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Colorectal cancer liver metastases organoids retain characteristics of original tumor and acquire chemotherapy resistance

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ABSTRACT

Background: Colorectal cancer (CRC) liver metastasis is highly unfavorable for patient outcome and is a leading cause of cancer-related death. Pre-clinical research of CRC liver metastasis predominately utilizes CRC cell lines grown in tissue culture. Here, we demonstrate that CRC liver metastases organoids derived from human specimens recapitulate some aspects of human disease.
Methods: Human CRC liver metastases pathological specimens were obtained following patient consent. Tumor disaggregates were plated and organoids were allowed to expand. CRC markers were identified by immunofluorescence. Stem cell genes were analysed by QPCR and flow cytometry. Response to drug therapy was quantified using time-lapse imaging and MATLAB analysis.
Results: Organoids showed global expression of the epithelial marker, EpCAM and the adenocarcinoma marker, CEA CAM1. Flow cytometry analysis demonstrated that organoids express the stem cell surface markers CD24 and CD44. Finally, we demonstrated that CRC liver metastases organoids acquire chemotherapy resistance and can be utilized as surrogates for drug testing.
Conclusion: These data demonstrate that CRC liver metastases organoids recapitulate some aspects of human disease and may provide an invaluable resource for investigating novel drug therapies, chemotherapy resistance and mechanism of metastasis.

1. Introduction

Colorectal cancer (CRC) is the 3rd most common cancer worldwide. Fatality is most commonly caused by metastasis, a multistep process which requires tumor cell dissemination and colonization of a foreign organ (Friedl and Wolf, 2003). Advantageous mutations which permit tumor cells to disseminate also contribute to chemotherapy resistance (Scheel and Weinberg, 2012). Furthermore, drug therapy in the metastatic setting provides little advantage due to the frequency of metastatic relapse and drug resistance (Wan et al., 2013; Yokobori et al., 2013).

Currently, pre-clinical investigation of metastasis relies on cell lines derived from CRC. While such studies have yielded invaluable insights into mechanisms which permit metastasis, these cell lines do not accurately represent all some aspects of advanced disease (Edwards et al., 2015; Marshall, 2014). Furthermore, novel drug therapies are commonly only tested on human-derived primary cultures. Collectively, this suggests that the development of human-derived cultures of CRC liver metastases would provide an invaluable resource for advancing our understanding of the mechanisms which permit tumor cell migration and invasion, and allow drug screening in cultures which recapitulate advanced stages of human disease. Here, we expand on previous work done on establishing CRC organoids (Ashley et al., 2014; Ashley et al., 2013; Fujii et al., 2016; Sato et al., 2011; Weeber et al., 2015). We demonstrate that human CRC liver metastases organoids can be rapidly cultured with mechanical dissociation, and these samples recapitulate some aspects of human disease. Furthermore, we show that these cultures can be utilized as surrogates for drug screening and acquire chemotherapy resistance.

2. Materials and methods

2.1. Human specimens and organoid culturing

Human CRC liver metastases pathological specimens were obtained from the Oxford Radcliffe Biobank following patient consent, institutional review and ethical approval. Pathological specimens were immediately placed in DMEM/F12 + GlutaMAX (ThermoFisher, USA, CAT #31331-028) containing 1% Penicillin/Streptomycin and 0.4% Ampicillin B. Pathological specimens were divided and embedded in Optical Cutting Temperature (OCT) for immunofluorescence, snap frozen for molecular analysis or transferred to fresh DMEM/F12 + GlutaMAX for tumor disaggregation as previously described (Ashley et al., 2014).

