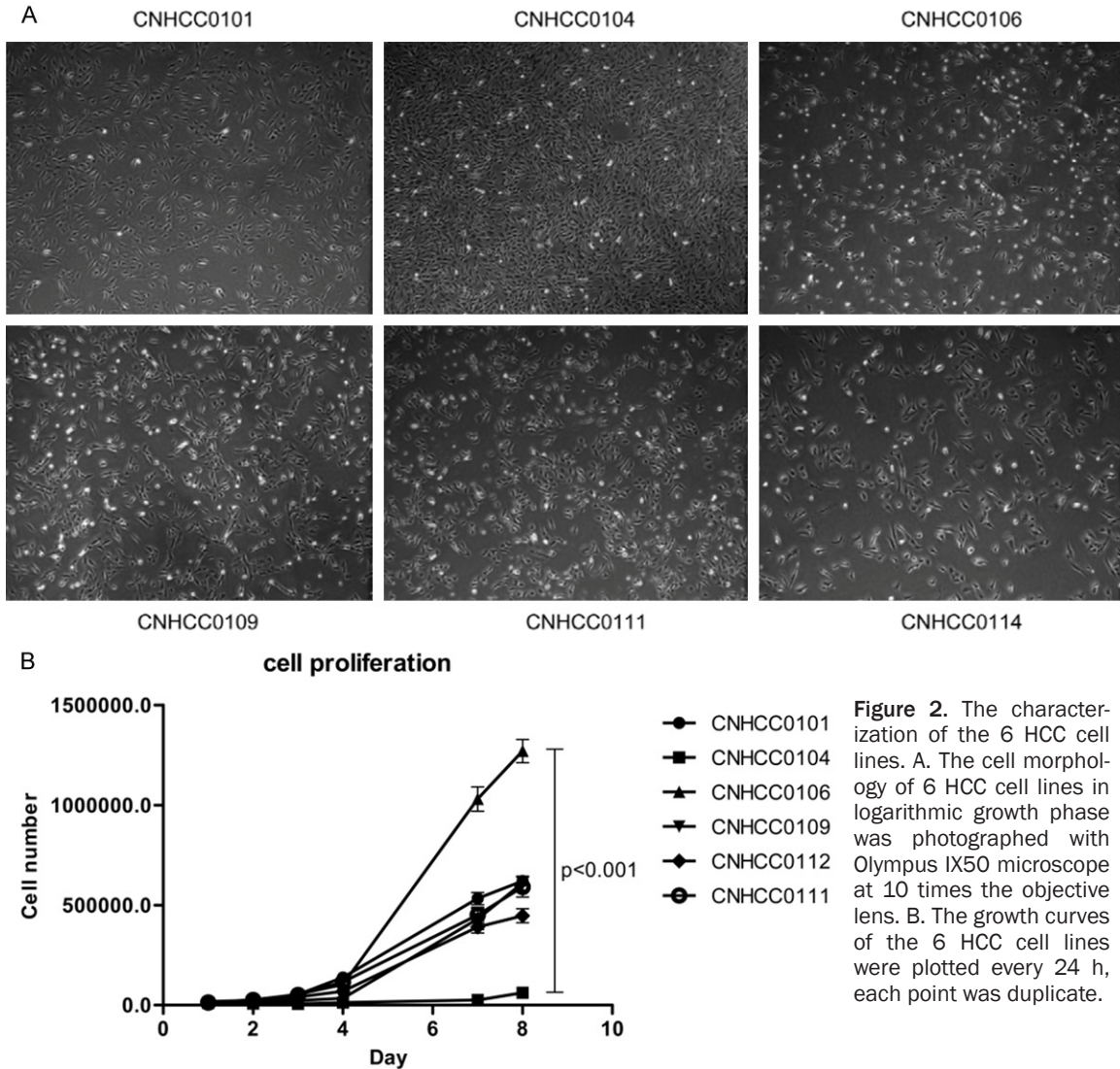
in CNHCC0106, which is consistent with the HBV negative diagnosis. Furthermore, the 6 novel cell lines are all HCV and HIV negative. STR (short tandem repeat) analysis revealed that all these 6 HCC cell lines were derived from the corresponding tissues and the STR loci profiles were all unique from the other HCC commercial cell lines (data not shown).

