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***Basic Study***

**Re-analysis of hepatitis B virus integration sites reveals potential new loci associated with oncogenesis in hepatocellular carcinoma**

Kojima R *et al*. Re-analysis of HBV integration sites

Ryuta Kojima, Shingo Nakamoto, Tadayoshi Kogure, Yaojia Ma, Keita Ogawa, Terunao Iwanaga, Na Qiang, Junjie Ao, Ryo Nakagawa, Ryosuke Muroyama, Masato Nakamura, Tetsuhiro Chiba, Jun Kato, Naoya Kato

**Ryuta Kojima, Shingo Nakamoto, Tadayoshi Kogure, Yaojia Ma, Keita Ogawa, Terunao Iwanaga, Na Qiang, Junjie Ao, Ryo Nakagawa, Ryosuke Muroyama, Masato Nakamura, Tetsuhiro Chiba, Jun Kato, Naoya Kato,** Department ofGastroenterology, Graduate School of Medicine, Chiba University, Chiba 260-8670, Japan

**Author contributions:** Kojima R and Nakamoto S contributed to the conception, design, and writing of the manuscript; Kojima R contributed to data management and analysis; Kogure T, Ma Y, Ogawa K, Iwanaga T, Qiang N, Ao J, Nakagawa R, Muroyama R, Nakamura M, Chiba T, Kato J, and Kato N contributed to manuscript review and editing; Kato N contributed to the project administration.

**Corresponding author: Shingo Nakamoto, MD, PhD, Assistant Professor,** Department of Gastroenterology, Graduate School of Medicine, Chiba University, Inohana 1-8-1, Chuo-ku, Chiba 260-8670, Japan. nakamotoer@faculty.chiba-u.jp

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**Abstract**

BACKGROUND

Hepatitis B virus (HBV) is a major cause of hepatocellular carcinoma (HCC). HBV DNA can get integrated into the hepatocyte genome to promote carcinogenesis. However, the precise mechanism by which the integrated HBV genome promotes HCC has not been elucidated.

AIM

To analyze the features of HBV integration in HCC using a new reference database and integration detection method.

METHODS

Published data, consisting of 426 Liver tumor samples and 426 paired adjacent non-tumor samples, were re-analyzed to identify the integration sites. Genome Reference Consortium Human Build 38 (GRCh38) and Telomere-to-Telomere Consortium CHM13 (T2T-CHM13 (v2.0) were used as the human reference genomes. In contrast, human genome 19 (hg19) was used in the original study. In addition, GRIDSS VIRUSBreakend was used to detect HBV integration sites, whereas high-throughput viral integration detection (HIVID) was applied in the original study (HIVID-hg19).

RESULTS

A total of 5361 integration sites were detected using T2T-CHM13. In the tumor samples, integration hotspots in the cancer driver genes, such as *TERT* and *KMT2B*, were consistent with those in the original study. GRIDSS VIRUSBreakend detected integrations in more samples than by HIVID-hg19. Enrichment of integration was observed at chromosome 11q13.3, including the *CCND1* promoter, in tumor samples. Recurrent integration sites were observed in mitochondrial genes.

CONCLUSION

GRIDSS VIRUSBreakend using T2T-CHM13 is accurate and sensitive in detecting HBV integration. Re-analysis provides new insights into the regions of HBV integration and their potential roles in HCC development.

**Key Words:** Carcinoma; Hepatocellular; Hepatitis B virus; Virus Integration

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**Core Tip:** To understand the role of hepatitis B virus (HBV) in hepatocellular carcinoma (HCC) development, we re-analyzed HBV integration sites using publicly available data. We found that chromosome 11q13.3 is a frequently observed HBV integration site. This region contains important cancer driver genes, such as *CCND1* and *FGF19*, which are amplified in HCC. This finding supports a mechanism of carcinogenesis promoted by HBV-induced genomic instability in the liver and provides insights into treating a subset of liver cancers.

