**Single Cell Spatial Transcriptomic Profiling Identifies a LINE1 Associated**

**Disarrayed Immune Microenvironment in Hepatocellular Carcinoma**

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**KEYWORDS**

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**ABSTRACT**

**Purpose**: Hepatocellular carcinoma (HCC) is a lethal malignancy driven by complex interactions between cancer cells, immune cells, and additional stromal cells in the tumor microenvironment (TME). The LINE-1 retrotransposon is a ubiquitous repeat RNA whose de-repression leads to significant cancer cell-intrinsic and TME changes that promote aggressive tumor characteristics. We leveraged single cell spatial transcriptomic profiling to characterize how the heterogeneous HCC TME varied depending on LINE-1 context.

**Experimental Design**: We applied our profiling methodology to a cohort of 23 tissue specimens collected from patients who had undergone liver resection or transplantation and validated it in a partially-overlapping similar cohort of 39 specimens using RNA in-situ hybridization (RNA-ISH).

**Results**: We found that LINE1-high tumors and LINE1-high single HCC cells exhibited a de-differentiated, stem-like, and inflammatory phenotype. Furthermore, within individual tumors, LINE-1 high cancer cells associated spatially with one another and excluded the larger, organized immune cell conglomerates seen in LINE-1 low tumors. Finally, we found that LINE-1 RNA expression correlated with worse overall survival in the larger expanded retrospective cohort.

**Conclusions**: Our study is the first to show a clearly disorganized immune TME in HCC driven by LINE-1 expression, and this observation correlated with poor survival for patients whose tumors expressed large amounts of the LINE-1 repeat RNA. These results provide further evidence of how effective anti-tumor immune responses contribute to cures after definitive surgery and may lead to novel biomarkers or drug targets in HCC.

**TRANSLATIONAL RELEVANCE**

The viral-like LINE1 retrotransposon is known to influence tumor cell state and the immune response in a variety of cancers. Here, we have used single cell spatial transcriptomic profiling to resolve repeat and coding gene RNA expression in a cohort of hepatocellular carcinoma (HCC) patients. LINE-1 RNA expression in HCC tumor cells was correlated with an undifferentiated stem-like cancer state and a disorganized, sparse immune infiltrate. Using in situ hybridization on an expanded validation cohort, we noted significantly worsened survival in the LINE-1 high group. Altogether, LINE-1 repeat RNA is a tumor-intrinsic biomarker of more aggressive features that can be used for risk stratification and as a potential biomarker for response to immunotherapies that merits further investigation.

**INTRODUCTION**

Hepatocellular carcinoma (HCC) is a leading cause of global cancer morbidity and mortality (1). Modern medical treatment regimens for advanced stage HCC have improved significantly over the past several years, with the emergence of multiple therapies inhibiting either pathologically activated vasculature, dysregulated immune checkpoints, or combinations thereof (2). These features have highlighted the importance of therapeutically targeting the HCC tumor microenvironment (TME). However, only a minority of patients derive significant clinical benefit from these regimens (3-5), and most patients eventually succumb to their disease (6,7). These factors highlight the importance of an improved understanding of the dynamic cellular interactions in the TME of HCC tumors, which has the potential to broaden our understanding of existing drug mechanisms and motivate the subsequent development of novel drug targets and biomarkers.

Our group and others have characterized the relationship of repeat RNA expression with the TME across cancers (8-15). These relationships appear to be linked to differences in repeat RNA sequence (16) and secondary structure (17), which both contribute to the activation of innate immune responses (17-26). Prior work has demonstrated the worsened prognosis of HCC patients with hypomethylation of the LINE1 repeat RNA (27-30) and evidence of retrotransposition events in viral and non-viral HCC (31). Further, there is mounting evidence of LINE-1 retrotransposition events in viral and non-viral interplay between HBV and HCV driven HCC oncogenesis with LINE1 (32,33). More generally, expression of specific repeat RNA species tends to correlate with distinct patterns of tumor immune infiltrates (8,9,15).

