A pre-clinical model combining cryopreservation technique with precision-cut slice culture method to assess the *in vitro* drug response of hepatocellular carcinoma

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Abstract. Models considering hepatocellular carcinoma (HCC) complexity cannot be accurately replicated in routine cell lines or animal models. We aimed to evaluate the practicality of tissue slice culture by combining it with a cryopreservation technique. We prepared 0.3-mm-thick tissue slices by a microtome and maintained their cell viability using a cryopreservation technique. Slices were cultured individually in the presence or absence of regorafenib (REG) for 72 h. Alterations in morphology and gene expression were assessed by histological and genetic analysis. Overall viability was also analyzed in tissue slices by CCK-8 quantification assay and fluorescent staining. Tissue morphology and cell viability were evaluated to quantify drug effects. Histological and genetic analyses showed that no significant alterations in morphology and gene expression were induced by the vitrification-based cryopreservation method. The viability of warmed HCC tissues was up to 90% of the fresh tissues. The viability and proliferation could be retained for at least four days in the filter culture system. The positive drug responses in precision-cut slice culture *in vitro* were evaluated by tissue morphology and cell viability. In summary, the successful application of precision-cut HCC slice culture combined with a cryopreservation technique in a systematic drug screening demonstrates the feasibility and utility of slice culture method for assessing drug response.

Introduction

Liver cancer is one of the most commonly diagnosed types of malignant cancer and there are approximately 850,000 new cases diagnosed yearly worldwide (1). The high incidence of HCC has induced the development of novel targeted and personalized therapies (2). Personalization of cancer treatment requires the reliable prediction of chemotherapy responses in individual patients. Various strategies have been applied to generate primary cultures from individual tumors which include 2D cell culture of dissociated tumor cells, 3-D spheroid cultures and patient-derived mouse xenograft cultures (3-7). However, the difficulties in replicating the heterogeneous microenvironment in the primary tumor reduce their efficiency in drug experiments (8). It was estimated that over 90% of novel anticancer drugs fail in clinical trials because these models could not simulate complete tissue structure and maintain the biological heterogenicity of the primary tumor (9). For these reasons, it is crucial for us to create novel models that are more predictive of *in vivo* efficacy.

Precision-cut slice is a new method of tissue culture *in vitro*, which is derived directly from the primary tumor (10). However, there is no preservation method applied to maintain

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Abbreviations: HCC, hepatocellular carcinoma; H&E, hematoxylin and eosin; IHC, immunohistochemistry; LDH, lactate dehydrogenase; REG, regorafenib; CCK-8, Cell Counting Kit-8

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living fresh tissue. Conventional preservation of fresh tumor tissue such as formalin-fixed paraffin-embedded samples and flash freezing in liquid nitrogen always leads to the absolute inactivation of the fresh tissue. Therefore, a reliable and efficient cryopreservation method for living tissue is indispensable. Vitrification-based cryopreservation method can be developed to preserve fresh tissue, by which the biological characteristics of the original tumor can be retained and the utilization of specimens may be markedly improved (11).

In the present study, we explored a precision-cut slice culture method combined with a cryopreservation technique to establish a preclinical model, which is derived from fresh tissues of HCC patients. In addition, we demonstrated systematic optimization of HCC slices *ex vivo* by comparing different culture conditions. Moreover, this culture system allowed the detection of tumor responses to REG chemotherapy.

Materials and methods

Collection of HCC specimens. From October, 2019 to February, 2020, surgically resected specimens were obtained from 30 HCC patients at the Renji Hospital Affiliated to Shanghai Jiao Tong University School of Medicine (Shanghai, China). Samples were maintained at 4˚C on ice and transported in preservation medium (Tissue Mate™; Celliver Biotechnology Co. Ltd.). Details are illustrated in Fig. 1. This investigation was approved by the Ethics Committee of Renji Hospital and followed the guidelines of The Declaration of Helsinki. All patients provided written informed consent. The inclusion criteria were: i) pathological diagnosis of HCC; ii) no patients had received any prior treatment; iii) the maximum diameter of a single tumor was more than 2 cm; iv) Child-Pugh score A or B. The exclusion criteria were: i) Child-Pugh score C; ii) exceptional circumstances, such as syphilis and acquired immune deficiency syndrome.

Cryopreservation and warming procedures. All specimens were cut into 1-mm-thick slices in a metal mold before cryopreservation (Fig. 2C). Cryopreservation solutions (LT2601; Tissue Mate™) and warming solutions (LT2602; Tissue Mate™) were provided by Celliver Biotechnology Co. Ltd. (Fig. 2B). For tissue cryopreservation, vitrification solution 1 (V1), vitrification solution 2 (V2) and vitrification solution 3 (V3) were pre-warmed in a 2~8˚C water bath. Fresh HCC tissues were cleaned twice with sterile PBS and transferred into 10 ml V1, 10 ml V2 and 10 ml V3 for 8, 8, 10 min, respectively. Tissues were then placed onto a thin metal strip and submerged into liquid nitrogen for at least 5 min. Finally, the strips with tissue were placed into frozen storage tubes and preserved in the nitrogen canister. The tissue samples were stored in the liquid nitrogen. For tissue warming, the frozen storage tubes were removed from the nitrogen canister and the strips with the cryopreserved biopsy tissues were quickly transferred into 30 ml warming solution 1 (T1), and incubated for 3 min in a 37˚C water bath. The tissues were then transferred into 10 ml warming solution 2 (T2) and 10 ml warming solution 3 (T3) for 5 and 10 min, respectively, at room temperature. Warmed tissues were cleaned twice with sterile PBS and kept on ice (Fig. 2D). The timeline of cryopreservation and warming procedures are depicted in Fig. 2A.

