**Aberrant integration of Hepatitis B virus DNA promotes major restructuring
of human hepatocellular carcinoma genome architecture**

Eva G. Álvarez^{1,2}, Jonas Demeulemeester^{3,4,†}, Paula Otero^{1,2, ‡}, Clemency Jolly^{3, ‡}, Daniel García-Souto^{1,2, ‡}, Ana Pequeño¹, Jorge Zamora¹, Marta Tojo⁵, Javier Temes¹, Adrian Baez-Ortega⁶, Bernardo Rodríguez-Martín^{1,2}, Ana Oitaben^{1,2}, Alicia L. Bruzos^{1,2}, Mónica Martínez-Fernández¹, Kerstin Haase³, Sonia Zumalave^{1,2}, Rosanna Abal¹, Jorge Rodríguez-Castro¹, Aitor Rodríguez-Casanova^{7,8}, Angel Diaz-Lagares^{7,9}, Yilong Li¹⁰, Keiran Raine¹⁰, Adam P. Butler¹⁰, Iago Otero^{1,2}, Atsushi Ono¹¹, Hiroshi Aikata¹¹, Kazuaki Chayama^{12,13,14}, Masaki Ueno¹⁵, Shinya Hayami¹⁵, Hiroki Yamaue¹⁵, Kazuhiro Maejima¹⁴, Miguel G. Blanco¹, Xavier Forns¹⁶, Carmen Rivas^{1,17}, Juan Ruiz-Bañobre^{1,9,18,19}, Sofía Pérez-del-Pulgar¹⁶, Raúl Torres-Ruiz^{20,21}, Sandra Rodríguez Perales²⁰, Urtzi Garaigorta^{17,†}, Peter J. Campbell^{10,22,†}, Hidewaki Nakagawa^{14,†}, Peter Van Loo^{3,†}
& Jose M. C. Tubio^{1,2,*}

¹Centre for Research in Molecular Medicine and Chronic Diseases (CiMUS), Universidade de Santiago de Compostela, Santiago de Compostela 15706, Spain.

²Department of Zoology, Genetics and Physical Anthropology, Universidade de Santiago de Compostela, Santiago de Compostela 15706, Spain.

³The Francis Crick Institute, London NW1 1AT, UK.

⁴Department of Human Genetics, University of Leuven, Leuven B-3000, Belgium.

⁵The Biomedical Research Centre (CINBIO), Universidade de Vigo, Vigo 36310, Spain.

⁶Department of Veterinary Medicine, University of Cambridge, Cambridge CB3 0ES, UK.

⁷Cancer Epigenomics, Translational Medical Oncology Group (Oncomet), Health Research Institute of Santiago (IDIS), University Clinical Hospital of Santiago de Compostela (CHUS/SERGAS), Santiago de Compostela 15706, Spain.

⁸**Roche-Chus Joint Unit**, Translational Medical Oncology Group (Oncomet), Health Research Institute of SantiagodeCompostela(IDIS), Santiago de Compostela 15706, Spain.

⁹Centro de Investigación Biomédica en Red Cáncer (CIBERONC), Madrid 28029, Spain

¹⁰Wellcome Sanger Institute, Wellcome Genome Campus, Hinxton, Cambridge, CB10 1SA, UK.

¹¹Department of Gastroenterology and Metabolism, Graduate School of Biomedical and Health Sciences, Hiroshima University, Hiroshima, Japan.

¹²**Collaborative Research Laboratory of Medical Innovation, Graduate School of Biomedical and Health Sciences, Hiroshima University, Hiroshima, Japan.**

¹³**Research Center for Hepatology and Gastroenterology, Graduate School of Biomedical and Health Sciences, Hiroshima University, Hiroshima, Japan.**

¹⁴RIKEN Center for Integrative Medical Sciences, Yokohama, Kanagawa 230-0045, Japan.

¹⁵Department of Surgery II, Wakayama Medical University, Wakayama, Japan.

¹⁶Liver Unit, Hospital Clínic, University of Barcelona, IDIBAPS, CIBERehd, Barcelona, Spain.

¹⁷Department of Molecular and Cellular Biology, Centro Nacional de Biotecnología – Consejo Superior de Investigaciones Científicas (CNB – CSIC), Madrid 28049, Spain.

¹⁸Department of Medical Oncology, University Clinical Hospital of Santiago de Compostela,
University of Santiago de Compostela, Santiago de Compostela 15706, Spain

¹⁹Translational Medical Oncology Group (Oncomet), Health Research Institute of Santiago de
Compostela (IDIS), Santiago de Compostela 15706, Spain.

²⁰Molecular Cytogenetics and Genome Engineering Group, Centro Nacional de Investigaciones
Oncológicas (CNIO), Madrid, Spain.

²¹Division of Hematopoietic Innovative Therapies, Centro de Investigaciones Energéticas,
Medioambientales y Tecnológicas (CIEMAT), Madrid, Spain.

²²Department of Haematology, University of Cambridge, Cambridge CB2 2XY, UK.

‡These authors contributed equally

†These authors contributed equally

*Correspondence to:

Dr Jose M. C. Tubio,

Genomes and Disease,

Centre for Research in Molecular Medicine and Chronic Diseases (CIMUS),

University of Santiago de Compostela,

Santiago de Compostela 15706,

Spain

Phone: +34 881 815 382

e-mail: jose.mc.tubio@usc.es

ABSTRACT

Most cancers are characterized by the somatic acquisition of genomic rearrangements during tumour evolution that eventually drive the oncogenesis. Here, using multiplatform sequencing technologies, we identify and characterize a remarkable mutational mechanism in human hepatocellular carcinoma caused by Hepatitis B virus, by which DNA molecules from the virus are inserted into the tumour genome causing dramatic changes in its configuration, including non-homologous chromosomal fusions, dicentric chromosomes and megabase-size telomeric deletions. This aberrant mutational mechanism, present in at least 8% of all HCC tumours, can provide the driver rearrangements that a cancer clone requires to survive and grow, including loss of relevant tumour suppressor genes. Most of these events are clonal and occur early during liver cancer evolution. Real-time timing estimation reveals some HBV-mediated rearrangements occur as early as two decades before cancer diagnosis. Overall, these data underscore the importance of characterizing liver cancer genomes for patterns of HBV integration.

INTRODUCTION

Human hepatocellular carcinoma (HCC) is the most common primary liver malignancy, resulting in over 700,000 deaths globally every year¹. Previous studies indicate that the disease has a complex genomic landscape, with frequent copy number changes and interchromosomal rearrangements^{2,3}. Hepatitis B virus (HBV) infection – a condition affecting 240 million people worldwide – is the second most frequent cause of cancer after tobacco, and a major cause of HCC. HBV infection has been associated with chromosomal instability in cancerous and non-cancerous liver genomes, and HBV DNA integration is known to be the cause of chromosomal rearrangements in HCC⁴⁻¹¹. However, we still ignore the full extent to which HBV DNA

integrations impact the structure (i.e., patterns and mechanisms of mutation) and function (i.e., driver events) of HCC genomes¹², which may have important consequences for the diagnosis, prognosis and treatment of the disease.

In this work we harness recent advances in DNA sequencing technologies using short and long-reads to characterize patterns of structural variation associated with HBV DNA integration in human HCC. Our analyses further illuminate a remarkable mutational mechanism, present in at least 8% of all HCC tumours, by which somatic integration of HBV DNA promotes non-homologous interchromosomal rearrangements coupled with megabase-size telomeric (i.e., that includes the telomere) deletions in one or two of the chromosomes involved, occasionally representing tumour driver events in HCC. We identify instances in which this process generates dicentric chromosomes, and removes relevant tumour suppressor genes in HCC evolution, such as *TP53*, *ARID1A*, *RBI*, *RPS6KA3* and *IRF2*. These events are clonal, and timing estimation reveals this mechanism is active in early stages of HCC evolution. Overall, these data underscore the importance of characterizing liver cancer genomes for patterns of HBV integration, and provide insights for the prevention of the disease in a subset of HCC patients.

