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A novel chemotherapeutic sensitivity-testing system based on collagen gel droplet embedded 3D-culture methods for hepatocellular carcinoma

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Abstract

Background: Patients suffering from advanced stage hepatocellular carcinoma (HCC) often exhibit a poor prognosis or dismal clinical outcomes due to ineffective chemotherapy or a multi-drug resistance (MDR) process. Thus, it is urgent to develop a new chemotherapeutic sensitivity testing system for HCC treatment. The present study investigated the potential application of a novel chemotherapeutic sensitivity-testing system based on a collagen gel droplet embedded 3D-culture system (CD-DST).

Methods: Primary cells were separating from surgical resection specimens and then tested by CD-DST. To identify whether HCC cell lines or cells separating from clinical specimens contain MDR features, the cells were treated with an IC_{50} (half maximal inhibitory concentration) or IC_{max} (maximal inhibitory concentration) concentration of antitumor agents, e.g., 5-fluorouracil (5-FU), paclitaxel (PAC), cisplatin (CDDP), epirubicin (EPI), or oxaliplatin (L-OHP), and the inhibitory rates (IRs) were calculated.

Results: HepG2 cells were sensitive to 5-FU, PAC, CDDP, EPI, or L-OHP; the IC_{50} value is $0.83 \pm 0.45 \mu\text{g/ml}$, $0.03 \pm 0.02 \mu\text{g/ml}$, $1.15 \pm 0.75 \mu\text{g/ml}$, $0.09 \pm 0.03 \mu\text{g/ml}$, or $1.76 \pm 0.44 \mu\text{g/ml}$, respectively. Only eight (8/26), nine (9/26), or five (5/26) patients were sensitive to the IC_{max} concentration of CDDP, EPI, or L-OHP; whereas only three (3/26), four (4/26), or two (2/26) patients were sensitive to the IC_{50} concentration of CDDP, EPI, or L-OHP. No patients were sensitive to 5-FU or PAC.

Conclusions: The in vitro drug sensitivity examination revealed the MDR features of HCC and examined the sensitivity of HCC cells from clinical specimens to anti-tumor agents. CD-DST may be a useful method to predict the potential clinical benefits of anticancer agents for HCC patients.

Keywords: Hepatocellular carcinoma, Multi-drug resistance, Collagen gel droplet embedded 3D-culture system, Chemotherapies

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Background

HCC is currently one of the most common causes of cancer-related deaths in China and the Asian-Pacific region [1–3]. In China, there are 466,100 new cases, and 422,100 deaths occur per year [2–5]. Although hepatic resection may be the first choice of treatment, only a small proportion (10–15%) of patients are suitable [6, 7]. Patients often suffer from advanced stage HCC upon their initial diagnosis and exhibit poor prognosis due to multi-drug resistance (MDR) features [8–10]. Thus, it is urgent and significant to develop a new chemotherapeutic sensitivity testing system for HCC treatment. Previously, the sensitivity of HCC cells to anti-tumor agents is often tested via MTT (3-(4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyl-2-H-tetrazolium bromide) assays or SRB (Sulforhodamine B) assays based on the two-dimensional (2-D) monolayer culture [11]. However, cancer cells in solid tumors grow in a three-dimensional (3-D) pattern [12]. In this respect, an in vitro 2-D cell cultural model is limited in evaluating the clinical efficacy of chemotherapies [13]. Therefore, it is urgent to establish an in vitro model reflecting the 3-D culture of tumor cells.

CD-DST is a mature test in which primary-culture cells are embedded in collagen droplets [11–16].