Tissue slice preparation and cultivation. Surgically resected specimens were cut into 300-μm-thick precision-cut slices using a microtome for slice preparation (Bio-Gene Technology, Ltd.) (Fig. 2E). A thickness of 300 μm was considered the most suitable thickness for HCC after several early slicing pre-experiments (Fig. 2F). Parameter settings, such as the frequency and amplitude of vibration slicing, were determined by the diverse cirrhosis degree and tumor stage. Tissue slices (diameter, 2 mm) were then prepared using a hand-held coring tool (Fig. 2G), and all the procedures were performed under sterile conditions. One-third of the precision-cut slices were maintained on Transwell inserts (pore size, 0.4 μm; Corning, Inc.) (Fig. 2I). One-third of the precision-cut slices were individually submerged in medium (Fig. 2H) and incubation was performed on a shaking platform (TYZD-III, QiQian Technology, Ltd.). The remaining precision-cut slices were cultured statically in medium as control (Fig. 2H). Cultivation was performed in 12-well plates containing 450 μl DMEM medium (Gibco™; Thermo Fisher Scientific, Inc.) with 10% fetal bovine serum (Gibco™; Thermo Fisher Scientific, Inc.), penicillin and streptomycin (100 U/ml; Gibco™; Thermo Fisher Scientific, Inc.), and kept at 37˚C in a humidified incubator with 5% CO_2.

Cell Counting Kit-8 (CCK-8) assay. A CCK-8 assay (Dojindo Molecular Technologies, Inc.) was used to evaluate the viability of tissue slices at each time point (24, 48, 72, 96 h). DMEM (90 μl/well) and CCK-8 solution (10 μl/well) were added into 96-well plates. The tissue slices were added one slice/well. The plates were maintained at 37˚C in a humidified incubator with 5% CO_2 for 2 h. The slices were removed from the 96-well plates and the plates were transferred to microplate reader (Multiskan GO; Thermo Fisher Scientific, Inc.). The absorbance at 450 nm was measured and three wells were tested for each sample at each time point.

Calcein-AM cell viability assay and Hoechst 33342 staining. The Live/Dead® Viability Assay kit (Nanjing KeyGen Biotech Co.) and Hoechst 33342 (Beyotime Institute of Biotechnology Co.) were stored at -20˚C and allowed to warm to room temperature prior to experimentation. The viability assay stock reagents (calcein-AM, 4 mM) were diluted to 1 μM in physiological solution and mixed with 2 μg/ml Hoechst 33342 stock reagents at room temperature for 30 min. Live cells are characterized by a bright green fluorescent and cell nucleus are blue. Representative images were captured with the Leica TCS SP8 confocal microscope (x20) (Leica Microsystems GmbH). The ratio of living cells in the calcein-AM cell viability assay/Hoechst 33342 staining were calculated based on manual counting within 10 random microscopic fields.