RESULTS

Analysis of HBV integration sites identifies non-canonical HBV insertions

We run our bioinformatic algorithms (**Online Methods**) to explore the landscape of HBV DNA integrations acquired somatically on Illumina paired-end whole-genome sequencing data from 296 HCC tumours from the Pan-Cancer Analysis of Whole Genomes (PCAWG) project¹³. Their

matched-normal samples derived from blood, were also sequenced. This analysis retrieved a total of 148 somatic HBV integration events in 51 tumour samples (**Fig. 1a**; **Supplementary Data 1**). Forty-two of these events represent canonical viral DNA insertions where the paired-end mapping data shows a classical pattern, characterized by two reciprocal – face-to-face oriented – read clusters delimiting the integration site, and whose mates support the presence of viral DNA (**Fig. 1b**). This result is consistent with an alternative study on the same dataset carried out by others¹⁴. However, in addition to these canonical insertions, our analysis revealed that a majority (72%, 106/148) of events followed an unexpected, non-canonical pattern. Here, paired-end mapping data showed single clusters of reads whose mates identify one extreme of the somatic viral integration only, while the reciprocal cluster supporting the other extreme of the insertion appeared to be missing. For instance, in one HCC tumour, SA501453, paired-end reads show a single cluster supporting one extreme of an HBV insertion event on chromosome 19, with no reciprocal cluster in the proximity of the integration site (**Fig. 1c**). Our algorithms successfully reconstructed the ends of these 106 non-canonical insertion events, confirming that they match HBV sequences (**Supplementary Data 2**).

Similar paired-end mapping patterns were previously identified in cancer genomes with high retrotransposition rates¹⁵, where this type of events represented hidden genomic rearrangements mediated by aberrant DNA integrations. This suggested that our findings could represent cryptic somatic rearrangements mediated by HBV DNA insertion. Actually, somatic rearrangements linked to HBV insertion sites have been recently identified using long-read sequencing technologies in human HCC cell-lines⁵ and primary tumours¹⁰. Hence, to illuminate the genuine configuration of the relevant rearrangement involved in the patterns described above, we

performed long-read whole-genome sequencing on the affected tumour, SA501453, using Oxford Nanopore Technologies (ONT) to a final coverage of 13.5X (median read size = 12 kb). The long reads revealed a cryptic translocation linking chromosomes 19 and 11, which is bridged by a 640 bp HBV DNA insert (**Fig. 2**). Although our algorithms had initially identified the missing reciprocal cluster on chromosome 11 in the paired-end data, the interchromosomal rearrangement remained undetectable due to size constraints of the Illumina sequencing library, which was too short to span the HBV insertion. Notably, the genomic breakpoints of this translocation remained unnoticed to a set of four different structural variation calling pipelines, which were employed in the identification of genomic rearrangements and in the PCAWG dataset^{13,16}.

Telomeric deletions mediated by HBV DNA insertions in HCC

Many non-canonical HBV insertions occur in association with megabase-size deletions that remove telomeric regions of a chromosome. For instance, in HCC tumour SA529726, the paired-end sequencing data revealed one single cluster of an HBV insertion on the short arm of chromosome 3. Here, the insertion boundary is associated with a large copy number loss (**Fig. 3a**), suggesting that the insertion event occurred in conjunction with a telomeric deletion that removed 21 Mb of chromosome 3p. We performed long-read sequencing on this sample, which revealed that the telomeric deletion occurred due to an unbalanced translocation between chromosomes 3 and X bridged by a 3.3 kb HBV insertion that shows a classical fragmented and rearranged form^{5,17} (**Fig. 3a; Supplementary Fig. 1a**). In the same sample, the ONT data showed a second, unrelated HBV insertion (3.5 kb long) that bridges a translocation between chromosomes 4 and 7, associated with a 20 Mb telomeric deletion on 4q (**Fig. 3a;**

Supplementary Fig. 1b). Similarly, in another remarkable HCC tumour, SA501511, up to three different HBV insertions were found associated with large deletions – 20.5, 33.6, and 76.7 Mb long – removing the telomeres on chromosome arms 10p, 4p and 13q, respectively (first circos plot in **Fig. 3b**). This time, the long-read sequencing data revealed three cryptic HBV-mediated translocations between the long arm of chromosome 8 and the relevant deletion breakpoints on chromosomes 4, 10 and 13 (**Fig. 3b; Supplementary Fig. 2a-c**).

We looked in the PCAWG HCC dataset for other HBV insertions demarcating huge telomeric copy number loss events, which could involve the same mutational mechanism, finding 26 additional events in 19 different HCC tumours (**Supplementary Fig. 3**). Hence, we find that ~8% (23/296) of all HCC samples in the PCAWG dataset bear the hallmarks of this mutational process (**Supplementary Data 1**). These 23 samples bear in total 40 telomeric deletions apparently caused by HBV integration. We analysed three of these samples (SA501424, SA501481 and SA529830) by whole-genome long-read sequencing with ONT, which confirmed cryptic interchromosomal rearrangements linked to telomeric deletion breakpoints in all of them (**Fig. 3b**), demonstrating that this aberrant mutational mechanism mediated by HBV insertions is recurrent in human HCC. Notably, in two of the samples sequenced with ONT (SA501481 and SA529830) the configuration of the rearrangement found supports a derived chromosomal fusion that generates a dicentric chromosome (i.e., a chromosome with two centromeres; **Fig. 3b**). These chromosomes are known to represent a potential source for breakage-fusion-bridge (BFB) repair^{15,18}, unless they become stabilized due to reduced intercentromeric distance or by means of inactivation of one of the two centromeres¹⁹. Here, the absence of copy number profiles and chromosomal rearrangements typically associated with BFB cycles supports the last scenario. In

179 this work we obtained the whole genomes from a total of 9 human of HCC samples in the
180 PCAWG dataset using ONT long-read sequencing, 7 of them bearing HBV-mediated telomeric
181 deletions. The reconstruction of the nucleotide sequences from the inserted HBV DNA shows
182 that, in general, insertions mediating telomeric deletions tend to have a more complex structure
183 than canonical ones, including duplications and inversions of the reference HBV genome,
184 including internal deletions, inversions and duplications (**Supplementary Fig. 4**).

186 **HBV insertion rate varies across the HCC genome**

187 To understand the properties of the insertion points of HBV DNA, we analysed the genome-wide
188 distribution of 148 somatic events (including 42 canonical and 106 non-canonical). We find
189 considerable variation in the rate of HBV integration (**Fig. 4a**). One region at chromosome 5p,
190 with 13 HBV insertions, stands out over the others, with a hotspot in the *TERT* gene (11 HBV
191 insertions in *TERT*, 9 of them located upstream to the gene close to the promoter region; **Fig. 4a**
192 and **Supplementary Data 3**). This cancer gene has been previously identified as a main target
193 for HBV insertions^{10,11,20,21}, which are the cause of *TERT* focal amplification and *TERT* promoter
194 activation¹⁰. Notably, we find that 72% (8/11) of the events in *TERT* represent non-canonical
195 insertions of HBV, which confirms previous data showing that this relevant gene can be targeted
196 by genomic rearrangements bridged by the virus¹⁰. Other cancer-related genes with recurrent
197 insertions within gene boundaries are *KMT2B* (n=4, three in coding exons) and *CCNE1* (n=2, all
198 intronic), both at chromosome 19q (**Fig. 4a**).

To further characterize the patterns of HBV integration in human HCC, we studied the association of HBV events and genomic features, including chromatin state, replication timing and gene expression (**Fig. 4b-c** and **Supplementary Fig. 5**). This analysis shows a significant enrichment of HBV events in genes ($P = 0.035$, **Fig. 4b**) and a depletion in intergenic regions ($P = 0.035$; **Fig. 4b**). Notably, 66% (93/148) of all HBV events fall within gene boundaries. When we split the HBV events into those linked to megabase-size telomeric deletions ($n=40$) and the remaining HBV insertions ($n=108$), we observe that while the category of HBV insertions with no telomeric deletions exhibits the expected pattern for canonical HBV insertions, characterized by an enrichment in regions of early replication timing²⁰, HBV insertions causing telomeric deletions are depleted in those regions ($P = 0.013$ and $P = 0.037$, respectively; **Fig. 4c**). This could obey to different DNA repair mechanisms driving the integration of HBV DNA at different stages of S phase²².