Table 1 Baseline characteristics of patients

Clinical features	Values (number (%))
Age(yr)	51.3 ± 7.4
Gender	
Male	20 (76.92%)
Female	6 (23.08%)
Aetiology	
Hbs-Ag positive	23 (88.46%)
HCV-Ab positive	3 (11.54%)
Child-Pugh score	
Class A	26 (100%)
Class B	0 (0%)
Tumour size	
< 3 cm	19 (73.07%)
3-5 cm	7 (26.93%)
Tumour number	
Single	16 (61.53%)
2–3	11 (38.47%)
BCLC staging	
Stage A	18 (69.23%)
Stage B	8 (30.77%)
Tumour differentiation	
well	9 (34.61%)
moderate	10 (38.46%)
poorly	7 (26.92%)
AFP	545 ± 892

Table 2 Concentration of the anticancer drugs

Compounds	Concentration				
	µg/ml				
5-PU	0.10	0.50	1.00	2.00	5.00
PAC	0.01	0.02	0.05	0.08	0.10
CDDP	0.10	0.20	0.50	1.00	2.00
EPI	0.01	0.05	0.10	0.50	1.00
L-OHP	0.10	0.50	1.00	2.00	5.00

Applications of CD-DST in chemotherapeutic sensitivity examination have been demonstrated in human cancers, e.g., lung cancer, gastric cancer, breast cancer, or colorectal cancer [11–16]. However, reports focused on potential applications and efficacy of CD-DST in HCC remain rare [17, 18]. This work aims to identify potential applications of CD-DST in HCC.

Methods

Cell lines and reagents

HepG2 (an HCC cell line) cells (Cat. 3111C001CCC000035) or HepG2/ADR (an HCC cell line resistant to Adriamycin), which were purchased from Cell Culture Center, Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences, or described previously [8], were cultured in complete DMEM (Invitrogen, Carlsbad, CA, USA) in a sterile incubator maintained at 37 °C, 5% CO₂. Anti-tumor agents, 5-fluorouracil (5-FU; Beijing Union Pharmaceutical Factory, China), Paclitaxel (PAC; Shanghai XudongHaipu Pharmaceutical CO., LTD., China), Cisplatin (CDDP; HaosohPharma, China), Epirubicin (EPI; Pfizer, USA), and Oxaliplatin (L-OHP; JiangshuHengrui Medicine CO, LTD, China) were purchased.

Human tissue samples

HCC specimens were obtained from surgical resections and preserved by our lab (from June 2015 and February 2016.). All patients with HBV (Hepatitis B Virus) - or HCV

Table 3 Growth rates of HepG2 cell in collagen gel droplets

Test Batch	Baseline (0-time)	Solvent Control (6 days)	Growth rate (GR)
	<i>O.D.</i> 540 nm		
1	22.18	97.95	4.1
	25.45	92.33	
	22.11	95.47	
2	26.29	108.51	4.56
	23.48	95.29	
	18.21	106.31	
3	19.83	111.03	4.62
	23.21	114.15	
	29.61	110.76	

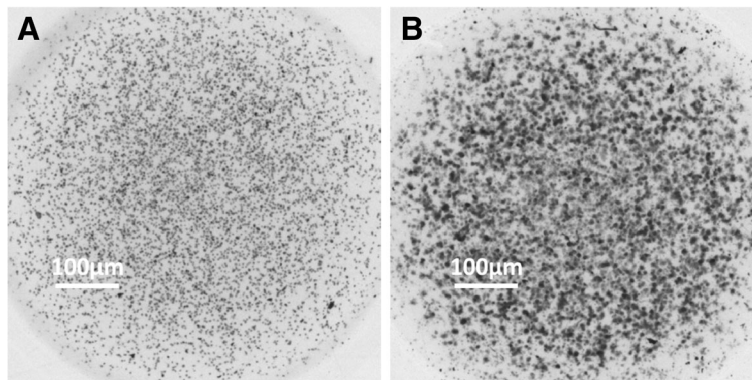


Fig. 1 HepG2 cell cultures in collagen gel droplets. HepG2 cells were seeded in collagen gel droplets and analyzed by a multifunctional microplate reader at 540 nm. **a** The represent grey scale photograph of baseline (0-time) HepG2 cells; **(b)** The represent grey scale photograph of HepG2 cells after 5–7 days culturing

(Hepatitis C Virus) -related HCC, diagnosed based on an imaging examination confirmed by needle core biopsy, were included. Tumor stages were classified following the Barcelona Clinic Liver Cancer (BCLC) staging classification system [19, 20]. The histological grades of HCC were identified based on Edmondson’s grading system: well-differentiated tumors, grade I or I–II; moderately differentiated tumors, grade II or II–III; and poorly differentiated tumors, grade III or III–IV. Table 1 showed the clinical features of 26 patients.