**INTRODUCTION**

The hepatitis B virus (HBV) is a major cause of hepatocellular carcinoma (HCC). When HBV infects liver cells, HBV DNA can be integrated into the human genome. Integration events typically occur during the early stages of an infection[1,2], and are known to promote carcinogenesis *via* several mechanisms: (1) Increasing the expression levels of neighboring genes; (2) induction of genomic instability and somatic copy number alterations of genes; (3) deletion of tumor suppressor genes through structural mutations[3]; and (4) inducing expression of HBV X protein (HBx) or HBx fusion proteins that contribute to carcinogenesis.

To investigate the effect of HBV on hepatocarcinogenesis, several studies have been conducted using next-generation sequencing technology to identify integration sites of HBV DNA. Examples of such technologies include whole genome sequencing[4] and HBV capture sequencing[5]. These studies revealed frequent integration into the promoter regions of *TERT* and *KMT2B* in tumor tissues and *FN1* in normal tissues. In an examination of an HBV-infected human-hepatocyte chimeric mouse model, mitochondrial DNA (mtDNA) was thought to be a frequent site of integration[1]. A European study reported a lower frequency of *KMT2B* insertion and a higher frequency of integration into *ADH* genes in normal tissues[6].

Most previous studies have used Genome Reference Consortium Human Build 37 (GRCh37) or human genome 19 (hg19) as the reference genomes. In GRCh37/hg19 and Genome Reference Consortium Human Build 38 (GRCh38)[7], tandem repeats, microsatellites, and minisatellites found in telomeres and centromeres remained unresolved. The complete human genome sequence, Telomere-to-Telomere Consortium CHM13 (T2T-CHM13 (v2.0)[8], was released in 2022.

Various methods have been used to detect integration breakpoints. High-throughput viral integration detection (HIVID), a detection method based on a pair-read assembly strategy[9], was applied in the analysis of 426 HCC cases[5]. GRIDSS is a multithreaded structural variant caller from a combination of assembly, split read, and read pair support[10]. VIRUSBreakend utilizes a virus-centric variant calling and assembly approach to identify viral integrations with high sensitivity and low false discovery rate, allowing the identification of integrations in repetitive host regions[11].

Here, we report new features observed by re-analyzing the published data using GridSS VIRUSBreakend based on GRCh38 and T2T-CHM13.

**MATERIALS AND METHODS**

Sequence data were obtained from the Sequence Read Archive (SRA) with accession number SRA335342[5]. The dataset consisted of 426 tumor samples and 426 paired adjacent non-tumor samples.

All reads in the dataset were aligned to the GRCh38 and T2T-CHM13 reference genomes using bwa\_mem2[12,13]. VIRUSBreakend was used to detect integration sites (Supplementary Figure 1), and the analysis was performed using Nextflow[14] on Amazon Web Service. HBV integration sites were detected using GRIDSS VIRUSBreakend[11]. Integration sites were compared with the count of fragments providing breakend for the variant allele (BVF) in the variant call format specification file. Statistical analysis and visualization were performed using R software, and statistical significance was set at *P* < 0.05.

**RESULTS**

***Comparison of HBV integration sites***

In total, 5361 and 5198 integration breakpoints were detected with T2T-CHM13 and GRCh38, respectively. The breakpoints were similar between the references using GRCh38 and T2T-CHM13 (Figure 1A and B). Consistent with previous studies, integration breakpoints were enriched in the *TERT* promoter region in tumor samples. In contrast, integration into *FN1* was frequently observed in non-tumor samples.

