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HBV Integration Affects the Efficacy of Systemic Drug Therapy after Radical Resection of Liver Cancer: A Prospective Cohort Study

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Research Article

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ABSTRACT

Objective: Hepatitis B Virus (HBV) gene integration is an important factor in the occurrence and development of HBV-Hepatocellular Carcinoma (HBV-HCC); however, its role in the clinical treatment of liver cancer is still unclear. This study aimed to investigate the effect of HBV integration on the prognosis of patients.

Method: Twenty patients with HBV-HCC were included based on strict inclusion criteria. Whole genome sequencing of HBV-HCC surgical specimens was performed to identify *HBV* gene integration events. After systemic drug treatment (tyrosine kinase inhibitors alone or in combination with immune checkpoint inhibitors), the therapeutic efficacy was evaluated based on RECIST 1.1 criteria. COX regression model was used to identify factors affecting Progression-Free Survival (PFS) and Overall Survival (OS).

Results: HCC tissue samples from 20 HBV-HCC patients were sequenced and matched with standard sequence. HBV integration was found in 10 out of the 20 patients. The highest frequency of HBV integration occurred on chromosome 5. Survival analysis showed that HBV integration was a risk factor for HCC recurrence (Hazard Ratio (HR): 3.366, P=0.019). However, there was no significant effect of HBV integration on the PFS after first-line systemic drug treatment (P=0.313). The overall survival of HCC patients with HBV integration was significantly shorter than their counterparts without HBV integration (HR (95% CI): 6.335 (1.237–32.446); P=0.027).

Conclusion: HBV integration event was found to be a risk factor for HCC recurrence in HBV-HCC patients after radical surgery. Patients with HBV integration are potential candidates for active intervention in the early postoperative period.

Keywords: Hepatitis B virus; Gene integration; Hepatocellular carcinoma; Recurrence; Systematic drug therapy

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INTRODUCTION

Primary liver cancer is one of the most common malignant tumors globally. According to the data from the international cancer research institute in 2021 [1], an estimated 906000 new cases of liver cancer are diagnosed worldwide each year, making it the sixth most commonly occurring malignant tumor; in addition, it accounts for 830000 deaths annually, making it the third most common cause of cancer related death. The main pathological type of primary liver cancer is Hepatocellular Carcinoma (HCC), accounting for 85%–90% of all cases. The other pathological types are mixed Intrahepatic Cholangiocarcinoma (ICC) and HCC-ICC. In China, chronic Hepatitis B Virus (HBV) infection is the main cause of HCC [2]. In recent years, the incidence rate and mortality of HCC have shown a downward trend. This is mainly due to the primary prevention strategy of vaccination against HBV infection in infancy [3,4].

Many cases of HBV-HCC in China are diagnosed at a relatively late stage, leading to poor prognosis [5-6]. After liver cancer resection and transplantation, patients with early liver cancer are prone to recurrence and metastasis due to the presence of background HBV infection [7]. Many studies have shown that *HBV* gene integration in the human host genome plays an important role in the occurrence and development of liver cancer. Many studies have documented the integration of HBV DNA in liver cancer samples [8,9]. For example, studies have shown that HBV genes can easily integrate into the human genome, such as *hTERT*, *MLL4*, *CTNNB1*, *TP53*, and other cancer related genes, thus promoting the occurrence and development of HCC [10-12]. After *HBV* gene integration, fusion proteins with important biological functions can also be generated, such as HBx-line, which can promote β-catenin indicates poor prognosis [13].

Several studies have investigated the role of HBV integration events in promoting the occurrence and development of HCC, but there is no robust evidence of its impact on the systemic drug treatment for liver cancer. Therefore, in this study, we performed an in-depth analysis of the effect of HBV integration events on the therapeutic efficacy of systemic therapy for HBV HCC. Our findings may provide a theoretical basis for the follow-up clinical treatment strategies for these patients.

MATERIALS AND METHODS

Data and methods

Research object: Using a cohort study design, we collected the diagnosis and treatment related information of patients with HBV-related liver cancer who had relapsed after radical surgery in Nanjing Jinling hospital from March 2017 to October 2021. Finally, 20 patients with HBV HCC were included, and their surgical HCC specimens were collected for Whole Genome Sequencing (WGS). Based on the results of WGS, 20 patients were divided into non-HBV integrated group (n-HI) and HBV integrated group (n-HI). These patients were closely followed up and the postoperative diagnosis and treatment related information were collected. This study was conducted in compliance with the principles enshrined in the 2013 Helsinki declaration. The study protocol was approved by the medical ethics committee of the Nanjing Jinling hospital (2020DZSKTZX-016).