Hematoxylin and eosin (H&E)/immunohistochemical (IHC) staining. Tumor slices were formalin-fixed, embedded in paraffin and cut into 4-μm-thick sections. Paraffin sections (4-μm) were stained with H&E at room temperature. IHC staining was carried out by standard protocols. Briefly, sections were de-waxed in xylene and rehydrated in graded ethanol, and heat-mediated antigen retrieval of tissue sections was carried out before being allowed to cool. Endogenous peroxidases were blocked using 0.9-3% hydrogen peroxide for 10 min, and non-specific antibody binding was blocked by
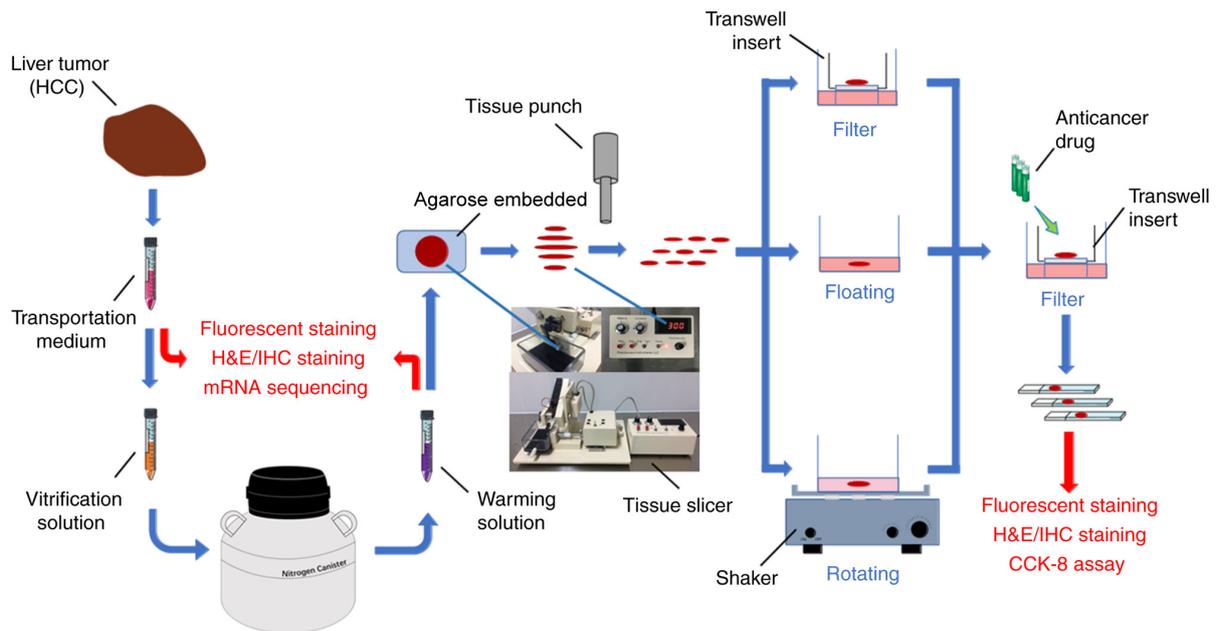
Figure 1. Workflow of the cryopreserved slice culture and drug testing. HCC tissues were obtained and transported to the laboratory in preservation medium at 4˚C within 2 h. Tissues were cryopreserved and stored in a nitrogen canister. Warmed tissues were used when required. The 1-mm-thick tissue slices were embedded in agarose and cut into 300-μm-thick slices using a VF-300 microtome. Tissue slices (2 mm in diameter) were maintained on different culture support. Slices were maintained on a Transwell insert and treated with REG at concentration of 20 μM. Experiments were performed to evaluate the efficacy of cryopreservation combined with slice cultivation in the assessment of anticancer drug responses by viability assay and fluorescent staining. HCC, hepatocellular carcinoma; REG, regorafenib; H&E, hematoxylin and eosin; IHC, immunohistochemical.
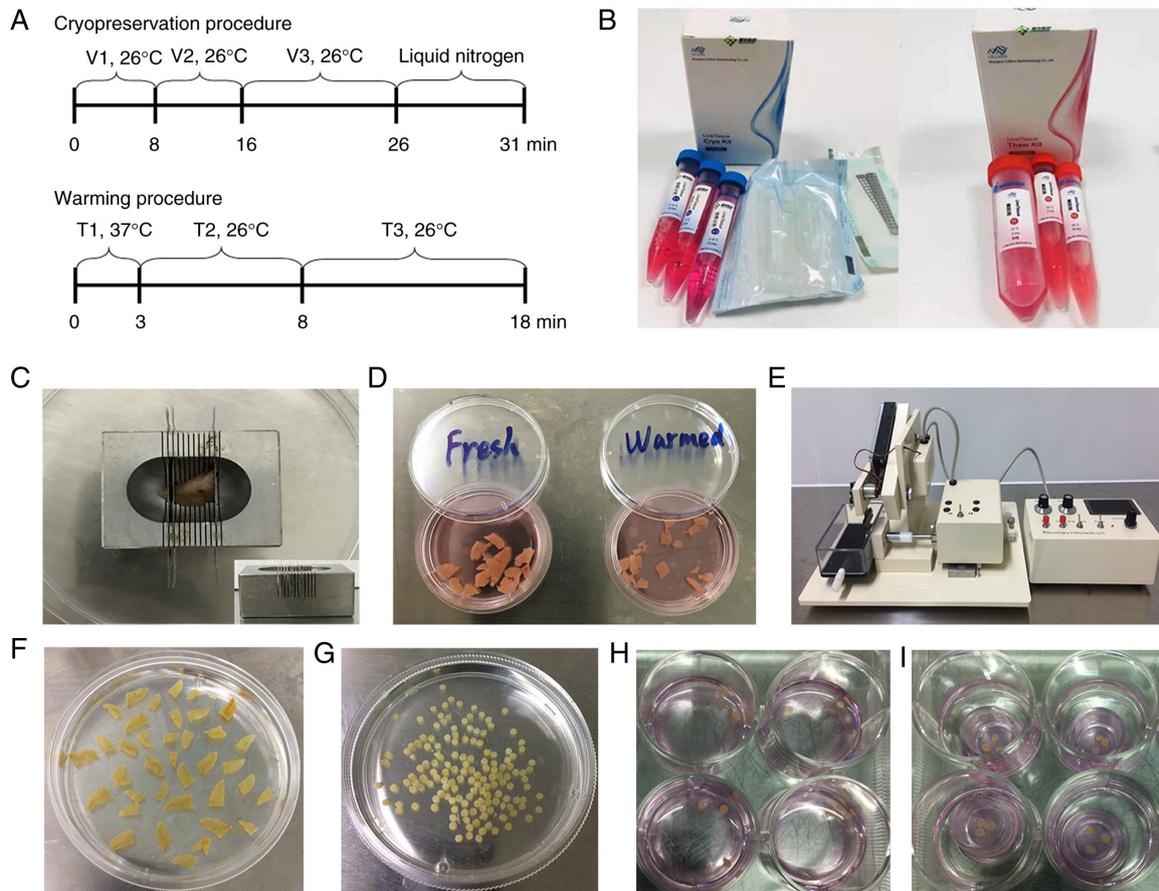
Figure 2. Cryopreservation technology and precision-cut tissue slice culture method. (A) The standardized time schedule of the cryopreservation and warming procedures. (B) Cryopreservation solutions and warming solutions. (C) Tissues were cut into 1-mm-thick slices by a stainless slicer in a metal mold. (D) Cryopreserved tissues and warmed tissues. (E) VF 300 microtome. (F) The 300-μm-thick slices. (G) The 2-mm-diameter slices. (H) Floating culture. (I) Filter culture (Transwell insert).