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Disaggregates were plated in a 96-well suspension plate (Sarstedt, GER, CAT #83.3924.500) or mixed with Matrigel Matrix (Corning, USA, CAT #354234) and plated into a 24-well suspension plate (Sarstedt, CAT #83.3922.500). For Matrigel mixed samples, 50 μL was added per well. DMEM/F12 + GlutaMAX containing StemPro, ROCKs inhibitor, R-Spondin-1 (RSPO-1), Noggin, WNT3A, Epithelial Growth Factor (EGF), Insulin-like Growth Factor 1 (IGF-1), Fibroblast Growth Factor 10 (FGF-10), Fibroblast Growth Factor basic (FGFβ) and Endothelin 3 (ET3) was added to the samples (Refer to Sup. Table 1 for concentrations). Media was changed 3 times/week and cultures were passaged every 1–2 weeks. Passaging was performed by incubating cultures in ice-cold PBS for 15 min, followed by 2 washes in ice-cold PBS. Cultures were then centrifuged and dissociated by pipetting. Culture disaggregates were then resuspended in PBS and counted under a light microscope. Approximately 50 crypt-like disaggregates were resuspended in Matrigel and plated in 4–8 wells of a 24-well suspension plate. DMEM/F12 + GlutaMAX containing all growth factors will be referred to as media with full supplementation. For all analysis and experiments, the passage number of cultures was between 8 and 16.

2.2. Hematoxylin and Eosin (H&E) staining and immunofluorescence

Organoid cultures were isolated from Matrigel and washed 3 times with ice cold PBS then immediately embedded in OCT freezing media and snap frozen in liquid nitrogen. 10 μm sections were collected and stored at −30 °C. Prior to staining, sections were dried for 1 h at 37 °C. For H&E staining, slides were hydrated in graded ethanol solutions then placed in Heamatoxylin Harris (VMR Chemicals, USA, CAT #3519455) for 2 min, rinsed in tap water and differentiated in 1% hydrochloride in 70% ethanol for 30 s. Following differentiation, samples were washed in tap water then incubated in Eosin solution (Sigma, USA, CAT #HT110132) for 2 min before being dehydrated in graded ethanol. Slides were incubated in xylene before being mounted with Vecta Mount (Vector, CAT #H-5000). For immunofluorescence, slides were fixed in 4% paraformaldehyde and permeabilized with 0.1% Triton. Slides were blocked for 1 h at room temperature (RT) and primary antibodies were left on overnight at 4 °C. The next day, slides were washed and secondary antibodies were added for 1 h at RT in the dark. Slides were then stained with Hoechst (1 in 250, 5 mg/mL ThermoFisher, CAT #33342) for 10 min and mounted with ProLong Diamond Antifade Mountant (ThermoFisher, CAT #P36961). Immunofluorescence images were captured with a Leica DM6000 confocal microscope. Antibodies were used at the following concentrations; Rabbit anti-human Pan-Laminin: 1 in 100 (Sigma, CAT #AB11575), secondary: Donkey anti-rabbit 555: 1 in 250 (ThermoFisher, CAT #21428); Mouse anti-human CEACAM5 (Santa Cruz, CAT # SC-23928): 1 in 100, secondary: Goat anti-mouse 546: 1 in 250 (ThermoFisher, CAT #A21123); Mouse anti-human EpCAM (AUA1 antibody raised in house) (Ashley et al., 2014): 1 in 100, secondary: Goat anti-mouse 546: 1 in 250 (ThermoFisher); Rabbit anti-human Ki67: 1 in 100 (Vector Labs, CAT #VP-RM04), secondary: Goat anti-rabbit 488: 1 in 250 (ThermoFisher CAT #A11008); Rabbit anti-human MUC2: 1 in 100 (Santa Cruz; CAT #SC-15334), secondary: Donkey anti-rabbit 555: 1 in 250 (ThermoFisher, CAT #21428). To quantify EpCAM and MUC2 staining, a Pathologist identified regions of tumor tissue within CRC liver metastases pathological specimens, and the percentage of EpCAM and MUC2 positive staining was measured. For CRC liver metastases organoids, the percentage of EpCAM and MUC2 positive staining was measured for the total organoid section. Measurements were obtained using ImageJ.