The characterization of the 6 novel HCC cell lines

Cells passaged at least 30-50 times were used for analysis of growth kinetics and chromosome aberrations. All cell lines were free of contamination by bacteria or mycoplasma. All cells grew as monolayer and the cell morphology was captured by phase contrast microscopy. The morphology of these 6 cell lines differed greatly with each other: CNHCC0111 and CNHCC0112 cells tend to form clusters, while the other four cell lines are uniformly dispersed on the plate, respectively (**Figure 2A**). The growth rates of the 6 cell lines were significantly different: CNHCC0106 cells grow at the fastest rate, their doubling time is 28 h; CNHCC0104 cells grow slowly, their doubling time is 77 h, nearly 3 times higher than that of CNHCC0106 cells; while the other four cell lines grow at moderate rate, their doubling time range from 32 to 34 hours (**Figure 2B**).

Chromosome aberrations could be found in all cell lines. Most cell lines had more than 46 chromosomes except CNHCC0101, which has 42 chromosomes (**Table 2**).

Figure 2. The characterization of the 6 HCC cell lines. A. The cell morphology of 6 HCC cell lines in logarithmic growth phase was photographed with Olympus IX50 microscope at 10 times the objective lens. B. The growth curves of the 6 HCC cell lines were plotted every 24 h, each point was duplicate.

The mRNA level of some cancer-related genes

The mRNA expression of some cancer-related genes (*CTNNB1, MET, RAF1, EGFR, BCL2, JAK2* and *TOP1*) was examined in these 6 HCC cell lines. Wnt-β-cantenin pathway was one of the most recurrently altered pathways in HCC, hence leading to the aberrant activation of this pathway [8, 9, 21, 22]. There were no significant difference in the expression of *β-cantenin* among those cell lines (**Figure 3**), although their expression was all higher than that in LO2 cell line, a normal liver cell line (data not shown). As to *EGFR* and *TOP1* genes, the situation was the same as *CTNNB1*. However, great difference existed in the expression of *RAF1, BCL2, MET* and *JAK2* genes between these 6 HCC cell lines. The expression of *RAF1, BCL2* and *MET*

genes was all the highest in CNHCC0106 cells, which were 3731, 3363 and 24-fold higher than that in the lowest expressed cells, respectively. Interestingly, *JAK2* expression cannot be detected in CNHCC0106 cells. There was 8-fold difference in *JAK2* expression between the other 5 cell lines.