HBV insertions are clonal events acquired early in HCC evolution

Our results illuminate a scenario where rearrangements mediated by viral DNA integration are important remodelers of human HCC genomes. The analysis of copy number profiles revealed that many HBV-mediated rearrangements occurred in chromosomes with copy number gains, providing opportunities for timing analyses^{23,24}. To pinpoint these rearrangements on a timeline from the fertilized egg to tumour diagnosis, we modified current timing algorithms to operate with single read-clusters only (see **Methods**). The method revealed that somatic insertions of HBV DNA in HCC are typically clonal events that have been acquired early in tumour evolution (i.e., prior to the copy number gain). For example, in one notable HCC, SA269680, which underwent whole-genome duplication (WGD), we identified eleven viral insertion events. All

but one were catalogued as early events (**Fig. 5a; Supplementary Data 4**), and five of these early events corresponded to single clusters associated with megabase-size copy number losses (**Supplementary Fig. 3**), supporting the notion that these large-scale rearrangements may be important in the initial stages of liver oncogenesis.

To further investigate the clinical relevance of HBV integration in HCC, we employed real time estimation data of WGD events from PCAWG²³ to perform a more precise timing estimation of HBV events along patients' lifetime. The method is based on the analysis of mutational clock signatures that correlate with patient age at diagnosis²⁵, which can be used for timing of WGDs and their associated variants²³. This approach allowed real-time timing of 37 HBV insertions (8 canonical and 29 non-canonical) embedded in WGD tumours (**Fig. 5b; Supplementary Data 5**), and revealed some of these rearrangements appear many years before diagnosis. For instance, in HCC SA501645, a cryptic HBV-mediated rearrangement in chromosome 10, coupled with a 7.3 Mb telomeric deletion on 10p, occurred over 21 years before the patient was diagnosed with HCC (**Fig. 5b-c**).

HBV-mediated telomeric deletions cause loss of tumour suppressor genes

We find 40 HBV-mediated rearrangements with telomeric deletions involving 23 HCC samples. Notably, there are instances in which essential tumour suppressor genes are lost by this mutational mechanism, which may provide the driver rearrangements that a cancer clone requires to survive and grow. In one remarkable HCC, SA529830, we identified one paired-end single cluster supporting an HBV insertion on the short arm of chromosome 17. The insertion

occurred in conjunction with a 14.9 Mb clonal telomeric deletion at the integration site, which removed one copy of tumour suppressor gene *TP53* (**Fig. 6**). Notably, the second copy of *TP53* in this tumour is inactivated by the missense point mutation C242S^{26,27} (**Supplementary Fig. 6**). The paired-end data showed a similar pattern on the short arm of chromosome 8, where a single cluster supporting an HBV insertion occurred together with a loss of the first 41 Mb of the chromosome. The patterns suggested that an HBV DNA molecule could be bridging an unbalanced translocation between chromosomes 17 and 8 that would generate a dicentric chromosome (see circos plot in **Fig. 3b**). We carried out whole-genome long-read sequencing, which confirmed the expected configuration of this relevant rearrangement (long-read plot in **Fig. 6**). In addition, we performed in-situ hybridization to identify the loss of *TP53* and the chromosomal fusion between chromosomes 17 and 8, which further validated these concomitant events (**Supplementary Fig. 7**).

Similarly, in one additional HCC tumour, SA501481, we identified an HBV insertion into chromosome 1 associated with the deletion of one copy of tumour suppressor gene *ARID1A* (**Fig. 7a**). Here, paired-end data shows a single cluster of reads, whose mates support the HBV insertion, demarcating a copy number loss of the first 57.2 Mb of 1p including *ARID1A*. Again, in this case, we initially lacked the DNA region on the other side of the rearrangement mediated by the virus, due to Illumina library insert size constraints. The paired-end data showed an analogous pattern in chromosome 9, with an independent cluster supporting an HBV insertion that occurred together with a telomeric deletion of the first 41 Mb of 9p at the integration site. This scenario suggested a cryptic unbalanced translocation between 1p and 9p, generating a

dicentric chromosome (see circos in **Fig. 3b**), which was confirmed by long-read sequencing with ONT (long-read plot in **Fig. 7a**).

ARIDIA is a relevant cancer gene harbouring monoallelic loss-of-function mutations in 10-15% of human HCC samples²⁸. Notably, in a different HCC, SA501424, we found a similar scenario to the one described above. This time, an HBV insertion demarcates a deletion of the first 31.5 Mb of chromosome 1p, which again involved loss of one copy of *ARIDIA* (**Fig. 7b**). Hence, we performed long-read sequencing with ONT, which revealed a cryptic interchromosomal rearrangement between chromosomes 1p and 11q bridged by HBV (see circos in **Fig. 3b**). The deletion and the chromosomal fusion were also validated by in-situ hybridization (**Supplementary Fig. 7**).

Overall, the analysis of the copy number data from the 23 HCC samples with telomeric deletions show that these alterations result in the loss of 244 genes classified as tumour suppressor or loss-of-function genes, according to Cancer Gene Census²⁹ and Intogen³⁰, respectively (**Supplementary Data 3**). Thirty-seven of these genes are found to be involved in human HCC in the mentioned databases. Notably, at least three of these events are reported in the Compendium of driver copy number alterations of the PCAWG Consortium¹³. These three events are described above and include the deletion of *TP53* in sample SA529830 (**Fig. 6**), the deletion of *ARIDIA* in sample SA501424 (**Fig. 7**), and the loss of one copy of the *RBI* gene in a 13q telomeric deletion from sample SA501511 (**Fig. 3b** and **Supplementary Fig. 2c**). In addition, although not included in the PCAWG Compendium, we find three additional clonal telomeric deletions removing one copy of two relevant cancer genes in human HCC. First, in

sample SA529726, a telomeric deletion at chromosome X removes one copy of the loss-of-function gene *RPS6KA3*³¹ (this rearrangement is described in **Fig. 3a**). Second, we find two unrelated deletions, in patients SA268027 and SA269383, that remove one copy of the *IRF2* gene (**Supplementary Fig. 3**). Although *IRF2* is not catalogued in Census nor Intogen, functional studies have identified it as a cancer suppressor gene in HCC³¹. These results provide evidence that this is a mutational mechanism that likely contributes to the development of human HCC.

DISCUSSION

Most cancers are characterized by somatic acquisition of genomic rearrangements during tumour evolution that, eventually, drive the oncogenic process³². These structural aberrations are caused by different mutational mechanisms that generate particular patterns or signatures in the DNA³³. Identification of these mechanisms and their associated patterns is necessary to understand the dynamic processes shaping the cancer genome. Here we described the patterns of a recurrent, quite remarkable mutational mechanism occurring in the early stages of human HCC development whereby HBV DNA integration mediates interchromosomal rearrangements contributing to megabase-size telomeric deletions, which may lead to loss of tumour suppressor genes. Our results demonstrate that the consequences of this mutational mechanism are dramatic for the architecture of HCC genomes and, on occasion, the resulting structural configuration can drive the oncogenic process. We have used two (related) ways of estimating the timing of HBV insertions and their mediated genomic rearrangements, which demonstrate this mutational mechanism is active early during tumour evolution and show that some HBV-mediated rearrangements can occur as early as 21 years before cancer diagnosis. Overall, these data

313 underscore the importance of characterizing liver cancer genomes for patterns of HBV
314 integration, and raise the question about the potential benefit of an earlier antiviral therapy
315 against HBV, in HBV-infected patients, to prevent the acquisition of early driver mutations
316 caused by the virus in the initial stages of liver cancer development. This is particularly relevant
317 for chronic hepatitis B patients in the “immune-tolerant” phase, a stage of the disease
318 characterized by no-fibrosis or minimal fibrosis for which antiviral therapy is not recommended
319 by current patient management guidelines³⁴⁻³⁶. However, levels of HBV replication in this stage
320 are very high^{34,35}, generating the template double-strand linear DNA (dsIDNA)³⁷ required for the
321 somatic acquisition of the HBV DNA insertions and HBV-mediated rearrangements that,
322 eventually, drive the oncogenic process.