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Inclusion and exclusion criteria

Inclusion criteria:

- Adult patients (age >18 years, male or female) who had undergone surgical resection of liver cancer and were pathologically diagnosed as HCC.
- HBsAg positivity detected in perioperative examination, with HBV DNA >500 IU/mL.
- Detection of at least one measurable lesion in the liver after surgery by imaging (according to the RECIST v1.1, the length and diameter of the measurable lesion on spiral CT scanning are ≥ 10 mm).
- Barcelona Clinic Liver Cancer (BCLC) stage B or C, and lesion not suitable for reoperation or local treatment.
- Relevant blood biochemical and liver function indicators (such as total bilirubin ≤ 1.5 × Upper Limit of Normal range (ULN), Alanine Transferase (ALT), Aspartate Transferase (AST), and Alkaline Phosphatase (ALP) ≤ 5 × ULN).
- ECOG: 0-1; expected survival time >12 weeks, and at least one imaging examination performed to evaluate the target lesions.
- Provision of written informed consent for participation in the study.

Exclusion criteria:

- Other liver tumors, including hepatocholangiocarcinoma, sarcomatous HCC, mixed cell carcinoma, and fibrous lamina.
- Patients who did not receive standardized first line systemic drug treatment for liver cancer for more than 2 months.
- Patients who received local treatment measures during first line treatment.
- Patients who were judged by clinical experts to be unsuitable for the study (such as poor compliance during the patient's visit, no follow-up imaging examination to assess the focus, etc.).

Data collection

Twenty patients qualified the above criteria and were included in the final analysis set (Figure 1). The baseline sociodemographic and clinical data were extracted from the hospital medical records, including age, sex, contact information, home address, history of smoking, alcohol consumption, and previous liver disease. The blood test results obtained within one week before systemic treatment were collected, such as liver function indicators, antibody and platelet counts, and a series of biochemical indicators. The relevant indicators of HBV infection in patients were recorded, including HBsAg, HBsAb, HBeAg, HBeAb, HBcAb and HBV DNA.

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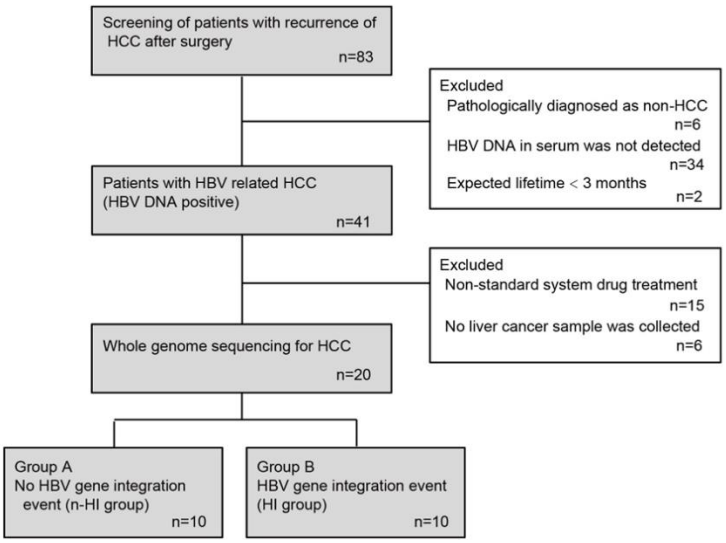
Figure 1. Flow chart of patient inclusion.



Questionnaires were designed to collect the following follow-up data:

- Basic information, clinical serological indicators, immune-related indicators, and pathological data.
- Focus information, including baseline target focus evaluation, non-target focus and other information.
- Follow-up treatment information, including all previous inpatient treatment plans and treatment duration.
- Information about therapeutic effect, including previous imaging examination results, target lesion evaluation, and non-target lesion evaluation.

Whole genome sequencing

The liver cancer tissue samples from 20 HBV-HCC patients were extracted using DNeasy Tissue Kit (QIAGEN) kit. According to the construction process of Illumina sequencing library, the genomic DNA was segmented, the end was repaired, A was added to the 3 'end, connector was connected, and library amplification and other steps were performed for construction of sequencing sample library. The library uses Qubit ® 2.0 Fluorometer to detect the concentration and Agilent2100 is used to detect the size of the library. The constructed library was sequenced by 2*150bp using Illumina Hiseq X. The original data was converted into Fastq file format with software recommended by Illumina, and the quality was evaluated with FastQC tool.

