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14. ABSTRACT
a The major goal of the research study is to enumerate biologically distinct liver cancer CTCs and investigate the association between the specific subtype of CTCs and liver cancer grade, stage, and progression after treatment. Successful completion of the project will improve disease monitoring, prognosis prediction, and selection of treatment in the era of precision medicine for liver cancer

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Hepatocellular carcinoma, circulating tumor cells, nanotechnology, mRNA, expression profiling

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1. **INTRODUCTION:**
The major goal of the research study is to enumerate biologically distinct liver cancer CTCs and investigate the association between the specific subtype of CTCs and liver cancer grade, stage, and progression after treatment. Successful completion of the project will improve disease monitoring, prognosis prediction, and selection of treatment in the era of precision medicine for liver cancer.

2. **KEYWORDS:**
Hepatocellular carcinoma, circulating tumor cells, nanotechnology, mRNA, expression profiling.

3. **ACCOMPLISHMENTS:**
-What were the major goals of the project?
<u>Specific Aim 1.</u> Conduct a prospective study on freshly collected peripheral blood samples of 50 HCC patients to isolate MAT2A CTCs and examine whether MAT2A CTCs are associated with HCC progression after adjusting for tumor grade and stage.

- **Major Task 1:** Prospective collection of blood specimen from a total of 50 HCC patients.
  - Timeline: Month 1-24 with 80% completion.
- **Major Task 2:** Statistical analysis to investigate the association between MAT2A CTC and HCC progression.
  - Timeline: Month 25-36 with 0% completion.
- **Milestone Achieved:**
  - IRB approval
  - An original HCC CTC paper entitled "Covalent Chemistry-Mediated Multimarker Purification of Circulating Tumor Cells Enables Noninvasive Detection of Molecular Signatures of Hepatocellular Carcinoma." was published on *Advanced Materials*.
    - Sun N, Lee YT, Kim M, Wang JJ, Zhang C, Teng PC, Qi D, Zhang RY, Tran BV, Lee YT, Ye J, Palomique J, Nissen NN, Han SB, Sadeghi S, Finn RS, Saab S, Busuttil RW, Posadas EM, Liang L, Pei R, **Yang JD**, You S, Agopian VG, Tseng HR, Zhu Y. Covalent Chemistry-Mediated Multimarker Purification of Circulating Tumor Cells Enables Noninvasive Detection of Molecular Signatures of Hepatocellular Carcinoma. Adv Mater Technol. 2021 May;6(5):2001056. doi: 10.1002/admt.202001056. Epub 2021 Apr 9. PMID: 34212072; PMCID: PMC8240468.

<u>Specific Aim 2.</u> To examine whether the ratio of *MAT2A/MAT1A* and their downstream gene mRNA expression in the CTCs measured by CTC-RNA Assay from patients in the Specific Aim 1 are associated with HCC progression after adjusting for tumor grade and stage.

- **Major Task 3:** CTC RNA extraction, and RNA quantification via CTC-RNA Assay.
  - Timeline: Month 1-24 with 80% completion.
- **Major Task 4:** Statistical analysis to investigate the association between CTC RNA result and HCC progression.
  - Timeline: Month 25-36 with 0% completion.
- **Milestone Achieved:** see above.

-What was accomplished under these goals?

1) Major activities
   - Sample collection from the Cedars-Sinai IRB protocol 00000066
   - Implement QA/QC protocols of NanoVelcro CTC Assay.
   - Preparation of artificial samples (SNU387, Hep3B, and PLC/PRF/5).
   - Automated fluorescent microscope and image cytometry analysis for CTC counting.
   - Calibration studies for CTC capture efficiency.
   - To carry out calibration studies to assess the performance of RNA quantification for with cDNA mixture of spiking different HCC cell lines into 5000 WBCs at densities of 200, 150, 100 and 50 cells per sample.
   - Bioinformatic pipeline for panel development in addition to the originally proposed genes.

2) Specific objectives
   - Technical validation using artificial and patient samples.
   - Path to implementation and initial clinical test of the HCC CTC-RNA assay.

3) Significant results

A. Sample collection to the Cedars-Sinai Biobank.

As the result of COVID-19 pandemic, research activity both in the lab and the clinical space has been quickly and severely halted since March 20th, 2020. The effects of the COVID-19 pandemic on the project include a delay in recruiting study subjects at Cedars-Sinai Medical Center and a delay in conducting laboratory-based research studies.

The COVID-19 pandemic has impacted patient enrollment and acquisition of patient blood samples due to a multitude of factors. There has been a transition to video visits to maintain social distancing, thereby limiting the ability enroll patients in clinic spaces. Additionally, the volume of procedures including liver resection, liver transplants, and locoregional therapy have decreased, hampering patient enrollment. As clinical protocols improve and the volume of patients presenting to clinic or undergoing procedures continues to expand, we anticipate increased enrollment.

In spite of the COVID-19 pandemic challenges described above, **patient enrollment has been robust**. The initial proposal anticipated enrollment of a total 50 patients in year into our study. From 7/1/2020 to 7/31/2021, 40 patients within the study criteria have been enrolled, and a total of 500 patients with a diagnosis of liver cirrhosis or HCC have been added to the Cedars-Sinai blood Biobank.

B. Covalent Chemistry-Mediated Multimarker Purification of CTCs Enables Noninvasive Detection of Molecular Signatures of HCC. Our HCC joint team demonstrated the feasibility of purifying HCC CTCs from patients' blood samples with improved purity and molecular integrity using Click Chips [1] in conjunction with a multimarker antibody cocktail [2] containing anti-EpCAM, anti-ASGPR1, and anti-GPC3 antibodies (**Figure 1a**). Such an HCC CTC purification approach combines two consecutive covalent chemistry-mediated reactions, i.e., 1) the click reaction-mediated HCC CTC capture from PBMC samples onto Click Chips, and 2) disulfide cleavage-driven HCC CTC release from Click Chips into phosphate-buffered saline (PBS) solution. Again, a pair of click chemistry motifs with high selectivity and reaction rates, i.e., tetrazine (Tz) and trans-cyclooctene (TCO), were grafted onto SiNWS (via chemical modification) and HCC CTCs (via antibody conjugation), respectively. By introducing a PBMC sample from an HCC patient into a Click Chip, click reaction between Tz and TCO enabled the rapid immobilization of HCC CTCs. Subsequently, 1,4-dithiothreitol (DTT) was employed to cleave the embedded disulfide bonds to release the captured HCC CTCs specifically. The released CTCs were then subjected to mRNA profiling by NanoString nCounter platform, targeting 64 HCC-specific genes, which were generated from an integrated data analysis framework with 8 tissue-based prognostic gene signatures from 7 publicly available HCC transcriptomic studies. After bioinformatics analysis and comparison, the HCC CTC-derived gene signatures in HCC CTCs showed high concordance with HCC tissue-derived gene signatures from TCGA database (**Figure 1b**), suggesting that HCC CTCs purified by Click Chips could enable the translation of HCC tissue molecular profiling into a noninvasive setting.