MAIN FIGURES

Fig. 1. The landscape of HBV insertions in 286 HCCs from the PCAWG dataset. (a)

Canonical (reciprocal) insertions are represented as red diamonds, and non-canonical insertions (single-clusters) as purple and green triangles for positive and negative clusters, respectively. In total, 148 integration events are shown of which 72% represent non-canonical events. (b) Classical pattern of canonical HBV insertions identified with Illumina paired-end mapping data are characterized by two reciprocal clusters of discordant reads, and clipped reads, in face-to-face orientation, demarcating the boundaries of the genomic integration. The mates of these reads map onto HBV consensus sequences. Clipped reads span the insertion site allowing base-pair resolution of the insertion breakpoints. (c) Most HBV insertion events in HCC tumours show a non-canonical pattern in which a single cluster of paired-end reads (short-reads in red) demarcates one of the two boundaries of the insertion only, while the second cluster is missing.

Fig. 2. Long-read sequencing reveals cryptic HBV-mediated translocations in human HCC.

In HCC SA501453, a hidden interchromosomal rearrangement between chromosomes 11 and 19 is identified using Oxford Nanopore Technologies (ONT). The copy number plot (CN) at the top shows the copy number profiles of the chromosomes involved in the rearrangement (note that the CN plot on chromosome 11 is flipped for illustrative purposes). The Illumina paired-end sequencing data (short-reads in red) shows two single clusters of discordant read pairs, one on 11q and a second on 19p, pointing to HBV insertion events that cannot be bridged due to Illumina library size constraints. The bottom shows four long-reads obtained with ONT that reveals the real configuration of the hidden rearrangement, consisting of a 640 bp HBV DNA

insertion bridging a translocation between 11q and 19p. ONT reads were cut (discontinued) for illustrative purposes. The long-read plot represents the alignment of one ONT read – 24 kb long – to chromosomes 11 and 19 of the human reference genome and to an HBV consensus sequence. The long-reads used to construct the long-read plots are annotated in Supplementary Table 1.

Fig. 3. HBV DNA integration mediates interchromosomal genomic rearrangements that lead to megabase-size telomeric deletions in HCC. (a) In tumour SA529726, two unrelated HBV-mediated interchromosomal rearrangements between chromosomes 3 and X, and between chromosomes 4 and 7, promote 21.2 Mb and 19.8 Mb telomeric deletions on the 3p and the 4q, respectively. The circos plot (left) represents the translocations (purple lines) revealed by ONT data. Single clusters identified with paired-end mapping data are denoted as triangles (green for positive orientation, purple for negative) on the chromosome ideograms. The copy number profiles are shown in yellow below the chromosome ideograms, with relevant telomeric deletions highlighted in red. The long-read plots (right) represent the alignment of one ONT long-read to chromosomes 3 and X, and chromosomes 4 and 7, of the human reference genome and an HBV consensus sequence, which validates the interchromosomal rearrangements mediated by the virus shown in the circos plot. Here, the analysis of the long-reads supporting the HBV events showed an HBV DNA insertion in a classical fragmented and rearranged form^{5,17}. The expected configuration of the rearranged chromosome is shown above each long read plot (the ideograms are for illustrative purpose only); ‘v’ denotes the HBV insertion. The long-reads used to construct the long-read plots are annotated in Supplementary Table 1. (b) Circos plots and chromosome diagrams of similar HBV-mediated non-homologous translocations

promoting megabase-size telomeric deletions in four additional HCC tumours. Again, the expected configuration of the rearranged chromosome is shown next to each circos (the ideograms are for illustrative purpose only). In SA501511, three unrelated HBV-mediated translocations involving different loci on chromosome 8 promote huge deletions involving telomeric regions on chromosomes 13q, 4p and 10p. In SA501424, one HBV insertion bridges a genomic translocation between chromosomes 1 and 11 that generates a terminal deletion at 1p. In SA501481 and SA529830, HBV-mediated translocations generate dicentric chromosomes and promote megabase-size terminal deletions.

Fig. 4. Distribution of HBV insertions across the HCC genome and association with genomic organization features. (a) Bars show the number of HBV insertions per 10-Mb window of the genome. Red bar represents the *TERT* hotspot at chromosome 5p. Other genes (*KMT2B* and *CCNE1*) with high rate of HBV insertion are shown at chromosome 19q. (b) HBV insertions are more frequent in genes than expected by chance (chi-squared test, $P = 0.0035$, $n=91$) and depleted in intergenic regions of the genome (chi-squared test, $P = 0.035$, $n=55$). False Discovery Rate “FDR” correction was applied (see Methods). (c) HBV events at telomeric deletion breakpoints are depleted in regions of the genome exhibiting early replication timing (chi-squared test, $P = 0.037$, $n=4$), while the remaining HBV insertions show the opposite pattern (chi-squared test, $P = 0.013$, $n=34$). “ns” stands for not significant. FDR correction was applied.

Fig. 5. HBV-mediated rearrangements are early clonal events in HCC evolution. (a) In SA269680, an HCC with a whole-genome duplication, HBV insertions are shown in the context

of point mutation burden for that sample. Colored dots above chromosome ideograms represent point mutations with different timing: early clonal (before the whole-genome duplication; green), late clonal (after the whole-genome duplication; purple), clonal (blue), subclonal (red). We identified nine HBV single clusters (black dots with red arrows), all but one catalogued as early clonal events. Five of these early HBV insertions (marked with red asterisks) are associated with megabase-size telomeric deletions (see copy number plots in **Supplementary Fig. 3**). The same sample bears two additional early clonal HBV canonical insertions (black dots with black arrows). Grey blocks below chromosome ideograms represent the copy number profile. MCN stands for Minor Copy Number. **(b)** Real-time timing estimation of HBV insertions along patients' lifetime in samples with whole-genome duplication events. The X axis shows the time interval when – before (green) and after (purple) – the somatic HBV insertions took place relative to the WGD event; thickness and strength of the green and purple bars correlates with the number of events. Black arrows represent when a WGD event took place, and numbers above arrows show the time – in years – before HCC diagnosis when the WGD event has occurred. Numbers within green and purple timelines represent the number of insertion events. Numbers at the end of the timeline represent the age of the patient at diagnosis. **(c)** Copy number plot showing a single cluster that supports an HBV insertion event (red triangle) associated with a 7 Mb telomeric deletion on chromosome 10 in SA501645 that, according to **Fig. 4b**, occurred at least 20.96 years before HCC diagnosis. The gold line represents total chromosome CN, and the grey line is the minor chromosome CN.

Fig. 6. HBV-mediated translocations may lead to loss of tumour suppressor gene *TP53*. In HCC SA529830, a cryptic interchromosomal rearrangement between chromosomes 17 and 8 is

bridged by a 4,829 bp HBV insertion associated with a 14.9 Mb telomeric deletion on chromosome 17 that removes one copy of the tumour suppressor gene *TP53*, and a second 43 Mb telomeric deletion on chromosome 8. Note that the CN plot on chromosome 8 is flipped for illustrative purposes. Single paired-end clusters (short-reads in red) on chromosomes 17 and 8 demarcate the boundaries of both deletions and support the insertion of HBV DNA. One ONT read of 36,845 bp evidences the extent of the rearrangement, whose alignment onto the reference genome – chromosomes 8 and 17 – and to a consensus HBV sequence is shown in the long-read plot. ONT reads were cut (discontinued) for illustrative purposes. The configuration of the rearrangement predicts the formation of a dicentric chromosome (**Fig. 3b**). The long-reads used to construct the long-read plots are annotated in Supplementary Table 1.