Compared with the original study, our analysis detected integrations in more samples (357 *vs* 328 in tumors; 288 *vs* 160 in non-tumors) (Table 1). In addition, we detected integration in the *TERT* region in 105 tumor samples, whereas the original study observed integration in 95 tumor samples (Table 2). In contrast, the number of breakpoints detected in tumors was lower than that in the original study (Table 1). In our study, only breakpoints validated by VIRUSBreakend were counted (Supplementary Figure 2). Integration of *DDX11L* was frequently detected in the original study, but no integration breakpoints were detected in our study (Table 2). The *DDX11L* gene family is frequently detected as a target for integration using a capture sequencing approach, but it is possible that fragments were mapped incorrectly owing to repetitive sequences[15,16]. In the non-tumor samples, our study detected more integrations, both in the number of samples and breakpoints. For example, we detected 97 integration breakpoints in the *FN1* gene from 56 non-tumor samples. The earlier analysis detected only 19 breakpoints from 17 non-tumor samples (Table 2). Few oncogenic regions were affected in the non-tumor samples. Breakpoints were most frequent around direct repeat 1 of the HBV genome (Figure 1C).

***Chromosome 11q13.3 is a frequent site for HBV integration***

When the chromosome region was explored, we found that the integration breakpoint at 11q13.3 was enriched with T2T-CHM13 and GRCh38 (Figures 2A-C). Breakpoints at 11q13.3 were more frequent in the tumor samples than in the non-tumor samples (16 (3.8%) of tumor samples compared to 1 (0.02%) of non-tumor samples, Figure 2B). 11q13.3 is characterized by the evolutionarily well-conserved genes *CCND1*, *FGF19*, *FGF4*, and *FGF3*[17], where copy number amplification frequently occurs in tumors (Figure 2D)[18,19]. Some breakpoints were within the genic and promoter regions of the genes, including *CCND1* and *FGF4*. Integration appeared to be distributed more in the non-genic regions (Figure 2B). When fragments from the integration site were counted using BVF, the values were higher in tumor samples than in non-tumor samples (Figure 2E). High BVF value formed a peak in the 11q13.3 in addition to the peak in the *TERT*, *KMT2B*, and *CCNE1* genes in the tumor samples (Figure 2E and Supplementary Figure 3).

***Mitochondrial DNA has sites where HBV DNA is frequently integrated***

There is some debate regarding whether mtDNA is a frequent site of HBV integration. A study using a mouse model by Furuta *et al*[1] found that mtDNA was frequently integrated early in infection. More recently, a preprint suggested that mtDNA is indeed a site for integration[20,21]. Although the original paper on which this study was based did not mention integration into mitochondria, we detected many integration breakpoints into mtDNA and identified repeat integration sites (Table 3 and Figure 3)[22]. Integration breakpoints in mtDNA were observed in both tumor and non-tumor samples. Recurrent integration events were observed in *ND4*. Of these, eight events were from non-tumor samples, and two from tumor samples. Microhomologous sequences were observed in some regions. For example, the GCCNTTCTCATC sequence, where N represents any nucleotide or gap, was observed at the junction of the *ND4* gene (Chromosome M:11079) and the HBV genome (HBV:1559). In contrast, the GCTTCACC sequence was observed at the junction of the *ND4* gene (Chromosome M:11104) and the HBV genome (HBV:1590). It is also possible that these integration breakpoints exist in nuclear-mitochondrial segments.

**DISCUSSION**

In this study, GRIDSS VIRUSBreakend, with an updated human reference genome, was used to detect HBV integration using public sequencing data from liver tumor and non-tumor samples. HBV integration was detected in more samples than in the original analysis (Table 1). The difference in methods could account for the discordant results. We investigated an example of HBV integration sites in the *TERT* region detected by GRIDSS VIRUSBreakend, but not in the original study (Supplementary Figures 4 and 5). In the original study, the HIVID pipeline, based on paired-end read assembly, was applied to detect integration[9]. In the sequencing data, some paired-end reads could not be assembled because of the absence of overlapping bases. These reads were also included in our analysis to detect integration sites more accurately. It should be noted that the GRIDSS VIRUSBreakend uses genotype D HBV for viral genome reference, whereas genotype C HBV is dominant in the current dataset, which may affect the sensitivity of virus detection.