Given the known viral drivers of cirrhosis and HCC (34), we hypothesized that endogenous repeat viral-like elements are important in HCC carcinogenesis and immunological response. To fully characterize the relationship of repeat elements with tumor cells and the surrounding microenvironment, we utilized high-plex spatial tissue profiling technologies. These technologies have many of the benefits of sequencing-based and proteomic cell profiling methodologies while retaining the spatial information inherent to conventional tissue profiling technologies such as in situ hybridization or immunohistochemistry (35). Our previous work in HCC utilized a regional whole transcriptome analysis of tumor cells, endothelial cells, and immune cells, revealing improved resolution of tumor cell subtyping and initial ligand-receptor signaling between tumor cells and the vasculature (36).

In this study, we have expanded on these previous studies by utilizing spatial molecular imaging of 957 genes (including several repeat RNA species) in HCC tumor resection samples to understand the relationship of repeat RNAs to tumor cell states and the microenvironment response. This is the first integrated dataset characterizing repeat RNA distributions and single cell spatial architecture in a cohort of samples derived from human HCC resection or transplantation specimens. We found that the HCC LINE1-high tumor cells are enriched for a stem cell phenotype with loss of hepatocyte differentiation markers. LINE1-high HCC tumors had a more disorganized immune response compared to LINE1-low HCC tumors, which had regional hubs of diverse immune cell infiltrates similar to tertiary lymphoid structures. Using RNA in situ hybridization (RNA-ISH) for LINE1, we found that LINE1-high compared to LINE1-low HCC tumors had significantly worsened survival after surgical resection or transplantation. Taken together, these findings support the importance of LINE1 and other repeat RNAs in HCC development and as prognostic markers of aggressive behavior.

**MATERIALS AND METHODS**

**Ethics statement, tissue procurement and annotation**

All patient-oriented research was conducted in accordance with both the Declarations of Helsinki and Istanbul. Patient tumor materials were obtained under Massachusetts General Hospital IRB protocol 2011P001236 and Dana-Farber Harvard Cancer Center IRB protocol 02-240. Archived FFPE samples in tissue microarray (TMA) format from who underwent surgical resection or liver transplantation for the treatment of hepatocellular carcinoma between March 2004 and December 2015 were obtained. Clinical and pathologic data was obtained through review of the Massachusetts General Hospital electronic medical record. There is no blinding, randomization, or power analysis relevant for this study.

**Spatial transcriptomics data acquisition and analysis**

A total of 23 patient FFPE HCC tumors on TMAs were evaluated with spatial molecular imaging (SMI) following published standard protocol (37). In brief, 5-µm FFPE sections were baked overnight at 60°C to ensure section adherence to the glass slides. Then the baked samples went through deparaffinization, proteinase K digestion, and heat-induced epitope retrieval (HIER) procedures to expose target RNAs and epitopes using Leica Bond Rx system. After rinsing samples with DEPC H2O twice, samples were incubated in 1:1000 diluted fiducials (Bangs Laboratory) in 2X SSCT (2X SSC, 0.1% Tween 20) solution for 5 min at room temperature. Excessive fiducials were removed by rinsing the samples with 1X PBS, followed by fixation with 10% neutral buffered formalin (NBF) for 5 min at room temperature. Fixed samples were rinsed with Tris-glycine buffer (0.1 M glycine, 0.1M Tris-base in DEPC H2O) and 1X PBS for 5 min each before blocked using 100 mM N-succinimidyl acetate (NHS-acetate, ThermoFisher) in NHS-acetate buffer (0.1 M NaP, 0.1% Tween 20, pH 8 in DEPC H2O) for 15 min at room temperature. Prepared samples were rinsed with 2X saline sodium citrate (SSC) for 5 min and then Adhesive SecureSealTM Hybridization Chamber (Grace Bio-Labs) was placed to cover the sample. ISH probe mix (1 nM ISH probes, 1X Buffer R, 0.1 U/µL SUPERase●In™ in DEPC H2O) was prepared by denaturing 980-plex RNA ISH probes at 95°C for 2 min and then placed on ice before making ISH probe mix. Hybridization occurred at 37°C overnight after sealing the chamber to prevent evaporation. After the overnight hybridization, samples were washed with 50% formamide (VWR) in 2X SSC at 37°C for 25 min for 2 times, rinsed with 2XSSC for 2 min for 2 times at room temperature, and then blocked with 100 mM NHS-acetate for 15 min. After blocking, the samples were washed twice using 2X SSC for 2 min at room temperature. A custom-made slide cover was attached to the sample slide to form a flow cell. Prepared samples were loaded to the CosMx SMI instrument and went through data collection, image processing, feature extraction, and cell segmentation procedures following published protocols (37). Transcript profiles of individual cell were generated by combining target transcript location and cell segmentation information and then fed into downstream data analysis.

