nature genetics

Supplementary information

https://doi.org/10.1038/s41588-025-02407-8

Spatially resolved multi-omics of human metabolic dysfunction-associated steatotic liver disease

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Supplementary Notes

Tissue dissociation and single-cell sequencing. A total of 29 liver samples were subjected to single-cell sequencing, including 6 controls, 7 MASL samples, 12 MASH samples with Fibrosis stages 1 and 2, and 4 MASH samples with Fibrosis stages 3 and 4 (**Fig. 1a,b and Supplementary Table 1**). Samples were immediately preserved in ice-cold sCelLive™ Tissue Preservation Solution (Singleron Biotechnologies), triple-washed with Hanks' Balanced Salt Solution (HBSS), finely minced, and digested in 3 mL of sCelLive™ Tissue Dissociation Solution (Singleron Biotechnologies) using the Singleron PythoN™ Tissue Dissociation System at 37°C for 15 minutes. The digested suspension was filtered through a 70-μm sterile mesh, washed with PBS containing 0.04% BSA, and subjected to red blood cell lysis using GEXSCOPE® RCLB (Singleron Biotechnologies). Hepatic parenchymal cells were collected by centrifugation at 50×g for 2 minutes, while the supernatant was further centrifuged at 300×g for 5 minutes to isolate hepatic non-parenchymal cells.

The collected cells were resuspended in 0.04% BSA/PBS to achieve a single-cell suspension with a final concentration of 2×10^5 cells/mL. The library of hepatic parenchymal cells was prepared following the GEXSCOPE® Single Cell RNA Library Kit (Singleron Biotechnologies) manual[1] to construct a 3′-end mRNA-seq library. In brief, 100 μL of the single-cell suspension was loaded onto a microfluidic chip with 55 μm pores. Barcoding beads were then captured using the Singleron Matrix® Single Cell Processing System, followed by reverse transcription to generate cDNA labeled with both cell and molecular identifiers. Subsequently, the amplified cDNA was fragmented and ligated with sequencing adapters suitable for the Illumina sequencing platform. The library for hepatic non-parenchymal cells was constructed in accordance with the sCircle® Single Cell Full Length Immuno_TCR/BCR Library Kit (Singleron Biotechnologies) manual. Briefly, 100 μL of the single-cell suspension was loaded onto a microfluidic chip with 40 μm pores to generate barcoded cDNA necessary for acquiring both gene expression data and the full-length VDJ sequences of TCR/BCR. After purification, a portion of the resulting cDNA was fragmented and adapter-ligated to construct 3′-end mRNA-seq library compatible with the Illumina sequencing platform. The remaining cDNA was subject to enrichment for immune receptors (TCR/BCR), and the enriched products were used to build sequencing libraries through PCR amplification for the Illumina platform. Finally, the libraries were sequenced on the NovaSeq 6000 system, utilizing a 150 bp paired-end read configuration (Singleron Biotechnologies, Nanjing, China).

Spatial transcriptomics. Liver tissue samples for spatial transcriptomics were collected from 35 donors, including 7 controls, 10 MASL samples, 13 MASH samples with Fibrosis stages 1 and 2, and 5 MASH samples with Fibrosis stages 3 and 4. (**Fig. 1a,b and Supplementary Table 1**). Fresh liver tissue samples were collected and subsequently embedded in optimal cutting temperature (OCT) compound. Sections of 10 μm were cut in a cryostat and placed within the capture areas of both the Visium Spatial Tissue Optimization Slides and Visium Spatial Slides. Following hematoxylin and eosin (H&E) staining and microscope brightfield imaging, the sections on Visium

Spatial Tissue Optimization Slides were subjected to a gradient permeabilization to determine the optimal premeabilization time. The sections on the Visium Spatial Slides were processed, and Visium Spatial Gene Expression libraries were constructed according to the 10x Genomics protocol. The libraries were then sequenced on a NextSeq 500 sequencer, using an R1 primer for the 16 bp spatial barcode and 12 bp UMI, along with an R2 primer for capturing the 90 bp cDNA insert (Singleron Biotechnologies, Nanjing, China).