incubation with serum-free blocking solution or 10% normal serum block for 30 min. Tissue sections were then incubated with the anti-Ki67 antibody (Ab15580; Abcam; 1:1,000 dilution), before being probed with the secondary antibodies Alexa Fluor® 488 (Ab150077; Abcam; 1:1,000 dilution). Antibodies were visualized using 3,3'-diaminobenzidine chromogen and counterstained with Meyer's Hematoxylin for 2 min. Sections were then dehydrated through graded alcohols, cleared in xylene and mounted. Confocal laser scanning microscopy (magnification, x20) was performed using an Olympus Corp. BX51 instrument. The ratio of proliferative cells in the Ki67 staining were calculated based on manual counting within 10 random microscopic fields.

Experimental methods for mRNA sequencing. RNA purity was assessed using the kaiaoK5500® Spectrophotometer (Beijing Kaiao Technology Development Co. Ltd.). RNA integrity and concentration were assessed using the RNA Nano 6000 Assay kit and the Bioanalyzer 2100 system (Agilent Technologies, Inc.). A total amount of 2 μg RNA/sample was used as input material for the RNA sample preparations. Sequencing libraries were generated using NEBNext® Ultra™ RNA Library Prep kit for Illumina® (E7530L; New England BioLabs, Inc.), following the manufacturer's recommendations, and index codes were added to attribute sequences to each sample. Briefly, mRNA was purified from the total RNA using poly-T oligo-attached magnetic beads. Fragmentation was carried out using divalent cations under elevated temperature in NEBNext® First Strand Synthesis Reaction Buffer (5X) (New England BioLabs, Inc.). First-strand cDNA was synthesized using random hexamer primer and RNase H. Second-strand cDNA synthesis was subsequently performed using buffer, dNTPs, DNA polymerase I and RNase H. The library fragments were purified with QiaQuick PCR kits (Qiagen, Inc.) and elution with EB buffer, then terminal repair, A-tailing and adapter adding were implemented. The products were retrieved and PCR was performed, and then the library was completed. The RNA concentration of the library was measured using a Qubit® RNA Assay kit in Qubit® 3.0 (Thermo Fisher Scientific, Inc.) for preliminary quantification, and then diluted to 1 ng/μl. Insert size was assessed using the Agilent Bioanalyzer 2100 system (Agilent Technologies, Inc.), and qualified insert size was accurately quantified using the StepOnePlus™ Real-Time PCR System (Thermo Fisher Scientific, Inc.; library valid concentration, >10 nM). The clustering of the index-coded samples was performed on a cBot cluster generation system using a HiSeq PE Cluster kit v4-cBot-HS (Illumina, Inc.) according to the manufacturer's instructions. After cluster generation, the libraries were sequenced on an Illumina, Inc. platform and 150-bp paired-end reads were generated. The variations in gene expression were detected by different colors in the heat map.

Metabolic activity of pH/glucose/LDH. For the testing of the potential of hydrogen (pH), we extract 15 μl culture medium from the slice culture system using a detecting instrument (InLab Ultra Micro-ISM, Mettler Toledo); Glucose was tested using a detecting instrument (GlucCell™, Brookfield), using 3 μl of culture medium; For LDH (lactate dehydrogenase), a detection kit (G1780, Promega Corp.) in a 96-well plate was

used. All the processes were conducted using the operation manuals provided by the suppliers.

Drug sensitivity test in vitro. Drug testing commenced after 24 h of slice culture and was performed for an additional 72 h. For drug testing of slices *in vitro*, regorafenib (REG; MedChemExpress LLC) was used and tested at a concentration of 5, 10, and 20 μM, respectively. To investigate cell proliferation and tissue morphology, the slices were incubated with CCK-8 solution and stained with H&E/fluorescent dyes.

Statistical analysis. Statistical evaluations were performed using one-way ANOVA with Scheffe's post hoc tests by IBM SPSS Statistics 22.0 (IBM Corp). $P<0.05$ was considered to indicate a statistically significant difference. Three repeats were performed.

Results

Biological characteristics of HCC tissues are maintained by vitrification-based cryopreservation and precision-cut slice method. All of the fresh HCC specimens were obtained from Renji Hospital Affiliated to Shanghai Jiao Tong University. The 30 patients included 23 men and 7 women with a mean age of 58 years. The workflow was strictly performed by standard procedures, as depicted in Fig. 1. The specific explanation is provided in the Materials and methods section. Human liver tissues were found to be very well sliceable and showed a good reproducibility as well as tissue viability. The 1-mm-thick HCC slices were cryopreserved and warmed according to the timeline in Fig. 2A, using cryopreservation solutions (Fig. 2B, left) and warming solutions (Fig. 2B, right). Thirty fresh specimens were derived from 30 HCC patients. Half of each specimen was processed by cryopreservation and warming procedures, and the remaining tissues were used as the control group. The 300-μm-thick precision-cut slices were made and cultured successfully (Fig. 2C-I). H&E staining of fresh tissue slices revealed no obvious differences in the morphology when compared to the warmed tissue slices. From the fluorescent and IHC staining, we found that the living cell ratio was 93% in fresh tissues and 90% in warmed tissues, which indicated that no obvious difference was detectable between the fresh HCC and warmed HCC tissues (Fig. 3A and B). The heat map of the cancer-associated genes indicated that the color of the left column was mostly consistent with the right column (Fig. 3E). Only a small part of differential gene expression was detected from the volcano plot (Fig. 3C) and distribution of sample expression (Fig. 3D). According to the GO analysis, it was found that the differential genes were closely related to cell metabolism (Fig. 3F). Original data were uploaded to the Gene Expression Omnibus database (accession number GSE194095). Therefore, the variations in gene expression between fresh and warmed tissues were limited. These results confirmed that the vitrification-based cryopreservation method was able to largely maintain the biological activity and histological features of the HCC tissues.