**RNA in situ hybridization (RNA-ISH)**

A total of 39 patient FFPE HCC tumor sections on TMAs were evaluated with RNA-ISH Automated chromogenic RNA-ISH assay was performed using the Advanced Cell Diagnostics (ACD) RNAscope 2.5 LS Duplex Reagent Kit - RED (Catalogue No. 322150) on the BondRx 6.0 platform (Leica Biosystems Inc., Buffalo Grove, IL). Assay was performed using custom probes from ACD in Channel 1 against HERV-K (Catalogue No. 469838) at original concentration, HERV-H (Catalogue No. 433558) at a 1:5 dilution, and LINE1 (Catalogue No. 565098) at a 1:50 dilution. 5 μm sections of FFPE embedded tumor microarrays were mounted on Fisherbrand Superfrost Plus glass slides, baked for 55 minutes at 60°C, and placed on the BOND RX for processing. The RNA unmasking conditions for the tissue consisted of a 15-minute incubation at 95°C in Bond Epitope Retrieval Solution 2 (Leica Biosystems) followed by 15-minute incubation with Proteinase K which was provided in the kit. Probe hybridization was done for 2 hours at 42C with RNAscope probes which were provided by ACD.

**RNA-ISH and IHC quantification**

The RNA-ISH slides were imaged with the Motic EasyScan Infinity Digital Pathology Scanner at 40x magnification. RNA quantification was performed with the Halo Image Analysis Platform by Indica Labs. Individual tissue areas on the TMA corresponding to each patient were annotated. In each tissue area, cellular segmentation was performed by detection of hematoxylin-stained nuclei and the RNA-ISH probe was detected by red chromogen. For HERV-K, HERV-H, and HSATII, the average number of probe copies per cell in the tissue area was quantified. For LINE1, due to the density of stain in the samples with the highest expression, accurate detection of individual red copies was challenging. LINE1 expression was therefore quantified as the probe detected per μm2 tissue area.

**Statistical and Computational analyses**

Survival analyses were done with Kaplan Meier analysis with log-rank test for significance using Graphpad Prism (v10).

**RESULTS**

**Single cell spatial transcriptomic profiling of HCC FFPE tissue**

We analyzed FFPE TMA sections derived from 23 patient resection specimens using spatial molecular imaging (**Fig. 1A**). The probe set utilized for this experiment leveraged a 950 gene panel with additional custom “spiked-in” probes targeting repeat RNAs (HERV-K, HSATII, LINE1-ORF1, LINE1-ORF2) as well as known HCC markers (*ASGR1*, *GPC3*, *LIN28B*) (**Table S1**). We utilized consecutive hematoxylin and eosin stained FFPE sections to guide the selection by an anatomic pathologist (BKP) of fields of view (FOVs) that contained representative areas of different TMA cores (**Fig. 1B, Fig. S1-2**). We then utilized immunofluorescent co-stains including a nuclear DAPI stain combined with fluorescent-conjugated primary antibodies targeting CD45, keratin 8/18 (CK8/18), pan-cytokeratin (panCK), and beta-2-microglobulin (B2M) to perform cell boundary segmentation (38) that is needed to identify and map each transcript to a specific cell (**Fig. 1C**). With this approach, after applying quality control filters to remove cells with adequate transcript density, we identified 158,971 cells with a per FOV median of 1081 cells (range 229-2812 cells) with an average of 244 transcripts/cell and 81 unique genes/cell (**Fig. 1D-E**).