HBV gene integration sequencing analysis

BWA tools were used to compare the reads in the FASTQ file with the human genome (hg19/GRCh37 version), and then repeated after samtools sorting and Picard marker duplicate. The proportion of all samples compared to the reference genome was more than 99%. Subsequently, GATK tool was used to conduct local realignment and base quality correction of the insertion deletion site. Finally, the pre-processed bam file was obtained, and BED tools and perl/python scripts were used to obtain the comparison information, including coverage and sequencing depth. In addition, through the standard sequence of HBV genome, the integrated information of HBV sequence in human genome and the integrated fragment information were compared.

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Statistical methods

All data were collated and analyzed using SPSS 25.0 software. Continuous variables were expressed as mean ± standard deviation, and between group differences were assessed using the t-test. Categorical variables were expressed as frequency and percentage, and between group differences were assessed using the *Chi-squared* test. The distribution of HBV integration events was mapped with Circos software. In addition, COX regression model was used for analysis of factors affecting patient outcomes, and to identify the independent risk factors. Kaplan-Meier method and Hazard Ratio (HR) were used to evaluate the influence of risk factors on disease outcomes. For all analyses, P values <0.05 were considered indicative of statistical significance.

RESULTS

Whole genome sequencing results of liver cancer tissues

The genome extracted from all 20 HBV-HCC specimens met the quality inspection requirements. The quality evaluation results of sequencing data showed that Q20 of all sequences was >97%, and the average sequencing depth was 28-30 times. The proportion of all samples compared to the reference genome was more than 99%, and the proportion of samples that could be compared with the reference genome was approximately 84% after removing possible PCR duplicates. HBV gene integration events were identified based on sequence alignment after whole genome sequencing. HBV integration events were found in 10 HBV HCC patients, as shown in Figure 2. In a single sample, the highest number of integration events was 7. In addition, in terms of chromosome distribution, chromosome 5 showed the highest frequency, and five HBV integration events were found. Among them, HBV integration events were found in genes such as *FRG1-DT*, *TERT*, *NOL4*, *SYNDIG1*, *GRM3*, *PSMA3-AS1*, and *TANGO6*, suggesting the involvement of these genes in the occurrence and development of HBV HCC.

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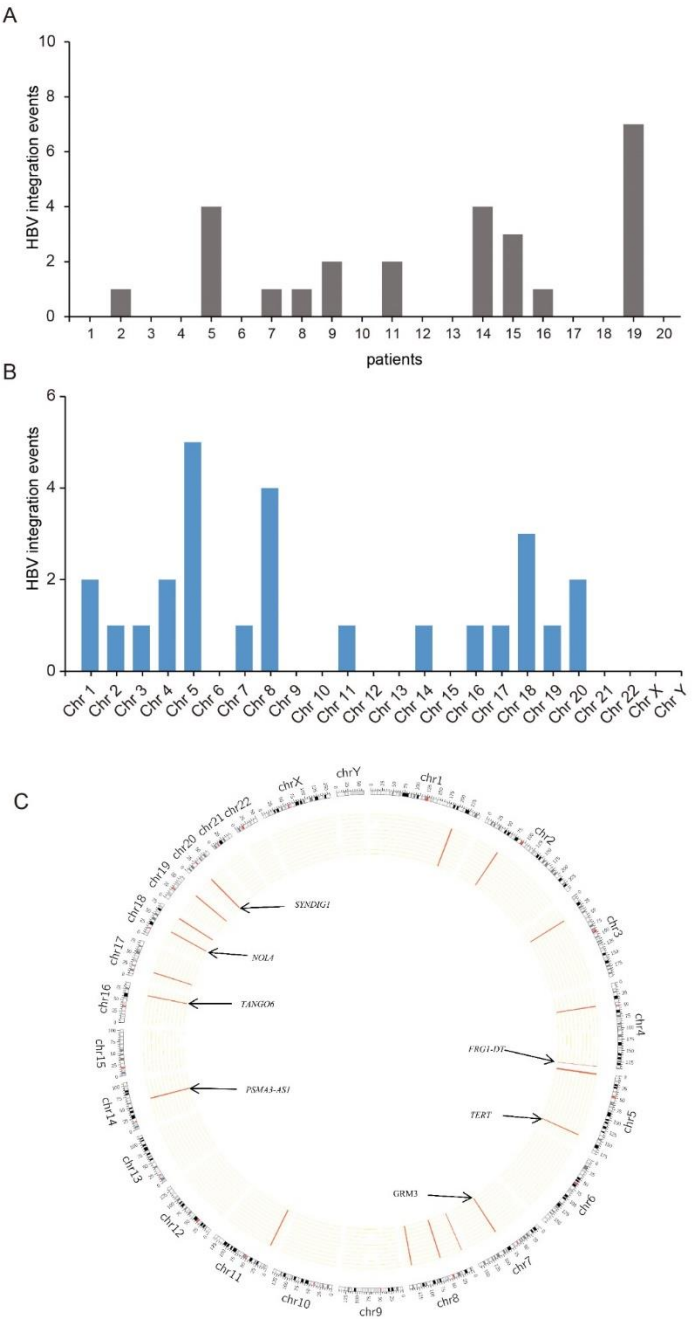
Figure 2. Whole genome sequencing results of tumor tissue; (A) Distribution frequency of HBV integration events in patients; (B) Distribution frequency of HBV integration events in chromosomes; (C) Distribution of HBV integration events in human genome.