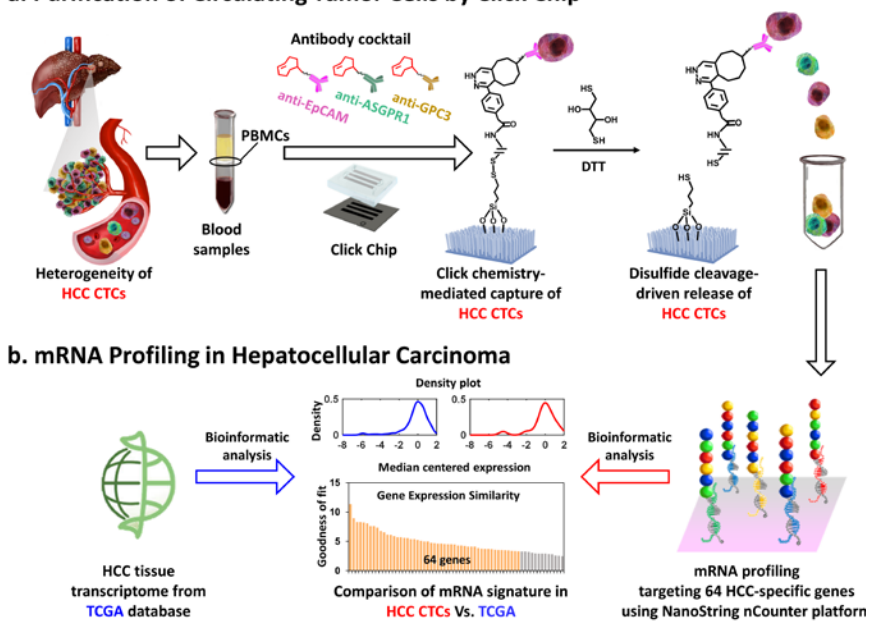

Figure 1. Multimarker purification of circulating tumor cells (CTCs) enables mRNA profiling of hepatocellular carcinoma (HCC). a) Schematic illustration of the workflow employed for HCC CTC purification (capture/release) by Click Chips in conjunction with an HCC-specific antibody cocktail (i.e., anti-EpCAM, anti-ASGPR1, and anti-GPC3). The HCC CTC purification approach combines two consecutive covalent chemistry-mediated reactions, i.e., 1) the click reaction-mediated HCC CTC capture from peripheral blood mononuclear cell (PBMC) samples onto Click Chips, and 2) disulfide cleavage-driven HCC CTC release from Click Chips to phosphate-buffered saline (PBS) by 1,4-dithiothreitol (DTT). b) Quantification of the 64 HCC-specific genes in the purified HCC CTCs were performed by Nanostring nCounter platform. After bioinformatic analysis and comparison, the gene signatures in HCC CTCs showed high concordance with HCC tissue gene signatures from The Cancer Genome Atlas (TCGA) database.

** The manuscript, Na Sun, Yi-Te Lee, Minhyung Kim, Jasmine J. Wang, Ceng Zhang, Pai-Chi Teng, Dongping Qi, Ryan Y. Zhang, Benjamin V. Tran, Yue Tung Lee, Jinglei Ye, Juvelyn Palomique, Nicholas N. Nissen, Steven-Huy B. Han, Saeed Sadeghi, Richard S. Finn, Sammy Saab, Ronald W. Busuttil, Edwin M. Posadas, Li Liang, Renjun Pei, **Ju Dong Yang**, Sungyong You, Vatche G. Agopian, Hsian-Rong Tseng, Yazhen Zhu*, "Covalent Chemistry-Mediated Multimarker Purification of Circulating Tumor Cells Enables Noninvasive Detection of Molecular Signatures of Hepatocellular Carcinoma" is currently published on *Advanced Materials Technology* (IF: 7.848).

(i) The fabrication of a Click Chip. Based on the previous demonstration of Click Chips, where covalent chemistry-mediated capture and release of CTCs were applied for purification of CTCs in non-small cell lung cancer (NSCLC), [1] we explored the combined use of Click Chip's device configuration with a multimarker cocktail for purification of HCC CTCs. A Click Chip (**Figure 1a**) is composed of two main functional components: 1) tetrazine (Tz)-grafted SiNWS: a patterned SiNWS [3] covalently modified by terminal Tz motifs with disulfide bridges, [1] and 2) an overlaid polydimethylsiloxane (PDMS) chaotic mixer [4] on which a network of microchannels

was designed to induce chaotic mixing.[5] They were housed in a custom-designed microfluidic chip holder to form a "Click Chip" device. The Tz-grafted SiNWS and PDMS chaotic mixer were fabricated according to the previously published procedures. [1,6]

(ii) General procedure for performing purification of HCC CTCs in Click Chips. The covalent chemistry-mediated HCC CTC purification approach [1] combines two consecutive steps, i.e., 1) the click reaction-mediated HCC CTC capture onto Click Chips and 2) disulfide cleavage-driven HCC CTC release from Click Chips. Prior to performing HCC CTC purification studies using Click Chips, trans-cyclooctene TCO motif was covalently conjugated onto the three antibody capture agents, i.e., anti-EpCAM, anti-ASGPR1, and anti-GPC3. Single or combined antibodies were then incubated with the artificial or clinical HCC CTC samples. After washing off the excess antibody, the HCC CTC samples in 200-μL PBS were introduced into Click Chips, where click reaction-mediated capture led to the capture of HCC CTCs on SiNWS. After CTC capture, the DTT solution (50 mM) was introduced to cleave disulfide bonds for specific CTC release.

