Patient-Derived Organoids from Colorectal Cancer with Paired Liver Metastasis Reveal Tumor Heterogeneity and Predict Response to Chemotherapy

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# Supplementary Methods

## KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Antibodies**      |        |            |
| Rabbit monoclonal anti-Ki-67 | Cell Signaling Technology | Cat# 9027; RRID:AB_2636984 |
| Rabbit monoclonal anti-CDX2 | Cell Signaling Technology | Cat# 12306; RRID:AB_2797879 |
| Rabbit monoclonal anti-β-Catenin | Cell Signaling Technology | Cat# 8480; RRID:AB_11127855 |
| Rabbit polyclonal anti-Cytokeratin Pan | Proteintech | Cat# 26411-1-AP; RRID:AB_2880505 |
| Rabbit monoclonal anti-Cytokeratin 20 | Cell Signaling Technology | Cat# 13063; RRID:AB_2798106 |
| Rabbit polyclonal anti-MLH1 | Proteintech | Cat# 11697-1-AP; RRID:AB_2145604 |
| Mouse monoclonal anti-MSH6 | Proteintech | Cat# 66172-1-Ig; RRID:AB_2881567 |
| Rabbit monoclonal anti-MSH2 | ABclonal | Cat# A8740; RRID:AB_2863598 |
| Rabbit polyclonal anti-PMS2 | Affinity Biosciences | Cat# DF4351; RRID:AB_2836719 |
| **Biological Samples** |        |            |
| Surgical tissues of CRLM patients | This study | N/A |
| **Chemicals, Peptides, and Recombinant Proteins** |        |            |
| Advanced DMEM/F12 | Gibco | Cat# 12634-010 |
| HEPES | Gibco | Cat# 15630080 |
|                | Supplier          | Catalog Number       |
|----------------|-------------------|----------------------|
| GlutMAX        | Gibco             | Cat# 35050-061       |
| Penicillin/streptomycin | Solarbio          | Cat# P1400           |
| N2             | Gibco             | Cat# 17502-048       |
| B27            | Gibco             | Cat# 17504-044       |
| EGF            | Sino Biological   | Cat# 50482-MNCH      |
| N-Acetyl-L-cysteine | Sigma-aldrich     | Cat# A9165           |
| Nicotinamide   | Sigma-aldrich     | Cat# N0636           |
| Normocin       | invivogen         | Cat# ant-nr-2        |
| Gentamicin/AmphotericinB | Gibco             | Cat# R01510         |
| A83-01         | Tocris            | Cat# 2939            |
| Prostaglandin E2 | Sigma-aldrich     | Cat# P6532           |
| Gastrin        | Sigma-aldrich     | Cat# G9145           |
| SB202190       | Sigma-aldrich     | Cat# S7067           |
| R-spondin-1    | Sino Biological   | Cat# 11083-HNAS      |
| Noggin         | Sino Biological   | Cat# 50688-M02H      |
| DMEM medium    | Hyclone GE        | Cat# SH30243.01      |
| Collagenase IV | Sigma-aldrich     | Cat# C9407           |
| Collagenase II | Solarbio          | Cat# C8150           |
| Bovine serum albumin | BBILife Science | Cat# A600332-0100   |
| Hyaluronidase  | Solarbio          | Cat# h8030           |
| Dispase type II | Sigma-aldrich     | Cat# D4693           |
| Y-27632 dihydrochloride | Sigma-aldrich | Cat# Y0503         |
| Matrigel       | Corning           | Cat# 356231          |
| TrypLE™ Express | GIBCO             | Cat#12605-010       |
| CELLBANKER™ 2  | ZENOAQ            | Cat#170905           |
| EDTA antigen retrieval solution | BIOTECH WELL | Cat# WH1034        |
| Donkey serum   | Solarbio          | Cat# SL050           |
| 5-Fluorouracil | Selleck           | Cat# S1209           |
| Item                                      | Supplier      | Catalog Number |
|-------------------------------------------|---------------|----------------|
| Irinotecan                                | Selleck       | Cat# S2217     |
| Oxaliplatin                               | Selleck       | Cat# S1224     |
| Leucovorin                                | Selleck       | Cat# S1236     |
| Phosphate buffered saline                 | BasalMedia    | Cat# B320KJ    |
| Fetal bovine serum                        | GIBCO         | Cat# 10270     |
| Hematoxylin solution                      | Servicebio    | Cat# G1005-1   |
| Eosin solution                            | Servicebio    | Cat# G1005-2   |
| Hydrochloric acid alcohol                 | Servicebio    | Cat# G1039     |
| Critical Commercial Assays                |               |                |
| NEBNext® UltraTM RNA Library Prep Kit     | New England   | Cat# E7530L    |
| GTVisionTM III Detection System/ Mo & RB   | Gene Tech     | Cat# GK500710  |
| CellTiter-Glo 3D Cell viability assay     | Promega       | Cat# G9683     |