Medium composition and culture mode are critical to tissue viability. In order to identify the best slice culture methods, we optimized the slicing process with different culture
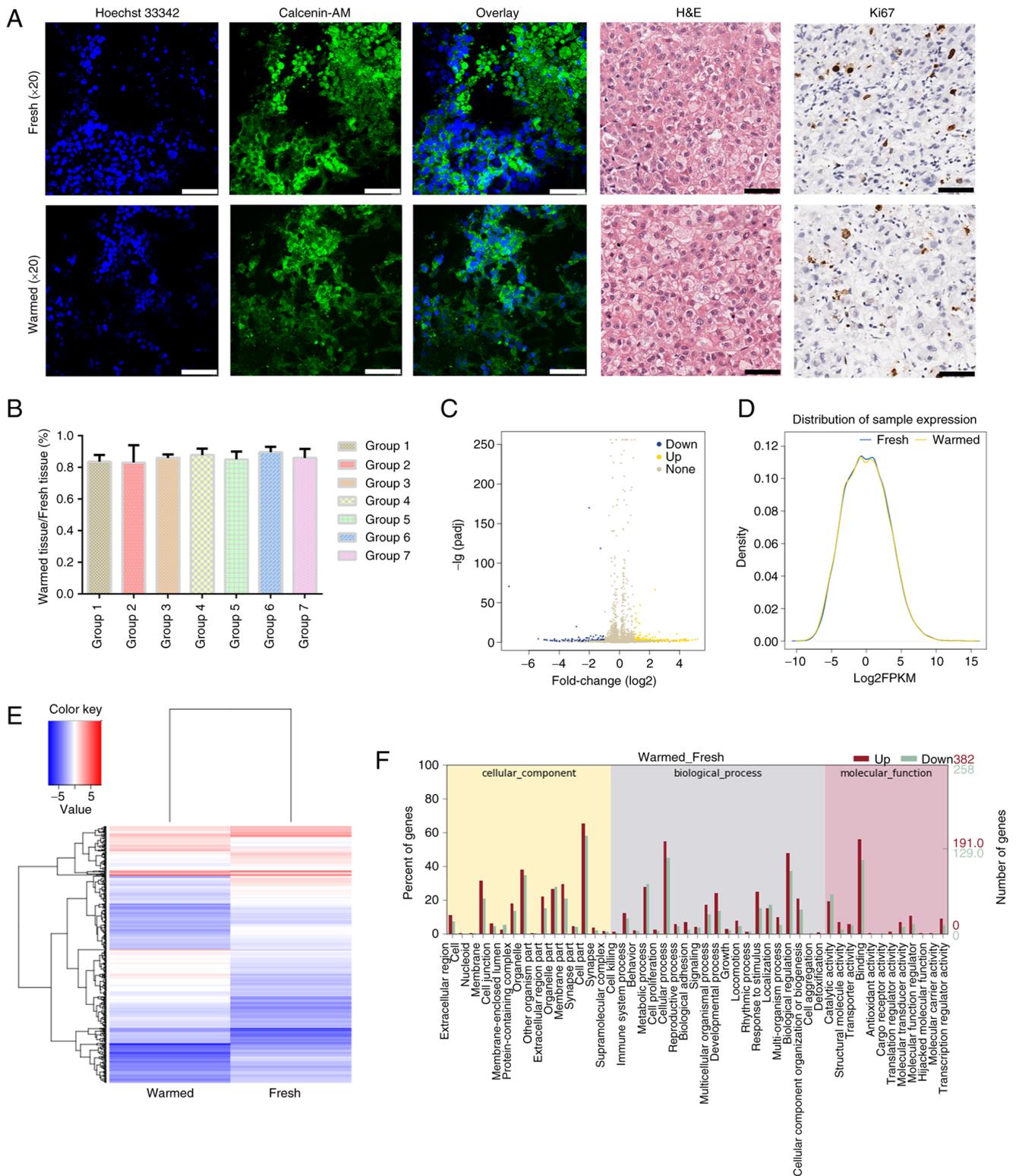
Figure 3. Effects of cryopreservation on the maintenance of the biological characteristics of tissues. (A) Calcein-AM/Hoechst staining and H&E/IHC staining. Blue nuclei indicate both living and dead nuclei, while green sections represent the cytoplasm of living cells. IHC staining was used to indicate the percentage of cells expressing the proliferation marker Ki67. Scale bars, 50 μm. (B) The viability of fresh HCC slices was more than 10% of the warmed tissues, which was determined by CCK-8 assay (n=7). (C and D) Volcano plot and distribution of sample expression showed the differential gene expression between fresh and warmed tissues. (E) Heat map of mRNA sequencing. The color change in the heat map is defined as the difference in gene expression between fresh and warmed tissues. The deeper the red, the more greatly increased the gene expression. The deeper the blue, the lower the gene expression. (F) The Gene Ontology (GO) analysis indicated that differential genes were closely related to cell metabolism. HCC, hepatocellular carcinoma; H&E, hematoxylin and eosin; IHC, immunohistochemistry.