MALDI-MSI. For integrated multi-omics analysis, 27 consecutive tissue sections from spatial transcriptomics samples, including 6 controls, 9 MASL samples, 8 MASH samples with Fibrosis stages 1 and 2, and 4 MASH samples with Fibrosis stages 3 and 4, were affixed to indium-tin-oxide coated conductive slides for MALDI imaging mass spectrometry (MALDI-MSI) analysis (**Supplementary Table 1**). These slides were subsequently desiccated in a vacuum chamber for 30 minutes. A matrix solution containing 15 mg/mL of 2,5-dihydroxybenzoic acid (DHB) in a 10:90 (v/v) mixture of water and acetonitrile was evenly deposited over the slides in 30 cycles utilizing a Bruker Daltonics HTX TM sprayer. The sprayer parameters were set at a nozzle temperature of 60°C, delivering the solution at a flow rate of 0.12 mL/min, and under a nitrogen pressure of 5 psi. MALDI-MSI data acquisition was performed on a prototype Bruker timsTOF fleX MS system (Bruker Daltonics, Bremen, Germany) equipped with a 10kHz smartbeam 3D laser, operated in positive ion mode. The laser intensity was calibrated to 70% of its full capacity. The acquisition of mass spectra spanned a mass-to-charge (m/z) range from 50 to 1300 Da and was conducted with a lateral resolution of 20 μm, compiling 400 laser shots per spectrum. Post-acquisition, MALDI-MSI data were normalized through Root Mean Square, with the resulting image signal intensities presented as normalized values. MS/MS fragmentations were executed on the timsTOF fleX MS system in the MS/MS mode for further detailed structural confirmation of the identified metabolites (Wuhan Metware Biotechnology, Wuhan, China). Peak annotation was conducted by cross-referencing the in-house developed MetWare Database (http://www.metware.cn/) and the comprehensive Human Metabolome Database (HMDB, https://www.hmdb.ca/).

Spatial proteomics. A total of 14 consecutive tissue sections from spatial transcriptomics samples, which included 4 controls, 3 MASL samples, 3 MASH samples with Fibrosis stages 1 and 2, and 4 MASH samples with Fibrosis stages 3 and 4, were mounted onto PEN membrane-coated slides for spatial proteomic profiling (**Supplementary Table 1**). The tissue sections underwent H&E staining and were imaged using the SLIDEVIEW VS200 digital slide scanner (Olympus) at a magnification of 20×. Regions of interest (ROIs), including the peri-central and peri-portal areas of the liver, were delineated by an experienced pathologist using OlyVIA software (Olympus, 3.3-24382). Laser-capture microdissection (LCM) was carried out on the PALM Laser-Capture Microdissection System (Zeiss). The system parameters were set to a 20× zoom, cut speed of 20, cut energy of 39, focus of 70, with a single cycle. After aligning the dried H&E-stained slides on the LCM microscope's slide

adapter, the ROIs were precisely excised. The retrieved tissue samples were deposited into Zeiss microtubes (415190-9201-000) for protein digestion. Microdissected samples were sonicated in lysis buffer using a contactless high-intensity ultrasonic processor (Scientz), followed by incubation at 95°C for 5 minutes. Subsequently, the samples were incubated at 37°C overnight to facilitate protein digestion. The resulting peptide solutions underwent reduction with 5 mM dithiothreitol for 30 minutes at 56°C and were subsequently alkylated with 11 mM iodoacetamide for 15 minutes at 25°C in darkness. Peptides were then purified using C18 ZipTips (Millipore) according to the protocol provided by the manufacturer.

Tryptic peptides were solubilized in solvent A (0.1% formic acid in water) and separated in solvent B (0.1% formic acid, 80% acetonitrile in water) using a Vanquish Neo UPLC system (ThermoFisher Scientific). The chromatographic gradient was set as follows: 0-1.6 min, 4%-22.5% B; 1.6-2.0 min, 22.5%-35% B; 2.0-2.1 min, 35.0%-35.1% B; 2.1-2.3 min, 35.1% B; 2.3-9.2 min, 35.1%-35.2% B; 9.2-9.6 min, 35.2%-55.0% B; 9.6-10.1 min, 55.0%-99.0% B; 10.1-12.0 min, 99% B, with a constant flow rate of 200 nL/min. After separation by the UPLC system, peptides were analyzed in Orbitrap Astral with a nano-electrospray ion source. The ion source voltage was set at 1.9 kV. Precursor ions were analyzed using the Orbitrap detector, while fragment ions were analyzed with the Astral detector. The scan range for the MS1 was set to 400–800 m/z, with a resolution of 240,000. The MS2 scan range was fixed starting at 150 m/z, with a resolution of 80,000. Data were collected in data-independent acquisition (DIA) mode, where after the MS1 scan, peptides from multiple consecutive m/z windows were fragmented in the HCD collision cell with 25% collision energy, followed by MS2 analysis. To enhance mass spectrometer efficiency, automatic gain control (AGC) was set to 800%, and the maximum injection time was set to 15 ms.

The resulting MS/MS data were processed with DIA-NN search engine (v.1.8), using software default parameters. The database applied was Homo_sapiens_9606_SP_20231220.fasta, which includes 20,429 sequences, with trypsin/P set as the cleavage enzyme and allowing for a maximum of one missed cleavage. The fixed modifications were defined as N-term M precision and C carbamidomethylation. To construct the theoretical spectral library, deep learning algorithms were applied, and a reverse library was incorporated to assess the FDR for random matching. The FDR threshold for precursor identification was set at 1% (Jingjie PTM BioLab, Hangzhou, China).

Reference

1. Dura, B. *et al.* scFTD-seq: freeze-thaw lysis based, portable approach toward highly distributed single-cell 3' mRNA profiling. *Nucleic Acids Res* **47**, e16 (2019).