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Disparity landscapes of viral-induced structural variations in HCC: Mechanistic characterization and functional implications

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Abstract

Background and Aims: HCC is the most common type of primary liver cancer and is a common malignancy worldwide. About half of all new liver cancers worldwide each year occur in China, including Hong Kong, due to a high prevalence of HBV infection. HBV DNA integrates into the human genome, disrupting the endogenous tumor suppressors/regulatory genes or enhancing the activity of proto-oncogenes. It would be useful to examine the different NGS-based databases to provide a more unbiased and comprehensive survey of HBV integration.

Approach and Results: We aimed to take advantage of publicly available data sets of different regional cohorts to determine the disparity landscapes of integration events among sample cohorts, tissue types, chromosomal positions, individual host, and viral genes, as well as genic locations. By comparing HCC tumors with non tumorous livers, the landscape of HBV integration was delineated in gene-independent and gene-dependent manners. Moreover, we performed mechanistic investigations on how HBV-TERT integration led to TERT activation and derived a score to predict patients' prognostication according to their clonal disparity landscape of HBV integration.

Conclusions: Our study uncovered the different levels of clonal enrichment of HBV integration and identified mechanistic insights and prognostic biomarkers. This strengthens our understanding of HBV-associated hepatocarcinogenesis.

Keywords: clonal disparity, clonal enrichment, genome instability, genomic landscape, prognosis

Abbreviations: ELF4, ETS transcription factor 4; N peak, NTL-specific peak; NTL, non-tumorous liver; T&C, telomeres and centromeres; TSS, transcription start site.

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INTRODUCTION

HCC is the most common type of primary liver cancer and is a common malignancy worldwide.[1] Indeed, 55% of all new liver cancers worldwide each year occur in China, including Hong Kong, due to a high prevalence of HBV infection. It is the second and third most common fatal cancer in Mainland China and Hong Kong, respectively.[2] Its incidence is also rapidly increasing in the West. Most (>80%) HCCs are diagnosed at an advanced stage and therefore not operable. Even after surgical resection, the long-term prognosis of HCC remains unsatisfactory due to high recurrence rates. It is also an extremely difficult-to-treat cancer.[3–6] Among the identified etiological risk factors for HCC, including chronic viral infections (HBV and HCV), chronic alcohol consumption, and metabolic dysfunction–associated steatohepatitis, chronic HBV infection accounts for around 50% of HCC cases worldwide[3] and about 80%–90% of HCC cases in Hong Kong. HBV DNA integrates into the human genome, which in turn disrupts the endogenous tumor suppressors and other regulatory genes or enhances the activity of proto-oncogenes, resulting in enhanced cell survival, proliferation, and reduced apoptosis, leading to HCC development.[7] Recently, different next-generation sequencing–based approaches have been used to provide a more unbiased and comprehensive survey of HBV integration.[8] Existing studies have used clinical HCC tissue samples to provide a comprehensive landscape of HBV integration and identified several recurrently affected genes, including *TERT*, *KMT2B*, *CCNE1*, and *CCNA2*.[9–13]

Noting distinctive enrichment patterns of HBV integration events in different aspects, we aimed to take advantage of our study to determine the disparity landscapes of integration events among sample cohorts, tissue types, chromosomal positions, individual host and viral genes, as well as genic locations. Moreover, we performed a mechanistic investigation on how *TERT* HBV integration led to *TERT* activation and derived a clonal disparity score that helps to predict patients' prognostication according to their clonal disparity landscape of HBV integration.

METHODS

Sample cohorts

The details of our in-house sample cohort were reported previously.[13] Moreover, we also downloaded the public data from 5 reported studies.[9–11,14,15] The details of sample cohorts included in our current study are summarized in Supplemental Table S1, http://links.lww.com/HEP/I676. We downloaded the sequencing data from the data repository. The aforementioned sample cohorts were used for determining HBV integration landscape.

Regarding the mechanistic investigation of HBV *TERT* integration, our in-house sample cohort and that of Sung and colleagues were used to study *TERT* gene expression modulated by HBV integration at the *TERT* promoter region. Both cohorts had gene expression data (Gene Expression Omnibus accession: GSE25097) available.

We also downloaded the data set from Sequence Read Archive (accession: PRJNA325415) and used it as training data for deriving the clonal disparity score.

Statistical analysis

Survival analysis was done by using R package survival. We used hypergeometric distribution to determine the significance of HBV integration enrichment at chromosomal intervals. A p value of <0.05 was recognized as significant.

Refer to the online Supplemental Methods, http://links.lww.com/HEP/I677, for the detailed methodology regarding HBV integration detection, dual luciferase reporter assay, clonal disparity score, genomic instability score, mutation detection, cell models, phosphor-H2AX staining, stable gene knockdown, quantitative real-time PCR, sphere formation assay, and cell proliferation assay.

RESULTS

Global geographical disparity of HBV integrations suggesting their difference in the degree of clonal viral insertion

HBV integration is regarded as a major risk factor for hepatocarcinogenesis.[16] Nevertheless, regarding HBV-associated HCCs, HBV integration events were detected in both tumors and the corresponding nontumorous liver (NTL). To explore the diversity in HBV integration landscape between HCC tumor and NTL samples among different regional cohorts, we examined various publicly available data sets with both HCC tumor and corresponding NTL tissues sequenced (Supplemental Table S1, http://links.lww.com/HEP/I676).[9–11,14,15] The data included whole-genome sequencing, whole-transcriptome sequencing, and target-capture sequencing and varied in sample size and ethnicity. In general, the samples demonstrated the expected male predominance, and patients were of similar ages. Although samples of different cohorts may have differences in library preparation and sequencing platform (Supplemental Table S1, http://links.lww.com/HEP/I676), it was still valid to compare HBV integration events between HCC tumors and the corresponding