(iii) Optimization of Click Chips for HCC CTC capture and release using artificial HCC CTC samples. To optimize the capture performance of Click Chips, artificial HCC CTC samples (**Figure 2a**) were prepared by spiking 200 DiO-labeled HCC cell lines (green color) into the DiD-labeled PBMCs (red color, 5×10^6 cells mL^{-1}) isolated from a healthy donor's whole blood. After incubating with single or combined antibody capture agents, the artificial HCC CTC samples were introduced into Click Chips for click chemistry-mediated capture of HCC CTCs. After staining with 4', 6-diamidino-2-phenylindole (DAPI), DiO-labeled HCC cells and background DiD-labeled WBCs were counted under a fluorescence microscope (Nikon 90i). The HCC CTC capture yields were calculated by dividing the counts of CTCs captured on the Click Chips by the counts of target cells that were initially spiked to the artificial HCC CTC samples. We first evaluated the effect of

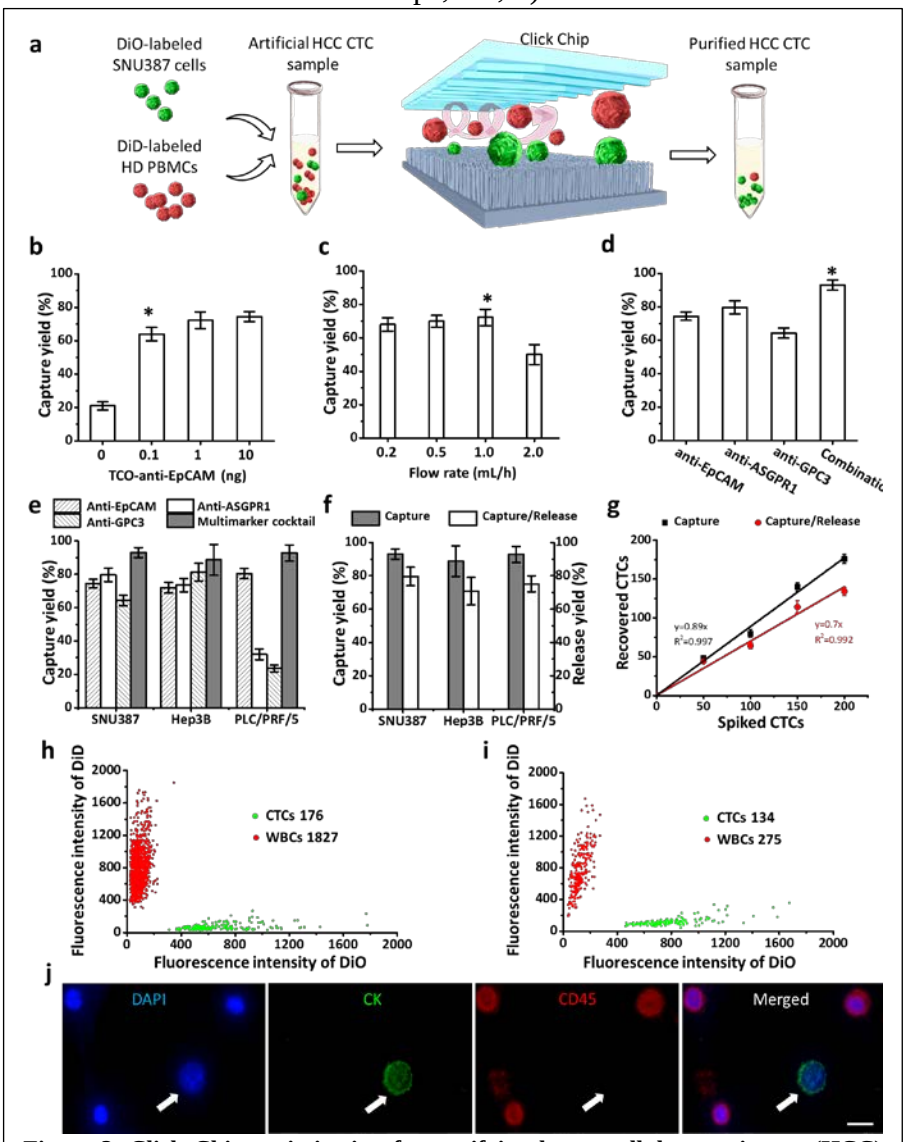

Figure 2. Click Chip optimization for purifying hepatocellular carcinoma (HCC) circulating tumor cells (CTCs) using artificial HCC CTC samples. a) DiO-labeled SNU387 HCC cells (green) and DiD-labeled peripheral blood mononuclear cells (PBMCs) (red) from a healthy donor (HD) were mixed to prepare the artificial HCC CTC samples for Click Chip optimization. **b)** The capture yields of Click Chips at different quantity of TCO-labeled anti-EpCAM. **c)** The capture yields of Click Chips at different flow rates. **d)** The capture yields acheived by using single and combined antibody, i.e., anti-EpCAM, anti-ASGPR1, anti-GPC3, and combination of the three capture agents. **e)** The capture yields achieved by using single and combined antibody capture agents. Different artificial HCC CTC samples containing three different HCC cells (i.e., SNU387, Hep3B, PLC/PRF/5) were validated. **f)** Capture/release yields of Click Chips observed for different artificial HCC CTC samples. **g)** Dynamic ranges of HCC CTC capture and capture/release yields of Click Chips. Here, artificial HCC CTC samples containing 0 to 200 SNU387 cells were tested. (**b**-**g**, The means ± SD of three independent assays are presented). **h)** A 2-parameter scatter plot showed the cell distribution of SNU387/White blood cell (WBC) observed for CTC capture in a Click Chip. **i)** A 2-parameter scatter plot showed the cell distribution of SNU387/WBC after CTC release, following the CTC capture (h). **j)** The representative fluorescent images of SNU387 (DAPI+/CK+/CD45-; white arrow)/WBC cells (DAPI+/CK-/CD45+) captured on the Click Chips. bar, 10 μm. *denotes the optimal condition.

different quantities of TCO-labeled anti-EpCAM (i.e., 0 ng, 0.1 ng, 1.0 ng, and 10 ng) on HCC CTC capture yields (**Figure 2b**). The capture yield of Click Chips was up to 72% when 1 ng of TCO-anti-EpCAM was used for HCC CTC capture at a flow rate of 1.0 mL h^{-1}. HCC CTC capture yields reached the plateau even with a higher quantity of TCO-labeled anti-EpCAM. Next, the effects of different flow rates (0.2, 0.5, 1.0, and 2.0 mL h^{-1}) on HCC CTC capture yields of Click Chips were evaluated (**Figure 2c**), and an optimal flow rate of 1.0 mL h^{-1} was identified, which was consistent with our works. [1] Our group has previously demonstrated the combined use of NanoVelcro Assay with the multimarker antibody cocktail (i.e., anti-EpCAM, anti-ASGPR1, and anti-GPC3 antibodies) for enumeration of HCC CTCs. [2] Under the optimal flow rate of 1.0 mL h^{-1}, we examined the capture yields (**Figure 2d**) using either single or antibody cocktails. Similarly, an optimal capture yield (93.02 $\pm$ 3.00 %) was achieved when the multimarker cocktail was used. We further validated the single antibodies versus the multimarker cocktail study using two additional HCC cell lines (i.e., Hep3B and PLC/PRF/5). As shown in **Figure 2e**, the HCC CTC capture performance observed for the multimarker cocktail group outperformed those from any single antibody groups.