Next, in combination with manual labeling, we utilized both unsupervised and supervised clustering techniques (39) to annotate cell identities (**Fig. 2A**). This resulted in classification of 96,677 tumor cells and 62,294 non-tumor cells (**Fig. 2B, Fig. S3**). For each cell type cluster, we found the top 1-5 genes for which average expression was uniquely greater than in the other clusters (insert ref) to confirm cell identity (**Fig. 2C**), and we further validated with differential expression analysis as done in previous studies (**Fig S4**) (insert ref). We found that the HCC tissue cellular composition was consistent with those from prior dissociated HCC single cell datasets (40). We noted high variability in the composition of individual FOVs, with ranges of 25-90% cancer cells, 10-30% stromal cells (fibroblasts, endothelial cells), and 10-50% immune cells (lymphoid cells, myeloid cells) (**Fig. 2D**). In summary, we utilized a subcellular resolution spatial transcriptomic technology to annotate and profile the in situ, spatially defined gene expression profiles of cells comprising systemic therapy-naïve HCC tumor microenvironment.

**LINE1 expression correlates with aggressive cancer phenotypes and disorganized immune niches**

Given prior work in other tumor types highlighting distinct immune niches associated with expression of various repeat RNAs, we then examined the spatial expression profiles of repeat RNA species using custom “spike in” oligonucleotide probes. Given prior literature highlighting the role of LINE1 methylation and retrotransposition in HCC (27-31,33), we focused our analysis on LINE1 ORF1 RNA (LINE1-ORF1) across the samples (**Fig. 3A**). Separating samples into the top tercile of LINE1-ORF1 (LINE1-high) and bottom two terciles (LINE1-low), we identified 579 differentially expressed genes, with 511 enriched in LINE1-high tumor samples and 68 enriched in LINE1- low tumor samples (**Fig. 3B**, **Table S2**). The gene set enriched in the LINE1-high subset included multiple additional repeat RNAs (LINE1-ORF2, HERV-K, HSATII) and many additional genes typically associated with aggressive HCC behavior: WNT pathway ligands (*WNT3*, *WNT7A*, *WNT7B*, *WNT9A*) and receptors (*FZD1*, *FZD5*, *FZD7*, *FZD8*), stemness associated genes (LEFTY1, NDRG1, POU5F1), and multiple interferon pathway genes (*IFNL2/3*, *IFNGR2*) (**Fig. 3C**). The gene set enriched in the LINE1-low subset included multiple genes associated with normal hepatocyte function (*APOA1*, *APOC1*, *FGG*, *GLUL*).

Next, we examined the individual cell variability in LINE1 expression within each FOV (**Fig. 3D**). Similarly to the patient-level data, differential expression analysis (**Fig. 3E-F, Table S3**) showed enrichment of co-regulated repeat RNAs (LINE1-ORF2, HSATII, HERV-K) and stemness related genes (*LEFTY1*, *POU5F1*) in the LINE1-high single cells. The LINE1-low cancer cells were enriched in markers of hepatocyte differentiation (*APOA1*, *FGG*, *SAA1/2*). Gene set enrichment analysis of the LINE1-high versus LINE1-low cells confirmed a diminution in well-differentiated gene signatures and an enrichment of growth factor signaling changes for LINE1-high cells. (**Fig. 3G**).

