Spatial transcriptomics reveals a low extent of transcriptionally active hepatitis B virus integration in patients with HBsAg loss

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Supplementary methods

Liver samples preparation

The spot diameter on the Visium Spatial Gene Expression Slide in this study was 55 μm, and each section contains up to 5000 gene expression spots in the capture area (6.5 mm by 6.5 mm). Each spot with primers that include Illumina TruSeq Read 1 (partial read 1 sequencing primer), 16-nucleotide (nt) spatial barcode (all primers in a specific spot share the same spatial barcode), 12-nt UMI; 30-nt poly(dT) sequence (captures polyadenylated mRNA for cDNA synthesis). The frozen samples were sectioned at 10μm thickness. The integrity of RNA was tested and RNA integrity number (RIN) ≥7 is qualified.

Optimization of permeabilization time

Tissue sections were placed on seven capture areas on a Visium Tissue Optimization slide. The sections were fixed, stained, and then permeabilized for different times ranging from 6 to 24 mins. The mRNA released during permeabilization binds to oligonucleotides in the capture areas. Fluorescent cDNA was synthesized on the slide and imaged. The permeabilization time that results in the maximum fluorescence signal with the lowest signal diffusion was optimal. The permeabilization time was 12 mins in this study.

Tissue fixation, staining, and imaging

Tissue sections on the Visium Slide were fixed using methanol, stained with hematoxylin-eosin, and then the stained tissue sections were imaged.

Tissue permeabilization and cDNA synthesis

A permeabilization enzyme was used for permeabilizing the tissue sections on the slide for incubating for the predetermined permeabilization time. The polyadenylated mRNA released from the overlying cells was captured by the primers on the spots. Incubation

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with RT Master Mix with the reverse transcription reagents produces spatially barcoded full-length cDNA from polyadenylated mRNA on the slide. Second Strand Mix was added to the tissue sections on the slide to initiate second strand synthesis. After denaturation, cDNA from each capture area was transferred to a corresponding tube for amplification and library construction.

Visium spatial gene expression library construction

A quantitative polymerase chain reaction (qPCR) using cDNA Primers was performed to determine the optimal number of PCR cycles needed. After determination, cDNA Amplification Mix was added to the remaining cDNA sample from denaturation for cDNA amplification, the product was then purified. Fragmentation was performed to purify the cDNA sample. P5, P7, i7, and i5 sample indexes and TruSeq Read 2 (read 2 primer sequence) were added via End Repair, A-tailing, Adaptor Ligation, and PCR. The final libraries contain the P5 and P7 primers used in Illumina amplification.