2.3. Flow cytometry (FACs)

Organoid cultures were isolated from Matrigel and washed 3 times with ice cold PBS. To generate a single cell suspension, organoids were incubated in TrypLE (ThermoFisher CAT #12604021) for 30 min at 37 °C before being passed through a 70 μm Nylon cell strainer. Single cell suspensions were washed with PBS and then stained with EpCAM, CEA CAM1, CD24, CD44, CD133, CD166, CD31 and CD45 for 30 min in the dark on ice. Samples were washed with 1 mL of PBS then resuspended in 2% Fetal Bovine Serum and ran through a Fortessa Flow Cytometer (BD Bioscience, USA). Analysis was performed using FlowJo V10 Software. Refer to Sup. Table 2 for flow cytometry antibody information.

2.4. RNA extraction and quantitative PCR (QPCR) analysis

RNA was harvested using TRIzol reagent (Life Technologies). RNA (1 μg) was reverse transcribed using Moloney murine leukemia virus reverse transcriptase (Promega) primed with oligo (dT). Quantitative PCR (QPCR) primers were designed using PRIMER EXPRESS (Applied Biosystems). SYBR green chemistry was used with Rpl32 as the internal reference gene. The conditions were 95 °C for 10 min, 40 cycles of 95 °C for 30 s and 60 °C for 1 min (Stratagene Mx3005P). QPCR analysis was performed on 4 technical replicates for each group, and results were analysed using sequence detector software, relative fold differences were determined using the ΔΔCt method. Human Rpl32-forward: 5-CATCTCCTTCTCGGCATCA-3′; human Rpl32-reverse: 5-ACCCTGTTGTC AATGCCTC-3′. Human ALDH1-forward: 5-TGTTAGCTGATGCCGACTTG-3′; human ALDH1-reverse: 5-TTCTTAGCCCGCTCAA CACT-3′. Human PROX1-forward: 5-CAGATGGAGAAGTA CGCAC-3′; human PROX1-reverse: 5-CTACTCATGAAGCAGCTCTTG-3′. Human LGR5-forward: 5-AA CAGTCCTGTGACTCAACTCAAG-3′; human LGR5-reverse: 5-TTAGAGA CATGGGACAAATGCCAC-3′. Human ABCG2-forward: 5-GGCCTTGGGA TACTTTGAATC-3′; human ABCG2-reverse: 5′-CTACTCATGAAGCAGC TCTT G-3′. Human CDH1-forward 5′-AGCTTGCGGAAGTCAGTTCA-3′; human CDH1-reverse: 5′-CAGAAACGGAGGCCTGATGG-3′; human CEA CAM7-forward: 5′-CACACAACGGTC GAGAGACA-3′; human CEA CAM7-reverse: 5′- TTGGGTGGCTCCGAGAATAC-3′; human EPHB2-forward: 5′-GACCCTCCTTTTGAGTGGGG-3′; human EPHB2-reverse: 5′-GAGTTTGCAGCAACACCCTG-3′.

2.5. Drug therapy and analysis

Organoid cultures were passaged 2 days prior to drug treatment. For drug therapy, organoid cultures were washed with PBS and fresh media containing full supplementation and different concentrations of chemotherapy agents was added. To assess chemotherapy resistance, cultures were pre-treated with chemotherapy agents for 4 days followed by 3 days of fresh media without any selective conditions. This process was repeated two times. To minimize growth differences caused by initial organoid size, organoids were only selected for time-lapse imaging if their longest length was between approximately 200–300 μM. Time-lapse imaging was performed on a Nikon Eclipse Ti-E inverted microscope system (Nikon, UK) and images were captured every 6 h for 60 h. At the completion of imaging, images were converted to TIF files and the area of organoids was measured using in-house software written in MATLAB R2015b software. Growth curves were generated by comparing the area of organoids to their starting size and data is represented as the percentage of growth from time point 0. Apoptosis analysis was measured following 6 days of chemotherapy treatment by staining with the eBioscience™ Annexin V Detection Kit APC in accordance with the manufacturer guidelines (Thermo-Fisher; 88-8007-72).

2.6. Statistical analysis

All data is expressed as mean ± SEM and statistical analysis was performed by one-way analysis of variance (ANOVA) and the appropriate parametric (student *t*-test) statistical test using Sigmastat (Jandel Scientific). For QPCR analysis, p-values were derived from comparison between organoid cultures and the corresponding tumor biopsy. For all organoid growth analysis, p-values were generated from comparing