CD-DST

collagen gel droplet embedded 3D–culture system (CD-DST) was performed using a Kit Tumor Chemosensitivity Assay (Collagen gel Droplet culture method) provided by

Guangzhou Darui Biotechnology Co., Ltd. (Guangzhou, China), according to the manufacturer’s instructions. For primary-culture cells of HCC, 0.2–0.5 g fresh-clinical specimens were digested by trypsin and incubated in collagen gel-coated flasks after eliminating blood cells or dead cells. Next, cells were collected and incubated in a collagen gel droplet embedded culture at 2×10^5 cells/ml of collagen gel droplet (30 µl per drop). Then, cells were treated with the indicted concentration of anticancer agents (Table 2) for 24 h. After treatment with the anticancer agents, cells were incubated in a serum-free medium (4 ml PCM-2 medium to inhibit fibroblast proliferation) for 5–7 days to form colonies. Living cells were stained with neutral red, fixed with neutral formalin buffer, and directly examined/quantified by the cultured

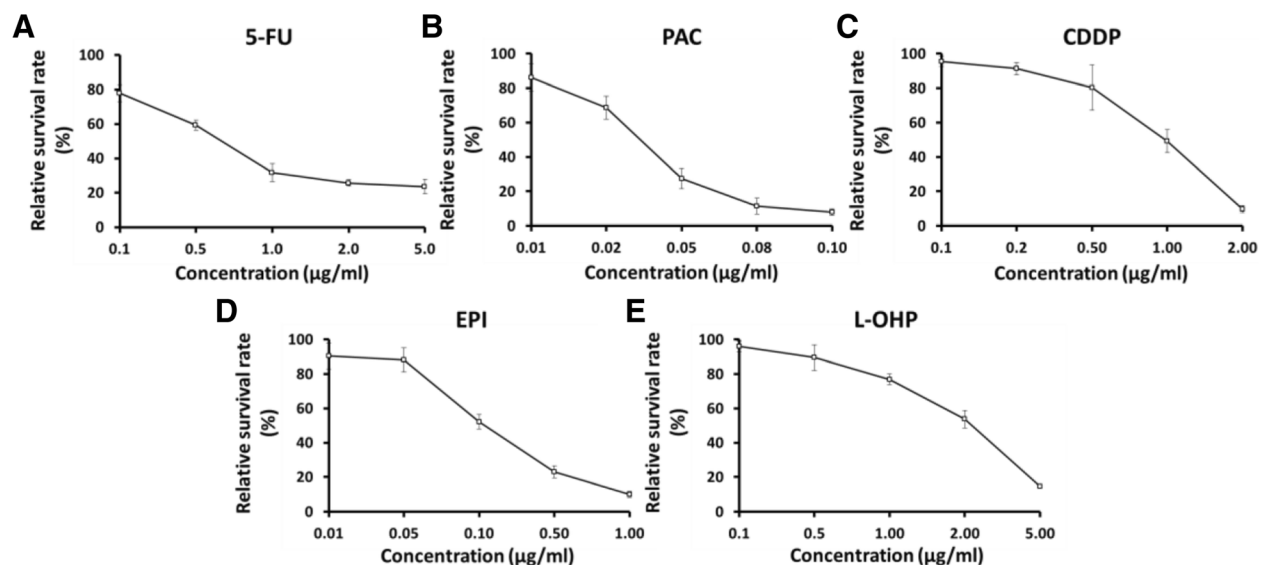


Fig. 2 Effects of anti-tumor agents on HepG2 cells. **a-e** HepG2 cells were treated with indicted concentration of 5-FU, PAC, CDDP, EPI or L-OHP and analyzed by CD-DST. The results are shown as mean ± SD from three experiments with similar results