We then sought to understand the spatial structure of the TME niches associated with LINE1 expression, and we utilized spatial colocalization analysis (41) to compare LINE1-high versus LINE1-low tumors. First, we noted that within each patient’s TME, LINE1-high cells tended to cluster with one another, and LINE1-low cells also clustered with one another (**Fig. 4A-B**). Next, we noted several significant differences in the immune niches of patients with LINE1-high tumors, compared to those of LINE1-low tumors. Cellular neighborhoods in the patients with low LINE1 tended to display large groups of co-localized immune cells, whereas neighborhoods in patients with high LINE1 tended to have more dispersed immune cells (**Fig. 4C-D**). Relative to LINE1-low niches, LINE1-high niches featured fewer correlated CD4+ T cell – NK cell pairs and myeloid dendritic cell – endothelial and – monocyte pairs. Overall, the structure of the immune niche within LINE1-high cancer micro-regions showed overall statistically significantly lower immune co-localization (**Fig. 4C-E**).

Taken together, our results show that LINE1 expression within hepatocellular carcinoma cells correlates with expression of genes associated with aggressive cancer features and with a significantly disarrayed immune microenvironment. LINE1 expression levels may also be implicated in explaining how tumor cells from the same neighborhood arrange themselves. We next sought to validate and extend our spatial transcriptomic data using orthogonal techniques.

**Validation and clinical significance of LINE1 expression in HCC tissue**

We validated repeat RNA expression using RNA in situ hybridization (RNA-ISH) across a cohort of 39 HCC tumor samples. Characteristics of the patient population from the expanded RNA-ISH cohort are summarized in **Table 1**. The median age of patients at the time of operation was 60 years old. Most patients had underlying cirrhosis (29/39; 74%) with the most common etiology being chronic HCV infection (15/39; 38%) followed by chronic HBV infection (6/39; 15%). A total of 30 patients underwent surgical resection (77%) and 9 patients had liver transplantation (23%). The five-year overall survival proportion of this cohort was 51%. The demographic data in our cohorts are generally representative of published epidemiologic and outcome data (42).

Digital imaging of RNA-ISH-stained TMA sections, followed by cellular segmentation and RNA-ISH signal quantification using the HALO AI platform (**Fig. S5B**), was used to quantify expression of HSATII, HERV-K, HERV-H, and LINE1 in the HCC tumor tissue (**Fig. 5A, Fig. S5C**)**.** Using this RNA-ISH expression data, patient samples were divided into terciles based on the expression of each repeat RNA element. Patients with LINE1 expression in the upper tercile were designated as “high” and samples in the middle and lower terciles were designated as “low” (**Fig. 5B, Fig. S6D**). In this repeat RNA-ISH dataset, the major repeat RNA species quantities were also well correlated with one another by RNA-ISH (**Fig. 5C**). The relationship between expression of LINE1 and overall survival (OS) was assessed using Kaplan Meier analysis and log-rank test to determine significance (**Fig. 5D**). High LINE1 expression was associated with worsened overall survival (median OS 2.04 vs. undefined years, p = 0.01).

**DISCUSSION**

The development, progression, and response to systemic therapy of hepatocellular carcinoma is influenced by complex interactions between cancer cells and the stromal/immune tumor microenvironment. In this study, we utilized a single cell resolution spatial transcriptomic profiling technology to characterize how the HCC tumor microenvironment (TME) varies in a manner dependent on cancer cell expression of the LINE1 repeat RNA. The spatial molecular imaging platform and downstream computational analytic pipeline enabled accurate cell boundary segmentation and transcript detection enabled robust gene expression profiling of thousands of single cells per sample with retained spatial context. We identified and profiled tens of thousands of cells in the HCC TME, with stromal (endothelial and fibroblast) cells and lymphoid cells being the predominant non-malignant components. These findings underscore the privileged roles of T cells and endothelial cells as the most pertinent druggable targets in the HCC TME (43).