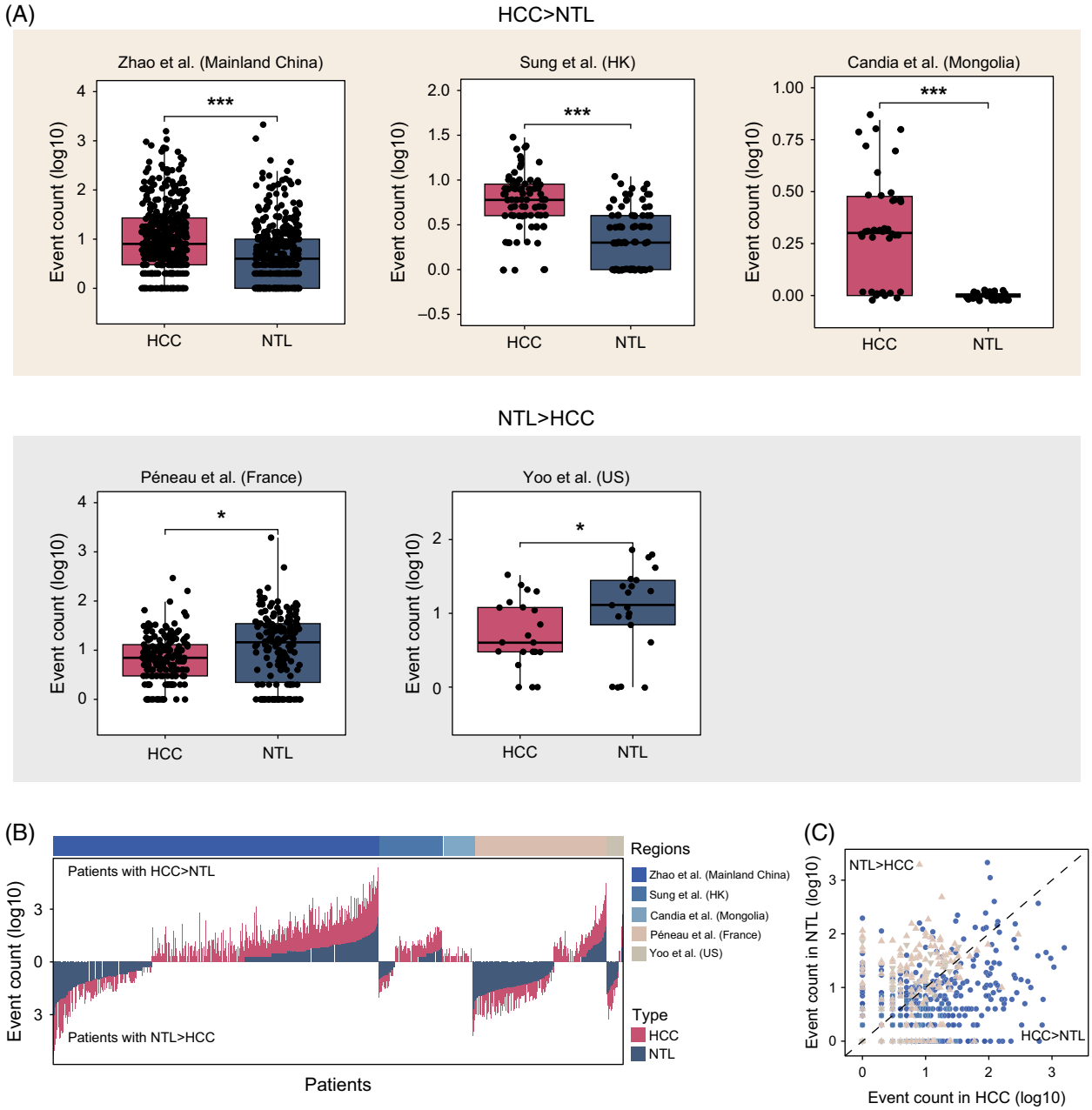


FIGURE 1 The geographical disparity of HBV integration in regional cohorts. (A) Observation of preferential enrichment of HBV integration events in HCC or NTL samples in different regional cohorts. (B) Relative event count distribution in HCC and NTL was revealed at the case level. Patients with preferentially higher HCC event count, that is, HCC > NTL were displayed above the *x* axis, whereas patients with preferentially higher NTL event count, that is, HCC < NTL were shown below the *x* axis. (C) Event count comparison between HCC and NTL for each patient with HCC. Patients with higher event counts in NTL were plotted above the dashed line, whereas patients with lower event counts in NTL were below the dashed line. *, **, and *** represent *p* values < 0.05, 0.01, and 0.001, respectively. Abbreviations: HK, Hong Kong; NTL, non-tumorous liver.

NTLs within the same individual cohorts. We detected significantly different numbers of HBV integration events in HCC tumors and NTL by Virus-Clip[17,18] with modifications. Due to the expected clonal enrichment of integration events in HCC tumors, initially, we expected relatively lower event counts in HCC tumors and higher event counts and lower supporting read counts in NTL. To our surprise, the HCC tumors in Mainland China, Hong Kong (HK), and Mongolia cohorts had higher numbers of events, whereas the reverse was true in the French and US cohorts (Figure 1A). This observation was consistently supported by per-case HCC-NTL count proportions (Figures 1B, C). This likely suggests underlying differences between patients of different regional cohorts leading to the apparently divergent modes of enrichment of events.

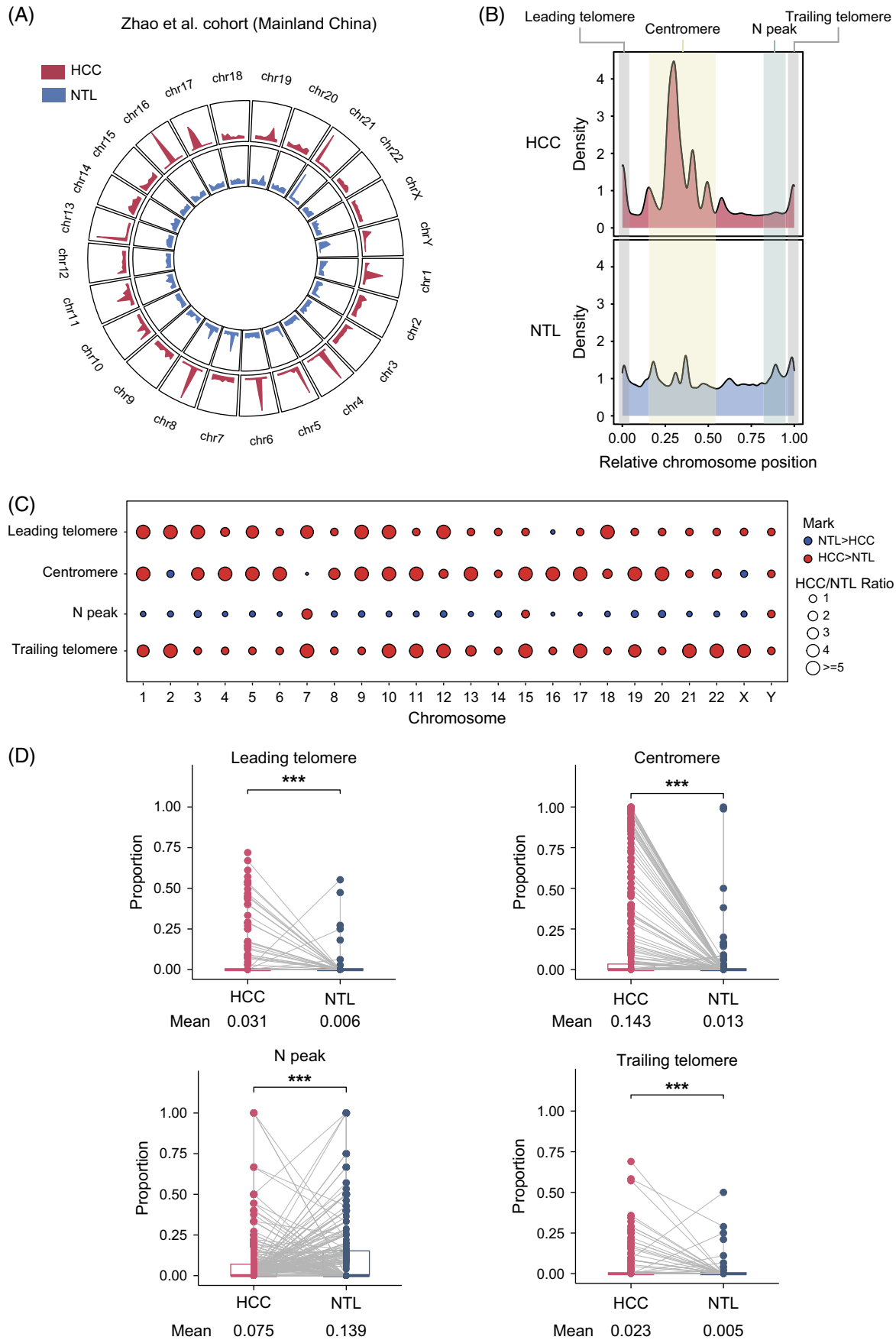

(A)
Zhao et al. cohort (Mainland China)
HCC
NTL
chr1
chr2
chr3
chr4
chr5
chr6
chr7
chr8
chr9
chr10
chr11
chr12
chr13
chr14
chr15
chr16
chr17
chr18
chr19
chr20
chr21
chr22
chrX
chrY
(B)
Leading telomere
Centromere
N peak
Trailing telomere
HCC
NTL
Density
Relative chromosome position
(C)
Leading telomere
Centromere
N peak
Trailing telomere
Chromosome
Mark
NTL>HCC
HCC>NTL
HCC/NTL Ratio
>=5
(D)
Leading telomere
Centromere
N peak
Trailing telomere
Proportion
HCC
NTL
Mean 0.031 0.006
Mean 0.143 0.013
Mean 0.075 0.139
Mean 0.023 0.005

FIGURE 2 HBV integration enrichment at telomeres and centromeres in a gene-independent manner. (A) The distribution of HBV integration breakpoint at the human genome across chromosomes in HCC (outer circle) and NTL (inner circle). (B) Distribution of HBV integration breakpoints by collating the relative chromosomal position ranging from 0 (leading telomere) to 1 (trailing telomere). Relative chromosome position was normalized by the corresponding length of chromosome. Peaks of HBV integration enrichment were observed at the leading telomere, centromere, N peak, and trailing telomere. (C) Significant enrichment of HBV integrations at telomeres (both leading telomere and trailing telomere) and centromeres in HCC and at N peak in NTL. The size and color of the dots indicate the extent and preference of enrichment of HBV integration, respectively. The red color indicates the enrichment of the event in HCC, while the blue color is for NTL. (D) Pairwise proportions of events at specific regions were compared between HCC and NTL. *, **, and *** represent p values < 0.05, 0.01, and 0.001, respectively. Abbreviations: N peak, NTL-specific peak; NTL, non-tumorous liver.