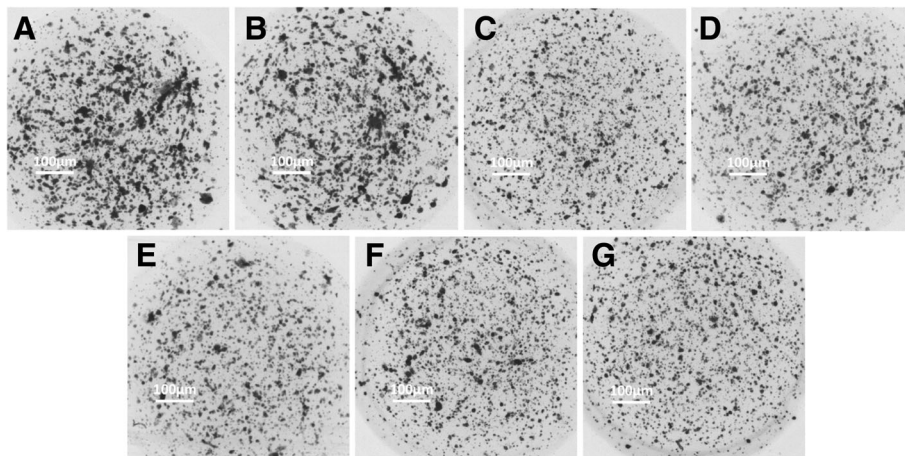


Fig. 3 The represent figures of IC_{50} concentration anti-tumor agents on 3-D cultured HepG2 cells. **a** HepG2 cells were cultured in a 3-D manner. **b-g** HepG2 cells were treated with solvent control (**b**), or IC_{50} concentration of agents, 0.8 µg/ml 5-FU (**c**), 0.3 µg/ml PAC (**d**), 1 µg/ml CDDP (**e**), 0.9 µg/ml EPI (**f**) or 1.7 µg/ml L-OHP (**g**)

cells analysis system DR6690 (Guangzhou Darui Biotechnology Co., Ltd., China). The baseline (0-time group) of cells was indicated as the blank group. Inhibitory rates (IRs) were calculated as (O.D. 540 nm control group – O.D. 540 nm administration group) / (O.D. 540 nm control group – O.D. 540 nm blank group) × 100%. Relative survival rates = 100% - IR. For the cell line test (HepG2 cells), cells were harvested and analyzed using CD-DST tests. An IR value >25% indicated that cells were sensitive to the compound.

Statistical analyses

Data are expressed as the mean ± SD. Statistical analyses were performed using the paired t-test or Fisher’s exact probability test. The IC_{50} and IC_{max} values were calculated using Origin 8.5 software.

Results

HepG2 cell cultures in collagen gel droplets

To establish the CD-DST methods, HepG2 cells were cultured in collagen gel droplets. The O.D. 540 nm values were