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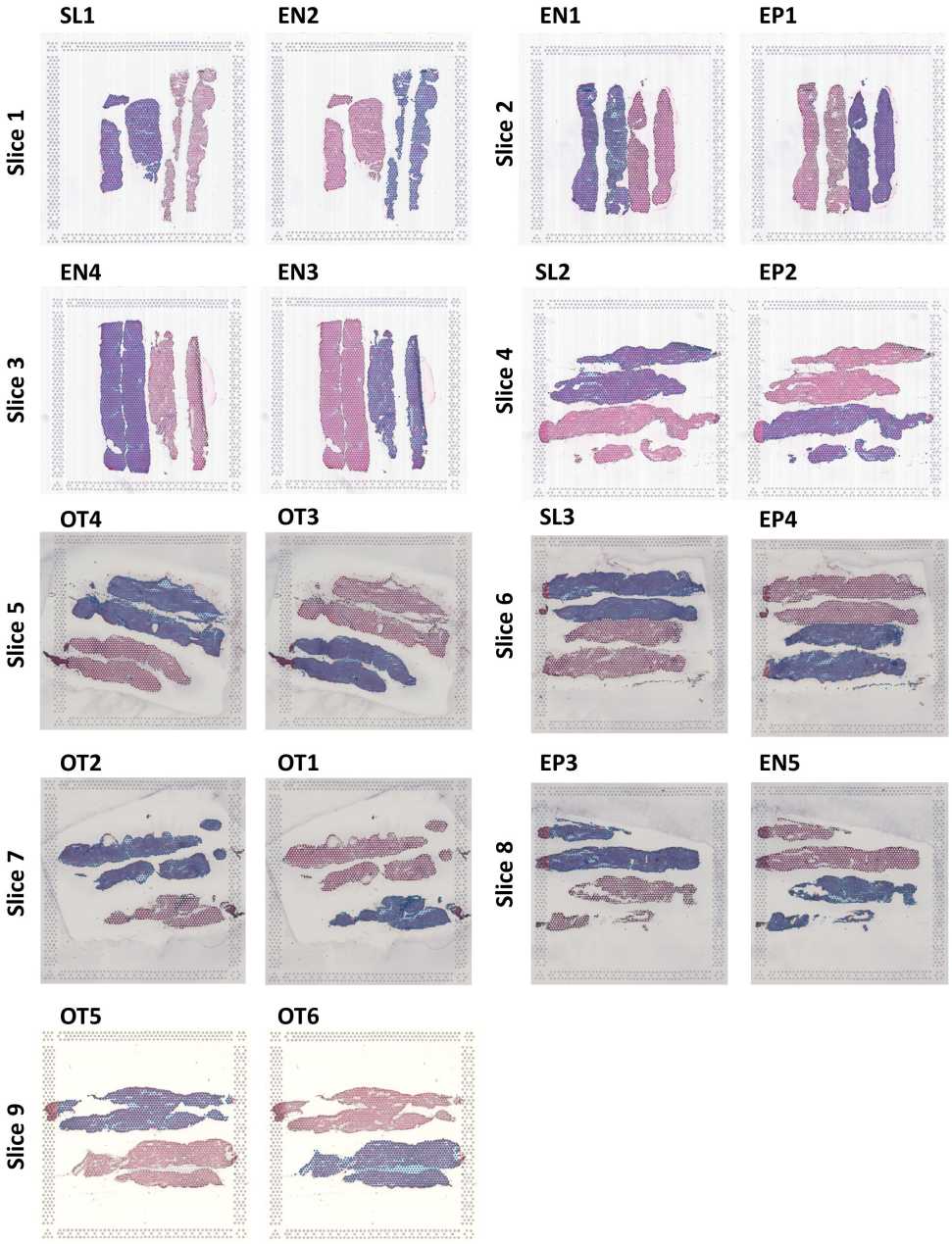