Given the prominent role of the LINE-1 retrotransposon in cancer (44), we chose to focus first on LINE-1 expression as a marker of aggressive cancer phenotype. We found that LINE-1 expression correlated with expression of repeat RNAs HSATII and HERV-K, consistent with coordinate de-repression of these critical repeat elements observed other cancer types (45). LINE1-high tumors and single cells exhibited an aggressive stem-like (POU5F1, TWIST2 (46)) and immune suppressive phenotype (PDCD1); LINE1-low tumor and single cell expression profiles showed less aggressive, more well-differentiated hepatocyte features (SAA1/2, APOA1). Leveraging the retained spatial data in combination with single cell expression profiling allowed detailed analysis of the microscopic cellular neighborhoods associated with LINE1 expression. We noted that LINE-1 cancer cell expression was correlated with significant differences in the TME. Whereas LINE-1 high cells tended to surround themselves with additional LINE-1 high cells, LINE-1 low cells associated with other LINE-1 low cells and immune cell aggregates. Other groups have shown that “tertiary lymphoid structures” (TLSs) in HCC (47) and “immunity hubs” in non-small cell lung cancer (41) correlate with effective anti-tumor immune responses. Our findings suggest that LINE-1 overexpression may serve as a biomarker for ineffectively organized immune responses to cancer and imply that disrupting LINE-1 effects may augment anti-tumor immunity.

We validated our findings with an extended cohort of RNA-ISH specimens, confirming coordinate expression of LINE-1 with other repeat RNA elements. We also noted that high expression of LINE-1 in HCC tissue specimens was associated with poorer survival, consistent with prior work in other tumor types (48-49) and in hepatitis B driven HCC (33). Other analyses have identified hypomethylation of LINE-1 as adverse prognostic factors in various HCC subsets (27-30). Our study is the first to identify LINE-1 transcript expression as a potential biomarker of poor prognosis in HCC. Given the observation that robust pre-resection immune infiltrates correlate with good surgical outcomes in HCC (47) and other (50) cancers, LINE-1 associated immune disarray may be driver of this effect.

Our study implies that LINE-1 – and perhaps additional co-regulated repeat RNAs such as HSATII and HERV-K – may also represent potential novel therapeutic targets in HCC. HERV-K is considered the most translationally active of the endogenous retroviruses, encoding for proviral Gag, Pro, Pol, and Env proteins (51). Specific targeting of HERV-K proteins with monoclonal antibodies (52), HERV-K reactive cytotoxic T-cells derived from patient serum (53), and CAR T-cells (54) have demonstrated anti-tumor effects in preclinical models. LINE1 encodes for ORF1, an RNA-binding protein, and ORF2, which has endonuclease and reverse transcriptase activity. Circulating LINE1-ORF1 has been proposed as a pan-cancer (including HCC) diagnostic marker (55), and LINE1-ORF1p has been explicitly identified as a potential therapeutic target in pancreatic cancer. These protein products of LINE1 and other repeat RNA elements may similarly be targetable in HCC.

The limitations of this study include the small sample size and the retrospective nature of the analyses. The tissue samples were operative samples from systemic therapy-naïve patients who underwent surgical resection or transplantation for management of their HCC, and therefore patients with unresectable or metastatic disease who may have different tumor biology at baseline or in response to systemic therapy regimens were not represented in the cohort. Additionally, while our spatial transcriptomic platform leveraged expression of thousands of genes, the majority of the (coding and non-coding) transcriptome was not assayed. Finally, while distinct tumor immune profiles were found to correlate with LINE-1 RNA expression, additional mechanistic experiments are needed to further assess potential causal relationships.

In summary, our study is the first to apply single cell spatial transcriptomic technologies simultaneously to coding and non-coding RNA elements in HCC. We identified expression of the LINE-1 retrotransposon as a potential adverse RNA prognostic biomarker and marker of tumor de-differentiation/stemness and disarrayed immune infiltrates. This work expands upon the existing literature describing the interplay between the coding and non-coding transcriptome in cancer and lays the groundwork for the development of novel biomarkers and mechanistic hypotheses to test in subsequent investigations. Ultimately, our data may motivate the development of additional therapeutic strategies to augment anti-tumor immunity and improve clinical outcomes for patients with HCC.