We found HBV integration clusters in the 11q13.3 region (Figure 2). Unlike previously known single gene integration sites, such as *TERT* and *KMT2B*, 11q13.3 spans multiple gene regions. Although these clusters can be observed in the supplemental data of the original paper, to our knowledge, it has not been previously mentioned. Enrichment of 11q13.3 was more significant in tumors than in non-tumor tissues. *CCND1*, *FGF19*, *FGF4*, and *FGF3* are located at 11q13.3, where copy number amplification frequently occurs in tumors.

Integration into *CCND1*, located at 11q13.3, is a potential driver event[23], but its frequency is not high. Although recent studies have not detected integration at 11q13.3[1,6], several studies have detected these events only as supplementary data[4,5,24] and they have been reported since 1988[25,26]. According to a study by Bok *et al*[27], the expression levels of cancer-related genes, including *CCND1* and *FGF19*, are elevated near the viral integration site on 11q13.3 in an HCC cell line. HBV integration at this locus may be linked to cancer gene activation, as *FGF19* amplification was associated with chronic HBV infection[28,29].

HBV integration may be associated with copy number alterations[3]. Chromosomal instability often leads to copy number alterations in the short and long arms of the chromosome. However, 11q13.3 causes strong copy number amplification in a localized region in the middle of the chromosome (Figure 2D). Previous results using whole genome sequencing indicated that the integration allele frequency was high in the tumor samples, especially in the recurrent integration in tumors such as *TERT*[4]. By comparing fragment counts from the integration site using BVF, the values were found to be higher in the tumor samples than in the non-tumor samples. Some of the integration breakpoints at 11q13.3 had extremely high fragment counts (Figure 2E and Supplementary Figure 3). If BVF correlates with the integration allele frequency, it is possible that these events reflect the clonal expansion of tumors with integration breakpoints or the amplification of integrated genes. *CCND1*-*FGF19* amplification occurred at later points in the evolution of HCC[30]. Further research is needed to investigate the relationships between integration, copy number alteration, and cancer gene activation at 11q13.3.

In our analysis, HBV integration in the mtDNA was observed in 2.3% (20/852) of the samples, and the *ND4* gene was a frequent target of HBV integration (Table 3). According to a previous study, HBV integration into mtDNA has occurred in only 0.1% of human clinical liver tissues[1]. Mouse model experiments have suggested that this integration primarily occurs during the early stages of HBV infection through microhomology-mediated end joining[1]. It is also possible that HBV integration occurs in nuclear copies of mtDNA sequences rather than in the mitochondria. Giosa *et al*[21] detected HBV integration in DNA isolated from mitochondria. The D-loop region is the target of HBV integration. Our analysis suggests that *ND4* genes may also be targeted for integration through microhomology-mediated mechanisms.

This study has several limitations. First, the analysis was conducted using existing data and the findings were not validated using independent data. Second, the original data were based on HBV capture sequencing, and gene copy numbers were not available. Finally, the integration data were obtained from short-read sequencing and have not been validated using long-read sequencing data.

**CONCLUSION**

HBV integration in HCC samples has been characterized using the complete human reference. GRIDSS VIRUSBreakend using T2T-CHM13 is accurate and sensitive in detecting HBV integration. HBV frequently integrates at the 11q13.3 region, where the *CCND1* gene is located, and this region is frequently amplified in several types of cancer, including HCC. Further research is needed to examine how HBV integration interacts with driver gene expression and copy number alteration.

**ARTICLE HIGHLIGHTS**

***Research background***

Many hepatitis B virus (HBV)-infected patients suffer from hepatocellular carcinoma (HCC), but a little focus is given to detect HBV integration pattern in the treatment of HCC. Detection of HBV integration can be improved by introducing a reliable detection method.