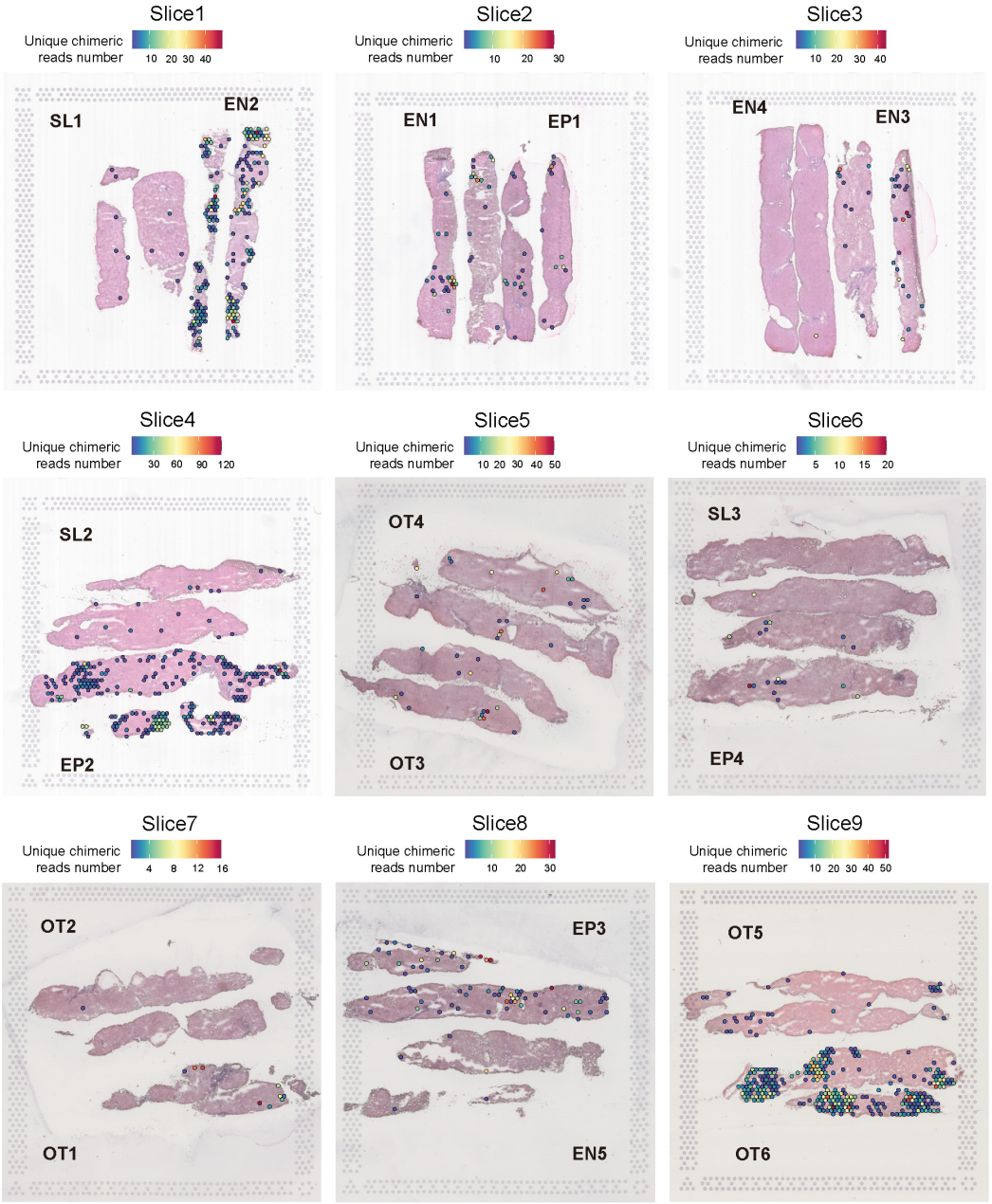
Fig.S1. Diagram of Visium Spatial Expression Slide for 18 patients