**RESOURCE AVAILABILITY**

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**Materials Availability:**

All RNA-ISH probes and sequences are available at ACD-Biotechne and custom CosMx probes from Bruker.

**Data and Code Availability:**

All code and statistical packages are detailed in the methods and will be made available upon request. All software for RNA expression and digital image data analysis is described in the methods above and all software will be provided upon request. All images and primary data will be made available upon request. Spatial transcriptomic expression matrices will be available upon request.

**AUTHORS’ DISCLOSURES**

JWF has received consulting fees from Eisai, Foundation Medicine, Guardant Health, Genentech, and Servier. JWF has received personal research funding from Genentech and institutional research funding from Abbvie, Alnylam, Genentech, Iterion Therapeutics, and Omega Therapeutics. None of these competing interests are related to this work. DTT has received consulting fees from ROME Therapeutics, Sonata Therapeutics, Leica Biosystems Imaging, PanTher Therapeutics, 65 Therapeutics, and abrdn. DTT is a founder and has equity in ROME Therapeutics, PanTher Therapeutics and TellBio, Inc., which is not related to this work. DTT is on the advisory board with equity for ImproveBio, Inc. and 65 Therapeutics. DTT has received honoraria from Astellas, AstraZeneca, Moderna, and Ikena Oncology that are not related to this work. DTT receives research support from ACD-Biotechne, AVA LifeScience GmbH, Incyte Pharmaceuticals, Sanofi, and Astellas which was not used in this work. DTT’s interests were reviewed and are managed by Mass General Brigham in accordance with their conflict of interest policies.

**AUTHOR CONTRIBUTIONS**

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**TABLES**

|  |  |  |  |
| --- | --- | --- | --- |
|  |  | **Spatial** | **RNA-ISH** |
| **Sex (n, %)** | *male* | 15 (65%) | 28 (72%) |
|  | *female* | 8 (35%) | 11 (28%) |
|  |  |  |  |
| **Age (years)** | *median* | 63 | 60 |
|  | *range* | 45-82 | 39-85 |
|  |  |  |  |
| **Cirrhosis (n, %)** | *absent* | 15 (65%) | 10 (26%) |
|  | *present* | 8 (35%) | 29 (74%) |
|  |  |  |  |
| **Cirrhosis etiology (n, %)** | *alcohol* | 3 (13%) | 3 (8%) |
|  | *HBV* | 2 (9%) | 6 (15%) |
|  | *HCV* | 8 (35%) | 15 (36%) |
|  | *metabolic* | 2 (9%) | 3 (8%) |
|  | *other, NOS* | 8 (35%) | 12 (31%) |
|  |  |  |  |
| **Operation (n, %)** | *resection* | 20 (87%) | 30 (77%) |
|  | *transplant* | 3 (13%) | 9 (23%) |
|  |  |  |  |
| **Tumor size (cm)** | *median* | 4 | 4.3 |
|  | *range* | 1.5 - 13 | 1.2 - 15 |
|  |  |  |  |
| **Differentiation (n, %)** | *well* | 4 (17%) | 7 (18%) |
|  | *well-moderate* | 0 (0%) | 1 (3%) |
|  | *moderate* | 13 (57%) | 25 (64%) |
|  | *moderate-poor* | 3 (13%) | 3 (8%) |
|  | *poor* | 3 (13%) | 3 (8%) |
|  |  |  |  |
| **Lymphovascular inv (n, %)** | *yes* | 11 (48%) | 16 (41%) |
|  | *no, NOS* | 12 (52%) | 23 (59%) |
|  |  |  |  |
| **Serum alpha-fetoprotein (ng/mL)** | *median* | 7.8 | 31 |
|  | *range* | 0 - 37510 | 0 - 37510 |
|  |  |  |  |