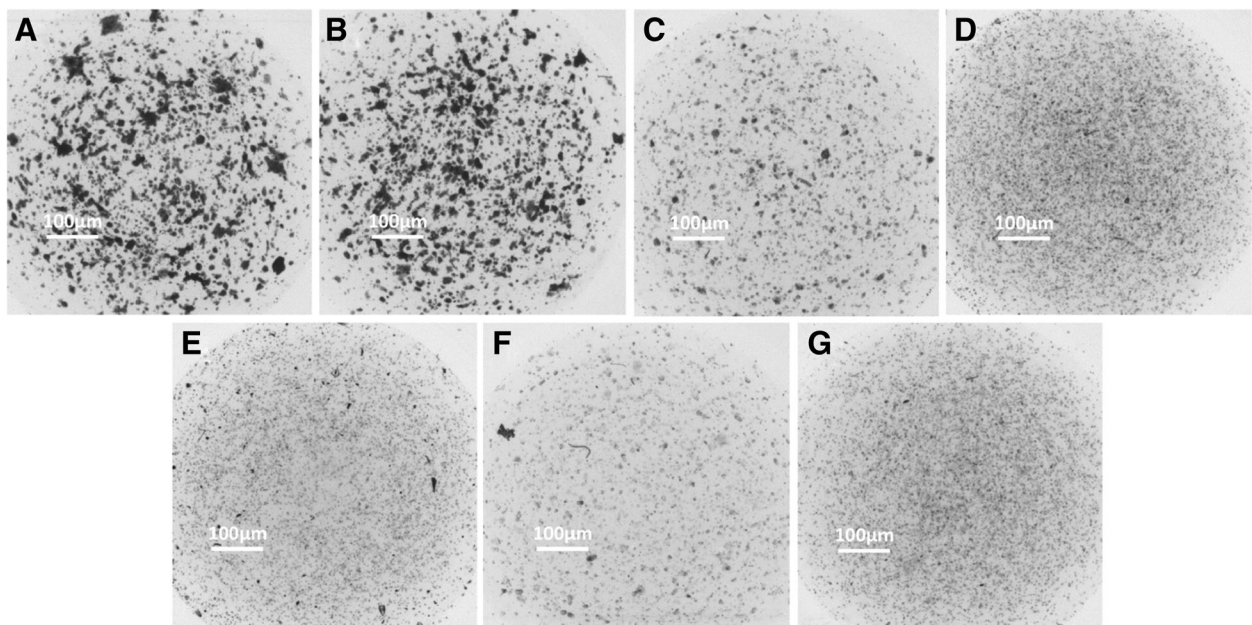


Fig. 4 The represent figures of IC_{max} concentration anti-tumor agents on 3-D cultured HepG2 cells. **a** HepG2 cells were cultured in a 3-D manner. **b-g** HepG2 cells were treated with solvent control (**b**), or IC_{max} concentration of agents, 2.0 µg/ml 5-FU (**c**), 0.08 µg/ml PAC (**d**), 2.0 µg/ml CDDP (**e**), 0.5 µg/ml EPI (**f**) or 5.0 µg/ml L-OHP (**g**)

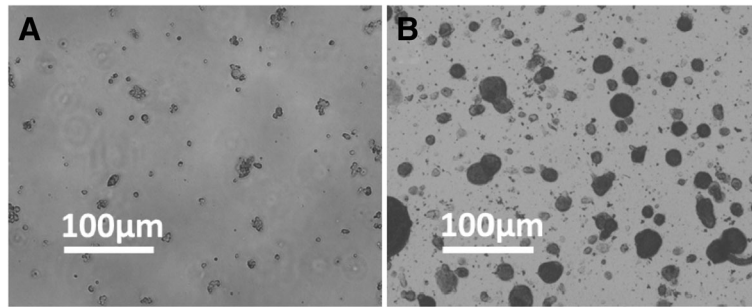


Fig. 5 Primary cells separated from clinical specimens culture in collagen gel droplets. Primary cells separated from clinical specimens were seeded in collagen gel droplets and analyzed by a multifunctional microplate reader at 540 nm. **a** The represent grey scale photograph of baseline (0-time) cells; **b** The represent grey scale photograph of cells after 5–7 days culturing

examined at indicated time-points from three independent experiments. As shown in Table 3, similar results were obtained from each experiment, and the growth rates of HepG2 were from 4.1 to 4.62-fold (Table 3). These results were further confirmed by representative photographs. As shown in Fig. 1, after 5–7 days' growth, the colony sizes were much larger than that at baseline.

HepG2 cells are sensitive to anti-tumor agents

Next, the effect of anti-tumor agents on HepG2 was detected by CD-DST (Fig. 2). The results showed that HepG2 was sensitive to 5-PU, PAC, CDDP, EPI, or L-OHP, and the IC_{50} values were $0.83 \pm 0.45 \mu\text{g/ml}$, $0.03 \pm 0.02 \mu\text{g/ml}$, $1.15 \pm 0.75 \mu\text{g/ml}$, $0.09 \pm 0.03 \mu\text{g/ml}$, and $1.76 \pm 0.44 \mu\text{g/ml}$, respectively. Moreover, the IC_{max} values were $2.0 \mu\text{g/ml}$, $0.08 \mu\text{g/ml}$, $2.0 \mu\text{g/ml}$, $0.5 \mu\text{g/ml}$, and $5.0 \mu\text{g/ml}$, respectively. The representative photographs are shown in Figs. 3 and 4.