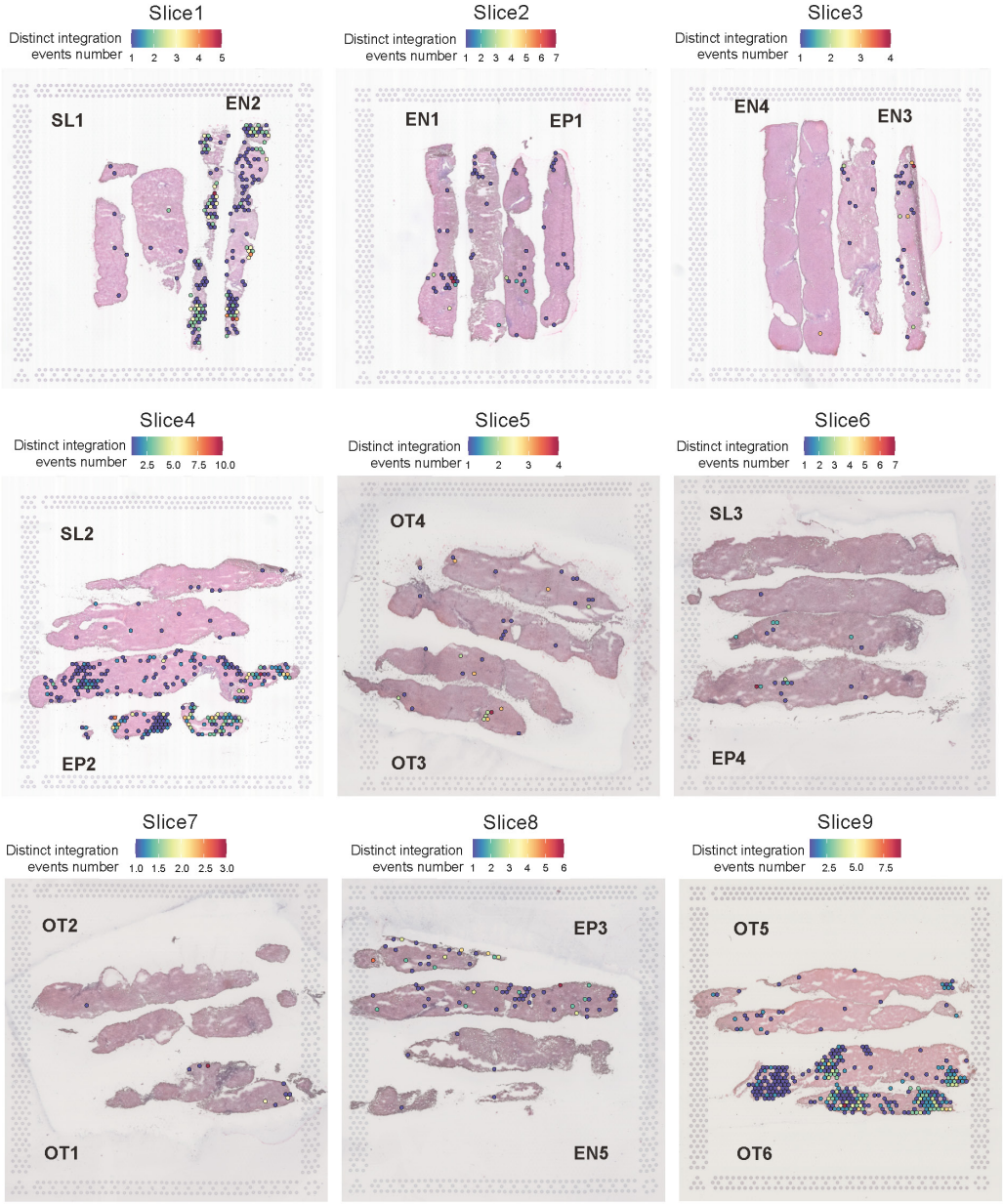
Each section contains liver biopsy tissues from two patients. Spots highlighted in blue represent the corresponding areas of liver tissue from the patient labeled in the figure. EP, HBeAg-positive; EN, HBeAg-negative; OT, on-treatment; SL, HBsAg loss.