Clinical information of the study population

Based on the detection of HBV integration events, the study population was divided into non-HBV Integration group (n-HI) and HBV Integration group (HI) (n=10 each). The baseline characteristics of patients in the two groups are shown in Table 1. There was no significant between group difference with respect to age, sex, antiviral therapy, tumor stage, cirrhosis, Model for End-stage Liver Disease (MELD) score, treatment methods or serological indicators (such as ALT, AST, TBIL, albumin, and AFP) (P>0.05). The serum HBV DNA titer (log IU/mL) in the HI group was higher than that in the n-HI group (4.45 ± 2.06 *vs.* 2.88 ± 1.62); however, the between group difference was not statistically significant due to insufficient sample size

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(P=0.075). Eight patients had previously used standardized antiviral therapy, and all drugs used against HBV were nucleotide analogues. There was no difference in the distribution between the two groups (P=0.650). The above results showed good comparability between the two groups with respect to the baseline characteristics.

Table 1. Clinical information of included patients.

Factors	n-HI group, n=10(%)	HI group, n=10(%)	P value
Age			
<55	5 (50.0)	5 (50.0)	
≥ 55	5 (50.0)	5 (50.0)	1
Gender			
female	1 (10.0)	1 (10.0)	
male	9 (90.0)	9 (90.0)	1
HBV DNA (log IU/mL)	2.88 ± 1.62	4.45 ± 2.06	0.075
antiviral therapy			
no	5 (50.0)	7 (70.0)	
yes	5 (50.0)	3 (30.0)	0.65
ALT			
<37 U/L	7 (70.0)	8 (80.0)	
≥ 37 U/L	3 (30.0)	2 (20.0)	0.606
AST			
<40 U/L	8 (80.0)	7 (70.0)	
≥ 40 U/L	2 (20.0)	3 (30.0)	0.606
TBIL			
<20.5 umol/L	10 (100.0)	8 (80.0)	
≥ 20.5 umol/L	0	2 (20.0)	0.136
Albumin			
<35 g/L	1 (10.0)	1 (10.0)	
≥ 35 g/L	9 (90.0)	9 (90.0)	1
AFP			
<20 ug/L	4 (40.0)	3 (30.0)	
≥ 20 ug/L	6 (60.0)	7 (70.0)	0.639
Cirrhosis			
no	5 (50.0)	7 (70.0)	
yes	5 (50.0)	3 (30.0)	0.65
MELD[a] score	3.05 ± 2.73	4.58 ± 3.11	0.26
Tumor stage			
BCLC A	0	2 (20.0)	
BCLC B	3 (30.0)	3 (30.0)	
BCLC C	7 (70.0)	5 (50.0)	0.32
TKIs			
TKIs	4 (40.0)	6 (60.0)	
TKIs+ICIs	6 (60.0)	4 (40.0)	0.371

Note: HBV: Hepatitis B Virus; ALT: Alanine Transaminase; AST: Alanine Transaminase; TBIL: Total Bilirubin; AFP: Alpha-Fetal Protein; MELD: Model for End-Stage Liver Disease; BCLC: Barcelona Clinic Liver Cancer; TKIs: Tyrosine Kinase Inhibitor; ICIs: Immune Checkpoint Inhibitors.
a. MELD score=3.78 × ln (T-BiL (mg/dl))+11.2 × ln (INR)+9.57 × ln (Cr (mg/dl))+6.43

In the whole genome sequencing results, based on the comparison information of COSMIC database, n-HI group was higher than the HI group (P<0.05). However, SNV, InDel and SVs showed no significant difference between the two groups (P>0.05). CNVs in the n-HI group were significantly higher than that in the HI group (P<0.05) (Figure 3).