methods and selected the optimal culture medium. Our results showed that Medium I (DMEM with high glucose +10% FBS) could obviously maintain cell viability, especially

from 48 h (Fig. 4A and B); Medium II (1640+10% FBS) and Medium III (DMEM/F12) were not suitable for the slice culture (Fig. 4A). The filter cultures were viable for up to
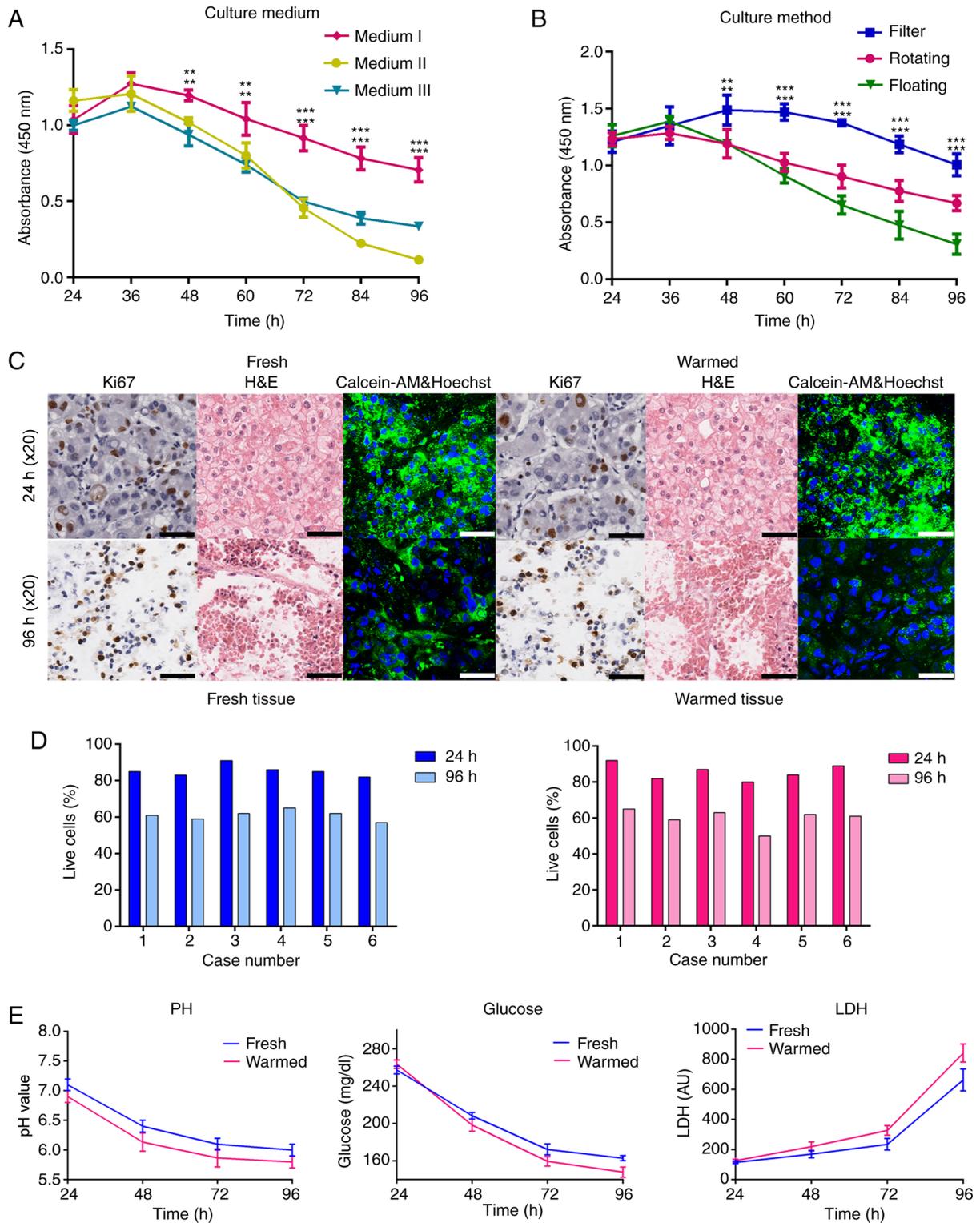
Figure 4. Optimization of medium composition and culture mode. (A) The adaption of Medium I (DMEM with high glucose +10% FBS) was able to provide a higher cell viability and a longer culture time than Medium II (1640+10%FBS) or Medium III (DMEM/F12). $^{**}P<0.01$ and $^{***}P<0.001$. vs. Medium I. (B) Filter cultures were viable for up to 4 days and were able to achieve a higher cell viability than the floating and rotating culture. $^{**}P<0.01$ and $^{***}P<0.001$. vs. Filter. (C) Calcein-AM/Hoechst staining and H&E/IHC staining. Scale bars, 50 μm. (D) The ratio of living cells in fresh and warmed slices after 96 h. (E) The variation in pH, glucose and LDH during the slice culture. H&E, hematoxylin and eosin; IHC, immunohistochemistry; pH, potential of hydrogen; LDH, lactate dehydrogenase.