Identification of positional enrichment of HBV integration at telomeres and centromeres

Next, by taking advantage of the large sample size of the Mainland Chinese cohort,[11] we further determined the landscape of HBV integration across the chromosomes in the human genome and compared the differences between HCC and NTL samples (Figure 2A). We noted different enrichment landscapes in the HCC and NTL samples, with some remarkable aggregations of HBV integration at certain chromosomes (HCC: chr 1, 4, 5, 6, 8, 13, 16, 17, and 21; NTL: chr 5, 7, 8, and 21). In addition, we observed frequent peaks of HBV integration at the middle or ends of the chromosomes, suggesting putative enrichment of events at telomere and centromere (T&C) regions. As T&C may contain repetitive elements that could result in mapping to multiple genomic locations, HBV integration events with evidence of multiple alignments were removed. Afterward, we confirmed that $>92\%$ of the events were free of repetitive elements by RepeatMasker, suggesting their very modest undesirable influence on the overall landscape (Supplemental Figure S1A, http://links.lww.com/HEP/I678). To confirm our observation, we normalized the events according to their relative positions in the chromosomes, with each chromosome being normalized into a range of 0–1 (Figure 2B). By overlaying the events into a single plot, the cumulative findings of HCC samples reinforced into peaks at both the telomere regions (15 kb interval at both ends of a chromosome) and centromere (according to UCSC definition) regions, though more prominent at the latter. On the other hand, in the NTL samples, the peaks of enrichment were at similar positions as the HCC samples, but the degree of enrichment at centromeres was obviously reduced. Intriguingly, we found a small NTL-specific peak (N peak; it was only detected in NTL samples) near the trailing telomere. Upon calculating the expected proportion of the peaks (leading telomere, centromere, N peak, and trailing telomere) according to the physical length of the intervals on the chromosomes and comparing to the corresponding observed proportion of the peaks detected, we were able to reveal prominent and significant enrichment of events at centromere and, to a lesser extent, the leading and trailing telomeres in HCC samples (Figure 2C, Supplemental Figure S1B, http://links.lww.com/HEP/I678, and Supplemental Table S2, http://links.lww.com/HEP/I679). In addition, N peak seemed to be more prominent in NTL samples (Figure 2C, Supplemental Figure S1B, http://links.lww.com/HEP/I678, and Supplemental Table S2, http://links.lww.com/HEP/I679).

The integrity of the telomere and centromere is crucial for genomic stability.[19–22] Our aforementioned findings demonstrated that both of them were significantly enriched with HBV integration; hence, we collectively considered telomere and centromere as T&C regions for subsequent analysis. Interestingly, although we found integration peaks in both HCC and NTL samples, they were essentially not in perfect concordance on the same chromosomes. During the malignant transformation from NTL to HCC, the enrichment preference dynamically shifted from N peak to T&C regions (Figures 2D and 3A, B). The aforementioned observation was confirmed in the French cohort (Figure 3C, Supplemental Figures S2, S3, http://links.lww.com/HEP/I678, and Supplemental Table S3, http://links.lww.com/HEP/I679). Phospho-H2AX staining detects DNA double-strand breaks and serves as a marker for DNA damage and repair processes. Indeed, the experimental results were in line with our expectations that HCC tissues carrying T&C events demonstrated prominent phospho-H2AX staining, while it was barely detected in NTL counterparts carrying N peak events (Figure 3D). This finding supports our hypothesis that HBV integrations at T&C regions contribute to promoting genome instability for HBV-associated hepatocarcinogenesis. Taken together, our findings suggest that HBV integrations may introduce genome instability in general, more than pinpointing particular genes located at specific positions of a certain chromosome in a gene-independent manner.

Coexistence of HBV integration revealed a novel landscape of HBV integration and further substantiated the importance of T&C regions

Reported studies generally investigated the landscape of HBV integrations affecting individual genes but neglected the possibility of their co-involvement.[9–11]

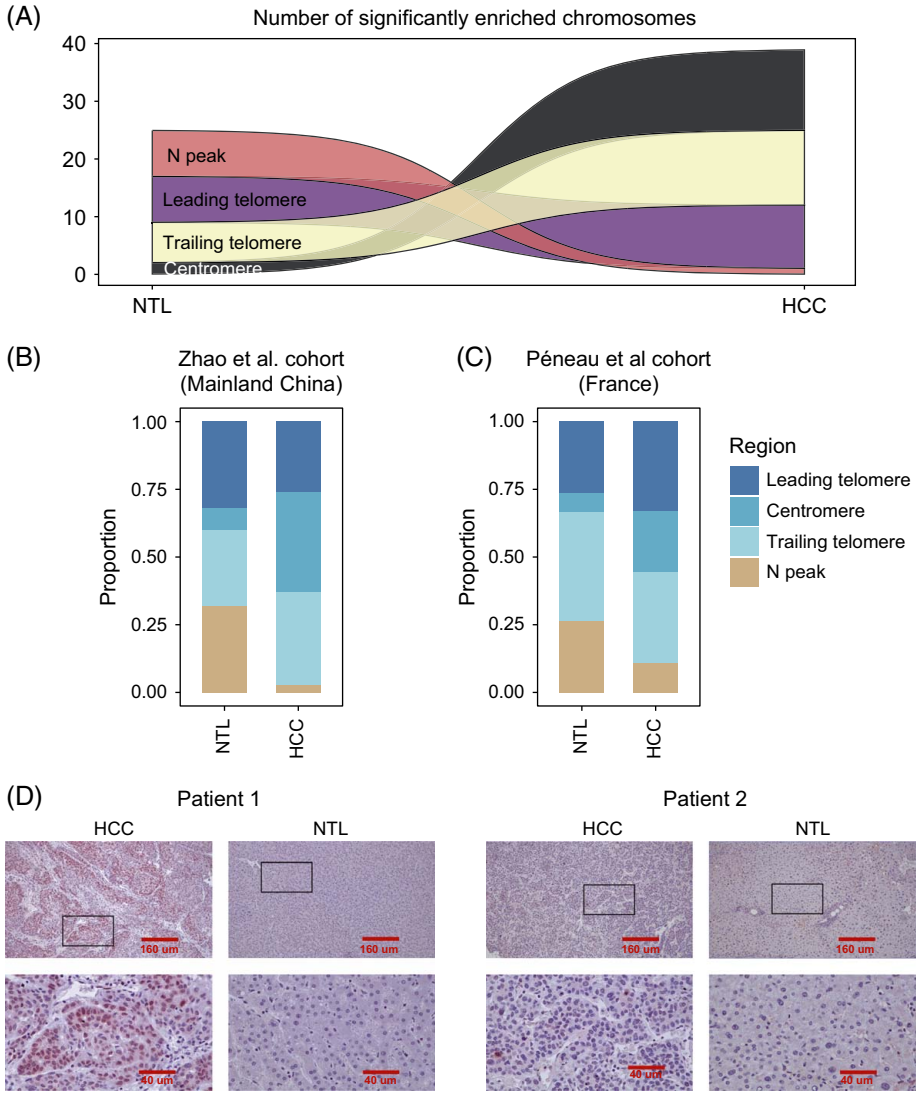


FIGURE 3 Enrichment preference dynamically shifted from N peak to T&C regions during the malignant transformation from NTL to HCC, introducing genome instability. (A) Dynamical shift of enrichment preference from NTL to HCC. (B, C) Distribution of significantly enriched regions in the cohorts of Zhao et al and Péneau et al. (D) Phospho-H2AX staining in HCC and NTL (upper panel: ×10 magnification; lower panel: ×40 magnification of the area denoted by a rectangle in the upper panel). Abbreviations: N peak, NTL-specific peak; NTL, non-tumorous liver.

To investigate their coinvolvement, we calculated the pairwise coexistence frequency of genes recurrently affected by HBV integration (occurred in ≥3 cases). We found a high concordance of HBV integration events at certain gene pairs in the HCC samples, and they tended to be enriched at the T&C regions (Figure 4A). However, the degree of gene concordance was very modest in the NTL samples (Supplemental Figure S4A, http://links.lww.com/HEP/I678). Specifically, we were curious about the coexistence status of genes that are frequently and recurrently identified with HBV integration (eg, *TERT*, *KMT2B*, *CCNE1*, and *FN1*). These genes had only low levels of coexistence with other genes (Supplemental Figure S5A, http://links.lww.com/HEP/I678). This may suggest their underlying functional sufficiency in eliciting their roles in hepatocarcinogenesis. Furthermore, upon examining the top 100 gene pairs in the NTL samples, we found no preference for coexistence at the T&C regions, whereas we observed obvious enrichment at T&C regions in the HCC samples (Supplemental Figure S4B, http://links.lww.com/HEP/I678), further implicating the functional importance of those chromosomal regions.