**Experimental Models: Cell Lines**

Human: CRLM organoids | This study | N/A

**Software and Algorithms**

R software | GNU project | [https://www.r-project.org/](https://www.r-project.org/)

SPSS 19.0 | IBM | [https://www.ibm.com/analytics/spss-statisticssoftware](https://www.ibm.com/analytics/spss-statisticssoftware)

Prism version 8 | GraphPad | [https://www.graphpad.com/scientific-software/prism/](https://www.graphpad.com/scientific-software/prism/)

**Other**

TCGA’s Study of Colorectal Carcinoma | NCI | [https://www.cancer.gov/about-nci/organization/ccg/research/structural-genomics](https://www.cancer.gov/about-nci/organization/ccg/research/structural-genomics)
Materials Availability

Distribution of organoids to third parties requires completion of a material transfer agreement and will have to be authorized by the Ethical Committee and Institutional Review Board of the Fudan University Shanghai Cancer Center. Use of organoids is subjected to patient consent; upon consent withdrawal, distributed organoid lines and any derived material will have to be promptly disposed of.

Data Availability

The dataset used during the study are available from the corresponding author on a reasonable request.

CRLM Organoids Culture Media

| Regent name                      | Company     | Cat No.  | Stock solution | Solvent | Final concentration |
|---------------------------------|-------------|----------|----------------|---------|---------------------|
| Advanced DMEM/F12               | GIBCO       | 12634-010| —              | 1 ×     | 1 ×                 |
| HEPES                           | Gibco       | 15630080 | 100 ×          | —       | 1 ×                 |
| GlutMAX                         | Gibco       | 35050-061| 100 ×          | —       | 1 ×                 |
| Penicillin/streptomycin         | Solarbio    | P1400    | 100 ×          | —       | 1 ×                 |
| N2                              | Gibco       | 17502-048| 50 ×           | —       | 1 ×                 |
| B27                             | Gibco       | 17504-044| 100 ×          | —       | 1 ×                 |
| EGF                             | Sino        | 50482-MN | 500μg/0.1%BS   | 50ng/mL |                     |
| N-Acetyl-L-cysteine             | Sigma-ald rich | A9165   | 500mM ddH2O    | 1mM     |                     |
| Nicotinamide                    | Sigma-ald rich | N0636   | 1M ddH2O       | 10mM    |                     |
| Normocin                        | invivogen   | ant-nr-2 | 500 ×          | —       | 1 ×                 |
Gentamicin/Amphotericin B

| Target          | Company     | Cat No. | Dilution |
|-----------------|-------------|---------|----------|
| A83-01          | Tocris      | 2939    | 5mM DMSO 500nM |
| Prostaglandin E2| Sigma-aldrich | P6532   | 100μM DMSO 10nM |
| Gastrin         | Sigma-aldrich | G9145   | 100μM 0.1%BS 10nM |
| SB202190        | Sigma-aldrich | S7067   | 30mM DMSO 3μM |
| R-spondin-1     | Sino Biological | 11083-HNA 50μg/mL 0.1%BS 500ng/mL |
| Noggin          | Sino Biological | 50688-M02 10μg/mL 0.1%BS 100ng/mL |