Fig.S2 Spatial distribution and the density of HBV-host unique chimeric reads



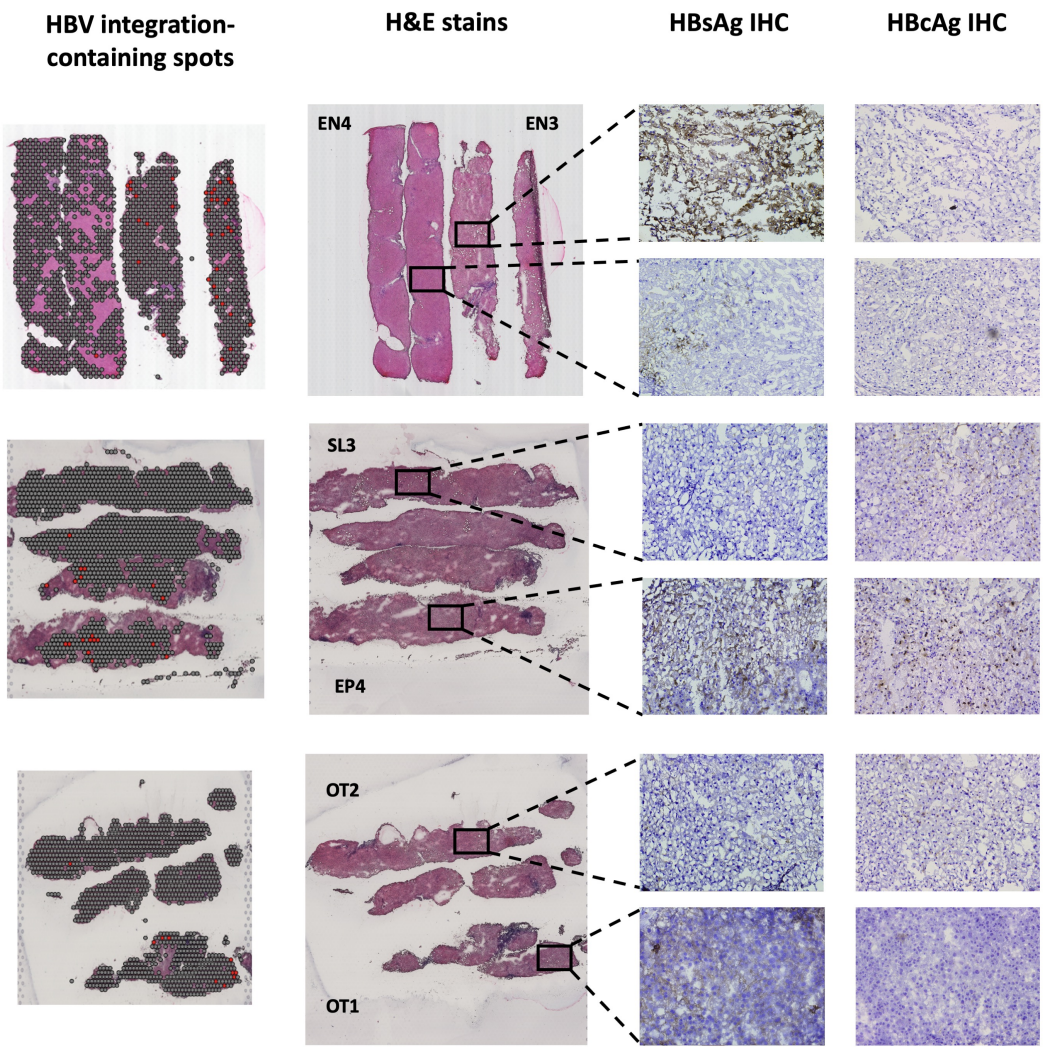
The density of unique chimeric reads in each spot was detected and displayed. EP, HBeAg-positive; EN, HBeAg-negative; OT, on-treatment; SL, HBsAg loss.