4 days and could receive a higher cell viability than the floating and rotating cultures (Fig. 4B). Subsequently, we conducted slice culture on Transwell insert with DMEM combined with 10% FBS for 72 h. As determined from the fluorescent and IHC staining, the living cell ratio was decreased slightly and a slight change in tissue morphological features was detected. In addition, these changes were observed both in fresh and warmed tissues (Fig. 4C and D). Moreover, we detected that the levels of pH and glucose were decreased while LDH was obviously increased (Fig. 4E).
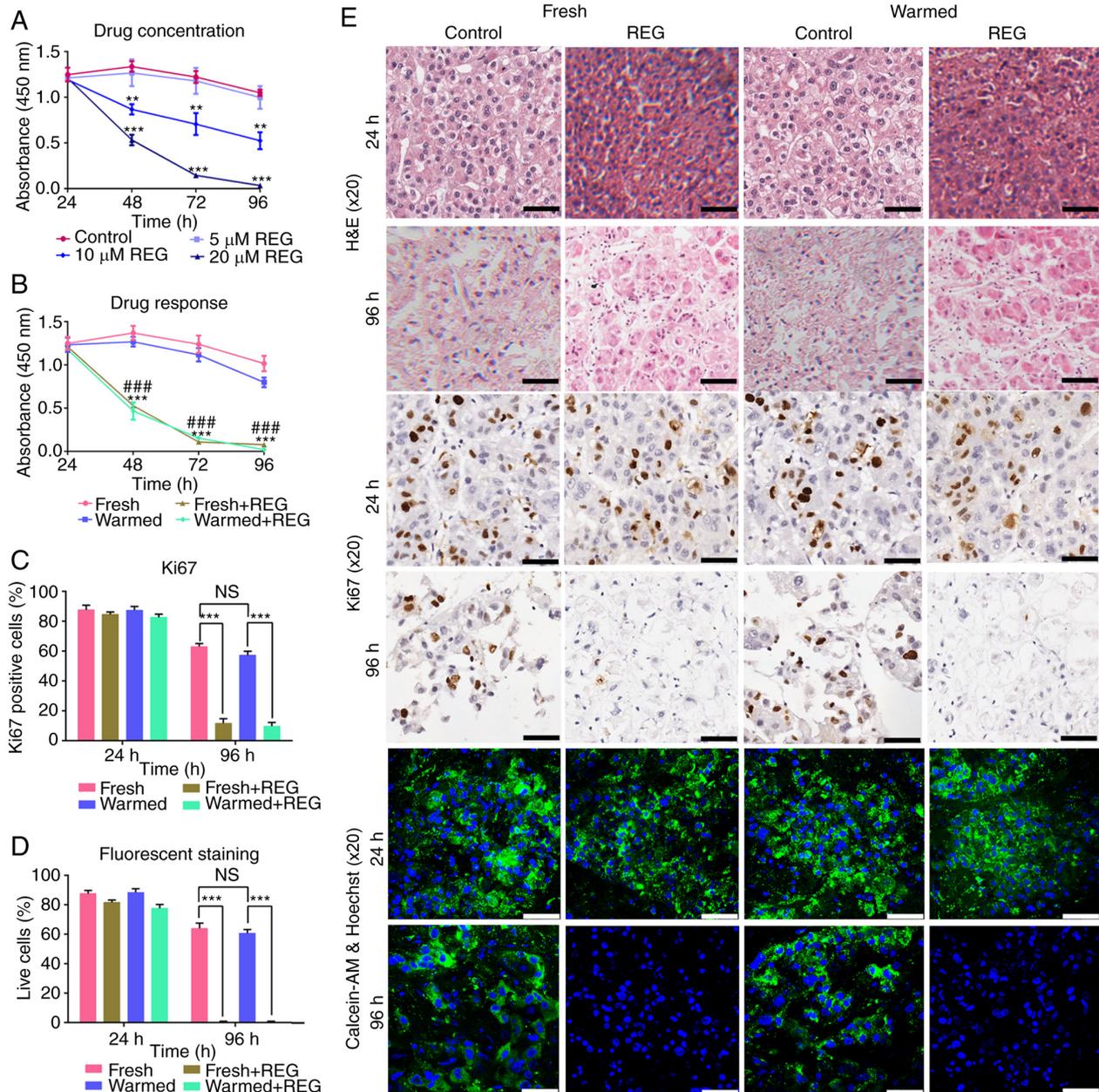
Figure 5. Positive drug responses in the slice culture model. (A) HCC slices were treated with different concentrations of REG (5, 10, and 20 μM) for 72 h. The control group was statistically different from the 10 μM group and 20 μM group, respectively. (B) CCK-8 cell viability assay was conducted for 72 h. **P<0.01 and ***P<0.001, Fresh group vs. the Fresh + REG group; $^{###}$P<0.001 Warmed group vs. Warmed + REG group. (C) The quantification of Ki67-positive cells, the proliferation rate before and after cryopreservation, was not statistically different as determined by Student's t-test. (D) The quantification of live cells, the number of live cells before and after cryopreservation, was not statistically different as determined by Student's t-test. ***P<0.001 and NS, not significant. The Fresh group was statistically different from the Fresh + REG group and the Warmed group was statistically different from the Warmed + REG group in 5B-D. (E) Morphological staining and fluorescent staining in fresh and warmed tissues during drug testing. Scale bars, 50 μm. HCC, hepatocellular carcinoma; REG, regorafenib.