***Research motivation***

HBV frequently integrates at the 11q13.3 region, where the *CCND1* gene is located, and this region is frequently amplified in several types of cancer, including HCC.

***Research objectives***

We aimed to analyze the features of HBV integration in HCC using a new reference database and integration detection method.

***Research methods***

Published data, consisting of 426 liver tumor samples and 426 paired adjacent non-tumor samples, were re-analyzed to identify the integration sites. Updated human reference genomes, Genome Reference Consortium Human Build 38 (GRCh38), and Telomere-to-Telomere Consortium CHM13 (T2T-CHM13 (v2.0) were used. In addition, GRIDSS VIRUSBreakend, which utilizes a virus-centric variant calling and assembly approach, was used to detect HBV integration sites.

***Research results***

A total of 5361 integration sites were detected using T2T-CHM13. In the tumor samples, integration hotspots in the cancer driver genes, such as *TERT* and *KMT2B*, were consistent with those in the original study. GRIDSS VIRUSBreakend detected integrations in more samples than original analysis. Enrichment of integration was observed at chromosome 11q13.3, including the *CCND1* promoter, in tumor samples. Recurrent integration sites were observed in mitochondrial genes.

***Research conclusions***

GRIDSS VIRUSBreakend using T2T-CHM13 is accurate and sensitive in detecting HBV integration and provides new insights into the regions of HBV integration and their potential roles in HCC development.

***Research perspectives***

Further research is needed to examine how HBV integration interacts with driver gene expression and copy number alteration.

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**Footnotes**

**Institutional review board statement:** This study is not applicable as it is a re-analysis of publicly available data.

**Conflict-of-interest statement:** All the authors have no conflict of interest related to the manuscript.

**Data sharing statement:** All the data supporting this study are stored in the SRA database with accession number SRA335342.

**ARRIVE guidelines statement:** The authors have read the ARRIVE guidelines, and the manuscript was prepared and revised according to the ARRIVE guidelines.

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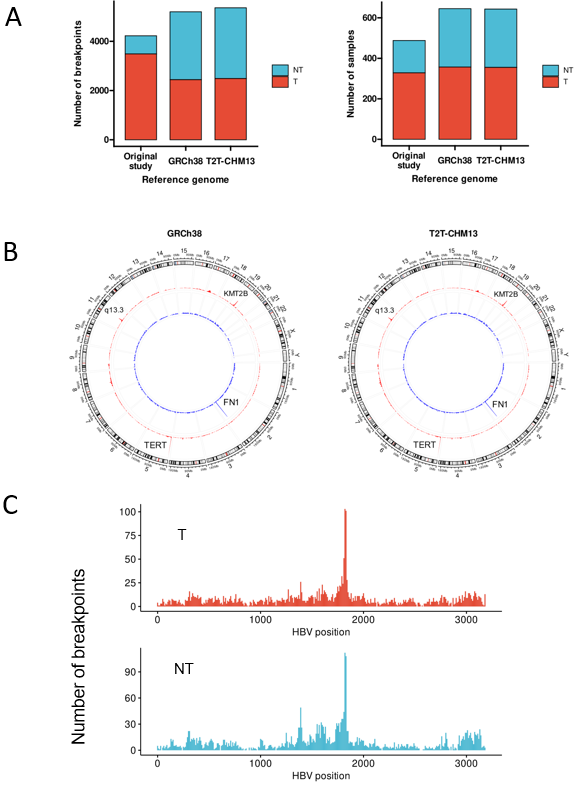
Grade C (Good): C, C

