

Integrated Immunological Analysis of a Successful Conversion of Locally Advanced Hepatocellular Carcinoma to Resectability with Neoadjuvant Therapy

SUPPLEMENTARY INFORMATION – METHODS

Trial design

This is an open-label single arm phase 1b study of neoadjuvant cabozantinib plus nivolumab in patients with borderline resectable or locally advanced HCC. Borderline resectable or locally advanced HCC was defined by, 1) Solitary tumor >5 cm, or 2) Unilobar multifocal disease either with >3 tumors or one tumor >3 cm, or 3) Bilobar disease with adequate future liver remnant, still technically resectable, or 4) High risk disease features (tumor >3 cm with macrovascular invasion or tumor >3 cm with AFP>400). Enrolled patients will receive a total of 8 weeks of cabozantinib therapy. After a two-week lead-in of cabozantinib monotherapy, patients receive concurrent nivolumab, one infusion every 2 weeks, for a total of 4 treatment doses. The primary endpoint is to characterize the safety and feasibility of preoperative cabozantinib plus nivolumab in locally advanced hepatocellular carcinoma (HCC).

Patient Samples

The evaluation of clinical samples was performed in accordance with the protocols approved by the Johns Hopkins Institutional Review Board (IRB). All specimens were obtained with written patient consent (IRB00149350). After receiving the surgical tumor tissue, mechanical mincing followed by enzymatic digestion in 0.1% collagenase in PBS at 37C for 30 minutes on an orbital shaker (60rpm) were performed. After centrifugation of the homogenate for 5 minutes at 1500rpm, the pellet was further processed using a Percoll gradient (GE Healthcare; 40% over 80% in PBS) centrifuged at 2000g for 25 minutes without break at room temperature (RT). The interface mononuclear layer was isolated into RPMI media. For peripheral blood samples, blood collection was done in two BD Vacutainer CPT – Cell Preparation Tube with Sodium Heparin and processed within two hours of collection. Tubes were centrifuged at RT for 30 minutes at 1800 rcf. Processed cells from tumor or blood were then counted and resuspended in AIM V (Gibco) and 10% DMSO. Cryovials were initially stored at -80C and transferred to liquid nitrogen for long term storage.

Antibodies

A list of Cytometry by Time-of-Flight (CyTOF) and Imaging Mass Cytometry (IMC) antibodies, isotopes, and concentrations used for immune cell subtyping is listed in Table S1 and S2, respectively. Conjugation of primary antibodies was performed using Maxpar Conjugation Kits according to the manufacturer's instructions. Briefly, purified antibodies were run through a buffer exchange protocol using 50kDa ultra filtration columns (Amicon) and then partially reduced with 4mM TCEP (Thermo Scientific). Polymers were loaded individually with isotopically enriched metals, ¹¹³In (Trace Sciences) and ¹¹⁵In (Sigma). Isotopically enriched Cisplatinium (194, 198) were directly conjugated onto the reduced antibodies (Mei, Leipold, & Maecker, 2016). Antibody concentrations in the wash buffer were quantified using Nanodrop. The final antibody concentrates were then diluted in a stabilization buffer (Candor) containing 0.3% sodium azide.

Cytometry by Time-of-Flight (CyTOF) Analysis

On the day of staining, peripheral blood samples were thawed rapidly in warm water bath and gently rinsed twice in RPMI media with 10% FBS and 1% penicillin-streptomycin according to standard protocols. Cells were rested at 37C in 5% CO₂ for at least 30 minutes prior to further manipulation. To permit simultaneous analysis of cytokine production capacity, all samples were

stimulated in 1X PMA/ionomycin/brefeldin A cocktail (Biolegend) for 2.5 hours in complete media. Live/dead staining was performed with 5-minute incubation in 500nM palladium chloride (Sigma) dissolved in DMSO and diluted in PBS, subsequently quenched with complete media. For multiplexing samples, five different metals conjugated to CD45 antibodies were used for a “5-choose-3” scheme for a total of 10 possible unique barcodes (Hartmann, Simonds, & Bendall, 2018). Multiplexed samples were then incubated in Fc block (Invitrogen) for 10 minutes at RT. Surface marker staining was first done with chemokine receptor antibodies for 10 minutes at 37C, followed by the rest of surface markers for 30 minutes at RT. After two washes, intracellular staining was performed using Cytotfix/Cytoperm kit (BD Biosciences) per manufacturer’s protocol. Just before data collection, all cells were labeled with rhodium (Fluidigm) at 1:500 for 45 minutes at room temperature. All events were acquired on a Helios™ mass cytometer (Fluidigm). Randomization, bead normalization, and bead removal of data collected were performed on CyTOF software (Fluidigm) v6.7. Using FlowJo (BD) v10.5, single cell events were identified by gating based on cell length and rhodium signal. Dead cell filtering and debarcoding were performed by manually gating. For all CyTOF analyses, a computational pipeline based on diffcyt was employed using R v3.5 (Weber, Nowicka, Soneson, & Robinson, 2019). Briefly, for unsupervised clustering, FlowSOM algorithm was used to identify 30 meta-clusters that were then annotated into specific immune cell subtypes (Van Gassen et al., 2015). Clustering was visualized using a two-dimensional uniform manifold approximation and projection (UMAP) dimensionality reduction algorithm (Becht et al., 2019).

