Tumor Initiating Label-Retaining-Cancer-Cells In Human Gastrointestinal Cancers Undergo Asymmetric Cell Division

Hong-Wu Xin[1*], Danielle M. Hari[1*], John E. Mullinax[1], Chenwi M. Ambe[1], Tomotake Koizumi[1], Satyajit Ray[1], Andrew J. Anderson[1], Gordon W. Wiegand[1], Susan H. Garfield[2], Snorri S. Thorgeirsson[2], Itzhak Avital[1]**

[1]Surgery Branch, [2]Laboratory for Experimental Carcinogenesis, Center for Cancer Research, National Cancer Institute, National Institutes of Health, Bethesda, MD 20892, USA.

* Authors contributed equally.

**Correspondence

SUPPORTING MATERIALS AND METHODS

Nota bene: All human surgical specimens were obtained and processed under NCI protocol 09-C-0079 approval by the NCI-IRB and after obtaining proper consent from the patients. All animal experiments wee approved by the NCI-IRB for animal experimentation.

Cells

All cells used in this study are listed in the following table with information of their source and culturing conditions.

Cell Source	Cell Name	Cell Source	Growth Media
Human hepatocellular	PLC/PRF/5	ATCC, CRL-8024	45% DMEM, 45% Ham's F-12

carcinoma (HCC)			supplemented with 10% FCS (Invitrogen Corp, Grand Isle, NY).
HCC	HuH-7	Japan Health Sciences Foundation (JHS), JCRB0403	As for PLC/PRF/5.
HCC	SK-Hep-1	ATCC, HTB-52	As for PLC/PRF/5.
Human Colon Cancer	CSCL-04-Ke	Derived from fresh surgical specimen in our lab	As for PLC/PRF/5.
Human Colon Cancer	CSCL-03-Ba	Derived from fresh surgical specimen in our lab	As for PLC/PRF/5.
Human Colon Cancer	CSCL-02-Ne	Derived from fresh surgical specimen in	As for PLC/PRF/5.

		our lab	
Human Benign Liver Cell	THLE-2	ATCC, CRL-2706	BEBM (CC-3171 Lonza, USA) supplemented with BEGM Singlequot Kit (Lonza, CC-4175).
Human Benign Liver Cell	THLE-3	ATCC, CRL- 11233	As for THLE-2.

Establishment of stable human cancer cell lines from fresh surgical specimen

In order to obtain fresh tumors, after obtaining consent patients were enrolled on our tissue procurement protocol, approved by the NCI/NIH IRB. As previously described, short term cultures were generated from fresh tumors harvested in the operating room from patients with colorectal cancers (Topalian et al., 1989; Yannelli et al., 1996). Tumors were harvested from patients (IRB Protocol: 09-C-0079). Tumors were brought to the laboratory from operation room immediately after resection and worked aseptically. They were washed briefly with 1X HBSS, minced into 1-3 mm chunks and transferred into gentleMACS C tubes of Miltenyi Biotec containing dissociation medium. Dissociation medium contained DMEM/F12 (1:1) growth medium supplemented with collagenase, type IV (1g/L) and pulmozyme (DNase, Dornase alfa inhalation solution) 10,000U/L. Minced tumors were dissociated gently by the programmable gentleMACS Dissociator of Miltenyi Biotec to yield cell suspensions with a high viability rate. Cell suspension was filtered through sterile 70 μm filter and washed with 1XHBSS. Cells were

resuspended with appropriate growth medium and plated into ultra low attachment tissue culture flask at a concentration of 1 million/ml for spheroid formation. Spheroids were collected after 6 days and either cryopreserved or cultured them on normal tissue culture flask. Spheroids thus harvested and grown in adherent were transplanted into nude mice (*nu-/nu-*). Xenografts were harvested, re-dissociated, cultured for one week, and re-injected into nude mice. Single cell suspensions from these xenografts were maintained and cultured for further experiment.