In vitro drug response

The 6 novel HCC cell lines were subjected to drug screening to investigate their response to antitumor drugs. 12 antitumor compounds were used, including 6 chemotherapeutic agents (Cisplatin, Docetaxel, Doxorubicin, Gemcitabine, Oxaliplatin, and Vinblastine) and 6 targeted compounds (Sorafenib, Everolimus, MK-
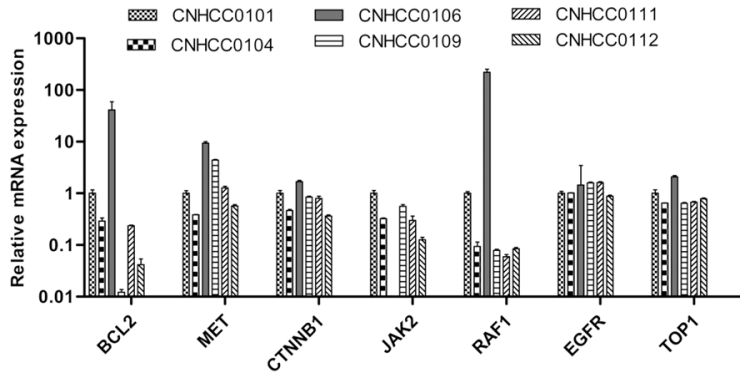
Figure 3. The mRNA levels of 7 cancer-related genes in the 6 HCC cell lines were investigated by qPCR. The expression of each gene was calculated relatively to the expression in CNHCC0101 cells.
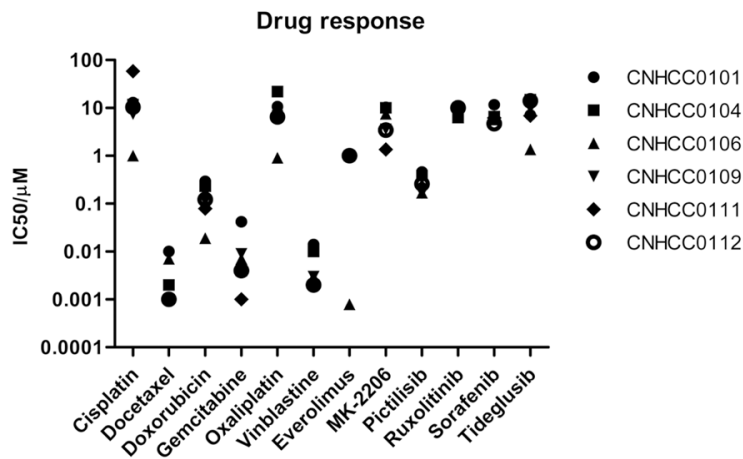

Figure 4. Response of 6 HCC cell lines to 12 antitumor agents. The IC50 values were determined by MTS assay and calculated with the aid of Graph-Pad Prism 5.0 software by nonlinear regression.

Discussion

Massive population with HCC in China and unmet clinical strategies makes it urgent to construct more Chinese-derived preclinical HCC models, including patient-derived xenografts (PDXs) and patient-derived cell lines (PDCs), to improve the therapy. On the other hand, the great intertumor heterogeneity of HCC patients and the development of precision cancer care needs more preclinical HCC models with distinct genomic signatures to test targeted compounds *in vitro*. In present work, 6 novel Chinese PDX-derived HCC cell lines were established and verified by STR loci analysis. They displayed great difference on cell morphology, growth rate, chromosomal number, mRNA expression of some cancer-related genes, and response to chemotherapy agents and targeted compounds.

CNHCC0106 cells grew at the rapidest rate among the 6 cell lines. Meanwhile, CNHCC0106 cells harbored the highest mRNA expression of *BCL2, RAF1,* and *MET. BCL2* gene encodes an integral outer mitochondrial membrane protein that blocks the apoptotic death of some cells to exert a survival function. High expression or activation of *BCL2* has been implicated in various cancers and been regarded as a potential therapeutic target in some kind of cancer [25-28]. Proto-oncoprotein RAF1 serves as a key signaling transduction molecule of RAS-RAF-MEK-ERK pathway and involved in the regulation of the cell division cycle, apoptosis, cell differentiation and cell migration. *RAF1* amplification, overexpression or activation in various cancers was correlated to tumor initiating cell regulation and cancer progression, and therefore been regarded as a resistant biomarker for some targeted therapy and a critical candidate target for combination therapy [29-31]. Proto-oncogene *MET* encodes a member of the

2206, Pictilisib, Ruxolitinib, and Tideglusib). These cell lines responded differentially to cisplatin, gemcitabine and everolimus, while they displayed not notable difference in sensitivity to the other compounds (**Figure 4** and **Table 4**). These cell lines were all sensitive to Docetaxel and Vinblastine, the IC50s of these cells to the two cytotoxic drugs ranged from 1-14 nmol/L. CNHCC0106 cells were the most sensitive to 5 drugs (Cisplatin, Doxorubicin, Oxaliplatin, Everolimus and Tideglusib), and moderately sensitive to the other drugs. CNHCC0111 was the most sensitive to gemcitabine. All 6 cell lines were resistant to sorafenib, although CNHCC0106 harbored the highest *RAF1* mRNA expression, and *RAF1* was suggested as a potential marker for Sorafenib response [23, 24].