**Table 1. Patient characteristics.**

**FIGURE LEGENDS**

**Figure 1. Overview of spatial molecular imaging (SMI) experiment utilizing HCC FFPE sections.** (**A**) Experimental workflow of spatial transcriptomic profiling experiment. (**B**) Schematic showing subset of TMA core tissue samples utilized in one spatial molecular imaging (SMI) “run.” H&E staining of a consecutive section on the left and immunofluorescent staining (green: pan-cytokeratin, red: CD45, yellow: CD68, cyan: beta-2-microglobulin) of the imaged slide section on the right. (**C**) Immunofluorescent “morphology marker” staining on a representative field of view (FOV). (**D**) Cell boundary demarcation and tumor versus immune cells shown in the same FOV as shown in C.

**Figure 2. Subcellular spatial molecular imaging (SMI) accurately** **identifies cellular subtypes in HCC tumor microenvironment**. (**A**) Schema of combined supervised and unsupervised computational workflow for cell type identification. (**B**) UMAP plot of cell types – with embedded spatial coordinate metadata allowing for mapping back onto the 2D slide surface – identified in the HCC SMI dataset. (**C**) Bubble plot showing expression of marker genes for each of the identified cell types. (**D**) Proportion of each major cell type category present within each FOV of the dataset.

**Figure 3. HCC expression of LINE-1 is associated with a de-differentiated, stem-like state**. (**A**) Bar plot of scaled LINE1-ORF1 counts within HCC cancer cells in “high” (top tercile) and “low” (middle plus bottom tercile) groups. (**B**) Volcano plot comparing LINE1-high and LINE1-low core (entire sample) groups. Statistical threshold set at false discovery rate corrected p-value of <0.05. (**C**) Heat map showing scaled expression of differentially expressed genes between LINE1-high and LINE1-low cores. (**D**) Box and whisker plots for each core showing LINE1-high and LINE1-low cores showing single cell expression of LINE-1, subdivided in each core into high and low expressing groups. (**E**) Volcano plot comparing LINE1-high and LINE1-low single cells. Statistical threshold set at false discovery rate corrected p-value of <0.05. (**F**) Normalized expression of HSATII, HERV-K, POU5F1 (Oct4), LEFTY1, and SERPINA3 between LINE1-high and LINE1-low single HCC cells. (**G**) Gene set over-representation analysis of LINE1-high versus LINE1-low single HCC cells.

**Figure 4. High LINE-1 expression within HCC tissue leads to a disorganized, sparse immune microenvironment**. (**A**) Paired plots of niche enrichment ratios of LINE-high and LINE1-low cells with one another. (**B**) Representative images of HCC LINE1-high (red dots) and LINE1-low (blue dots) neighborhoods, with all other cell types represented as white dots. (**C**) Heatmaps of enrichment ratios of the microenvironment niches of LINE1-high tumor tissues (left) versus LINE1-low tumor tissues (right). (**D**) Observed immune organization structure (red dashed vertical line) versus simulated random distrubtion of \_\_\_\_\_\_. (**E**) Representative images of LINE1-high (left two columns) versus LINE1-low (right two columns), with immune cells represented as red dots and all other cells as white dots (first row), with paired immunofluorescent images shown in the second row (red: CD45, yellow: keratin 8/18, blue: DAPI).

**Figure 5. Validation of LINE-1 expression by RNA-ISH**. (**A**) Representative LINE-1 RNA-ISH image. (**B**) Plot of LINE1 RNA ISH quantitative expression in the “high” (upper tercile) group versus the “low” (bottom terciles) group. (**C**) Correlation of HERV-H and HERV-K with LINE-1 expression by RNA-ISH. (**D**) Kaplan-Meier overall survival analysis for patients with LINE1-high (red) versus LINE1-low (blue) tumor tissues (p = 0.01 by log-rank test).