Positive drug responses could be detected in slice culture model. Drug testing commenced after 24 h of slice culture and was performed for an additional 72 h. To study the activity of anti-cancer drug REG in this tissue culture model, HCC slices were treated with different concentrations of REG (5, 10 and 20 μM) for another 72 h as depicted in Fig. 5A. Our results revealed that 20 μM was the most obvious concentration with which to significantly decrease the cell viability. Morphological staining and viability assays both indicated that no obvious differences were detectable after 24 h of slice culturing. However, compared with the control group, both fresh and warmed tissue slices in the drug treatment group cultured for 72 h showed a significant decrease in cell viability. In addition, tissue slices in the drug treatment group evidently lost the morphological structure of the original tumor (Fig. 5B-E).

Discussion

Research has indicated that a tissue slice culture system can be applied to perform preclinical and clinical studies for medical research (12-15). In our research, we describe precision-cut slice cultures as a novel model to perform *ex vivo* experiments on

hepatocellular carcinoma (HCC) tumors, which preserves the three-dimensional structure of the tumor and provides an alternative to *in vivo* experiments. Our study was performed using standard procedures (Fig. 1). Studies have shown that there may be a drastic difference between a drug effect on cancer cells in a normal monolayer cell culture vs. a 3-D cell culture (13,16,17). This evidence indicates the importance of normal tissue architecture and cell-cell communications that clearly exist *in vivo*.

One way to maintain these features is the tissue slice method, which was originally described for culture of breast and colon tumors (18-20). It has several important advantages. Firstly, the slice culture system provides the possibility to investigate the relationship between tumor cells and specific tumor microenvironments, which is suitable for the evaluation of drug effects and many other biological studies (21). Secondly, slice culture systems may reduce the need for animal testing, since they provide a biologically relevant platform for screening compounds. Normally, the exact control of thickness will be beneficial for full diffusion of nutrients and oxygen. The optimal thickness of slices was found to be related to the different type of tissue (10,15,22). In order to determine the optimal thickness of slicing and culturing, we optimized the slicing process with a slicer and found that 300 μm was the most suitable thickness for the HCC tumor slice after pre-experiments. Previous studies have reported that viability and proliferation could be retained for 3 to 7 days (10,23-25). Our results demonstrated that slices (300-μm) cultured on filter inserts were viable for up to 4 days. We did not characterize later time points, but there were no significant signs of tissue deterioration after 4 days, suggesting that extended incubation may be possible if required for a specific functional assay.

In order to maintain the viability of the tissue and improve the utilization of specimens, a standardized vitrification-based cryopreservation method was developed. The cryopreservation and warming procedures should be implemented strictly in accordance with the time schedule. In fact, several types of cells, such as embryo and stem cells have been successfully vitrified (26,27). The results of our research showed that no obvious difference was detected in the cell viability and morphological characteristics of the original tumor before and after cryopreservation. Gene expression analysis also showed that no significant alterations in gene expression were introduced by this cryopreservation method, except a slight alteration associated with cell metabolism. As determined by pre-experiments, no difference was induced by different lengths of preservation time in liquid nitrogen after cryopreservation. These findings further support the conclusion that vitrification is less damaging to cell viability and function due to the minimal ice crystallization in the process of cryopreservation (28).

To test and optimize the culture condition, the different composition of medium and the different growth support were compared. We adapted the culture medium for long-term expansion of the slice, because composition of the culture medium is highly important to maintain tumor slice viability. Similar as observed with other slices (24), the filter culture was superior to the rotating culture and floating culture. The reason may be attributed to a better oxygen supply of the tissue in the filter cultures. In the present research, tissue slices processed by a microtome all showed evident responses to anticancer drugs. The slice model therefore has tremendous potential in selecting sensitive anticancer drugs via examining the cell morphology and proliferation rate.

The present research demonstrated that HCC tissue slices could be effectively cryopreserved, and the tumor biological characteristics were well retained. The tissue slice model provides a better predictability of cancer drug response and improves the efficiency of precision or personalized treatment. Similar assays can be developed to investigate other drugs. At present, human tissue slice cultures have their limitations in regard to *in vitro* cultivation time and low throughput. Accordingly, further development is required to allow for high throughput analysis which is not possible in the current experiments. In addition, the cryopreserved method can also detect the toxicity of drugs to normal cells, which can be an area of future research.

Availability of data and materials

The datasets used and/or analyzed during the current study are available in the Gene Expression Omnibus database (accession number GSE194095).

Authors' contributions

YZ made substantial contributions to the conception and design of the study. ZYW made substantial contributions to the analysis and interpretation of the data. HSJ was involved in drafting the manuscript, and HDZ revised the draft critically for important intellectual content, and these authors also contributed to manuscript drafting and critical revisions on the intellectual content. HXY made substantial contributions to the conception and design of the study. JXF gave the final approval of the version to be published. YZ, JXF and BZ validated the data generated in this study. BZ 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.

Ethics approval and consent to participate

Patient-derived specimens were used in the research. The manuscript does not contain experiments using animals. The investigation was approved (2019-09-15) by Ethics Committee of Renji Hospital, School of Medicine, Shanghai Jiao Tong University (Shanghai, China) and all patients provided written informed consent.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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