Fig.S3 Spatial distribution and the density of distinct HBV integration events



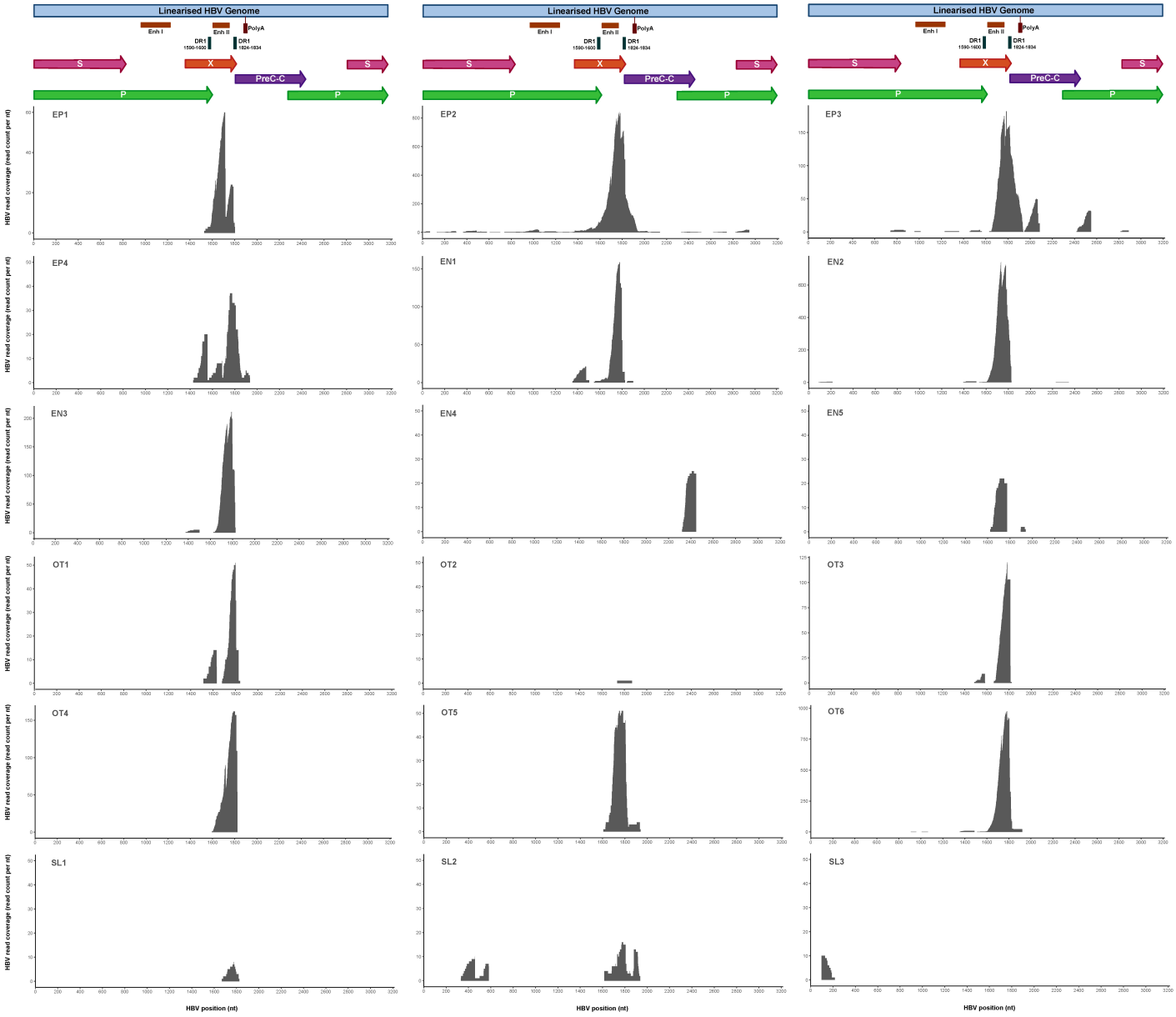
The density of distinct viral integration events in each spot was detected and displayed. EP, HBeAg-positive; EN, HBeAg-negative; OT, on-treatment; SL, HBsAg loss.

Fig.S4 Intrahepatic HBsAg and HBcAg immunostaining



HBsAg and HBcAg IHC were performed on adjacent slices from the same OCT-embedded samples of patients with adequate liver sections. The IHC results are displayed with matched H&E staining and spatial plots showing the distribution of transcriptionally active HBV integration. Spots containing viral integration are highlighted in red. Brown, HBsAg or HBcAg; blue, nuclei. IHC, immunohistochemistry. EP, HBeAg-positive; EN, HBeAg-negative; OT, on-treatment; SL, HBsAg loss.

Fig.S5 The transcriptionally active HBV integrations sites on viral genome.



Histogram shows the frequency of transcriptionally active viral integration at each nucleotide position in the HBV genome for each patient. The locations of EnhI, EnhII, DR1, DR2, and the genes encoding HBV polymerase (green), core (violet), S (pink), and X (red) proteins are shown. Enh: enhancer; DR: direct repeat.

Table S1. Primers and probes used for total HBV DNA and cccDNA quantification

Amplicon size (bp)	Name	Sequence
149	total HBV Fwd	TGTGCCTTCTCATCTGCCG
	total HBV probe	FAM-CGTGTGCACTTCGCTTCACCTCTGC-BHQ1
	total HBV Rev	GCCTCAAGGTCGGTCGTTGAC
308	cccDNA Fwd	CCGTGTGCACTTCGCTTCA
	cccDNA probe	FAM-CATGGAGACCACCGTGAACGCCC-BHQ1
	cccDNA Rev	GCACAGCTTGGAGGCTTGA
86	ACTB Fwd	GGAAATCGTGCGTGACATTAAG
	ACTB probe	FAM-CTACGTCGCCCTGGACTTCG-BHQ1
	ACTB Rev	AGGAGCTGGAAGCAGCC