Functional and clinical characterization of coexistence of HBV integration

To further confirm the functional and clinical relevance of the coexistence of HBV-integrated gene pairs, we used an in-house HCC patient cohort (n = 43) to detect such a phenomenon. We detected recurrent coexistence of multiple HBV-integrated gene pairs that were consistently identified in both the Mainland

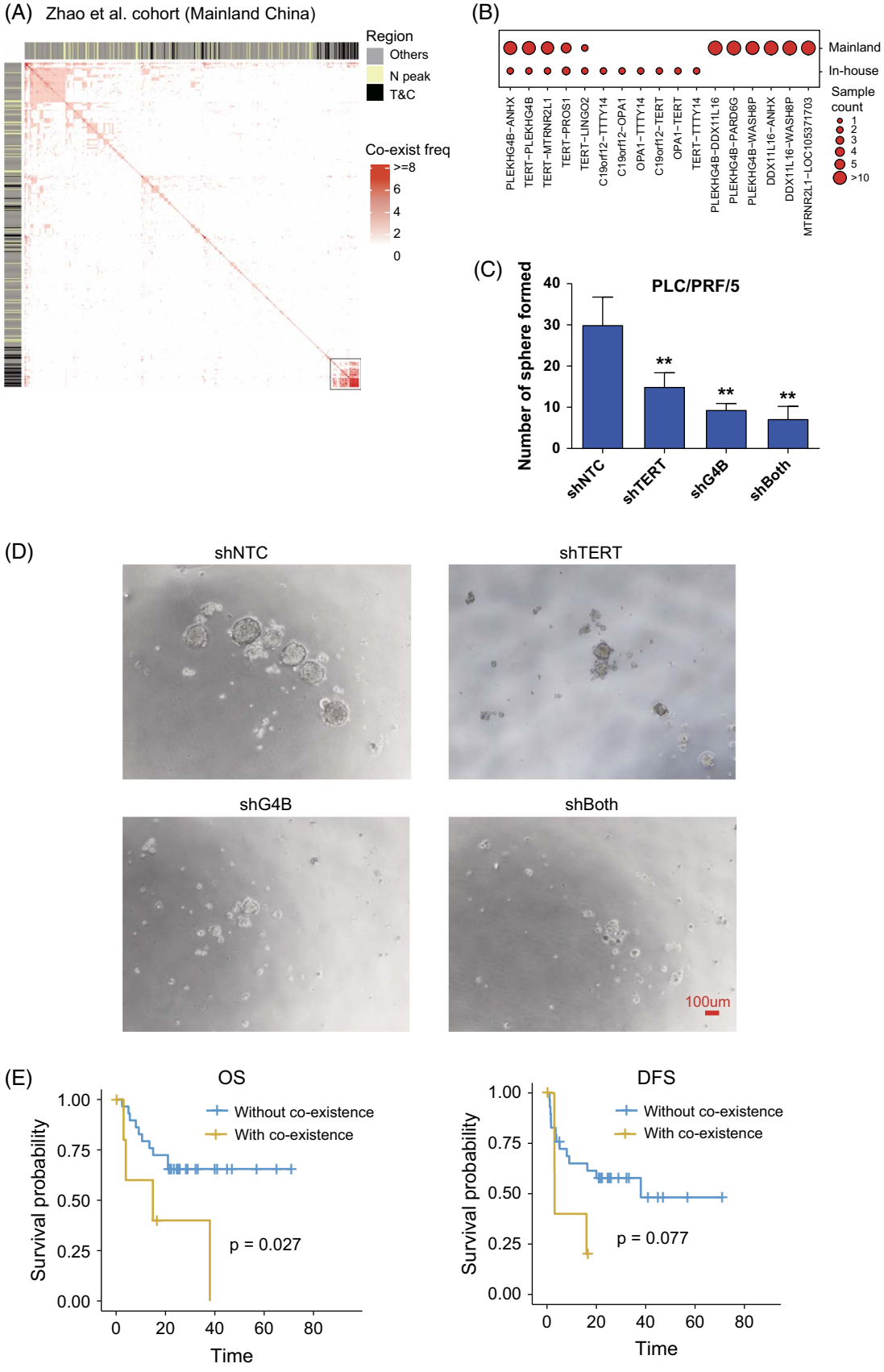

(A)
Zhao et al. cohort (Mainland China)
Region
Others
N peak
T&C
Co-exist freq
>=8
6
4
2
0
(B)
Mainland
In-house
Sample count
1
2
3
4
5
>10
PLEKHG4B–ANHX
TERT–PLEKHG4B
TERT–MTRNR2L1
TERT–PROS1
TERT–LINGO2
C19orf12–TTTY14
C19orf12–OPA1
OPA1–TTTY14
C19orf12–TERT
OPA1–TERT
TERT–TTTY14
PLEKHG4B–DDX11L16
PLEKHG4B–PARD6G
PLEKHG4B–WASH8P
DDX11L16–ANHX
DDX11L16–WASH8P
MTRNR2L1–LOC105371703
(C)
PLC/PRF/5
Number of sphere formed
40
30
20
10
0
**
**
**
shNTC
shTERT
shG4B
shBoth
(D)
shNTC
shTERT
shG4B
shBoth
100um
(E)
OS
Survival probability
1.00
0.75
0.50
0.25
0.00
Without co-existence
With co-existence
p = 0.027
0
20
40
60
80
Time
DFS
Survival probability
1.00
0.75
0.50
0.25
0.00
Without co-existence
With co-existence
p = 0.077
0
20
40
60
80
Time

FIGURE 4 Novel perspective of HBV integration landscape revealed by the coexistence of HBV integrations in patients. (A) The frequency of gene pairs with the coexistence of HBV integration in HCC. Coexistence indicates the genes in the gene pairs were simultaneously affected by HBV integration in the same patients. (B) Recurrently coexistent events between the in-house cohort and the cohort of Zhao et al (Mainland China). (C, D) Sphere formation assay on PLC/PRF/5 cells that stably knockdown on both *TERT* and *PLEKHG4B* (shBoth), *TERT* only (shTERT), *PLEKHG4B* only (shG4B), and nontarget control (shNTC). (E) Overall survival and disease-free survival of patients with HCC stratified by the presence of coexistence of HBV integration. *, **, and *** represent p values < 0.05, 0.01, and 0.001, respectively. Abbreviation: T&C, telomeres and centromeres.

Chinese cohort and our current in-house cohort (Figure 4B). Notably, as *TERT* and *PLEKHG4B* were among the topmost genes recurrently affected by HBV integrations and both of them had substantial expression in the PLC/PRF/5 HCC cell line, we chose the gene pair of TERT-PLEKHG4B as our candidate for further experimental characterization.

Regarding functional characterization, we performed in vitro proliferation and sphere formation assays using PLC/PRF/5 HCC cells that stably knockdown on both *TERT* and *PLEKHG4B* (shBoth), *TERT* only (shTERT), *PLEKHG4B* only (shG4B), and nontarget control (shNTC) (Supplemental Figures S5B, C, http://links.lww.com/HEP/I678). Results showed that the proliferation rate was significantly affected by the shG4B knockdown but not for shTERT (Supplemental Figures S5D, E, http://links.lww.com/HEP/I678), whereas the sphere formation outcome (a sign of self-renewal ability that indicates cancer stemness) was significantly reduced upon individual gene knockdown (shTERT or shG4B) and reached minimum upon co-knockdown (shBoth) (Figures 4C, D). Moreover, we noted the effect of co-knockdown (shBoth) was not significantly different from the individual knockdown counterparts, suggesting the unlikeliness of prominent functional synergism of the coexistence of their HBV integration. This evidence suggests that the coexistence of HBV integration at *TERT* and *PLEKHG4B* may just individually elicit proliferation-related or stemness-related oncogenic effects in promoting tumor initiation in HBV-associated HCC. This may somehow be in line with our previous hypothesis that *TERT* HBV integration may demonstrate functional sufficiency in driving hepatocarcinogenesis that the majority of *TERT* HBV integration events did not coexist with other events, but there could still be a minority of them coexisting but without demonstrated functional synergism in their gene-dependent effect (Supplemental Figure S5A, http://links.lww.com/HEP/I678).

Regarding clinical relevance, we stratified our in-house HCC patient cohort according to the presence of coexistence of HBV-integrated gene pairs and examined their prognostication outcome. Cases having coexistence of HBV-integrated gene pairs had significantly poorer overall survival as compared to those without. Consistently, those cases having coexistence of HBV-integrated gene pairs also demonstrated a trend for poorer disease-free survival as well (Figure 4E). Given the demonstrated genome instability consequence elicited by T&C events (Figure 3D) and the enrichment of coexistence of HBV integration at T&C regions in the HCC samples (Supplemental Figure S4B, http://links.lww.com/HEP/I678), they collectively implicate the potential gene-independent influence on hepatocarcinogenesis through abrogation of genome stability by preferential coexistence of HBV integration at T&C regions.

Landscapes of the preferential human and viral breakpoints of integration events using a meta-cohort of multiple ethnicities

On the other hand, we examined the gene-dependent perspective of HBV integration events. To determine the most frequently and recurrently HBV-integrated human genes, we used the combined cohort (n = 848) of several recently reported studies[9–11,13–15] to delineate the landscape of events. We identified key ($\geq 5\%$) HBV-integrated human genes in HCC (*TERT*, *KMT2B*, *PLEKHG4B*, *LOC441666*, and *MTRNR2L1*) and NTL (*FN1*), respectively (Figure 5A, Supplemental Figure S6, http://links.lww.com/HEP/I678). In general, these key HBV-integrated genes detected in HCC tumors were also found in NTL, but the frequency was much lower. This possibly indicates that those events may likely originate from NTL but may not be easily detectable unless they have been clonally enriched. Intriguingly, *FN1* was the only frequently integrated gene that was detected in NTL. It suggests that it may carry some biological functions, rendering it to be preferentially selected in premalignant NTL stage but disappearing in the subsequent HCC stage. Moreover, we also identified low levels of HCC-enriched events in the cyclin family (*CCNE1* and *CCNA2*, each at 2%), whereas there were some NTL-enriched events in cadherin (*CDH2* and *CDH12*, each at 2%) and contactin (*CNTN5*, *CNTNAP2*, and *CNTNAP5*, each at 1%) families. In accordance with previous reports,[8] viral breakpoints were frequently located at around position 1800 of the HBV genome (the DR region of HBV) (Figure 5B).