After determining the optimal condition for click creaction-mediated capture of HCC CTCs, we tested the performance of HCC CTC release in Click Chips, in which 200 μL of DTT (50 mM) soluiton was flowed through [1] at a rate of 1.0 mL h^{-1} to release the immobilized HCC CTCs. The HCC CTC release yield was evaluated by dividing the CTC counts released from the Click Chips by the counts of target cells that were initially spiked to the artificial HCC CTC samples. Furthermore, the universal applicability of Click Chips for purification (capture/release) of HCC CTCs was evaluated using three artificial HCC CTC samples spiking with three HCC cell lines, i.e., SNU387, Hep3B, or PLC/PRF/5. **Figure 2f** showed that the HCC CTC capture/release yields across three HCC cell lines were ranging from 88.75% to 93.02% (capture yields), and 70.74% to 79.57% (release yields), respectively. The dynamic range of Click Chips was also tested using artificial HCC CTC samples by spiking 0 to 200 SNU387 cells into a healthy donor's PBMCs. Consistent capture yields ($y = 0.89x$, $R^2 = 0.997$) and release yields ($y = 0.70x$, $R^2 = 0.992$) were observed for Click Chips that are sufficient for testing clinical samples (**Figure 2g**). A representative study using an artificial sample spiked with 200 SNU387 cells demonstrated (**Figure 2h,i**) a minimal background (1,827 WBC-capture and 275 WBC-release) of Click Chips in CTC-capture (176/200) and release (134/176) . **Figure 2j** depicted the representative fluorescent images of SNU387 (DAPI+/CK+/CD45-)/WBC cells (DAPI+/CK-/CD45+) captured on the Click Chips for cell enumeration and capture yield calculation. The CTC purification data indicate that the disulfide cleavage-driven release mechanism can further improvethe purity of the purified CTCs (ca. one order of magnitude reduction in WBC contamination), providing robust and reproducible samples with high purity for downstream molecular characterization. Overall, these results suggest that the HCC CTCs can be purified by Click Chips effectively (click chemistry-driven CTC capture followed by disulfide cleavage-driven CTC release).

(iv) Development of HCC-specific gene panel with 64 genes. Before performing mRNA profiling on the HCC CTCs purified by Click Chips, we adopted an integrated data analysis framework to select and develop an HCC-specific gene panel, capable of detecting HCC CTCs in the presence of non-specifically purified WBCs. We started HCC-specific gene panel selection by assembling 8 tissue-based prognostic gene signatures (Table S1, Supporting Information) from 7 publicly available HCC transcriptomic studies, [7-13] including Hoshida's HCC subtype gene signature, [7] cholangiocarcinoma-like (CCL) HCC gene signature, [8] Hippo pathway inactivation associated gene signature, [10] risk score classifier based on 65 genes for HCC survival prediction, [11] NCI proliferation signature, [12] hepatoblastoma-like tumor signature, [13] iCluster1signature, and IDH1-like signature. A total of 1,535 candidate genes were collected, and serially and selectively pared down in a step-wise fashion to enrich for genes with high expression in HCC CTCs and low expression in immune cells in order to avoid the signals from non-specifically trapped WBCs in the Click Chips. [14] To this end, an integrated data analysis framework (**Figure 3a**) was applied for the selection of the HCC-specific gene panel. This framework consists of four major steps: 1) Select genes involved in at least two independent tissue-based HCC prognostic signatures to further support their role in HCC tumor biology, this selection step results in 273 candidate genes; 2) Select the subset of genes also included in IDH1-like signature, known to be associated with aggressive HCC subtypes, resulting in 201 genes; 3) Select genes that are highly expressed in HCC cell lines from the Cancer Cell Line Encyclopedia (CCLE), [15] further enriching genes specific to HCC compared to other cancer cell lines, resulting