Fig. 7. HBV-mediated translocations lead to recurrent loss of tumour suppressor gene *ARID1A*. (a) In HCC tumour SA501481, the Illumina paired-end data (short-reads in red) shows two clusters, one on chromosome 1 and another on chromosome 9, which point to both extremes of an HBV insertion. The copy number (CN) plot at the top shows the total (gold line) and minor (grey line) chromosomes' copy number profiles. The CN plot reveals two telomeric deletions associated with HBV events, one that removes 57.2 Mb on 1p, including one copy of the *ARID1A* tumour suppressor gene, and a second deletion that removes 21.2 Mb on 9p. Note the CN plot from chromosome 1 is flipped for illustrative purposes. The long-read plot shows a 2,688 bp HBV insertion that bridges an interchromosomal rearrangement between chromosomes 1p and 9p. The configuration of the rearrangement predicts the formation of a dicentric chromosome (**Fig. 3b**). (b) A similar scenario, in tumour SA501424, where an HBV DNA insertion induces an interchromosomal translocation between chromosomes 1 and 11. The

Illumina paired-end data (short-reads in red) shows two single clusters, one on chromosome 1 and another on chromosome 11, which point to both extremes of an HBV insertion. The CN plot at the top reveals a 31.5 Mb telomeric deletion on 1p associated with the HBV insertion event (note that the CN plot from chromosome 1 is flipped for illustrative purposes). Here, the associated telomeric deletion on chromosome 1 removes one copy of tumour suppressor gene *ARID1A*. The long-read alignment plot demonstrates an interchromosomal rearrangement between chromosomes 1 and 11 mediated by an HBV insertion. The long-reads used to construct the long-read plots are annotated in Supplementary Table 1.

METHODS

Materials and Methods

Sequencing dataset and DNA sources

We analyzed Illumina whole-genome paired-end sequencing reads, 100-150 bp long, from 286 hepatocellular carcinoma (HCC) tumours and their matched-normal samples from the Pan-Cancer Analysis of Whole Genomes (PCAWG) project dataset³⁸. The tumour specimens consisted of a fresh frozen sample, while the normal specimens consisted of a blood sample. The average coverage was 44 reads per bp for tumour samples and 33 reads per bp for matched normal samples. BWA-mem algorithm v0.78.8-r455³⁹ was used to align sequencing reads to human reference genome build GRD37, version hs37d5. Tumour's DNA for additional long-read sequencing and FISH were transferred from the HCC tumours collection at RIKEN (Japan) within the framework of the International Cancer Genome Consortium (ICGC). The ethics oversight for the PCAWG protocol was undertaken by the TCGA Program Office and the Ethics

and Governance Committee of the ICGC. Each individual ICGC and TCGA project that contributed to the PCAWG dataset had their own local arrangements for ethics oversight and regulatory alignment. We performed additional whole-genome sequencing with Oxford Nanopore (ONT) on nine HCC tumours from the PCAWG project (all are ICGC tumours only). The tumour specimens consisted of a fresh frozen sample. The average ONT sequencing coverage was 8 reads per bp (range 3.83-13.97), and the mean read length was 7.9 kb. All tumours sequenced and analysed in this project have written informed consent for the usage of DNA samples for whole-genome sequencing (for either short-reads or long-reads) and for publication of their genomic data. All patients agreed to participate in the ICGC study and provided informed consent following ICGC guidelines.

Detection of viral insertions using v-TraFiC

v-TraFiC represents a modified version of former algorithm TraFiC⁴⁰, for the identification of somatic insertion events of viral DNA using paired-end sequencing data in three main steps: (i) selection of candidate reads; (ii) reads clustering; and (iii) identification of viral DNA events.

1) Candidate reads selection

v-TraFiC (v0.23) identifies reads from BWA-mem mapping that are likely to provide information pertaining to viral DNA site inclusion. Two different read-pair types are considered for the identification of viral insertions, named SINGLE_END (i.e., one end of the pair – called anchor is mapped onto the reference genome while the other is unmapped), and ABERRANT (i.e., both reads of the pair are improperly mapped to a chromosome, where the read with the highest MAPQ is considered the anchor). In both cases, the anchor's MAPQ must be higher than zero, its mapping pattern must not be 'soft clipping–alignment match–soft clipping' (i.e., CIGAR

481 string must not be #S#M#S, where # represents the number of nucleotides), and must not map
482 onto decoy sequences, mitochondrial DNA or Y chromosome. In addition, the pair is also
483 excluded if any of the reads is not a primary alignment, fails platform/vendor quality checks, or
484 is PCR or optical duplicate. Non-anchor reads must not contain unsequenced nucleotides ('N')
485 and MAPQ of non-anchor ABERRANT reads must be < 20 . The algorithm dustmasker v2.6.0⁴¹
486 is used to identify non-anchor read-pairs containing low complexity sequences, which are later
487 discarded.

488 2) Clustering

489 Anchor reads are clustered together if (i) they share the same orientation, and (ii) the distance
490 relative to the nearest mapped read of the same cluster is ≤ 200 bp. Two main cluster categories
491 are defined, namely POSITIVE and NEGATIVE (i.e., anchor reads are mapped onto the positive
492 and negative strand, respectively). A preliminary range of genome coordinates is associated with
493 each single cluster – final breakpoint coordinates are refined in a later step –. Ranges are defined
494 by a lower (left) coordinate (P_L_POS and N_L_POS, respectively for positive and negative
495 clusters) and an upper (right) coordinate (P_R_POS and N_R_POS, for positive and negative).
496 Only clusters consisting of ≥ 4 supporting reads are considered for further analysis. To avoid
497 miscalls due to alignments in complex regions, the full set of reads mapping within cluster
498 coordinates [P_L_POS, N_R_POS] are further analysed, and clusters are removed if: (i) the
499 proportion of reads with $\text{MAPQ} \leq 10$ relative to the total reads mapped within cluster boundaries
500 represents > 0.3 (30%), and/or (ii) the proportion of reads with CIGAR string #S#M#S relative to
501 the total reads mapped within cluster boundaries represents < 0.15 (15%). Clusters in the tumour
502 are removed if a syntenic cluster in the matched-normal sample is detected with the same
503 orientation and mapping the same locus less than 500 bp away. Finally, one positive and one

negative clusters are reciprocal if $P_R_POS \geq N_L_POS$ and $abs(N_L_POS - N_R_POS) \leq 350$ bp, otherwise clusters are catalogued as single (or independent).

3) Identification of viral DNA events

Non-anchored reads from each cluster were de novo assembled using Velvet v1.2.10⁴², and contigs were used as queries of BLAST v2.6.0 searches against the RVDB Reference viral database⁴³ v12.2 containing 2,467,269 viral DNA sequences, of which 91,455 correspond to human Hepatitis B virus (HBV). Only contigs matching human HBV DNA are considered, and reciprocal clusters pointing to HBV DNA are catalogued as canonical HBV DNA insertion events, while single, independent clusters are catalogued as candidates for aberrant HBV DNA integration events. Finally, we used the algorithm MEIBA v.0.8.8¹⁵, to identify and reconstruct HBV DNA insertion breakpoints to base-pair resolution, with the following non-default parameters: ‘Maximum number of clipped read clusters in the insertion region’ = 20 (default = 10), and ‘Window size to search for clipped read clusters from discordant read-pair clusters ends’ = 100 bp (default = 50 bp).