Regarding the genic positions, integration events were enriched at the intergenic regions in general, whereas the *X* gene interval of HBV was most frequently affected (Figure 5C). Particularly, we focused on the *TERT* and *FN1* genes, which were the topmost integrated ones in HCC and NTL samples, respectively. While their HBV breakpoints were consistently found at

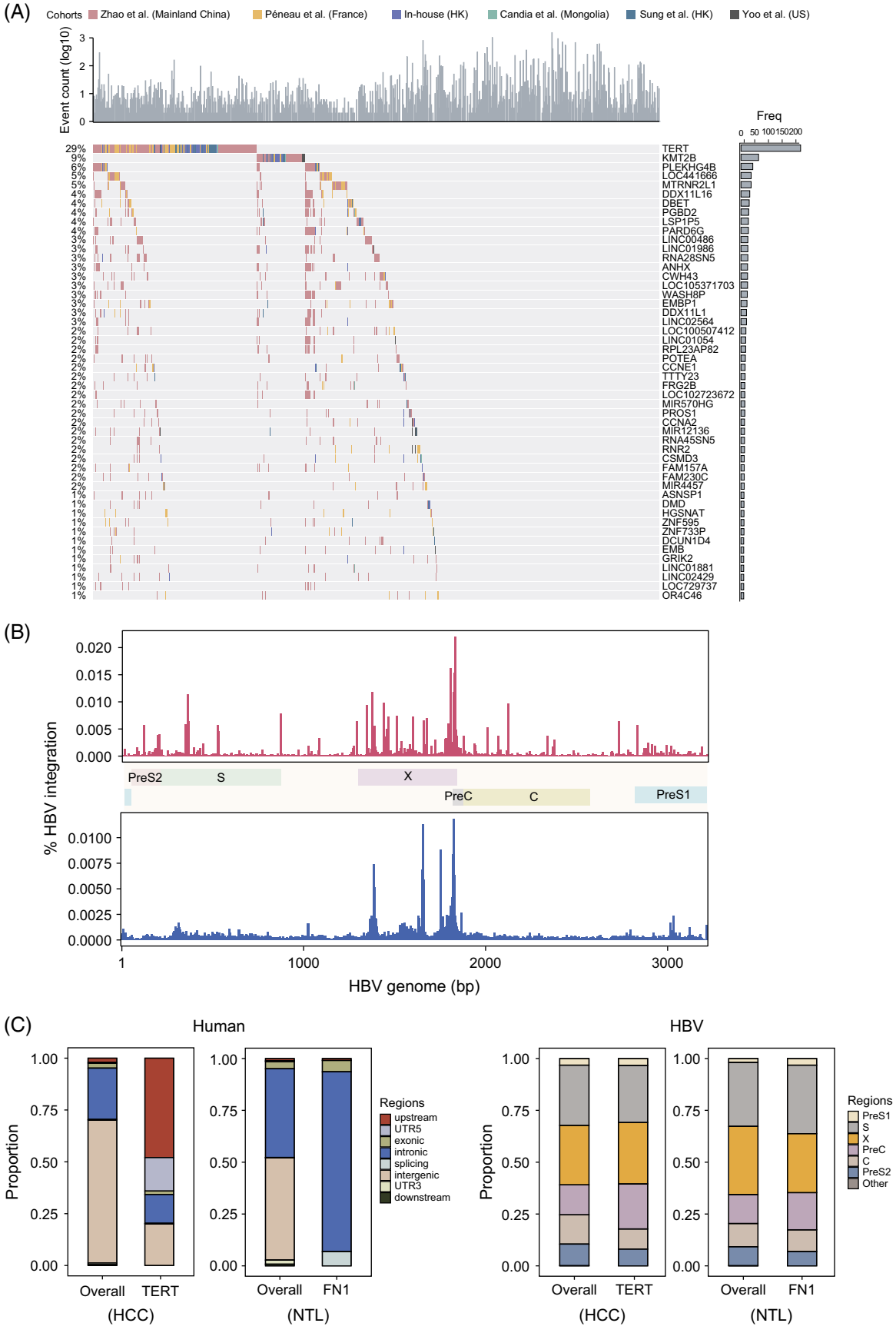

(A)
Cohorts
Zhao et al. (Mainland China)
Péneau et al. (France)
In-house (HK)
Candia et al. (Mongolia)
Sung et al. (HK)
Yoo et al. (US)
Event count (log10)
Freq
TERT
KMT2B
PLEKHG4B
LOC441666
MTRNR2L1
DDX11L16
DBET
PGBD2
LSP1P5
PARD6G
LINC00486
LINC01986
RNA28SN5
ANHX
CWH43
LOC105371703
WASH8P
EMBP1
DDX11L1
LINC02564
LOC100507412
LINC01054
RPL23AP82
POTEA
CCNE1
TTTY23
FRG2B
LOC102723672
MIR570HG
PROS1
CCNA2
MIR12136
RNA45SN5
RNR2
CSMD3
FAM157A
FAM230C
MIR4457
ASNSP1
DMD
HGSNAT
ZNF595
ZNF733P
DCUN1D4
EMB
GRIK2
LINC01881
LINC02429
LOC729737
OR4C46
(B)
% HBV integration
PreS2
S
X
PreC
C
PreS1
HBV genome (bp)
(C)
Human
HBV
Proportion
Regions
upstream
UTR5
exonic
intronic
splicing
intergenic
UTR3
downstream
PreS1
S
X
PreC
C
PreS2
Other
Overall
TERT
FN1
(HCC)
(NTL)

FIGURE 5 HBV integration landscape of human and HBV breakpoints investigated using a large meta-cohort of patients with HCC (n = 848). (A) The landscape of HBV integration at human genome in HCC. The frequency of the top 50 recurrently integrated genes. (B) The distribution of HBV integration breakpoints at HBV genome. The upper and lower panels represent the distribution in HCC and NTL, respectively. (C) Distribution of genic positions for the overall events and those affecting the top genes (*TERT* in HCC, *FN1* in NTL). Abbreviation: NTL, non-tumorous liver.

the *X* gene, in contrast, the *TERT* and *FN1* integrated events were preferentially located upstream (ie, promoter) and intronic regions, respectively (Figure 5C). This may implicate their underlying mode of influence. Promoter/upstream *TERT* integrated events may elicit their mechanistic functions by introducing transcription factor binding sites at the *TERT* promoter to activate *TERT* transcription,[13] whereas intronic *FN1* integrated events could potentially modify the coding sequence through alternative splicing by intronic modifications.[12] Similarly, other viral integrated events with breakpoints enriched at certain genic positions may possibly exert their functional alterations through similar mechanisms.

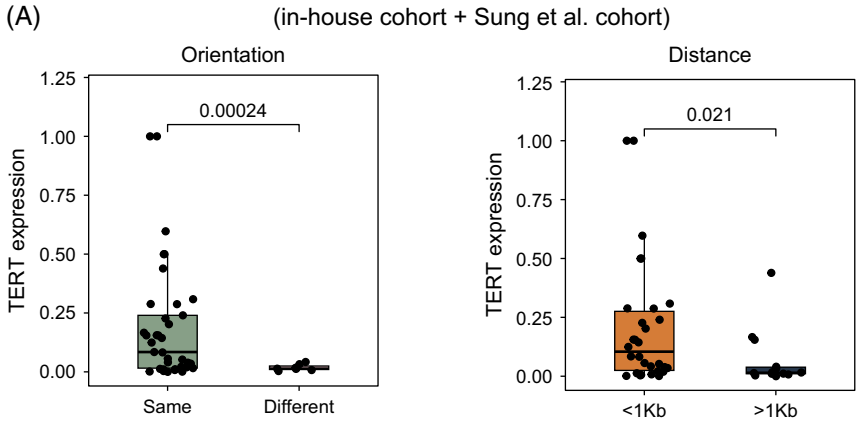
Concurrent modulating effect of orientation and relative distance of HBV integration on *TERT* transcription activation