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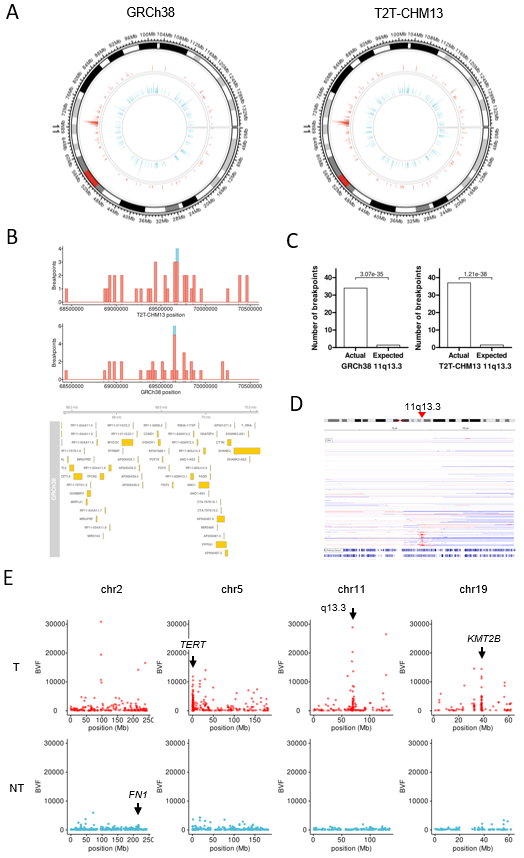
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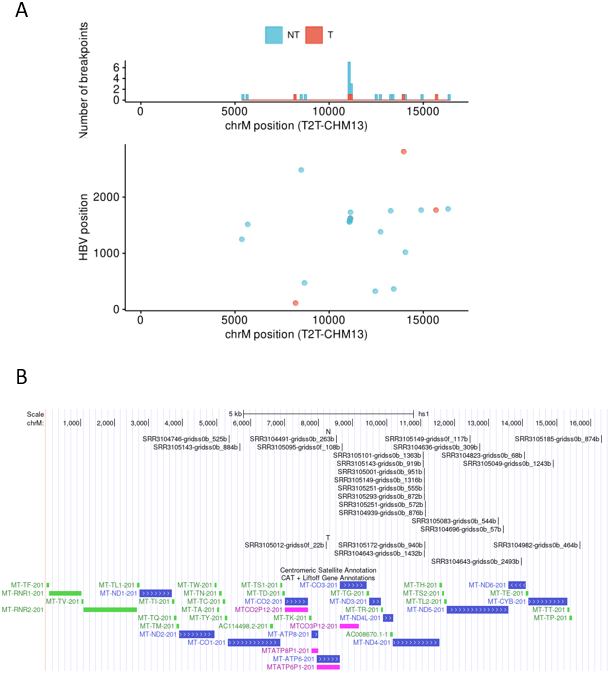
**Figure Legends**



**Figure 1 Hepatitis B virus integration breakpoints across the reference genomes.** A: Integration breakpoints in the human reference genomes in tumor and non-tumor samples; B: Circos plot of integration breakpoints. Red represents tumor samples, and blue represents non-tumor samples; C: Hepatitis B virus genome integration breakpoints. T: Tumor; N: Non-tumor.



**Figure 2 Integration breakpoints at chromosome 11.** A: Circos plot of breakpoints at chromosome 11 in the human reference genomes; B: Integration breakpoints around 11q13.3 in relation to coding genes retrieved from Ensembl. Red represents tumor samples, and blue represents non-tumor samples; C: Comparison of integration breakpoints around 11q13.3 in the tumor samples. Actual represents actual number of integration breakpoints. Expected represents expected number of integration breakpoints assuming random distribution; D: Copy number of liver cancer samples from cBioPortal[18,19]. Red represents amplification, and blue represents deletion. E: Distribution of the number of fragments that provide breakend for the variant allele. T: Tumor; NT: Non-tumor.



**Figure 3 Integration breakpoints in the mitochondrial genome.** A: The upper panel displays integration breakpoints across mitochondrial genomes according to tumor and non-tumor samples, and the lower panel shows integration breakpoints along the human and hepatitis B virus genomes. Red represents tumor samples, and blue represents non-tumor samples; B: Integration breakpoints on the mitochondrial genome annotated using UCSC genome browser (NT: Non-tumor; T: Tumor)[22].