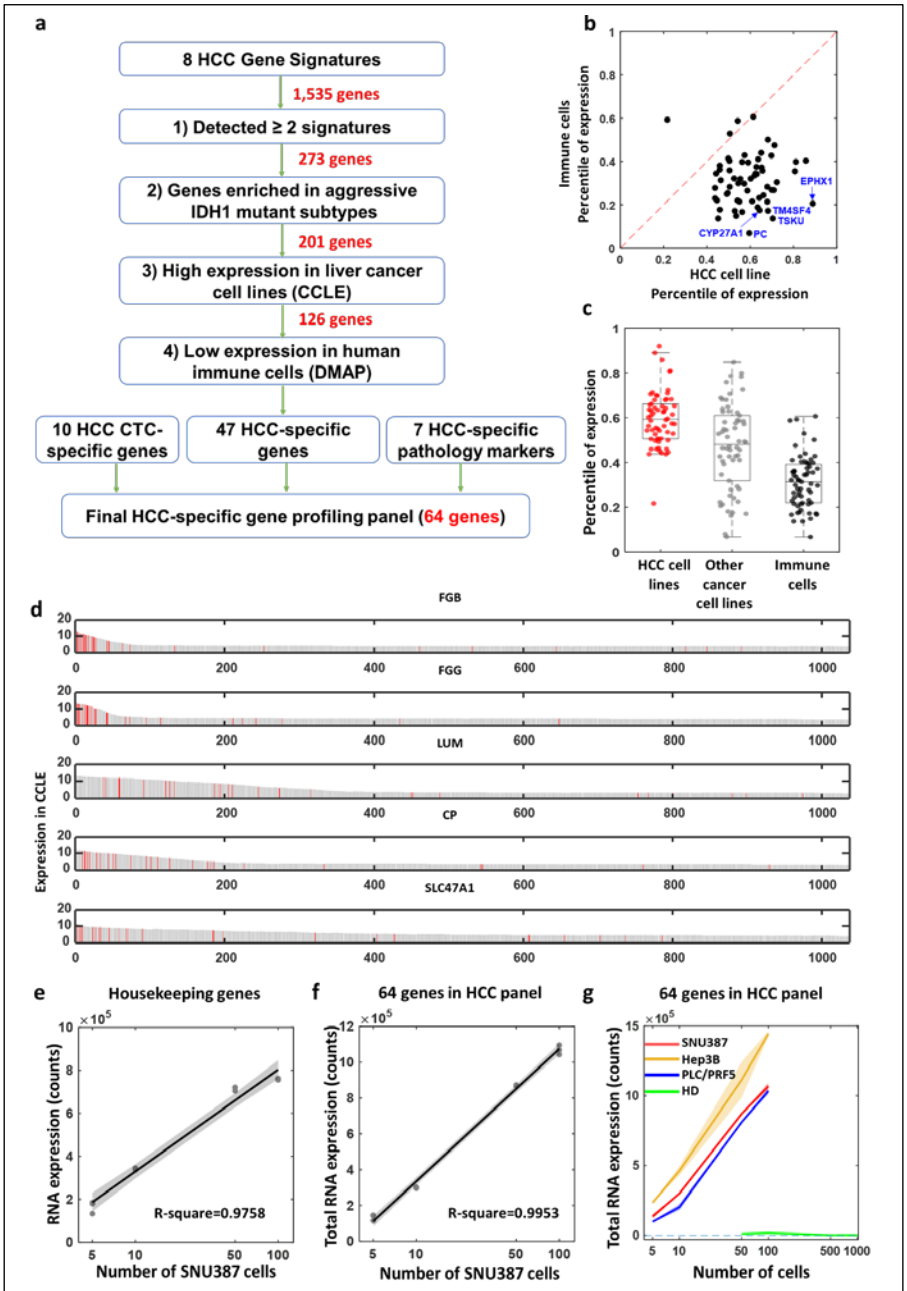

Figure 3. Development and validation of hepatocellular carcinoma (HCC)-specific gene panel with 64 genes. a) An integrated data analysis framework adopted for selecting and developing the 64 genes of HCC-specific gene panel. **b)** Scatter plot shows expression of the 64 genes comparing HCC cell lines to humane immune cells. Dotted line indicates the same expression between HCC cells and other cancer cells which is y=x axis. **c)** Box plot depicts differential expression of 64 genes between HCC cell lines (red dots), other cancer cell lines (gray dots) from Cancer Cell Line Encyclopedia (CCLE), and humane immune cells from Differentiation MAP dataset (DMAP) (black dots). **d)** Bar charts show distribution of all the cells in CCLE based on the expression level of the genes (i.e., *FGB, FGG, LUM, CP, SLC47A1*). Red bar indicates HCC cell lines. **e, f)** Dots with regression line depict expression dynamics of housekeeping genes (e, R^2 = 0.976) and 64 genes (f, R^2 = 0.995) was assessed with difference SNU387 cell counts from 0 to 100. **g)** Line plots with distinct colors represent dynamic changes of the 64 gene expression counts from SNU387, Hep3B, PLC/PRF/5 and healthy donor peripheral blood mononuclear cells (PBMCs) with different cell numbers. Slopes of the curve- SNU387: 9,829.5 counts/cell, Hep3B: 12,744.9 counts/cell, PLC/PRF/5: 9,862.5 counts/cell, healthy donor PBMCs: -8.8 counts/cell. Click Chip. **i)** A 2-parameter scatter plot showed the cell distribution of SNU387/WBC after CTC release, following the CTC capture (h). **j)** The representative fluorescent images of SNU387 (DAPI+/CK+/CD45-; white arrow)/WBC cells (DAPI+/CK-/CD45+) captured on the Click Chips. bar, 10 µm. *denotes the optimal condition.

in 126 genes; and 4) Select genes with low expression in various immune cells using Differentiation MAP dataset (DMAP), [16] to minimize the signal from non-specifically captured WBCs. This yielded 47 genes that are specific for detecting HCC. Finally, we incorporated the 10 HCC-specific genes[17] (i.e., *AFP*, *AHSG*, *ALB*, *APOH*, *FABP1*, *FGB*, *FGG*, *GPC3*, *RBP4*, and *TF*), that were used to specifically detect HCC CTCs and monitor disease progression, and 7 markers (i.e., *ARG1*, *ASGR1*, *ASGR2*, *CPS1*, *KRT8*, *KRT18*, and *ERRFI1*) that are commonly used in clinical pathology diagnosis, [18,19] resulting in a 64-gene HCC-specific gene panel.

(v) Analytical validation of HCC-specific gene panel. We first confirmed that the 64 genes of the HCC-specific gene panel are HCC cell-specific by performing bioinformatics analysis using the CCLE and DMAP. As shown in **Figure 3b**, 61 out of 64 genes (95%) are below the regression line, indicating that these genes are highly expressed in HCC cell lines and lowly expressed in immune cells. We then compared expression ranks (percentiles) of the 64 genes in HCC and other cancer cell lines from the CCLE and immune cells from the DMAP. Expression ranks of the 64 genes are significantly higher in HCC cells compared to other cancer cell lines (P <0.001) or immune cells (P <0.001) (**Figure 3c**). The top 5 genes (i.e., *FGB*, *FGG*, *LUM*, *CP*, *SLC47A1*) with the highest expression in HCC cell lines was assessed in all CCLE cancer cell lines (**Figure 3d**). This result demonstrates the predominant expression of the 64 genes in HCC cell lines.

To validate the feasibility of applying Nanostring nCounter platform for profiling the 64 genes of the HCC-specific gene panel, we then prepared the cell mixture by spiking different numbers of SNU387 cells with serial dilution (i.e., 5, 10, 50, 100 cells) into PBMCs (5×10^6 cells mL^{-1}) isolated from a healthy donor's blood. The cell

mixtures were then subjected to RNA extraction and Nanostring nCounter platform for the 64 gene expression analysis. Five housekeeping genes (i.e., *DYNLL1*, *GAPDH*, *RPL13A*, *RPS11*, and *RPS16*) were tested simultaneously as an internal control of the samples. The dynamic ranges of mRNA expression detected for housekeeping genes (**Figure 3e**, $R^2 = 0.976$) and the 64 genes (**Figure 3f**, $R^2 = 0.995$) showed excellent linearity of the Nanostring nCounter platform. We then tested the general applicability of the Nanostring nCounter platform for the 64 gene expression analysis using three cell mixtures spiked with three HCC cell lines, i.e., SNU387, Hep3B, or PLC/PRF/5. PBMCs (5×10^6 cells mL^{-1}) isolated from a healthy donor's blood served as the negative control. Results summarized in **Figure 3g** showed that the Nanostring nCounter platform for the 64 gene expression analysis exhibited consistent performances across all three cell mixtures spiked with three HCC cell lines.