Identification of HBV-mediated translocations and validation of v-TraFiC calls using single-molecule sequencing with Oxford Nanopore

We performed long-read whole-genome sequencing with Oxford Nanopore Technologies (ONT) on nine native HCC tumours with relevant HBV DNA insertion events (i.e., SA501491, SA529726, SA529759, SA529830, SA501424, SA501453, SA501481, SA501511, SA501534). Libraries were constructed using the Oxford Nanopore Sequencing 1D ligation library preparation kit (SQK-LSK109, Oxford Nanopore Technologies Ltd) according to the manufacturer's protocol, including an initial DNA repair step with NEBNext FFPE DNA Repair

527 Mix (New England BioLabs) and NEBNext Ultra II Ligation Module (New England BioLabs).
528 Two low DNA yield samples (SA529726 and SA501481) were whole-genome amplified using
529 ϕ 29 DNA polymerase (REPLI-g midi kit, Qiagen) prior library construction. Amplified DNA
530 was then digested with t7 endonuclease I (New England BioLabs) for linearization of branched
531 amplicons and deproteinized with Proteinase K (New England BioLabs). Next, unbranched DNA
532 underwent size selection of fragments longer than 20 Kb by means of a Short Read Eliminator
533 buffer (Circulomics) precipitation step and was further purified with Ampure XP Beads
534 (Beckman Coulter Inc). Then, libraries were obtained according the manufacturer's protocol as
535 described above.

536 Sequencing was performed onto MinION R9.4 flowcells (FLO-MIN106 rev-D, Oxford
537 Nanopore Technologies Ltd), controlled by the Oxford Nanopore MinKNOW software
538 v18.12.09 to v19.12.5. Base-calling and post-processing of the ONT raw fast5 files was
539 conducted with ONT software Albacore v2.3.4 or Guppy v2.3.1 to obtain fastq files. Files with
540 quality scores below the recommended values were dropped at this point from further analysis.
541 Reads for each library were then independently mapped to the hs37d5 human reference genome
542 with minimap2 v2.14-r883⁴⁴ and the resulting SAM files were converted to BAM files, sorted
543 and indexed using Samtools v1.7⁴⁴. All partial BAM files were merged, sorted and indexed to
544 the final BAM files.

545 We performed validation of 47 putative somatic HBV insertion events (36 single clusters and 11
546 reciprocal insertions) identified with v-TraFiC in the 9 HCC tumours that were sequenced using
547 Illumina paired-end and ONT long-reads. For each one of the HBV events we interrogated the
548 long-read tumour BAM file to seek for long-reads validating the event. Two types of supporting
549 reads were employed, namely (i) 'spanning-reads', composed of ONT reads completely spanning

the HBV insertion, hence they can be identified as a standard insertion on the reference genome, and (ii) ‘clipped-reads’, composed of ONT reads spanning only one of the HBV insertion ends, hence they get clipped the alignment onto the reference genome. HBV events supported by at least one ONT read were considered true positive events, while those not supported by such reads were considered false positive calls. Overall, we find ~10% (5/47) of false positive events (note that this rate could be overestimated due to low coverage in the ONT data). Spanning-reads were used to identify 11 cryptic translocations.

Copy-number dataset

We analyzed copy number profiles obtained by the PCAWG Working Group 11 (PCAWG-11) using a consensus approach combining six different state-of-the-art copy number calling methods⁴⁵. GC content corrected LogR values were extracted from intermediate Battenberg algorithm results⁴⁶, smoothed using a running median, and transformed into copy number space according to $n = [2^{\log R}(2(1 - \rho) + \psi\rho) - 2(1 - \rho)] / \rho$, where ρ and ψ are the PCAWG-11 consensus tumour purity and ploidy, respectively.

Identification of telomeric deletions associated to HBV insertion events

Single read clusters, identified with v-TraFiC, supporting an HBV insertion event (i.e., clusters of discordant read-pairs – Illumina – with apparently no reciprocal cluster within the proximal 500 bp, and whose mates support a somatic HBV event), were interrogated for the presence of associated telomeric deletions. Briefly, we looked for copy number loss calls from PCAWG (see “Copy number dataset” above) where: (i) the copy number loss extends from the HBV insertion breakpoint up to the end of the chromosomal arm, involving the telomere, and (ii) one

independent cluster, which supports the integration of the HBV event, unequivocally demarcates the copy number loss boundary. We used MEIBA v0.8.8¹⁵ to reconstruct the relevant insertion breakpoint.

HBV rate across genomic features

We analysed the frequency of HBV events across genomic features. Chi-squared was employed to test significance. For each test, we randomly generated in-silico HBV insertions in the genome, as many as observed in real dataset, with the regioneR package v1.22.0⁴⁷. In cases where chi-squared test assumptions were not met due to the low number of observations (i.e., cells with less than five observations), the chi-squared test was not performed. False Discovery Rate (FDR) correction was applied. Chromatin segments: We used the ENCODE segmentation of the HepG2 cell line genome into a set of major genome states⁴⁸, considering the following two predicted categories: repressed and transcriptionally active; Replication timing: Replication timing was defined using HepG2 Repli-seq data (GEO:GSM923446). Regions with gene expression signals higher than 70 were defined as early, below 20 were defined as late, and between 20-70 as middle, in the same way described by²⁰; Expression: Transcripts Per Kilobase Million (TPM) from 208 liver tissues⁴⁹ were used to evaluate HBV insertion rate within genes classified according to different gene expression categories in the liver [0-0.5), [0.5-10), [10-10000].

Timing of viral insertions

We have used two (related) ways of estimating the timing of HBV insertions and their mediated telomeric deletions. The first is a relative timing approach, which classifies insertion events as

clonal early/late/NA or subclonal, depending on their allele frequency and the local copy number state. This approach does not look at the whole-genome doubling state of the tumour and only provides timing information relative to a chromosomal gain (if any). The second approach takes this one step further. When a whole-genome duplication has generated the gain of the HBV-derivative chromosome, we can time the gain itself much more precisely by aggregating information from the allele frequencies of small variants across the genome. By focusing on the clock-like mutations, this relative timing can be anchored and turned into a real-time timing.

We employed the SVclone algorithm v0.2.2²³ to obtain the number of reads supporting and non-supporting HBV DNA insertions. To deal with HBV insertions supported by single-read clusters only, a modification of the method was implemented to accept structural variants with only one break-end side as follows: (i) relevant filters were switched off in order to allow insertion events with one breakpoint only to be considered by SVclone; (ii) only two types of reads were extracted from the BAM file: split reads (soft-clipped reads that cross each break-end) and normal reads (reads that cross or span the break-ends but match the reference), being spanning reads removed (read pairs that align either side of the break-ends but match the reference). Read counts from SVclone, together with tumour purity and copy number states, were used as input of MutationTime.R v0.1²³ for the classification of HBV insertions into four different timing categories, namely clonal [early], clonal [late], clonal [NA] or subclonal. Then, real-time estimates for whole-genome duplication (WGD) events, based on CpG>TpG mutations analysis²³, were used to place particular HBV insertion within a chronological time-frame – in years – during a patient's lifespan, depending on whether mutations occurred before or after a WGD event.

Probe synthesis and fluorescence in situ hybridization

Two sets of bacterial artificial chromosome (BAC) clones (RP5-1125N11 and RP11-891N16 for t(1;11); and RP11-125F4 and RP11-652N13 for t(8;17)) were obtained from the BACPAC Resources Center (<https://bacpacresources.org/>) to develop two-color single-fusion FISH probes to detect chromosome translocations. *ARID1A* deletion probe was developed with RP5-696E2 and RP11-372B18 BAC clones, and Metasystems #D-5103-100-OG probe was used to study *TP53* gene deletion. RP5-1125N11, RP11-125F4, and RP5-696E2 BACs were labelled with Spectrum-Orange, and RP11-891N16, RP11-652N13 and RP11-372B18 with Spectrum-Green. FISH analyses were performed using the Histology FISH Accessory Kit (DAKO) following the manufacturer's instructions (PMID: 25798834 DOI: 10.1038/onc.2015.70) on 5mm TMA sections mounted on positively charged slides (Thermo Scientific). Briefly, the slides were first deparaffined in xylene and rehydrated in a series of ethanol. Slides were pre-treated in 2-[N-morpholino]ethanesulphonic acid (MES), followed by a 30 min protein digestion performed on proteinase-K solution. After dehydration, the samples were denatured in the presence of the specific probe at 73°C for 5 min and left overnight for hybridization at 37°C. Finally, the slides were washed with 20×SSC (saline-sodium citrate) buffer with detergent Tween-20 at 63°C, and mounted on fluorescence mounting medium (DAPI in antifade solution). Cells were imaged with a Leica DM 5500B fluorescence microscope equipped with a 100x oil-immersion objective, Leica DM DAPI, Green and Orange fluorescence filter cubes and a CCD camera (Photometrics SenSys camera) connected to a PC running the Zytovision image analysis system (Applied Imaging Ltd., UK) with Z stack software v7.4. The z-stack images were manually scored by two independent investigators by counting the number of co-localized signals, representing fused transcripts, or missing signals, representing deletions, all over the tissue.