**H&E, Immunohistochemistry, and Immunofluorescence Staining**

The primary antibodies used for immunohistochemistry and immunofluorescence were listed below:

| Target          | Company     | Cat No. | Dilution |
|-----------------|-------------|---------|----------|
| Ki-67           | Cell Signaling Technology | 9027 | 1:400 |
| CDX2            | Cell Signaling Technology | 12306 | 1:2000 |
| β-Catenin       | Cell Signaling Technology | 8480 | 1:100 |
| CK20            | Cell Signaling Technology | 13063 | 1:500 |
| CK-pan          | Proteintech  | 26411-1-AP | 1:1500 |
| MLH1            | Proteintech  | 11697-1-AP | 1:100 |
| MSH6            | Proteintech  | 66172-1-Ig | 1:500 |
| MSH2            | ABclonal     | A8740   | 1:100 |
Whole Exome Sequencing, Mutation and Copy Number Analysis

DNA degradation and contamination (RNA and protein contamination) were monitored on 1% agarose gel, and DNA concentration was accurately measured using Qubit DNA analysis kit in Qubit 2.0 Flurometer (Invitrogen, USA). More than 0.6μg genomic DNA with concentration over 20 ng/μL was used to construct the whole exome sequencing library. Genomic DNA was randomly interrupted by Covaris fragmentation apparatus into fragments with 180-280bp in length. Sequencing library construction and capture experiments were carried out by using Agilent SureSelect Human All Exon kit (Agilent Technologies, CA, USA) following manufacture’s recommendations and index codes were added to each sample. After sequencing library was qualified, Illumina Hiseq platform was used for whole exome sequencing and 150bp paired-end reads were generated, according to the effective concentration and data output requirements of the library.

Sequencing data were mapped against human reference genome GRCh37 by Burrows-Wheeler Alignment (BWA)\(^1\) and Samblaster\(^2\) to get the initial comparison results of BAM format. The BAM file is marked and repeated by Samblaster to get the final comparison result of BAM format. If one or a pair of read (s) can have multiple alignment positions on the gene, BWA’s processing strategy is to choose the best one from them. If there are two or more best alignment positions, then one from them is randomly selected. We used MuTect software\(^3\) to search for somatic single nucleotide variant (SNV) sites in organoids and paired tissues. Somatic insertion and deletion (InDel) information of organoids and paired tissues was detected by Strelka\(^4\).

Control-FREEC\(^5\) was used to detect somatic copy number variation (CNV) in paired tumor and normal samples. Somatic CNV was based on the depth distribution of reads compared to the reference genome. Considering the base types of 1 bp upstream or downstream of point mutation site, point mutation can be divided into 96 types. Based on the frequency of 96 mutation types in each tumor sample, mutation feature analysis
is to decompose point mutation into multiple different mutation features through Nonnegative Matrix Factorization (NMF) method\textsuperscript{[6]}. Each mutation feature represents one or more tumor mutation process. We compared somatic mutation with the known driver genes in the database to screen out driver genes in organoid and paired tumor tissue samples\textsuperscript{[7-10]}. Significant mutated genes (SMG) take into account the SNV and InDel mutations in somatic cells. MuSiC\textsuperscript{[11]} was used for analysis of SMGs in organoid and corresponding tumor tissue samples.

**Clonal Heterogeneity and Tumor Evolution Analysis**

SuperFreq\textsuperscript{[12]} was used to track clones across the organoid and tissue. SuperFreq analysis infers and tracks subclones of individual samples by using somatic SNV, InDel and CNV information from cancer exome data. Analysis was performed using variants from GATK UnifiedGenotyper and corresponding BAM files from three groups of normal tissue sample and paired organoid samples from the same patient. Each mutation in a specific sample of each individual is given a clonal value and a specific clone determined by SuperFreq algorithm, thus tracking the clone evolution in the paired organoid samples.