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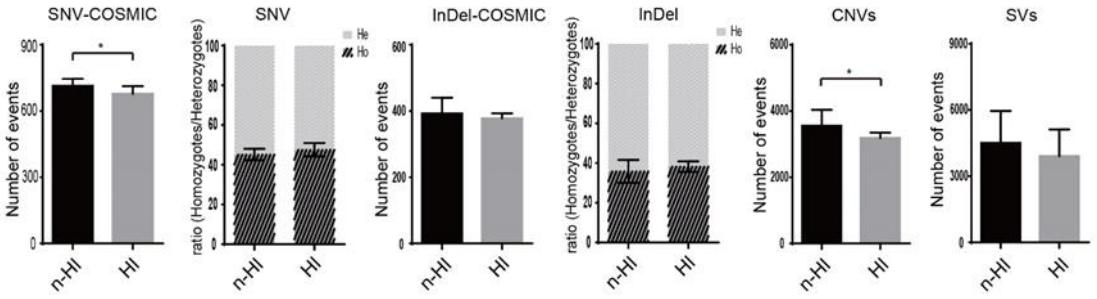
Figure 3. Distribution of gene mutations in the two groups.

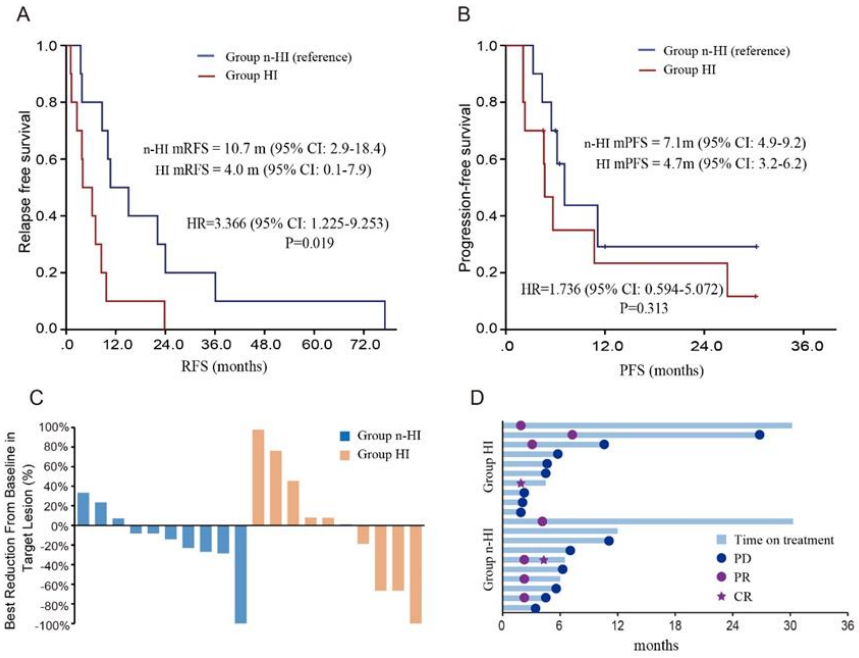


Impact of HBV integration on the efficacy of clinical treatment for HCC

Based on the results of univariate COX regression analysis, different HBV integration events were used as comparative factors to compare their impact on Recurrence-Free Survival (RFS) and Progression-Free Survival (PFS). The median RFS in the HI group was 4.0 months as compared to 10.7 months in the n-HI group. Therefore, HBV integration was identified as a risk factor for HCC recurrence (HR: 3.366, P=0.019). There was no significant difference between n-HI group and HI group with respect to PFS after first-line systemic drug therapy for HBV-HCC (HR: 1.736 (95% CI: 0.594–5.072), P=0.313) (Figure 4).