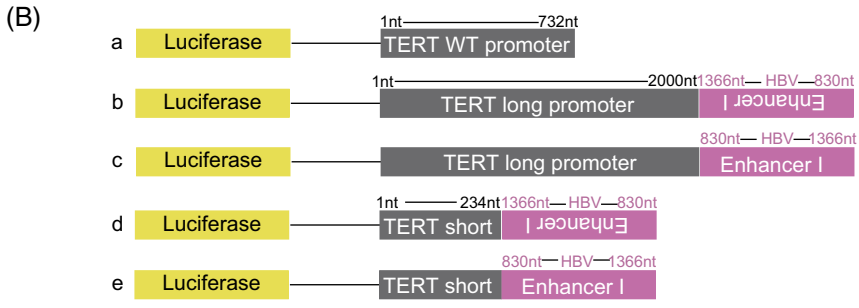
In our previous report[13] using a target-panel sequencing approach to survey HBV integration in our in-house patient cohort (n = 95), we found that HBV enhancer I (EnhI) was the key viral component leading to *TERT* activation upon HBV integration at the *TERT* promoter. In addition, we identified a molecular mechanism of *TERT* activation through the E74-like ETS transcription factor 4 (ELF4), which normally can drive HBV gene transcription. ELF4 bound to the chimeric HBV EnhI at the *TERT* promoter, resulting in *TERT* activation.[13] In this study, we combined our in-house sample cohort (n = 95) with that of Sung and colleagues (n = 81, HBV-positive cases), which had both genomic and transcriptomic data available. We were able to validate in this combined cohort our previous observation that the orientation of HBV integration could modulate the extent of transcription activation on the *TERT* gene, with better transcription activation effect elicited by events integrated at the same orientation as the direction of transcription (Figure 6A). Notably, using this large, combined sample cohort, we additionally evaluated the influence of the relative distance, that is, the extent of physical separation between the transcription start site (TSS) of *TERT* and the integrated HBV fragment. As we understood that *TERT* promoter-integrated cases harnessed the HBV EnhI to recruit host transcription factors to activate *TERT* transcription, we hypothesized that the distance between HBV EnhI and TSS of *TERT* could modify the extent of transcription activation. Interestingly, we detected significantly higher *TERT* expression in cases having HBV integration <1 kb from TSS of *TERT*, as compared to those beyond 1 kb (Figure 6A). To further substantiate our findings, we constructed luciferase reporter constructs in either orientation (same or opposite to the direction of *TERT* gene transcription) and with different lengths of the *TERT* promoter sequence (long [2 kb] and short [234 bp]) (Figure 6B). They represented HBV integration scenarios with different orientations of integration and relative distances to the TSS of *TERT*. Consistent with our hypothesis, constructs with shorter *TERT* promoter sequences had significantly higher transcription activation, and the right orientation of integration, that is, the same as the direction of *TERT* transcription, further exacerbated the activation effect (Figure 6C, Supplemental Figure S7, http://links.lww.com/HEP/I678).

Extent of clonal diversity informed patients' prognosis

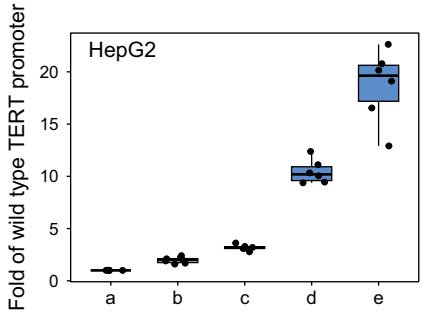
Based on our findings, there were multiple levels of enrichment of HBV integration detected within or among patients with HBV-associated HCC. Intriguingly, clonal HBV integration events were found in some but not all patients.[9,23] We speculated that the presence of clonal events might represent another layer of disparity landscape in patients. We stratified patients according to their clonal disparity landscape and determined their prognostic outcomes. Taking advantage of the multisite sampling data set by Lin et al,[24] we deliberately combined the multisite data of the same patient as individual pseudo-bulk data. Therefore, events that were shared among multiple tumor sites had enriched read counts, as compared to the remaining events (Supplemental Figures S8–S10, http://links.lww.com/HEP/I678). They served to represent clonal HBV integration events, for example, *TERT* and *KMT2B*, and as such, we defined clonal and nonclonal events in the patients. Next, we defined events that were shared at multiple tumor sites and with enriched read counts as clonal events, whereas those that did not as nonclonal events. Patients were classified according to the presence of clonal events (Figure 7A).

Taking the multiple levels of disparity into account, we derived a clonal disparity score that reflected the clonal diversity status of patients, with a high score (above median) indicating cases with clonal events, while a low score (below median) for those without. Indeed, cases with clonal events had significantly higher clonal disparity scores as compared to those without (Figure 7B). Using





Plasmid	a	b	c	d	e
TERT promoter	WT	long	long	short	short
Orientation	–	different	same	different	same

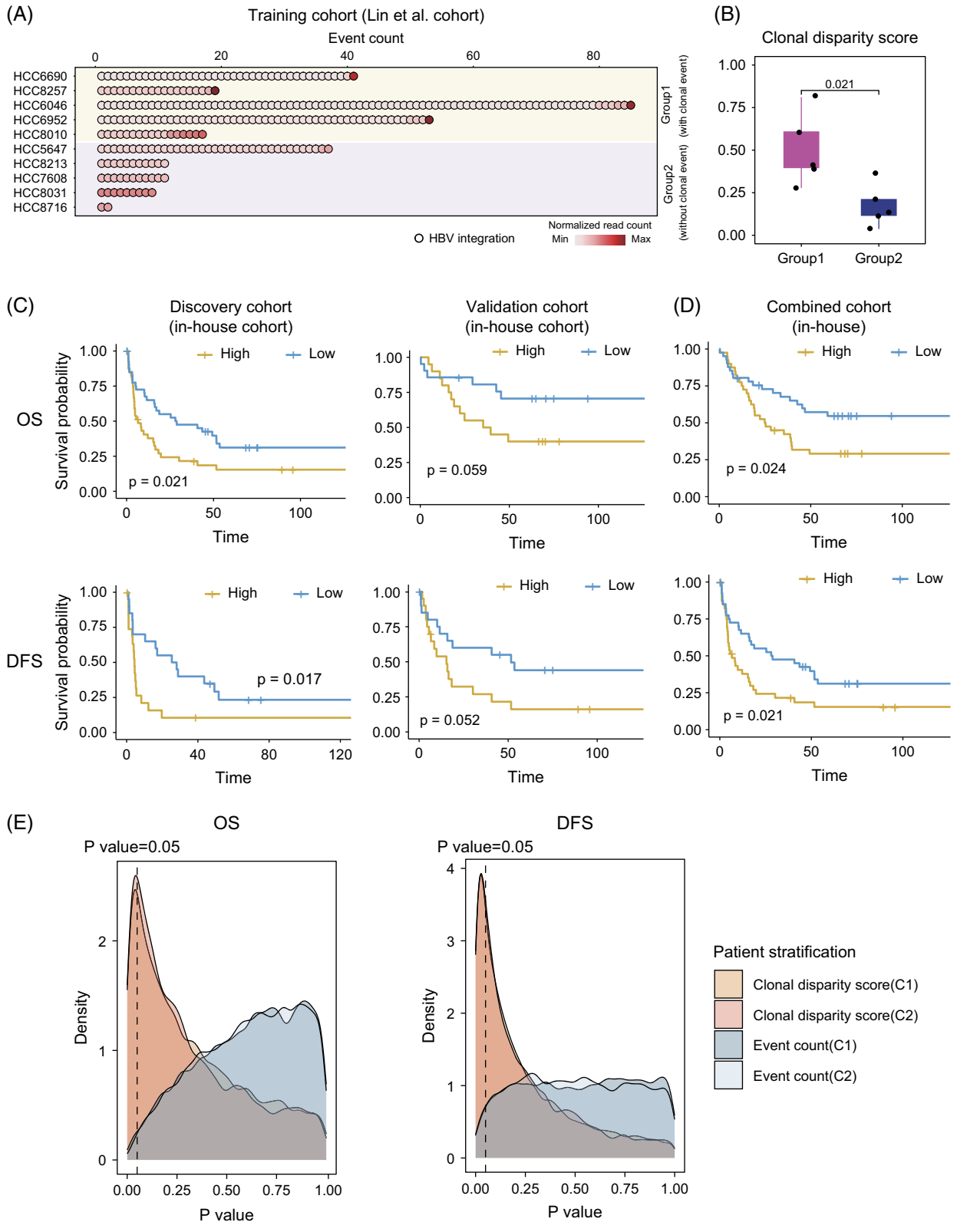


Factor	G1	G2	Fold change (G2/G1)	P value
Distance	b	d	5.28	<0.0001
	c	e	5.86	0.0001
Orientation	b	c	1.61	<0.0001
	d	e	1.78	0.0014
Distance + Orientation	a	e	18.68	<0.0001

FIGURE 6 Mechanistic investigation demonstrated the effects of both orientation and relative distance of the HBV integration in modulating *TERT* transcription activation. (A) Both orientation (HBV as compared to *TERT*) and relative distance (from the TSS of *TERT*) significantly modulated *TERT* transcription activation. (B) Panel of luciferase reporter constructs. Constructs consisted of different combinations of the length of the *TERT* promoter (long and short) and the orientation of HBV integration (same and different). (C) There was a differential level of transcription according to the relative distance and orientation of HBV insert in HepG2 cells. *p* value was calculated by comparing group 2 (G2) against group 1 (G1) samples. Abbreviation: TSS, transcription start site.

the clonal disparity score to further investigate our in-house sample cohort, we observed poorer overall and disease-free survival in patients with higher scores, that is, carrying clonal events (Figure 7C). We also combined the discovery-stage and validation-stage samples into a merged cohort. Consistent with our previous findings, the merged sample cohort demonstrated significantly poorer survival rates in cases with higher clonal disparity scores (Figure 7D). To minimize potential sample ascertainment bias, we simulated the sample allocation process 5000 times and found that the simulated *p* value distribution peaked similarly as the observed *p* values, indicating the reproducibility of our findings (Figure 7E).