**RNA-seq Analysis**

A total amount of 1 μg RNA per sample was used as input material for the RNA sample preparations. Sequencing libraries were generated using NEBNext\textsuperscript{®} UltraTM RNA Library Prep Kit for Illumina\textsuperscript{®} (NEB, USA) following manufacturer’s recommendations and index codes were added to attribute sequences to each sample. The clustering of the index-coded samples was performed on a cBot Cluster Generation System using TruSeq PE Cluster Kit v3-cBot-HS (Illumia) according to the manufacturer’s instructions. After cluster generation, the library preparations were sequenced on an Illumina Novaseq platform and 150 bp paired-end reads were generated. Reference genome and gene model annotation files were downloaded from genome website directly. Index of the reference genome was built using Hisat2 v2.0.5 and paired-end clean reads were aligned to the reference genome. featureCounts v1.5.0-p3 was used to count the reads numbers mapped to each gene. And then FPKM
of each gene was calculated based on the length of the gene and reads count mapped to this gene. Spearman correlation between samples was calculated using the normalized read counts from all 15000 most variable genes and samples were clustered using hierarchical clustering with complete linkage on the correlation matrix. Stage IV CRC cases with gene expression information from TCGA dataset were downloaded from UCSC Xena (https://tcga.xenahubs.net). To subtype all organoid and TCGA samples, we used the consensus molecular subtypes (CMS) of colorectal cancer published by Guinney et al\textsuperscript{[13]}.

**RECIST Guideline (version 1.1)**

Evaluating change of tumor burden before and after treatment is an important feature of clinical evaluation of cancer therapeutics: tumor shrinkage (objective response) and disease progression are useful endpoints in both clinical practice and clinical trials. RECIST guideline (version 1.1)\textsuperscript{[14]} was used to evaluate tumor response in this study. The definition of response criteria in RECIST guideline (version 1.1) was listed below.

| Response                  | Description                                                                 |
|---------------------------|-----------------------------------------------------------------------------|
| Complete Response (CR)    | Disappearance of all target lesions. Any pathological lymph nodes (whether target or non-target) must have reduction in short axis to $<$10 mm |
| Partial Response (PR)     | At least a 30\% decrease in the sum of diameters of target lesions, taking as reference the baseline sum diameters |
| Progressive Disease (PD)  | At least a 20\% increase in the sum of diameters of target lesions, taking as reference the smallest sum on study (this includes the baseline sum if that is the smallest on study). In addition to the relative increase of 20\%, the sum must also demonstrate an absolute increase of at least 5 mm |
| Stable Disease (SD)       | Neither sufficient shrinkage to qualify for PR nor sufficient increase to qualify for PD, taking as reference the smallest sum diameters while on study |