(vi) Comparative analysis of the 64-gene expressions between HCC CTCs purified by Click Chips and HCC tissues from TCGA. We performed HCC CTC purification by Click Chips using 20 patient blood samples with HCC across all clinical stages. Subsequently, the purified HCC CTCs were subjected to analyze the 64 genes of the HCC-specific gene panel using the Nanostring nCounter platform. The 64-gene expressions from HCC CTCs were compared with the ones from HCC tissues from TCGA. We first examined whether the two 64-gene expression profiles (one from HCC CTCs and the other from HCC tissues) came from populations with a common distribution. The quantile-quantile plot (Q-Q plot) suggests that both are from HCC patient populations with a common gene expression distribution (**Figure 4a**). In addition, the principal component analysis (PCA) score plot spanned by the first two principal components based on the 64-gene expression data showed that 20 patient samples of HCC CTCs (red dots) were overlapped with HCC tissues from TCGA (gray dots), supporting the high concordance of expression variance between the HCC CTCs and HCC tissues from TCGA (**Figure 4b**). Further assessment of expression concordance was performed using the goodness of fit measure based on the Kolmogorov-Smirnov (KS) distance between the HCC CTCs and HCC tissues from TCGA, and 51 out of the 64 genes (80%) have a significantly high level of expression concordance (KS distance<0.3, **Figure 4c**). Empirical cumulative distribution function (CDF) analysis of 64 gene expression from the HCC CTCs and HCC tissue from TCGA reveals the expression concordance of 64 genes between HCC CTCs (red) and HCC tissues from TCGA (blue). The density plots of the 6 most representative genes having the best concordance expression between HCC CTCs and HCC tissues from TCGA were shown in **Figures 4d-i**. Collectively, these results demonstrate the high expression concordance of 64 genes of HCC CTCs by Click Chips with HCC tissues.

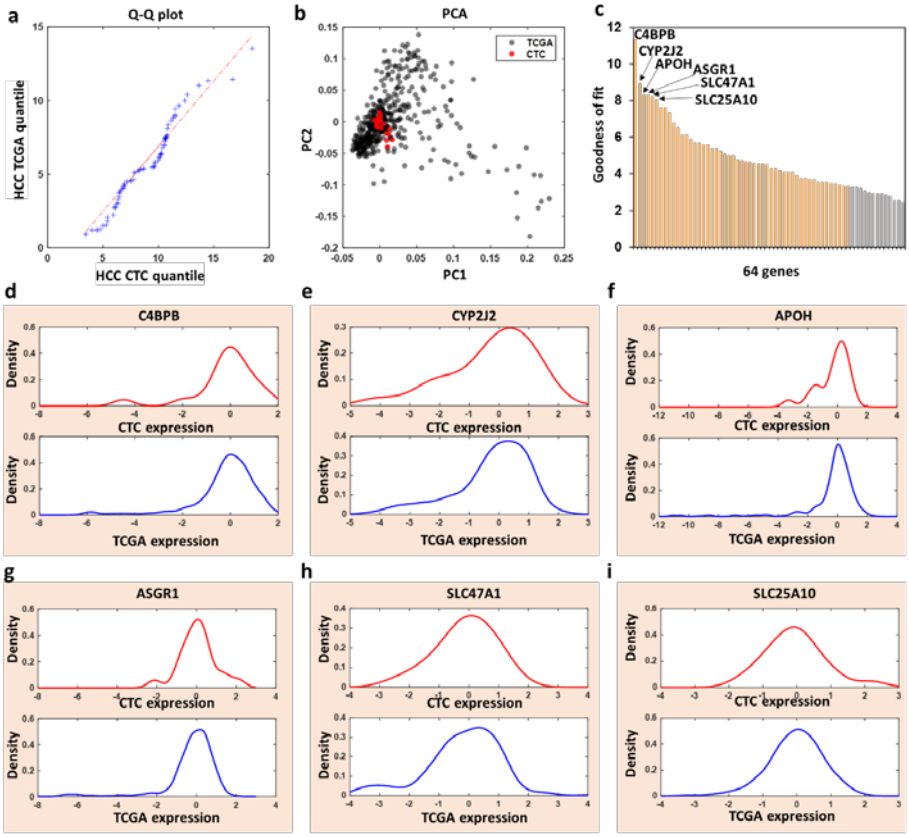

Figure 4. Bioinformatic analysis and comparison of the 64-gene mRNA signatures of hepatocellular carcinoma (HCC) circulating tumor cells (CTCs) purified by Click Chips from HCC patients and HCC tissue mRNA signatures from The Cancer Genome Atlas (TCGA) database. a) Q-Q Plot showed that the 64-gene expression profiles from the HCC CTCs and HCC TCGA came from populations with a common distribution. b) PCA plot based on the 64-gene expression from the HCC CTCs from 20 patients (red dots) and HCC TCGA (gray dots). c) Bar charts showed the goodness of fit (inverse KS distance) of the 64 genes between the HCC CTCs and HCC TCGA. d-i) Kernel density plot of the 6 representative genes with the highest expression concordance between HCC CTCs and HCC TCGA.

-What opportunities for training and professional development has the project provided?

1) Didactics including coursework, short courses, seminars.

I attended a series of basic and translational programs/workshops to acquire the necessary skills, competence, and expertise to complete the proposed study and prepare myself for independent career development. I completed the clinical scholar program, a mentored 2-year program offered to assist junior faculties at Cedars-Sinai Medical Center (CSMC) in obtaining extramural research funding and success in their academic career. I attended the bioinformatics and integrative genomics cluster meeting offered at CSMC to expand my understanding of bioinformatics skills.

2) Mentoring.

I have been meeting with Dr. Shelly Lu (mentor) weekly and Drs. Edwin Posadas and Hsian-Rong Tseng (co-mentors) every 2-4 weeks to discuss data and my progress on the project. I attended weekly liver cancer biology lab meetings run by Dr. Lu (mentor) and weekly CTC research meetings run by Dr. Posadas (co-mentor). I learned from my mentors solid background knowledge of molecular/cancer biology, clinical oncology, bioinformatics, and nanotechnology. I will continue to learn liver cancer biology related to MATs, which are dysregulated in liver cancer from my primary career mentor, Dr. Shelly Lu. Dr. Hsian-Rong Tseng (UCLA) and Dr. Edwin Posadas (CSMC), my co-mentors, will cover concepts of the NanoVelcro assay in conjunction with CTC RNA analysis. I will continue to learn computer science and laboratory methods in the analysis of NanoVelcro CTC assay data from my co-mentors and co-investigator.