Sensitivity of primary cells separated from HCC clinical specimens

Next, the sensitivity of primary cells separated from HCC clinical specimens to anti-tumor agents was detected by CD-DST methods (Fig. 5). Cells were treated with an IC_{max} or IC_{50} concentration of antitumor agents. As shown in Table 4, 8/26, 9/26, and 5/26 patients may be sensitive to IC_{max} values of CDDP, EPI, or L-OHP, and only 3/26, 4/26, and 2/26 patients may be sensitive to IC_{50} . However, no patients were sensitive to 5-Fu or

PAC. The represent photographs are shown in Fig. 6. The characteristics of the tumor (histological grade, BCLC grade number of nodules) in relation to the sensitivity to chemotherapeutic agents are shown in Table 5. Taken together, patients suffering from HCC were not sensitive to PAC or 5-Fu, and only a few obtained clinical benefits from CDDP, EPI, or L-OHP.

2-D cultured HepG2 cells were more sensitive to anti-tumor agents

Moreover, to confirm the sensitivity of HCC cells to anti-tumor agents, the effect of agents on HepG2 or HepG2/ADR, an MDR cell line, was examined by MTT assays and the IC_{50}/IC_{max} concentrations were calculated. The results are shown in Table 6. The IC_{50} value of agents on HpeG2 cells obtained from the MTT assays were much lower than those obtained from CD-DST. Moreover, the IC_{50} value of agents on HpeG2/ADR cells obtained from the MTT assays were similar with those obtained from CD-DST. These results indicated that sensitivity of 2-D cultured HepG2 cells to anti-tumor agents was much higher than that in 3-D-cultured HepG2 cells.

Discussion

HCC is currently one of the most common malignancies, especially in China and the Asia-Pacific region. Although an oral multi-targeted kinase inhibitor, sorafenib, has been used as a curative approach, cytotoxicity chemotherapeutic agents are still treatment choices, e.g., oral medication or hepatic artery chemotherapy and transcatheter arterial chemoembolization (TACE) [21–25]. 5-FU, CDDP, EPI, and L-OHP can induce double DNA strand breaking (DSB) and block DNA replication via multi-mechanisms [26]. PAC targets microtubules and disrupts cell division [27]. It is well known that HCC may be resistant to almost all kinds of cytotoxicity chemotherapeutic agents due to its multi-drug resistance (MDR) features. During the MDR process, breast cancer

Table 4 the sensitivity of patients to anti-tumor agents

Compounds	Total patients	Patients were sensitive to compounds	
		IC_{max} group	IC_{50} group
5-FU	26	–	–
PAC	26	–	–
CDDP	26	8	3
EPI	26	9	4
L-OHP	26	5	2

Table 6 the sensitivity of HepG2 or HepG2/ADR cells to antitumor agents

Compounds	2-D HepG2 <i>IC</i> ₅₀ values (μg/ml)	2-D HepG2/ADR	3-D HepG2
5-FU	0.45 ± 0.06	0.77 ± 0.33	0.83 ± 0.45
PAC	0.01 ± 0.00	0.02 ± 0.01	0.03 ± 0.02
CDDP	0.26 ± 0.23	1.39 ± 0.40	1.15 ± 0.75
EPI	0.02 ± 0.01	0.16 ± 0.04	0.09 ± 0.03
L-OHP	0.55 ± 0.12	1.85 ± 0.44	1.76 ± 0.44