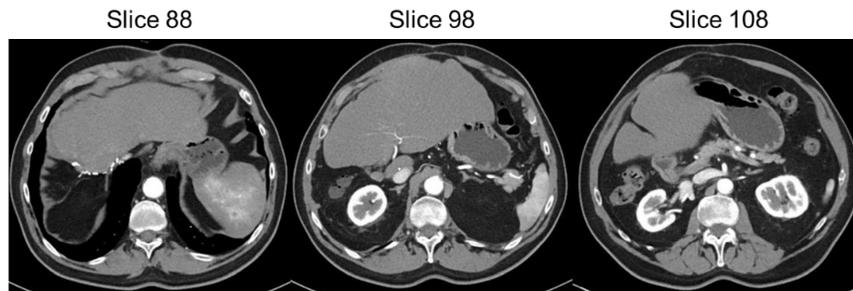
Imaging Mass Cytometry (IMC) Analysis

After constructing a tissue microarray (TMA) containing 1.5mm-diameter cores, including two from a deidentified normal liver and three from the patient described, it was dewaxed in xylene and rehydrated in alcohol gradient. The TMA slide was heated at 95C in Antigen Retrieval Agent pH 9 (Agilent) for 30 minutes. It was then blocked with 3% BSA in Maxpar PBS for 45 minutes at room temperature, followed by staining overnight at 4C with the antibody cocktail listed in Table S2. Ir-Intercalator in Maxpar PBS was used for DNA labeling. Images were acquired using a Hyperion Imaging System (Fluidigm) and data was preprocessed using a commercial software (Fluidigm). Image segmentation was performed using CellProfiler v.3.1.8. based on added image of Ir191 and Ir193 DNA staining, primary objects were identified using 5-15 pixel diameter (threshold strategy: global; method: minimum cross entropy; smoothing scale: 1.3488; correction factor 1.0) followed by secondary object identification with 3-pixel expansion. Resulting objects were converted to image in uint16 format. The single-cell segmentation mask was overlaid and the spatial information along with mean metal intensities of the markers were extracted into csv files. Individual files from each of the cores were converted into fcs files (<https://github.com/sydneycytometry/CSV-to-FCS>) to be input into the aforementioned computational pipeline. For neighborhood analysis, Single cell CoGAPS analysis (Stein-O’Brien et al., 2019) was performed on 26 mass intensities with the R/Bioconductor package version 3.5.8 to identify 13 patterns in these data. The PatternMarker statistic was applied to the inferred protein weights to define a set of unique protein markers associated with each pattern. The neighbors for each cell within surrounding 4 pixels were obtained using histoCAT (Schapiro et al., 2017). For each annotation in each core, the number of different cell-type and functional neighbors were calculated. The resulting numbers were combined for tumor and normal cores and the proportion of different neighbors were calculated. Moran’s I autocorrelation coefficient was computed with R package ape version 5.3. To compute the autocorrelation coefficient, 10 nearest neighbors of each cell, computed based on the Euclidean distance of the geographic coordinates of each cell using FastKNN package version 0.0.1, were assigned a weight of 1 and the rest were assigned a zero weight.

SUPPLEMENTARY INFORMATION – RESULTS

Supplementary Figures 1-3
Supplementary Table 1

Supplementary Figure 1.