Growth curves and doubling times

In order to maximize the quantitative and qualitative detection rate of asymmetrically dividing cells, we had to synchronize the cell cycle according to each cell line's and the fresh tumors doubling times. To determine cell doubling times, in 15 to 18 wells of 6-well plates, 5 x 10^4 cells were plated per well and allowed to attach for 24 hours. On each time point, 8 hours apart, the numbers of live cells were determined in three wells. Acquired numbers were averaged and converted into percentage relative to the average acquired at the first time point. These percentages were plotted using Excel spreadsheet. A best fit exponential trend line with y-axis interception at 100% was generated and by using regression analysis of this trend line the doubling time was computed (**Table S1**). Correlation value $R^2 \geq 0.9$ was considered adequate for computations of doubling times. Doubling times were calculated for all cells tested.

Isolation of live LRC and non-LRC

Live LRC and Non-LRC were isolated as follows (Fig. S1A) from HCC cell lines (Hari et al, 20:1649-1658, 2011), established fresh primary colon and pancreas tumor cells as well as benign

liver cells. Human cancer cells grown in flasks were first arrested at G0/G1 phase with serum-free media for one cell cycle, re-plated at 5e6 cells/T175 flask in growth media without antibiotics, then labeled with dUTP (100 uM) as a control or with the Cy5-dUTP (100 uM) from VWR before S phase by microporation (Invitrogen). Microporation was done according to the manufacturer's instruction. Briefly, 60e6 cells cultured in antibiotics-free growth media were trypsinized, harvested and washed with PBS once. 5e6 cells were resuspended in 108 ul of R-buffer purchased from Harvard Apparatus and mixed with 12 ul of either dUTP or Cy5-dUTP (100 uM). Cells were loaded into 100 ul tip and into the microporation tube containing 3 ml of microporation buffer, and microporated at 1400 V for 20 millisecond twice, then transferred immediately to growth media without antibiotics for culture at 37°C. After one complete cell cycle Cy5+ cells were sorted by FACS (BDFacsAriaII, BD Biosciences, San Jose´, CA). A population of Cy5+ cells comprising approximately 60% of the total viable cells was sorted and re-ran to make sure Cy5+ high cells were more than 99% pure. The Cy5+ high subpopulation was propagated in log phase in culture for six, eight or fifteen cell cycles. Then Cy5+ cells were gated based on the unlabeled dUTP-microporated cells and sorted as LRC, and 8-10% of total viable cells with lowest Cy5 were sorted as Cy5- cells or non-LRC for subsequent analysis.

Ki67 and pHH3 detection by FACS analysis

The human cancer and benign cells were labeled with Cy5-dUTP or dUTP control as above, cultured in log growth phase for 8 cell cycles and stained for Ki67 and pHH3 as follows. Cells were trypsinized, then 5e5 cells were added in each FACS tube, washed once with 2ml PBS, fixed with 200ul of 4% paraformaldehyde for 20 minutes and 1800ul of cold (-20°C) absolute methanol for 10 minutes at room temperature, washed once with 2ml PBS with 0.1% Tween-20

(PBST), heated at 120°C in 10ml of sodium citrate buffer (10mM sodium citrate, 0.05% tween 20, pH 6.0) in a pressure boiler, washed with PBST, permeablized with 500ul of 1.0% Triton-X-100 in PBST for 10 min at RT, washed once, blocked with 80ul of PBST with 10% FBS, incubated with antibodies (Ki67-FITZ from Dako at 2ug/1e6 cells; P-Histone H3-Alexa-488, S-10, from Cell Signaling at 0.4ug/1e6 cells; FITZ-isotope control antibody from BD biosciences) in blocking buffer in the dark at 4 °C for 30 minutes, washed once, then analyzed on BD FACS aria II by acquiring 1e5 cells/sample. Each condition was done triplicates. FACS data were analyzed with FlowJo.

Cell cycle analysis of LRCC and Non-LRCC

After sorted into LRCC and non-LRCC, cells were fixed with ice-cold 70% ethanol for 30 min on ice, washed with PBS, stained with a 1:1 ratio of Vindelov's PI and plain PI (1ml per sample) for 10 min and acquired on FACS machine. Data were analyzed with ModFit LT software to assess cell cycle. All conditions were performed in triplicate.