Note: the appearance of one or more new lesions is also considered progression
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**Figure S1.** Generation of PDOs from CRLM Patients Enrolled in This Study. A) Flow chart indicating the number of CRLM patients, the number of evaluable patients, the success rate of establishing cultures from patients, and the multiomics analysis of CRLM organoids. B) Overview of clinical parameters in PDO cohorts (left). The pie chart shows the failure factors and proportion of CRC and LM derived organoids (right).
Figure S2. Preservation of CRLM Histopathology in Organoids. H&E comparison of 8 CRC and 8 LM organoids as noted with the corresponding tumor from which they were derived (8 CRLM patients). All representative images of these CRLM organoids in bright field were displayed (bottom). Black scale bar, 200 μm. Red scale bar, 100 μm.
Figure S3. Conservation of Enterocyte Markers. Sixteen PDOs (8 CRC and 8 LM organoids) are compared to their respective tumors for ki-67, CDX2, β-catenin, CK-pan, and CK20 staining. T, tumor tissue; O, organoid. Black scale bar, 200 μm. Red scale bar, 100 μm.
Figure S4. Comparison of Nuclear Mismatch Repair Proteins between CRLM Patient and PDO Samples. Immunohistochemistry of the nuclear mismatch repair (MMR) proteins MLH1, MSH6, MSH2, and PMS2. Displayed are Six (3 CRC and 3 LM organoids) MMR-proficient PDOs, P2, P3, and P4 CRLM patients. Black scale bar, 200 μm. Red scale bar, 100 μm.
Figure S5. The Mutational Fingerprint in CRLM Organoids and Corresponding Primary Tumors. Overview of somatic tumor driver gene mutations found in CRLM organoids and corresponding primary tumors.
Figure S6. Riverplots Generated by SuperFreq Analysis Showed The Clonal Evolution of CRLM Organoids Derived from CRC and Paired LM Tumor Tissues. The y-axis represents the proportion of tumor cells in each subclone. The different color lines represent different subclones present in CRC and / or LM organoids. The thickness of the color lines corresponds to the number of mutations obtained in the population.
Figure S7. Ki67 positive rates of CRC and LM organoids from P3 and P13 CRLM patients cultured for the same days (Day9) were compared. **p<0.01, Red scale bar, 100 μm
Figure S8. Correlation analysis based on 2000 variable features via FindVariableFeatures function of Seurat package supported the correspondence between the four samples of organoids.
Figure S9. Single Cell RNA Sequencing Profiling in CRLM Organoids, related to Figure 4. A) CMS signatures at the single-cell level (left) and Pearson’s correlation to CRC molecular subtype (right) are shown. B) RNA velocities of single cells in each organoid.
**Figure S10.** Response of CRLM Organoids to 5-Fluorouracil, Irinotecan, and Oxaliplatin, related to Figure 5. Sixteen CRLM organoids (8 CRC and 8 LM organoids) dose-response to 5-FU, irinotecan, and oxaliplatin. Red scale bar, 100 μm.
Figure S11. Drug Sensitivity in PDOs Derived from 25 CRLM Patients. The standardized IC50 values of 50 PDOs (25 CRC and 25 LM organoids) from 25 CRLM patients were displayed in the form of bar chart (left) and heat map (right).
Figure S12. Organoid Drug Sensitivity of Different CMS Subtypes. A) 10 cases of CMS1 organoids dose-response to 5-FU, irinotecan and oxaliplatin. B) 22 cases of CMS2 organoids dose-response to 5-FU, irinotecan and oxaliplatin. C) 17 cases of CMS4 organoids dose-response to 5-FU, irinotecan and oxaliplatin. D) Comparison of drug sensitivity among CMS1, CMS2 and CMS4 organoids.
Figure S13. Swimmer’s Plot of Each CRLM Patient Whose PDO Has Been Analyzed
for Chemosensitivity *ex vivo*. Clinical treatment information and postoperative prognosis information of 25 CRLM patients have been displayed.
Figure S14. Responses of CRLM Organoids to FOLFOX or FOLFIRI Treatment. A) *Ex vivo* chemosensitivity of 13 CRC (left) and 13 LM (right) organoids to FOLFOX in the form of dose response curves are displayed for each CRLM organoid (3 independent experiments for each). B) The standardized IC50 values of CRC and LM organoids were analyzed by paired t-test to compare FOLFOX sensitivity between them. C) Correlation between the standardized IC50 values of CRC and LM organoids are displayed (Two-tailed Spearman correlation: Spearman r = 0.752, p=0.003 for FOLFOX). The linear regression line is plotted. D) *Ex vivo* chemosensitivity of 10 CRC (left) and 10 LM (right) organoids to FOLFIRI in the form of dose response curves are displayed for each CRLM organoid (3 independent experiments for each). E) The standardized IC50 values of CRC and LM organoids were analyzed by paired t-test to compare FOLFIRI sensitivity between them. F) Correlation between the standardized IC50 values of CRC and LM organoids are displayed (Two-tailed Spearman correlation: Spearman r = 0.943, p<0.001 for FOLFIRI). The linear regression line is plotted.