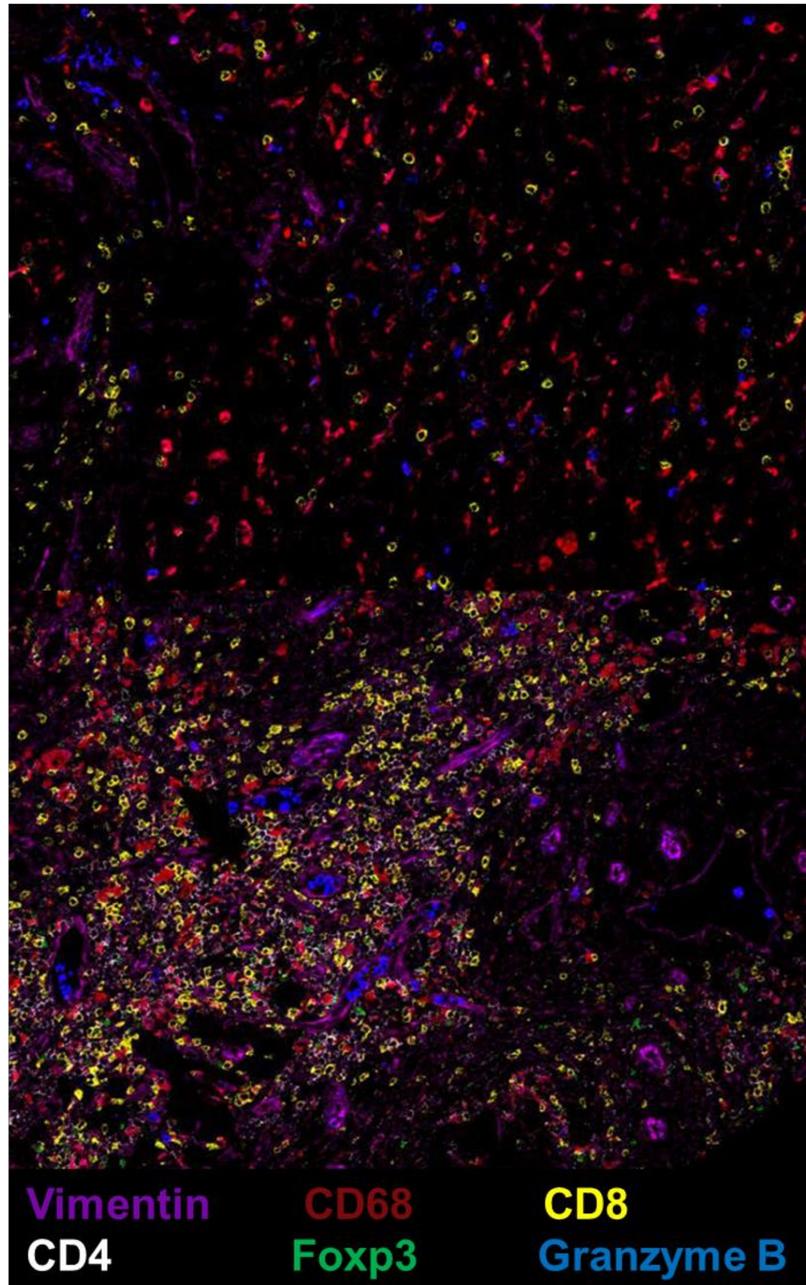
Three representative slices from the most recent CT scan (approximately 2 years from the date of the tumor resection) are shown.

Supplementary Table 1.

Timepoint	Total Prot g/dL	Alb g/dL	ALT U/L	AST U/L	Alk Phos U/L	Total Bili mg/dL	AFP ng/mL
At presentation	7.8	4.9	21	24	89	0.4	17,528.30
1 mo post-surgery	5	3.1	144	92	55	0.7	4.5
2 yr post-surgery	7.5	4.6	18	17	84	0.5	2.6

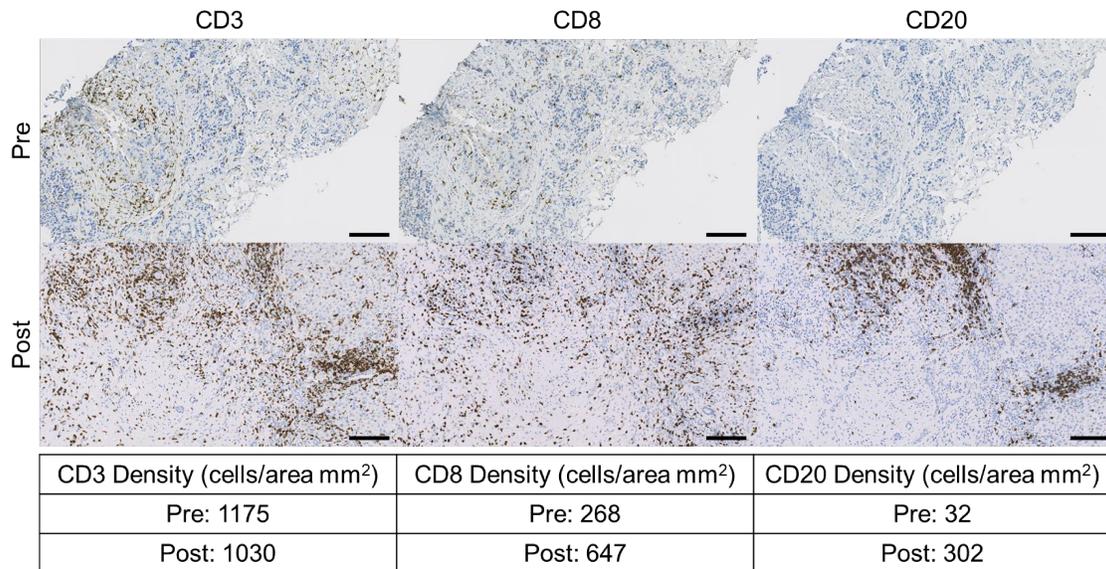
Liver function and AFP at presentation, 1 month post-surgery, and 2 years post-surgery

Supplementary Figure 2



Higher resolution zoomed in images for IMC results for normal liver (Top) and surgically resected hepatocellular carcinoma (Bottom).

Supplementary Figure 3.



Representative single immunohistochemistry results against CD3, CD8, and CD20 in the tumor microenvironment between pre-treatment core biopsy and the post-treatment surgical resection samples. Quantified positive cell densities as determined by HALO are summarized in the table below. 10X magnification is shown (scale bar: 200 μ m).