3) Practical experience.

I started building up a liver cancer biorepository for the proposed study. I attended the virtual annual meetings of the American Association for Cancer Research, the American Association for the Study of Liver Diseases, and and networked with leading researchers.

-How were the results disseminated to communities of interest?

The latest version of NanoVelcro Chips for purification of CTCs and extracellular vesicles (EVs) are utilized by our multi-institutional HCC team including investigators from Cedars-Sinai Medical Center (CSMC) and University of California, Los Angeles (UCLA). In addition, our collaborators use the NanoVelcro CTC and EV purification technology for melanoma, pancreatic cancer, prostate cancer, and breast cancer-related research projects at UCLA, and at the Translational Oncology Program and Urologic Oncology Program at CSMC led by Dr. Edwin M. Posadas. This collaboration network has provided crucial feedback to further improve the CTC and EV isolation technology.

-What do you plan to do during the next reporting period to accomplish the goals?

The goals of the project in the upcoming year are the following: 1) complete patient enrollment and expand the Cedars-Sinai Biobank of peripheral blood samples from HCC patients to evaluate associated translational and clinical questions, 2) further improve on a novel HCC CTC-RNA assay for bioinformatic analysis specific to this project's patient cohort.

I aim to expand patient recruitment within the scope of this project to evaluate the translation and clinical questions described below.

In the laboratory, our group is expanding on the previously developed HCC CTC-RNA assay in order to develop an assay specific to the project's study population. This involves a multi-disciplinary approach with specialists amongst the bioinformatic, basic science, technology platform, translational, and clinical spheres of the project.

We will conduct both retrospective and prospective study on a cohort of patients over the course of their disease progression to test HCC CTC-RNA assay for prognosis in HCC. Statistical modeling on a digital score resulting from the HCC CTC-RNA assay at baseline and longitudinally to discriminate HCC patients with different clinical outcomes will be further evaluated and established.

4. IMPACT:

-What was the impact on the development of the principal discipline(s) of the project?
HCC is a highly lethal cancer. Disease burden of HCC has been increasing in the United States. Abdominal cross-sectional imaging with intravenous contrast is often used to monitor the recurrence or progression of the tumor, which can be time-consuming, costly, resource intensive, and harmful to patients from repeated doses of radiation or contrast agents. Importantly, images do not reflect the tumor biology and are unable to predict the treatment response. With regard to blood-based biomarkers for HCC, alpha-fetoprotein (AFP) is a traditional diagnostic and prognostic biomarker with limited accuracy. Noninvasive, prognostic and predictive biomarkers will accelerate the biomarker-integrated enrichment clinical trials in HCC, which will provide evidence to improve clinical practice based on precision medicine for an individual patient with a specific tumor biology. Therefore, there is a significant, unmet need to develop accurate and clinically relevant biomarkers to optimize the management of HCC.

-What was the impact on other disciplines?
There are many platforms for enrichments or purifications of CTCs, which belong to the field of engineering. However, the subsequent studies of clinical applications are few. Our clinical validation of the CTC-RNA assay can provide positive feedback to the platform development. Indeed, our group has developed newer generations of NanoVelcro Chips which can purify CTCs with higher purity and throughput.[1,20]

Based on the success with HCC, we could also utilize this platform in other diseases including melanoma[21], lung cancer[1], pancreatic cancer[22] and noninvasive prenatal diagnostics.[23]

-What was the impact on technology transfer?
Nothing to Report

-What was the impact on society beyond science and technology?
The successful development of the proposed CTC-RNA assay is rapidly translatable, enabling a sensitive and biologically relevant CTC-based assay for prognostication, prediction of cancer progression, and evaluation of treatment response. Such an approach will improve costs of care and, most important, quality of life for patients with HCC. Furthermore, NanoVelcro Chips are expected to enable purification of CTCs from other solid tumors by targeting the corresponding surface markers, paving the way for the realization of a CTC-based RNA assay for cancer detection.

5. CHANGES/PROBLEMS:

-Changes in approach and reasons for change
Expansion of the CTC-RNA assay: As a result of this project, we have strengthened our interactions with Dr. Sungyong You, an expert in HCC computation biology, we have been revising our approach to molecular characterization of CTCs using the CTC-RNA assay. While the original proposal focused on MAT-related genes as a primary focus, we have gained the capacity to conduct other bioinformatics approaches that we will explore to further optimize the performance of the assay.

-Actual or anticipated problems or delays and actions or plans to resolve them
Nothing to Report

-Changes that had a significant impact on expenditures
Nothing to Report

-Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents
Nothing to Report

-Significant changes in use or care of human subjects
Nothing to Report

-Significant changes in use or care of vertebrate animals
Nothing to Report

-Significant changes in use of biohazards and/or select agents
Nothing to Report

6. PRODUCTS:

- **Publications, conference papers, and presentations**

Journal publications.

1. Sun N, Lee YT, Kim M, Wang JJ, Zhang C, Teng PC, Qi D, Zhang RY, Tran BV, Lee YT, Ye J, Palomique J, Nissen NN, Han SB, Sadeghi S, Finn RS, Saab S, Busuttil RW, Posadas EM, Liang L, Pei R, **Yang JD**, You S, Agopian VG, Tseng HR, Zhu Y. Covalent Chemistry-Mediated Multimarker Purification of Circulating Tumor Cells Enables Noninvasive Detection of Molecular Signatures of Hepatocellular Carcinoma. Adv Mater Technol. 2021 May;6(5):2001056. doi: 10.1002/admt.202001056. Epub 2021 Apr 9. PMID: 34212072; PMCID: PMC8240468.

Books or other non-periodical, one-time publications.
Nothing to Report

Other publications, conference papers and presentations.
Nothing to Report

- **Website(s) or other Internet site(s)**
  Nothing to Report

- **Technologies or techniques**
  Nothing to Report

- **Inventions, patent applications, and/or licenses**
  UCLA Technology Development Group filed the first patent application entitled “Click Chemistry-Mediated Rare-Cell Sorting in Microfluidic Devices” (UCLA # 2018-441) to cover the IPs associated with the Click Chips and the related research and clinical applications.

- **Other Products**
  Nothing to Report

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

-What individuals have worked on the project?