642

643 ACKNOWLEDGEMENTS

644 We thank the Supercomputing Centre of Galicia (CESGA) for providing complementary
645 computational resources. **Funding:** J.M.C.T is supported by European Research Council Grant
646 ERC-2016-STG – ERC Starting Grant 716290, and Ministerio de Ciencia, Innovación y
647 Universidades, Grants PGC2018-102245-B-100 and RYC-2014-14999. E.G.A. and P.O. are
648 supported by Ministerio de Educacion Cultura y Deporte, fellowships FPU17/05396 and
649 FPU18/03421, respectively. B.R-M. and S.Z. are supported by Xunta de Galicia fellowships
650 ED481A-2016/151, ED481A-2017/306, and ED481A-2018/199, respectively. This work was
651 supported by the Francis Crick Institute, which receives its core funding from Cancer Research
652 UK (FC001202), the UK Medical Research Council (FC001202), and the Wellcome Trust
653 (FC001202). For the purpose of Open Access, the authors have applied a CC BY public
654 copyright licence to any Author Accepted Manuscript version arising from this submission. This
655 project was enabled through access to the MRC eMedLab Medical Bioinformatics infrastructure,
656 supported by the Medical Research Council (grant number MR/L016311/1). M.M-F. is funded
657 by the Spanish Association for Cancer Research (AECC, grant207mart). S.R-P. is supported by
658 grants from the Spanish National Research and Development Plan, Instituto de Salud Carlos III,
659 and FEDER (PI17/02303, PI20/01837 and DTS19/00111); AEI/MICIU EXPLORA Project
660 BIO2017-91272-EXP and AECC_Lab_2020 Project (Asociación Española Contra el Cáncer).
661 J.R-B. is supported by a Rio Hortega Fellowship from the Institute of Health Carlos III
662 (CM19/00087) and a 2020 TTD Research Grant from the Spanish Cooperative Group for the
663 Teatment of Digestive Tumours (TTD). J.D. is a postdoctoral fellow of the Research Foundation
664 – Flanders (FWO). PVL is a Winton Group Leader in recognition of the Winton Charitable

Foundation's support towards the establishment of The Francis Crick Institute. AD-L is supported by a Juan Rodés contract from ISCIII (JR17/00016). AR-C is supported by GAIN and Xunta de Galicia (IN853B 2018/03).

AUTHOR CONTRIBUTIONS

E.G.A., P.V.L., P.J.C., J.M.C.T. conceived the study. E.G.A., J.D., Y.L., J.T., J.Z., C.J., A.B-O., B.R-M., K.H., S.Z., K.R., A.P.B., K.M., I.O., P.V.L., J.M.C.T. contributed to pipelines and/or data management. E.G.A., J.D., J.Z., Y.L., C.J., M.T., K.H., P.V.L., P.J.C., J.M.C.T. analyzed the sequencing data. J.R-B, E.G.A., P.O., M.M-F., J.M.C.T. analysed the clinical data. J.T., D.G-S., A.P., J.R-C, A. R-C., A. D-L. performed sequencing. M.T., M.M-F., R.A., A.O., A.L.B. performed laboratory experiments. M.T., H.N., X.F., S.P.P, A.O., H.A., K.C., M.U., S.H., H.Y. provided tumour specimens and/or performed pathological diagnosis. D.G-S., U.G., C.R., M.G-B., J.M.C.T. build a comprehensive biological model for HBV-mediated rearrangements. R.T., S.R.P. performed cytogenetics. E.G.A., P.V.L., P.J.C., J.M.C.T. wrote the manuscript with assistance from all authors. M.T., P.V.L., P.J.C., J.M.C.T. supervised the project.

COMPETING INTERESTS

The authors declare no competing interests.

CODE AVAILABILITY

A preliminary version of the code v-TraFiC for the identification of somatic HBV insertions, is available at <http://gitlab.com/mobilegenomesgroup/v-TraFiC>.

DATA AVAILABILITY

All genomic datasets generated for this manuscript were deposited in public databases. Somatic and germline variant calls, mutational signatures, subclonal reconstructions, transcript abundance, splice calls and other core data generated by the ICGC/TCGA Pan-cancer Analysis of Whole Genomes Consortium¹³ are available at <https://dcc.icgc.org/releases/PCAWG>. Additional information on accessing the data, including Illumina and ONT sequencing raw read files, can be found at <https://docs.icgc.org/pcawg/data/>. In accordance with the data access policies of the ICGC and TCGA projects, most molecular, clinical and specimen data are in an open tier, which does not require access approval. To access potentially identifying information, such as germline alleles and underlying sequencing data, researchers will need to apply to the TCGA Data Access Committee (DAC) via dbGaP for access to the TCGA portion of the dataset, and to the ICGC Data Access Compliance Office (DACO) for the ICGC portion. In addition, to access somatic SNVs derived from TCGA donors, researchers will also need to obtain dbGaP authorization.

The analyses in this paper used a number of datasets that were derived from the raw sequencing data and variant calls. The individual datasets are available at Synapse (<https://www.synapse.org/>), which are also mirrored at DCC portal (<https://dcc.icgc.org>). Donor clinical data, tumor histopathology, consensus CNA, consensus SV calls, driver mutational events, purity and ploidy, timed copy number segments and real time inferences of MRCA and WGD are available at Synapse with the following accession numbers: syn10389158,

syn1038916, syn8042988, syn7596712, syn11639581, syn8272483, syn14778989 and
syn14778990, respectively. Hyperlinks are show below.

syn10389158 [<https://www.synapse.org/#!/Synapse:syn10389158>]

syn1038916 [<https://www.synapse.org/#!/Synapse:syn1038916>]

syn8042988 [<https://www.synapse.org/#!/Synapse:syn8042988>]

syn7596712 [<https://www.synapse.org/#!/Synapse:syn7596712>]

syn11639581 [<https://www.synapse.org/#!/Synapse:syn11639581>]

syn8272483 [<https://www.synapse.org/#!/Synapse:syn8272483>]

syn14778989 [<https://www.synapse.org/#!/Synapse:syn14778989>]

syn14778990 [<https://www.synapse.org/#!/Synapse:syn14778990>]

REFERENCES

1. Bray, F. *et al.* Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J Clin* **68**, 394-424 (2018).
2. Midorikawa, Y. *et al.* Molecular karyotyping of human hepatocellular carcinoma using single-nucleotide polymorphism arrays. *Oncogene* **25**, 5581-90 (2006).
3. Wong, N. *et al.* A comprehensive karyotypic study on human hepatocellular carcinoma by spectral karyotyping. *Hepatology* **32**, 1060-8 (2000).
4. Meyer, M., Wiedorn, K.H., Hofschneider, P.H., Koshy, R. & Caselmann, W.H. A chromosome 17:7 translocation is associated with a hepatitis B virus DNA integration in human hepatocellular carcinoma DNA. *Hepatology* **15**, 665-71 (1992).
5. Meng, G. *et al.* TSD: A Computational Tool To Study the Complex Structural Variants Using PacBio Targeted Sequencing Data. *G3 (Bethesda)* **9**, 1371-1376 (2019).
6. Wang, Y. *et al.* Characterization of HBV integrants in 14 hepatocellular carcinomas: association of truncated X gene and hepatocellular carcinogenesis. *Oncogene* **23**, 142-8 (2004).
7. Rogler, C.E. *et al.* Deletion in chromosome 11p associated with a hepatitis B integration site in hepatocellular carcinoma. *Science* **230**, 319-22 (1985).