We then assessed the underlying mechanistic differences among patients with differential clonal disparity scores. Merged sample cohorts of high and



low scores were examined for their underlying intrinsic differences (integration event count, presence of events at T&C regions, presence of *TP53* mutations [as a token of genome instability], and presence of HBV *TERT* integrations [a key HBV integration]) (Figure 8A). We detected a significant association between the clonal disparity score and these intrinsic differences (Figure 8B). We also stratified the patients by an event

FIGURE 7 The clonal diversity of HBV integration determined patients' prognostication outcomes. (A) Patients with HCC were classified into 2 groups according to clonal diversity status, namely with clonal integration events (G1) and without clonal integration events (G2). Each dot represents 1 integration event, and the color indicates the normalized number of read counts supporting the event. A dot with a darker color represents a clonal event (uneven enrichment of read distribution among the events for a patient). (B) Significant difference in clonal disparity score between G1 and G2 patients. (C) Clonal disparity score stratified patients' prognostication outcomes, including OS and DFS, in the in-house discovery and validation cohorts. (D) Stratification of patients' prognostication outcomes in the combined in-house sample cohort. (E) *p* value distribution of patients' OS and DFS stratified by clonal disparity score and event count, with random sample allocation into discovery and validation cohorts repeated 5000 times using our in-house sample cohort. Abbreviations: DFS, disease-free survival; OS, overall survival.

count cutoff of 3, or the presence of *TP53* mutations or HBV *TERT* integrations, and tested their prognostic outcomes. Compared to the clonal diversity score, the event count and HBV *TERT* integration did not have prognostic significance, whereas *TP53* mutations, and *TP53* mutations and/or events at T&C regions had (Supplemental Figure S11, http://links.lww.com/HEP/I678). This may imply the possible relationship between clonal HBV integration and genome instability. Indeed, we further observed a significantly higher level of genome instability in the subjects of Sung et al, carrying integration events at the T&C regions and/or *TP53* mutations (Supplemental Figure S12, http://links.lww.com/HEP/I678). This further supports our findings that the preferential enrichment of HBV integrations at T&C regions might likely result in genome instability to promote hepatocarcinogenesis.

DISCUSSION

Oncovirus genomic integration is generally regarded as a random process when it initially occurs.[8,25] However, despite the fact that viral integration events arise irregularly without preference, nonrandom enrichment leads to apparent disparity landscapes. In our study, we have investigated various disparity patterns of HBV integration, which importantly suggests the retention of events is probably the consequence of the clonal selection of the favorable ones. Therefore, we inspected the viral integration and identified the uneven distribution of events at multiple levels, including geographical cohorts, individual patients, and among chromosomal or even genic localizations. They could shed light on indicating different functional and mechanistic biases that favor the hepatocarcinogenesis process.

To our concern, it was generally believed that driver events for hepatocarcinogenesis likely possess proliferative and/or functional advantages that render them to be favorably selected in HCC tissue, that is, reduction in event counts but with concomitant increase in supporting read counts, as compared to the corresponding NTL counterparts. Nevertheless, this assumption was not always valid and there were discrepancies in the preference of event occurrence in HCC and NTL. Controversially, some studies reported higher event counts in HCC,[10,11,15] whereas others advocated the reverse.[9,14] Interestingly, by intentionally analyzing various publicly available sample cohorts of different ethnic groups, we could identify preferential clonal selection of events in NTL tissue in some but not all of the cohorts examined. We noted significantly higher HCC-related event count in the cohorts (Mainland Chinese, HK Chinese, and Mongolian) of mainly Asian ethnicity, while higher NTL-related event count in the cohorts (French and American) of Western ethnicity. Although there is preferential regional prevalence of HBV genotype,[26–28] we found that it is not a major factor causing the difference in event count between HCC and NTL (Supplemental Figure S13A, http://links.lww.com/HEP/I678). We speculate that the relative preference of event occurrence (HCC > NTL or NTL > HCC) may potentially hinge upon the mode of HBV infection. With a high prevalence of HBV infection in Asian populations, and over 90% of them were infected at birth or early childhood, they were usually chronic HBV infections.[29] On the other hand, most of the people in Western populations got infected with HBV in adulthood, and they usually underwent acute hepatitis.[27,30] We believe that the degree of clonal enrichment of integration events is a reflection of the underlying ages of premalignant viral incubation in the livers. Due to this subtle difference in their mode of infection, there could be substantial differences in their period of viral incubation in the body that results in the apparently diverse extent of clonal enrichment of integration events in their premalignant stage NTL tissues (Supplemental Figure S13B, http://links.lww.com/HEP/I678). We further calculated the normalized clonality statistics[9] (it is a measure of the extent of clonal insertion of the virus) using the public cohorts (HK and France; both had human-capture and viral-capture genomic data available) and noted a significantly higher clonality value in the NTL tissues in the HK cohort, suggesting its relatively higher level of clonal enrichment, as compared to the French counterparts. In addition, the normalized clonality was also higher in HCC tissues of the HK cohort than that of the French cohort, suggesting another level of clonal enrichment that could possibly favor oncogenic hepatocarcinogenesis (Supplemental Figure S13C, http://links.lww.com/HEP/I678). Notably, the observation in the HK cohort was in line with the existing knowledge[28,31,32] on the seroconversion time course of chronic hepatitis B that at the early HBeAg-positive stage, there were more