**Table 1 Comparison of hepatitis B virus integration breakpoints among reference genomes**

|  | **GRCh38** | **T2T-CHM13** | **Original** |
| --- | --- | --- | --- |
| Tumor |  |  |  |
| Number of breakpoints | 2439 | 2487 | 3486 |
| Number of samples | 357 | 355 | 328 |
| Non-tumor |  |  |  |
| Number of breakpoints | 2759 | 2874 | 739 |
| Number of samples | 288 | 288 | 160 |

**Table 2 Comparison of frequent integration breakpoints in the samples**

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Gene | GRCh38 | | Original | |
| **Breakpoints (*n*)** | **Samples (*n*)** | **Breakpoints (*n*)** | **Samples (*n*)** |
| Tumor |  |  |  |  |
| *TERT* | 150 | 105 | 160 | 95 |
| *KMT2B* | 56 | 33 | 55 | 30 |
| *DDX11L1* | 0 | 0 | 36 | 23 |
| *CCNA2* | 12 | 7 | 14 | 8 |
| *CCNE1* | 13 | 9 | 14 | 7 |
| Non-tumor |  |  |  |  |
| *FN1* | 97 | 56 | 19 | 17 |
| *TERT* | 12 | 10 | 8 | 3 |
| *IQGAP2* | 7 | 5 | 1 | 1 |
| *KMT2B* | 7 | 4 | 5 | 3 |

**Table 3 Hepatitis B virus integration breakpoints in mitochondrial DNA**

| **Sample** | **Tumor/Non-tumor** | **Chromosome** | **Position** | **HBV** | **Quality score** |
| --- | --- | --- | --- | --- | --- |
| SRR3104746 | NT | chrM | 5367 | 1247 | 6933.33 |
| SRR3105143 | NT | chrM | 5682 | 1513 | 11817.58 |
| SRR3105012 | T | chrM | 8220 | 112 | 2080.04 |
| SRR3104491 | NT | chrM | 8524 | 2482 | 33322.16 |
| SRR3105095 | NT | chrM | 8694 | 471 | 4209.06 |
| SRR3105101 | NT | chrM | 11079 | 1559 | 2937.33 |
| SRR3105143 | NT | chrM | 11079 | 1559 | 14876.83 |
| SRR3105001 | NT | chrM | 11104 | 1590 | 45538.21 |
| SRR3105149 | NT | chrM | 11104 | 1590 | 16156.63 |
| SRR3105251 | NT | chrM | 11104 | 1590 | 2276.31 |
| SRR3105293 | NT | chrM | 11104 | 1590 | 24562.69 |
| SRR3104643 | T | chrM | 11104 | 1590 | 13683.16 |
| SRR3105172 | T | chrM | 11126 | 1621 | 2610.44 |
| SRR3105251 | NT | chrM | 11130 | 1625 | 6793.43 |
| SRR3104939 | NT | chrM | 11139 | 1729 | 30830.54 |
| SRR3105149 | NT | chrM | 12453 | 323 | 2345.03 |
| SRR3104636 | NT | chrM | 12735 | 1381 | 26840.64 |
| SRR3105083 | NT | chrM | 13273 | 1755 | 4230.85 |
| SRR3104696 | NT | chrM | 13433 | 363 | 2963.36 |
| SRR3104643 | T | chrM | 13964 | 2809 | 2222.86 |
| SRR3104823 | NT | chrM | 14052 | 1017 | 1788.18 |
| SRR3105049 | NT | chrM | 14892 | 1768 | 21102.87 |
| SRR3104982 | T | chrM | 15679 | 1768 | 32371.78 |
| SRR3105185 | NT | chrM | 16319 | 1788 | 2013.53 |

HBV: Hepatitis B virus.