Time lapse movie for real-time detection of ACD-NRCC in live cells

To detect ACD-NRCC in real-time, we labeled cell DNA with Cy5-dUTP and did time lapse imaging using confocal microscope as described in details below and Fig. S2. Human cancer cells were developed from surgically dissected fresh patient tumors in our lab. It's doubling time was determined as described above (doubling time=30.1h, R^2=0.989). Cells cultured in growth media were arrested in serum-free media for one cell cycle, then sub-cultured at 5e6 cells per T175 flask, arrested with double-thymidine. After thymidine was removed, cells were washed

three times with PBS, and labeled with Cy5-dUTP (VWR) by microporation (Invitrogen). Microporation was done according to the manufacturer's instruction. Briefly, cells cultured in antibiotics-free growth media were trypsinized, harvested and washed with PBS once. 5e6 cells were resuspended in 108 ul of R-buffer purchased from Harvard Apparatus, mixed with 12 ul of Cy5-dUTP (50 uM), loaded into 100 ul tip and into the microporation tube containing 3 ml of microporation buffer, microporated at 1400 V for 20 millisecond twice, then transferred immediately to growth media without antibiotics for culture at 37°C. After cells passed the first labeling cycle, Cy5+ cells were sorted by FACS (BDFacsAriaII, BD Biosciences, San Jose´, CA) to get the Cy5 high cells. Then Cy5+ cells were plated in collagen-IV coated 8-well chamber slide (Ibidi) at 5e4 cells/ml and 300ul/well, and cultured. After attached, the cells were washed with PBS, and feeded with growth media containing nucleic acids staining dye Cyto9 at 0.5uM concentration (Invitrogen). Then cells were imaged with time lapse confocal microscopy in real-time. As a control, Cy5+ cells were also plated in collagen-IV coated 8-well chamber slide, fixed and stained with DAPI for confocal imaging to confirm that Cy5-dUTP labels nuclei (Fig. S2).

Time lapse imaging was performed on either the Zeiss LSM 510 NLO confocal equipped with an environmental chamber (Precision Plastics, Beltsville, MD) or the Zeiss LSM 710 NLO confocal equipped with an environmental chamber (Carl Zeiss, Thornwood, NY) where temperature (37 C), humidity (80%) and CO2 (5%) were controlled and monitored. Images were acquired every 15 or 30 min for 65 hours using either the Zeiss AIM software on the Zeiss 510 or the ZEN software on the Zeiss 710. Time lapse movies were made in either the Zeiss AIM or ZEN software from the original images (8 bit, 512 x 512 pixels) acquired at each time point on either

the Zeiss 510 or 710 confocal. The movie was then exported as an avi file at 2 frames per second.

Gene expression analysis

Live LRC and Non-LRC cells were first isolated from HCC cell lines, established fresh primary colon and pancreas tumor cells as well as benign liver cells. They were lysed in lysis buffer, and total RNAs were isolated and treated with DNase using miRNeasy Mini kit and RNase-Free DNase Set (QIAGEN) following the manufacturer's protocol. Total RNAs were quantified using spectrophotometry (Nanodrop, Wilmington, DE). All reagents for genomic DNA elimination, reverse-transcription, pre-amplification and real-time qPCR were purchased from SABiosciences (Frederick, MD) and all real-time qRT-PCR experiments for Human Stem Cell Pathway and Human Wnt Pathway analyses were done in triplicates following the manufacturer's protocol. Before genomic DNA elimination and cDNA synthesis, 2µl of 5X genomic DNA elimination buffer was added to 8µl of RNA and the mixture was incubated at 42ºC for 5 minutes and immediately chilled in ice. The RT cocktail was made with the following materials: 4µl of BC3 (5X reverse transcription buffer 3), 1µl of P2 (primer and external control mix), 1µl of RE (cDNA Enzyme Synthesis Mix), 1µl RI (RNase Inhibitor), and 3µl of RNase free water for a total volume of 10µl. 10µl of the RNA from which genomic DNA had been eliminated was then added to 10µl of the RT cocktail and incubated at 42ºC for 30 minutes. The reaction was stopped by heating at 95ºC for 5 minutes.