Table 4. Response of 6 HCC cell lines to 12 antitumor agents

IC50 (μM)	CNHCC0101	CNHCC0104	CNHCC0106	CNHCC0109	CNHCC0111	CNHCC0112
Cisplatin	12.966	11.829	1.007	7.442	58.695	10.41
Docetaxel	0.01	0.002	0.007	0.001	0.001	0.001
Doxorubicin	0.292	0.23	0.019	0.114	0.079	0.122
Gemcitabine	0.042	0.004	0.007	0.009	0.001	0.004
Oxaliplatin	10.691	21.914	0.904	6.826	6.357	6.505
Vinblastine	0.014	0.01	0.003	0.003	0.002	0.002
Everolimus	>1	>1	0.000793	>1	>1	>1
MK-2206	10.349	10.101	7.524	3.553	1.361	3.445
Pictilisib	0.459	0.389	0.168	0.167	0.221	0.26
Ruxolitinib	>10	6.245	>10	>10	>10	>10
Sorafenib	11.67	6.539	5.733	6.58	6.19	4.706
Tideglusib	7.12	14.877	1.355	9.662	6.862	14.087

receptor tyrosine kinase, which is activated by binding of its ligand, hepatocyte growth factor, and plays a role in cellular survival, embryogenesis, and cellular migration and invasion. Amplification and overexpression of this gene are associated with multiple human cancers [32-34]. Co-overexpression of BCL2, RAF1, and MET in CNHCC0106 might render these cells great growth advantage over the other 5 HCC cell lines.

Furthermore, CNHCC0106 was resistant to sorafenib in spite of RAF1 overexpression. RAF1 is one of main target of sorafenib, previous study has suggested that breast tumor initiating cells harboring recurrent RAF1 amplification was very sensitive to sorafenib [31]. The insensitivity of CNHCC0106 to sorafenib might be in the co-overexpression of MET and BCL2, two genes promote cancer cell proliferation or survival. Firtina Karagonlar et al proposed that Met activation is the reason for acquired resistance of HCC cells to sorafenib [35], which may help explain the insensitivity of CNHCC0106 to sorafenib.

Interestingly, CNHCC0106 was the only sensitive cell line to everolimus among the 6 novel HCC cell lines. Everolimus is a specific mTOR inhibitor approved by FDA for treatment of breast cancer, pancreatic cancer, gastrointestinal cancer, lung cancer, renal cell carcinoma, subependymal giant cell astrocytoma. TSC1 mutation has been correlated with everolimus respones [36], which suggests that the genomic profiling should be examined among those cell lines to identify more occult biomarkers of drug sensitivity in patients. Hence, the 6 novel HCC cell lines established, especial CNHCC0106, warrant a comprehensive detection of genetic alterations to explain the sensitivity to everolimus and to figure out more possible responsive drugs for these novel cell lines.

Taken together, 6 novel HCC cell lines were established from PDX and verified by STR loci analysis. These cell lines showed significant difference in cell morphology, growth kinetics, chromosomal number, mRNA levels of some cancer-related genes, and the response to chemotherapy and targeted agents. Among which, CNHCC0106 grows at the fastest rate, harbors the highest expression of BCL2, RAF1, and MET, and responds well to everolimus but not to sorafenib, which may serve as a useful model for new mechanism of action exploration of some anticancer drugs. Our work enlarges the number of HCC cell lines that can be used for further exploring the molecular mechanism of HCC and anticancer drug screening.

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Disclosure of conflict of interest

None.

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