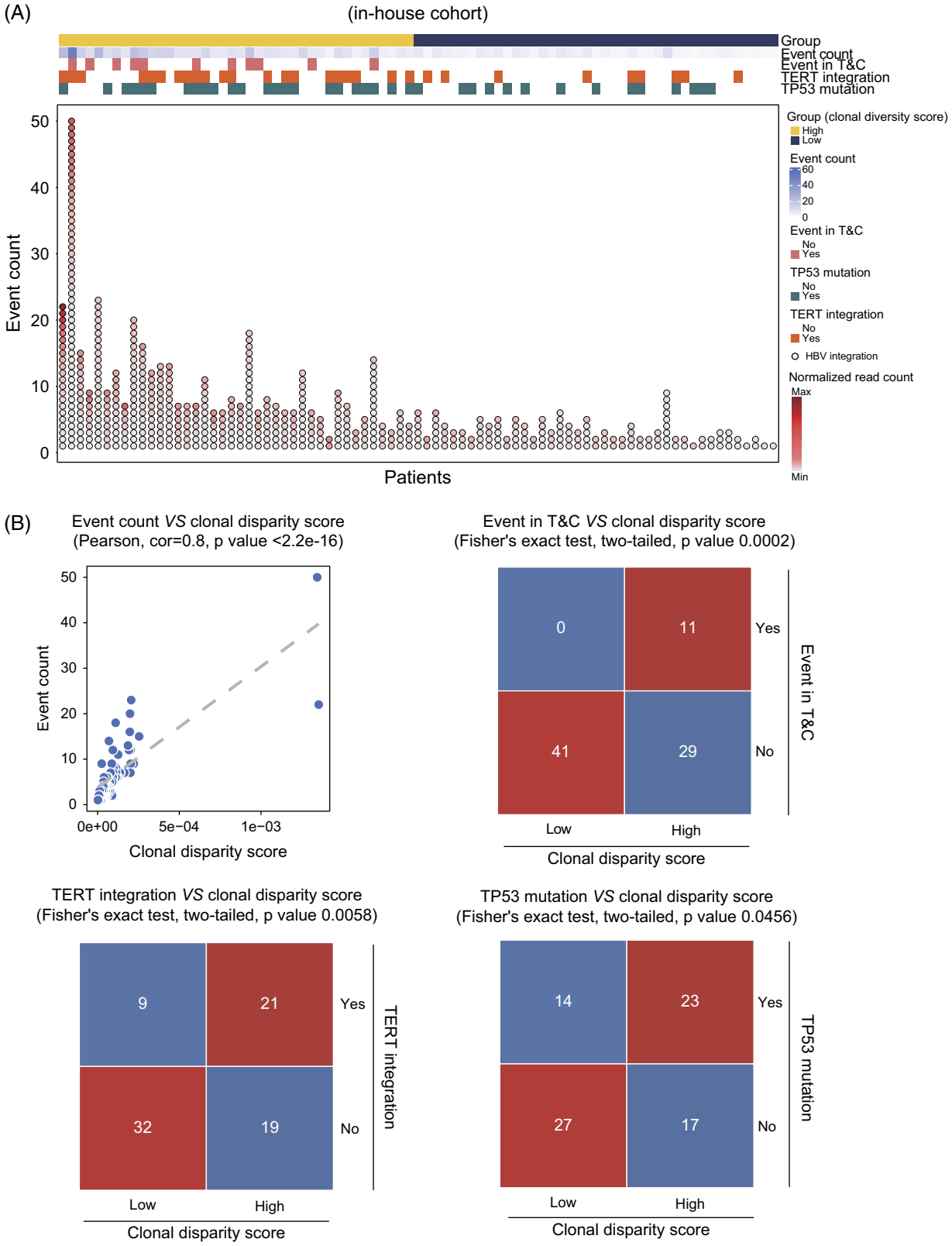


FIGURE 8 Underlying mechanistic differences among patients of differential clonal disparity scores. (A) Distribution of clonal disparity among patients and (B) its correlation with different molecular parameters (HBV integration event count, presence of integration event at T&C region, presence of *TP53* mutation, and presence of integration at *TERT*) in our in-house sample cohort. Abbreviation: T&C, telomeres and centromeres.

events of smaller clone size, whereas, at the subsequent HBeAg-negative stage (after years of chronic infection and immune clearance), there were fewer events but of larger clone size.

In the reported literature, studies predominantly focused on the identification of human and viral breakpoint landscapes. Notwithstanding the fact that they identified various key driver genes for hepatocarcinogenesis, the usual focus on individual affected human genes and their frequencies of viral integration may easily neglect the less reported nonsingle gene endeavors of events. Regarding the coexistence of HBV integration, our experimental investigation of co-knockdown of *TERT* and *PLEKHG4B* suggests the low possibility of their functional synergism, which may somehow strengthen the belief of functional sufficiency of certain frequently and recurrently HBV-integrated genes like *TERT*. On the other hand, we revealed the coexistence of HBV integration and their particular enrichment at T&C regions may further advocate the importance of gene-independent deterioration of genome stability of viral integration at T&C regions. We may use HBV integration-specific genome instability as a useful indicator for explaining the underlying driving force for liver malignancy. In fact, our results also pinpointed multiple axes involving *PLEKHG4B*. Although HBV integration at *PLEKHG4B* has been reported by studies, it is still less characterized as compared to the better known genes, for example, *TERT*, *KMT2B* and cyclin family genes, and existing literature only reported copy number gain of *PLEKHG4B* in neuroblastoma.[33] *PLEKHG4*, a paralog of *PLEKHG4B4*, could function as a guanine nucleotide exchange factor that facilitates activation of the small GTPases Rac1, Cdc42, and RhoA. Overexpression of *PLEKHG4* in NIH3T3 cells induced rearrangements of the actin cytoskeleton, specifically enhanced formation of lamellopodia.[34] Regarding the coexisting gene targets, *PARD6G* was suggested to be the negative regulator for PI3K/AKT signaling, and its inactivation was frequently observed in epithelial cancers.[33] Interestingly, other major coexisting gene targets were pseudogene or have uncharacterized biological functions (*DDX11L16* and *WASH8P*), which are worthy of further experimental delineation.

Our analysis revealed that there were preferential enrichments of HBV integrations at *FN1* in NTL tissue, whereas it was also detected in HCC tissue but at a much lower frequency. Our findings were in line with reported literature.[9,10] Importantly, no substantial mRNA expression changes were associated with the presence of HBV integration at FN1. Apart from protein coding and transcriptional consequences, HBV integration may also elicit potential effects on modifying mRNA stability, gene regulation machinery, and activation of cryptic sites.[9,12,13] In addition, we detected a peak of sequencing reads at the *FN1* intronic region in the proximity of HBV integration sites (Supplemental Figure S14, http://links.lww.com/HEP/I678), suggesting the possibility of alternative splicing or generating of novel isoforms of transcripts. Interestingly, HBV integration at *FN1* has also been identified in liver cirrhosis, suggesting its potential role in liver fibrogenesis.[35]

HBV integration can lead to oncogenic transcription activation. In our recent publication,[13] we adopted a target-panel sequencing approach to survey HBV integration in our patients' HBV-associated HCCs (n = 95). HBV integration at the human telomerase reverse transcriptase (*TERT*) gene promoter was frequent (35.8%, n = 34/95) in the HCC tumors and was associated with increased *TERT* mRNA expression and more aggressive tumor behavior. More importantly, we identified a molecular mechanism of *TERT* activation through the E74-like ELF4, which normally could drive HBV gene transcription. ELF4 bound to the chimeric HBV EnhI at the *TERT* promoter, resulting in *TERT* activation. Stable knockdown of ELF4 significantly reduced the *TERT* expression and sphere-forming ability in HCC cells. Along this direction, in our current study, we combined our in-house sample cohort with that of Sung et al, to strategically delineate the paired genomic and transcriptomic landscapes of *TERT*. By using the sequencing data complemented by luciferase reporter experimental validation, we discovered another important modulating mechanism regarding transcription activation elicited by HBV *TERT* integration. We also highly anticipated that other integration events at the promoter of other host genes may harness a similar mechanistic basis for oncogenic transcriptional enhancement, which awaits future studies.

Another observation that is worthy of notice is the uneven distribution of supporting read counts at different affected genes. On one hand, this is probably an indication of the clonal selection process that favors the enrichment of events possessing proliferative and/or functional advantages. We further believe such disparity of read distribution may probably reflect the clonal diversity of viral integration and they are likely to result in differential prognostic consequences in patients. Indeed, we demonstrated in our current study that clonal disparity is a significant biomarker that measures the underlying complexity of HBV integration landscape that can result in different treatments and/or survival advantages for the patients. Those carrying higher clonal disparity scores were associated with a poorer prognosis, and we believe disparity landscapes of viral integration could likely represent an independent aspect of biomarkers that is worthy of further investigation for their predictive and translational applications for clinical management of HCC and other oncovirus-induced human cancers. However, the clonal disparity score is more likely applicable to Asian cohorts than the Western cohorts (Supplemental Figure S15, http://

links.lww.com/HEP/I678). Similar to our observation on the global geographical disparity of HBV integration that preference of event occurrence differs between Asian and Western cohorts, we strongly believe that the subtle and intrinsic differences between the sample cohorts, namely the ethnicity of patients, age of HBV infection, and the prevalence of different HBV genotypes, could possibly result in the variability of performance of our clonal disparity score in different cohorts. We admit this potential limitation of our scoring system and we humbly await for further verification of our work in future studies.

In summary, our study investigated different disparity landscapes of HBV integration in HCC. Notably, our study may likely address the controversy on preferential occurrence of viral integration in HCC and NTL tissues that could likely be explained by the mode of HBV infection. Our findings also support that gene-independent enrichment of viral integration at T&C regions leads to genome instability, and there were also frequent and nonrandom coexistence of integration events that could consistently pinpoint the involvement of T&C regions. These could have profound meaning in suggesting the collective importance of chromosomal regions in addition to particular key driver genes. Moreover, we further delineated the mechanistic characterization of orientation-dependent and relative distance–dependent *TERT* HBV integration in modulating *TERT* activation. Last but not least, the clonal disparity landscape of viral integration can also have translational applications as useful biomarkers for predicting patients' prognosis.[36,37] We anticipate our findings may likely be applicable to other human cancers involving oncovirus and their genomic integration. Our insights will open up new avenues for the exploration of oncovirus-induced structural variations in human cancers.

AUTHOR CONTRIBUTIONS

Daniel Wai-Hung Ho and Irene Oi-Lin Ng conceived, supervised, and funded the study. Xueying Lyu and Sandrine Imbeaud performed the data analyses. Karen Man-Fong Sze and Joyce Man-Fong Lee performed experiments. Daniel Wai-Hung Ho, Xueying Lyu, Abdullah Husain, Lu Tian, and Jessica Zucman-Rossi interpreted the data. All authors contributed to the writing of the manuscript, reviewed the results, and approved the final version of the manuscript.

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CONFLICTS OF INTEREST

The authors have no conflicts to report.

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