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Table S2. Patient characteristics

Patient-ID	Sex	Age(y)	HBV DNA (IU/mL)	HBsAg (IU/mL)	HBeAg (S/CO)	ALT (IU/mL)	HBV Genotype	Inflammation Grade	Fibrosis Stage	Antiviral treatment	Duration of treatment(y)	Known Duration of infection(y)
Untreated												
Indeterminate grey area												
EP1	Male	42	1.47E+03	1.88	37.497	14	B	1	1	Peg-IFN 1year (>1 year before liver biopsy)		>3
HBeAg-positive chronic hepatitis B												
EP2	Female	38	>1e8	40815	1528.762	56	C	2	1	/	/	19
EP3	Male	38	>1e8	88381	1510.695	52	C	2	1	/	/	21
EP4	Female	25	>1e8	91085	1292.453	45	B	2	1	/	/	>7
HBeAg-negative chronic HBV infection												
EN1	Male	60	1.16E+04	>250	/	NA	B	1	0 ~ 1	/	/	5
HBeAg-negative chronic hepatitis B												
EN2	Female	29	6.62E+03	1994.03	/	16	C	2	2	/	/	>6
EN3	Female	56	3.53E+03	10986	/	35	C	2	3 ~ 4	/	/	31
EN4	Male	45	1.54E+04	262.66	/	19	B	2	0	NUC 1year (6 years before liver biopsy) & Peg-IFN 1year (>1 year before liver biopsy)		21

EN5	Male	34	5.39E+04	186.38	/	64	B	2	1	Peg-IFN 1year (>1 year before liver biopsy)		34
On treatment												
OT1	Male	47	<50	500.83	1.388	39	C	1	0	Peg-IFN &NUC	9.7	19
OT2	Male	38	<50	387.82	/	29	C	0	0	NUC	8.1	>10
OT3	Male	40	<50	4933.31	5.635	16	C	0	3	NUC	4.4	6
OT4	Female	44	<50	652.66	/	13	B	1	0	NUC	8.0	9
OT5	Female	37	<50 (7.91E+02)*	2622.61 (5837.25)	/ (/)	65 (24)*	C	1 (2)*	1 (1)*	Peg-IFN	1.6	20
OT6	Female	54	<50 (>1e8)*	690.65 (8628.81)*	2.166 (1987.395)*	23 (115)*	C	0 (2)*	0 (1)*	NUC	4.2	25
HBsAg loss												
SL1	Female	45	<50	/	/	20	B	0	0	NUC	15.8	>20
SL2	Male	39	<50	/	/	48	B	1	1	NUC	9.0	23
SL3	Male	34	<50	/	/	24	B	2	0	Peg-IFN &NUC	15.4	>16

ALT, alanine aminotransferase; NUC, nucleos(t)ide analogues; Peg-IFN, Pegylated infection. EP, HBeAg-positive; EN, HBeAg-negative; OT, on-treatment; SL, HBsAg loss.

* Values in parentheses represent the baseline levels of each indicator before antiviral treatment.

Table S3. Sample spatial transcriptomics sequencing data summary

Slice	Number of Spots Under Tissue	Mean Reads per Spot	Median Genes per Spot	Number of Reads	Total Genes Detected	Median UMI Counts per Spot
Slice1	1158	162375	2335	188030823	19538	9022
Slice1.1	1471	135898	2158	199906112	20552	7556
Slice1.2	1928	121060	1086	233403706	18987	2558
Slice1.3	1742	141606	2278	246677994	21125	9016
Slice1.4	1872	115519	1019	216251180	18851	2808
Slice1.5	2091	86071	839	179973749	18218	2104
Slice1.6	1213	248348	1065	301246328	17934	2943
Slice1.7	1274	151990	1346	193634774	18364	3882
Slice1.8	1574	127584	2877	200817668	21073	12032

UMI, Unique Molecular Identifier.

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