Name:	Ju Dong Yang (no change)
Project Role:	Contact-PI
No change	
Name:	Manaf Alsudaney
Project Role:	Clinical research coordinator
No change	

-Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?
Nothing to Report

-What other organizations were involved as partners?
Organization Name: University of California, Los Angeles (UCLA)
Location of Organization: 500 Westwood Plz, California NanoSystems Institute (CNSI)
Partner's contribution to the project

- In-kind support and Facilities: Equipment for HCC CTC isolation and transcriptomic profiling.

8. SPECIAL REPORTING REQUIREMENTS

- **NONE**

9. APPENDICES:

Literature Cited:

1. Dong J, Jan YJ, Cheng J, et al. Covalent chemistry on nanostructured substrates enables noninvasive quantification of gene rearrangements in circulating tumor cells. *Sci Adv.* 2019;5(7):eaav9186.
2. Court CM, Hou S, Winograd P, et al. A novel multimarker assay for the phenotypic profiling of circulating tumor cells in hepatocellular carcinoma. *Liver Transpl.* 2018;24(7):946-960.
3. Wang S, Wang H, Jiao J, et al. Three-dimensional nanostructured substrates toward efficient capture of circulating tumor cells. *Angew Chem Int Ed.* 2009;48(47):8970-8973.
4. Wang S, Liu K, Liu J, et al. Highly efficient capture of circulating tumor cells by using nanostructured silicon substrates with integrated chaotic micromixers. *Angewandte Chemie.* 2011;50(13):3084-3088.
5. Stroock AD, Dertinger SKW, Ajdari A, Mezić I, Stone HA, Whitesides GM. Chaotic mixer for microchannels. *Science.* 2002;295(5555):647-651.
6. Sun N, Lee YT, Zhang RY, et al. Purification of HCC-specific extracellular vesicles on nanosubstrates for early HCC detection by digital scoring. *Nat Commun.* 2020;11(1):4489.

7. Hoshida Y, Nijman SM, Kobayashi M, et al. Integrative transcriptome analysis reveals common molecular subclasses of human hepatocellular carcinoma. *Cancer Res.* 2009;69(18):7385-7392.
8. Woo HG, Lee JH, Yoon JH, et al. Identification of a cholangiocarcinoma-like gene expression trait in hepatocellular carcinoma. *Cancer Res.* 2010;70(8):3034-3041.
9. Cancer Genome Atlas Research Network. Electronic address wbe, Cancer Genome Atlas Research N. Comprehensive and Integrative Genomic Characterization of Hepatocellular Carcinoma. *Cell.* 2017;169(7):1327-1341 e1323.
10. Sohn BH, Shim JJ, Kim SB, et al. Inactivation of Hippo Pathway Is Significantly Associated with Poor Prognosis in Hepatocellular Carcinoma. *Clin Cancer Res.* 2016;22(5):1256-1264.
11. Kim SM, Leem SH, Chu IS, et al. Sixty-five gene-based risk score classifier predicts overall survival in hepatocellular carcinoma. *Hepatology.* 2012;55(5):1443-1452.
12. Lee JS, Chu IS, Heo J, et al. Classification and prediction of survival in hepatocellular carcinoma by gene expression profiling. *Hepatology.* 2004;40(3):667-676.
13. Cairo S, Armengol C, De Reynies A, et al. Hepatic stem-like phenotype and interplay of Wnt/beta-catenin and Myc signaling in aggressive childhood liver cancer. *Cancer Cell.* 2008;14(6):471-484.
14. Jan YJ, Yoon J, Chen JF, et al. A Circulating Tumor Cell-RNA Assay for Assessment of Androgen Receptor Signaling Inhibitor Sensitivity in Metastatic Castration-Resistant Prostate Cancer. *Theranostics.* 2019;9(10):2812-2826.
15. Barretina J, Caponigro G, Stransky N, et al. The Cancer Cell Line Encyclopedia enables predictive modelling of anticancer drug sensitivity. *Nature.* 2012;483(7391):603-607.
16. Novershtern N, Subramanian A, Lawton LN, et al. Densely interconnected transcriptional circuits control cell states in human hematopoiesis. *Cell.* 2011;144(2):296-309.
17. Kalinich M, Bhan I, Kwan TT, et al. An RNA-based signature enables high specificity detection of circulating tumor cells in hepatocellular carcinoma. *Proceedings of the National Academy of Sciences of the United States of America.* 2017;114(5):1123-1128.
18. Choi WT, Ramachandran R, Kakar S. Immunohistochemical approach for the diagnosis of a liver mass on small biopsy specimens. *Hum Pathol.* 2017;63:1-13.
19. Choi WT, Kakar S. Immunohistochemistry in the Diagnosis of Hepatocellular Carcinoma. *Gastroenterol Clin North Am.* 2017;46(2):311-325.
20. Shen MY, Chen JF, Luo CH, et al. Glycan Stimulation Enables Purification of Prostate Cancer Circulating Tumor Cells on PEDOT NanoVelcro Chips for RNA Biomarker Detection. *Adv Healthc Mater.* 2018;7(3).
21. Hou S, Zhao L, Shen Q, et al. Polymer nanofiber-embedded microchips for detection, isolation, and molecular analysis of single circulating melanoma cells. *Angew Chem Int Ed Engl.* 2013;52(12):3379-3383.
22. Court CM, Ankeny JS, Sho S, et al. Circulating Tumor Cells Predict Occult Metastatic Disease and Prognosis in Pancreatic Cancer. *Ann Surg Oncol.* 2018;25(4):1000-1008.
23. Hou S, Chen JF, Song M, et al. Imprinted NanoVelcro Microchips for Isolation and Characterization of Circulating Fetal Trophoblasts: Toward Noninvasive Prenatal Diagnostics. *ACS Nano.* 2017;11(8):8167-8177.

Reprints of manuscripts:

1. Sun N, Lee YT, Kim M, Wang JJ, Zhang C, Teng PC, Qi D, Zhang RY, Tran BV, Lee YT, Ye J, Palomique J, Nissen NN, Han SB, Sadeghi S, Finn RS, Saab S, Busuttil RW, Posadas EM, Liang L, Pei R, **Yang JD**, You S, Agopian VG, Tseng HR, Zhu Y. Covalent Chemistry-Mediated Multimarker Purification of Circulating Tumor Cells Enables Noninvasive Detection of Molecular Signatures of Hepatocellular Carcinoma. Adv Mater Technol. 2021 May;6(5):2001056. doi: 10.1002/admt.202001056. Epub 2021 Apr 9. PMID: 34212072; PMCID: PMC8240468.