For pre-amplification of cDNA target templates, the Nano PreAmp PCR cocktail mix was prepared by mixing 12.5µl of the RT^2 PreAmp PCR master mix and 7.5µl of the RT^2 Nano PreAMP cDNA synthesis Primer Mix for either Human Stem Cell pathway or Human Wnt pathway. 5µl of the first strand cDNA synthesis reaction mix were added to 20µl of the nano PreAmp PCR cocktail mix, pre-amplified at the following condition: 95ºC for 10 minutes followed by 12 cycles of 95ºC 15 seconds and 60ºC 2 minutes. After PCR, the tubes were put on ice. 2µl of the side reaction reducer (SR1) was then added to each pre-amplified reaction, incubated at 37ºC for 15 minutes followed by heat inactivation at 95ºC for 5 minutes. 84µl of Rnase-DNase free water was then added to each 27µl of nana PreAMP PCR reation.

Real-Time qPCR was accomplished using the SABioscience RT^2 master mix and a 384 well plate for either Human Stem Cell pathway or Human Wnt pathway using ABI 7900 HT system (Applied Biosystems, Foster City, CA) following the supplier's protocol. 1665µl of the 2x SABioscience RT^2 qPCR master Mix, 50µl of the diluted first strand cDNA syhtnesis reaction water were mixed in the total volume to 2700µl. 10µl of the above mixture was then added into each well of a 384 well PCR array plate. The plate was then placed in the real time thermal cycler for qPCR amplification at the following condition: 95ºC for 10 minutes followed by 40 cycles of 95ºC 15 seconds and 60ºC 2 minutes.

Ct values were analyzed using the SABioscience company's online software. Gene pathway analysis were done using Ingenuity Pathway Analysis online software (IPA 9.0).

Mouse xenogeneic transplantation

To test the tumor initiation capacity of LRC and non-LRCC, the human liver cancer cell line (PLC/PRF/5) and the fresh primary human pancreas cancer cells (CSCL-04-Ke) were labeled with either Cy5-dUTP or dUTP control (VWR) by microporation (Invitrogen). Cy5+ high cells were sorted and cultured in log growth phase. After 6 or 8 generations, cells were then sorted again for LRC (Cy5+) and non-LRC (Cy5-). Sorted cells were either fixed for confocal microscopy or injected into nude/SCID mice (SHO, Jackson Lab) subcutaneously in 25% of Matrigel with 10 cells per injection in 100 ul and 2 sites per mouse. Each mouse was injected with a mice- tracking electronic transponder (Bio Medic Data Systems, Inc) to track mouse ID and mice were mixed among cages. Therefore neither the persons who injected the cells nor the persons who measured the tumors knew what mouse belongs to what group and what cells were injected. Tumor growth was monitored weekly at two dimensions with a ruler and in a double blinded manner for 16 weeks. Mice with tumors were examined and photographed.

Statistics

The objective was to determine the statistical significance of observing asymmetric cell division via non-random chromosomal cosegregation (ACD-NRCC) (Fig. 2). The theoretical probability of detecting a single cell that underwent asymmetric cell division via non-random chromosomal cosegregation would be extremely small (one-in-2^{23} cell divisions), <0.00001 for any given experiment. For any given experiment in which one or more asymmetric cell divisions via non-random chromosomal cosegregation are identified, the two-tailed p-value for the exact binomial test of whether the observed fraction is equal to any value of 0.00001 or less is <0.0001. Thus, any instances in which at least a single asymmetric cell division via non-random chromosomal cosegregation would be identified would be extremely unlikely to occur by chance. For the

combined experiments, detecting ACD-NRCC with 85/4106 having non-random asymmetric cell divisions, by an exact binomial test against a null hypothesis of 0.00001 as a potential fraction, the p-value is <0.0001.