Traditionally, the biological behavior of cancer cells was determined using 2-D methods, e.g., MTT or colony formation. However, 2-D methods may be insufficient to reflect the *in vivo* behavior of cancer cells in solid tumor tissues [7]. Thus, it is valuable to develop and validate a rapid method to examine the biological behavior of cancer cells in a 3-D manner. Tissue engineering research, e.g., 3-D spatial array, investigated the involvement of the extracellular matrix (ECM) in solid tissues [7]. The interaction between cells and the ECM modulates the behavior and features of solid tumors [8–10]. Collagen is a main constituent of the ECM and supports the formation of the microenvironment in solid tumors [8–12]. CD-DST is a mature test in which primary-culture cells of HCC are separated from surgical resection specimens embedded in collagen droplets [7–12]. Applications of CD-DST in chemotherapeutic sensitivity examination have been demonstrated in some kinds of human cancers, e.g., lung cancer, gastric cancer, breast cancer, and colorectal cancer [8–12]. Higashiyama et al. (2010) predicted the chemotherapeutic effect on postoperative recurrence in non-small cell lung cancer patients [9]. Sun et al. (2013) established a 3-D gastric tumor culture model [10]. Lin et al. (2015) revealed the effect of pyruvate kinase M2 on Epirubicin and 5-Fluorouracil in breast cancer by CD-DST [11]. Takahashi et al. (2015) examined the anti-tumor activity of fucoxanthin and fucoxanthinol on cell lines or primary cells from colorectal cancer clinical specimens [12]. In the present work, for the first time, we developed and validated an effective method to predict the resistance of HCC patients to anti-tumor agents. The result showed that 2-D cultured HepG2 cells were more sensitive to anti-tumor agents than cells cultured in a 3-D manner but similar to a 2-D cultured resistance cell line, HepG2/ADR. Moreover, primary cells separated from clinical specimens were much more resistant to anti-tumor agents. Therefore, CT-DST, a 3-D tumor model, is valuable for evaluating and mimicking the *in vivo* efficacy of antitumor agents.

Conclusions

The *in vitro* drug sensitivity examination validated the MDR features of HCC. CD-DST may be a useful method

to predict the potential clinical benefits of anticancer agents for HCC patients.

Abbreviations

5-FU: 5-fluorouracil; BCRP: Breast cancer resistance protein; CDDP: Cisplatin; CD-DST: Collagen gel droplet embedded 3D-culture system; EPI: Epirubicin; HCC: Hepatocellular carcinoma; IR: Inhibitory rate; JAK: Janus kinase; L-OHP: Oxaliplatin; MDR: Multi-drug resistance; MDR-1: Multi-drug resistance 1; MRP2/3: Multi-drugs resistance relative protein2/3; mTOR: Mammalian target of rapamycin; PAC: Paclitaxel; PI3K: Phosphatidylinositol 3-kinase; STAT: Signal transducers and activators of transcription; TACE: Transcatheter arterial chemoembolization

Acknowledgements

We thank Dr. Juan-juan Zhao for her advice and excellent technical assistances to this study.

Funding

This work was supported by direct grants from the Chinese Government: the Dean Innovation Fund (YNKT2014039) from the 302nd Hospital of Chinese PLA, Beijing 100,039, People's Republic of China. These funding sources or funding bodies had no role in the design of this study and will not have any role during its execution, analyses, interpretation of the data, or decision to submit the results.

Availability of data and materials

Additional data and materials may be requested from the corresponding author on reasonable request.

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Authors' contributions

ZXH, JFB and ZZ made substantial contributions to conception and design. JH, ZXH, FF, YTC, YKZ QYJ, SQW, and YH made substantial contributions to acquisition of data, or analysis and interpretation of data. JH, FF and ZZ have been involved in drafting the manuscript or revising it critically for important intellectual content. YSW, XNG, QC and YW have given final approval of the version to be published. Each author should have participated sufficiently in the work to take public responsibility for appropriate portions of the content; and agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Our studies are in compliance with the Helsinki Declaration. The collection of tissues and the study protocol were approved by the Ethics Committee of No. 302 Military Hospital of China, with informed written and verbal consent from 26 patients. The methods did not relate to the clinical trial or methods.

Consent for publication

Not applicable

Competing interests

The authors declare that they have no competing interests. Author Xunian Gao works at/is employed by Guangzhou Darui Biotechnology as the supplier of the chemosensitivity assay for this research.

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Received: 8 November 2016 Accepted: 26 October 2017

Published online: 08 November 2017

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