- 740 8. Pineau, P. *et al.* A t(3;8) chromosomal translocation associated with hepatitis B virus
741 intergration involves the carboxypeptidase N locus. *J Virol* **70**, 7280-4 (1996).
- 742 9. Jiang, Z. *et al.* The effects of hepatitis B virus integration into the genomes of
743 hepatocellular carcinoma patients. *Genome Res* **22**, 593-601 (2012).
- 744 10. Peneau, C. *et al.* Hepatitis B virus integrations promote local and distant oncogenic driver
745 alterations in hepatocellular carcinoma. *Gut* (2021).
- 746 11. Fujimoto, A. *et al.* Whole-genome mutational landscape and characterization of
747 noncoding and structural mutations in liver cancer. *Nat Genet* **48**, 500-9 (2016).
- 748 12. Tu, T., Budzinska, M.A., Shackel, N.A. & Urban, S. HBV DNA Integration: Molecular
749 Mechanisms and Clinical Implications. *Viruses* **9**(2017).
- 750 13. Consortium, I.T.P.-C.A.o.W.G. Pan-cancer analysis of whole genomes. *Nature* **578**, 82-
751 93 (2020).
- 752 14. Zapatka, M. *et al.* The landscape of viral associations in human cancers. *Nat Genet* **52**,
753 320-330 (2020).
- 754 15. Rodriguez-Martin, B. *et al.* Pan-cancer analysis of whole genomes identifies driver
755 rearrangements promoted by LINE-1 retrotransposition. *Nat Genet* **52**, 306-319 (2020).
- 756 16. Li, Y. *et al.* Patterns of somatic structural variation in human cancer genomes. *Nature*
757 **578**, 112-121 (2020).
- 758 17. Nagaya, T. *et al.* The mode of hepatitis B virus DNA integration in chromosomes of
759 human hepatocellular carcinoma. *Genes Dev* **1**, 773-82 (1987).
- 760 18. Li, Y. *et al.* Constitutional and somatic rearrangement of chromosome 21 in acute
761 lymphoblastic leukaemia. *Nature* **508**, 98-102 (2014).
- 762 19. Sullivan, B.A. & Willard, H.F. Stable dicentric X chromosomes with two functional
763 centromeres. *Nat Genet* **20**, 227-8 (1998).
- 764 20. Furuta, M. *et al.* Characterization of HBV integration patterns and timing in liver cancer
765 and HBV-infected livers. *Oncotarget* **9**, 25075-25088 (2018).
- 766 21. Sung, W.K. *et al.* Genome-wide survey of recurrent HBV integration in hepatocellular
767 carcinoma. *Nat Genet* **44**, 765-9 (2012).
- 768 22. Scully, R., Panday, A., Elango, R. & Willis, N.A. DNA double-strand break repair-
769 pathway choice in somatic mammalian cells. *Nat Rev Mol Cell Biol* **20**, 698-714 (2019).
- 770 23. Gerstung, M. *et al.* The evolutionary history of 2,658 cancers. *bioRxiv* (2018).
- 771 24. Jolly, C. & Van Loo, P. Timing somatic events in the evolution of cancer. *Genome Biol*
772 **19**, 95 (2018).
- 773 25. Alexandrov, L.B. *et al.* Clock-like mutational processes in human somatic cells. *Nat*
774 *Genet* **47**, 1402-7 (2015).
- 775 26. Jordan, J.J. *et al.* Altered-function p53 missense mutations identified in breast cancers
776 can have subtle effects on transactivation. *Mol Cancer Res* **8**, 701-16 (2010).
- 777 27. Tate, J.G. *et al.* COSMIC: the Catalogue Of Somatic Mutations In Cancer. *Nucleic Acids*
778 *Res* **47**, D941-D947 (2019).
- 779 28. Sun, X. *et al.* Arid1a Has Context-Dependent Oncogenic and Tumor Suppressor
780 Functions in Liver Cancer. *Cancer Cell* **32**, 574-589 e6 (2017).
- 781 29. Sondka, Z. *et al.* The COSMIC Cancer Gene Census: describing genetic dysfunction
782 across all human cancers. *Nat Rev Cancer* **18**, 696-705 (2018).
- 783 30. Martinez-Jimenez, F. *et al.* A compendium of mutational cancer driver genes. *Nat Rev*
784 *Cancer* **20**, 555-572 (2020).

31. Amaddeo, G., Guichard, C., Imbeaud, S. & Zucman-Rossi, J. Next-generation sequencing identified new oncogenes and tumor suppressor genes in human hepatic tumors. *Oncoimmunology* **1**, 1612-1613 (2012).
32. Stratton, M.R., Campbell, P.J. & Futreal, P.A. The cancer genome. *Nature* **458**, 719-24 (2009).
33. Li, Y. *et al.* Patterns of structural variation in human cancer. *bioRxiv* (2017).
34. Lok, A.S., Zoulim, F., Dusheiko, G. & Ghany, M.G. Hepatitis B cure: From discovery to regulatory approval. *J Hepatol* **67**, 847-861 (2017).
35. Fanning, G.C., Zoulim, F., Hou, J. & Bertoletti, A. Therapeutic strategies for hepatitis B virus infection: towards a cure. *Nat Rev Drug Discov* **18**, 827-844 (2019).
36. European Association for the Study of the Liver. Electronic address, e.e.e. & European Association for the Study of the, L. EASL 2017 Clinical Practice Guidelines on the management of hepatitis B virus infection. *J Hepatol* **67**, 370-398 (2017).
37. Yang, W. & Summers, J. Integration of hepadnavirus DNA in infected liver: evidence for a linear precursor. *J Virol* **73**, 9710-7 (1999).
38. Campbell, P.J., Getz, G., Stuart, J.M., Korb, J.O. & Stein, L.D. Pan-cancer analysis of whole genomes. *bioRxiv* (2017).
39. Li, H. & Durbin, R. Fast and accurate long-read alignment with Burrows-Wheeler transform. *Bioinformatics* **26**, 589-95 (2010).
40. Tubio, J.M. *et al.* Mobile DNA in cancer. Extensive transduction of nonrepetitive DNA mediated by L1 retrotransposition in cancer genomes. *Science* **345**, 1251343 (2014).
41. Morgulis, A., Gertz, E.M., Schaffer, A.A. & Agarwala, R. A fast and symmetric DUST implementation to mask low-complexity DNA sequences. *J Comput Biol* **13**, 1028-40 (2006).
42. Zerbino, D.R. & Birney, E. Velvet: algorithms for de novo short read assembly using de Bruijn graphs. *Genome Res* **18**, 821-9 (2008).
43. Goodacre, N., Aljanahi, A., Nandakumar, S., Mikailov, M. & Khan, A.S. A Reference Viral Database (RVDB) To Enhance Bioinformatics Analysis of High-Throughput Sequencing for Novel Virus Detection. *mSphere* **3**(2018).
44. Li, H. *et al.* The Sequence Alignment/Map format and SAMtools. *Bioinformatics* **25**, 2078-9 (2009).
45. D'Amico, S.C. *et al.* Portraits of genetic intra-tumour heterogeneity and subclonal selection across cancer types. *bioRxiv* (2018).
46. Nik-Zainal, S. *et al.* The life history of 21 breast cancers. *Cell* **149**, 994-1007 (2012).
47. Gel, B. *et al.* regioneR: an R/Bioconductor package for the association analysis of genomic regions based on permutation tests. *Bioinformatics* **32**, 289-91 (2016).
48. Consortium, E.P. An integrated encyclopedia of DNA elements in the human genome. *Nature* **489**, 57-74 (2012).
49. Consortium, G.T. The GTEx Consortium atlas of genetic regulatory effects across human tissues. *Science* **369**, 1318-1330 (2020).

Figure 1

a

b

c

Figure 2

SA501453

Figure 3

Figure 4**a** **b** **c**

Figure 5

a

b

c

Figure 6

Figure 7