At the maximum baseline response rate, the n-HI group had 2 cases of Progressive Disease (PD), 2 cases of Partial Response (PR), 1 case of Complete Response (CR), and 5 cases of Stable Disease (SD) maintenance. In the HI group, there were 3 cases of PD, 2 cases of PR, 1 case of CR, and 4 cases of SD maintenance. The above results suggest that HBV integration events tend to lead to PD at the early stage of treatment, but there was no significant difference with n-HI group at the stage of treatment (Figures 4C and 4D).

Figure 4. Effect of HBV integration events on the clinical treatment of HCC.



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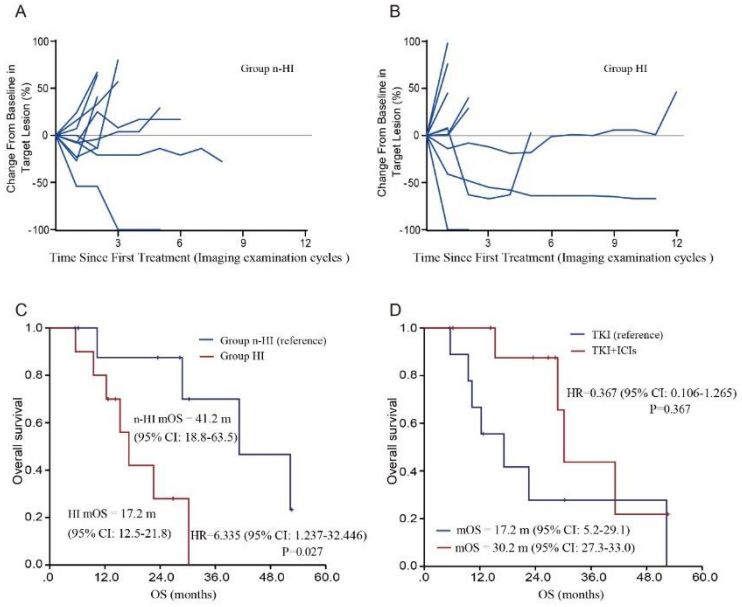
Impact of HBV integration events on the survival period of HCC patients

After first line treatment, there was no significant difference between n-HI group and HI group with respect to all previous tumor evaluation results. However, HCC patients with HBV integration were found to be more prone to disease progression in the early stage of first-line systemic drug treatment (Figure 5). We used Overall Survival (OS) as the primary endpoint to analyze the impact of HBV integration events and treatment methods on disease outcomes. COX regression analysis showed significantly shorter survival time in the HI group (HR (95% CI): 6.335 (1.237–32.446); P=0.027). However, the results showed that there was no dose dependent effect between HCC recurrence and HBV integration events (p>0.05). The first-line treatment of HCC was divided into Tyrosine Kinase Inhibitors (TKI) group and TKI+Immune Checkpoint Inhibitors (ICIs) group, and its impact on OS was analyzed. The median OS of patients treated with TKI+ICIs was 30.2 m (95% CI: 27.3–33.0), which was 13.0 months longer than that of patients treated with TKI (17.2 m (95% CI: 5.2–29.1)). However, the between group difference was not statistically significant due to the limited sample size (P=0.367) (Figures 5C and 5D).

Figure 5. The impact of HBV integration events on the survival of HCC patients.



DISCUSSION

Chronic HBV infection is one of the most important causes of HCC. An increasing number of studies have shown that the integration of HBV DNA in the host cell genome is a potential mechanism of viral gene activity [14,15]. HBV integration into the host cell genome is one of the mechanisms of immune escape, and also an important reason for the long term persistence of HBV *in vivo* [16]. The integrated HBV DNA fragments can be used as templates for gene transcription to express corresponding gene proteins.

Currently, there is no direct evidence of the impact of HBV integration events on the outcomes of systemic drug therapy for liver cancer; however, some studies have preliminarily explored this issue. A study demonstrated that the occurrence of HBV-DNA integration events, which can be targeted by specific T cells *in vivo*, provides a new approach for cell therapy of

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HCC [17]. HBx-LINE1 was detectable in 23.3% of HBV-HCC tumors, and this integration event was associated with worse patient survival. In this study, the authors did not assess the difference in the prognosis of systemic therapy for HCC, but the results showed that this integration event was an independent risk factor for therapeutic efficacy (HR (95% CI): 2.649 (1.194–5.876); P=0.016) [18]. A case report described the first transgenic expression of HBsAg-specific T cell receptor in HCC autologous T cells for treatment of chemotherapy resistant extrahepatic metastasis based on the occurrence of HBV-DNA integration events. This demonstrated that the expression of HBV antigen in HCC metastases can enable the recognition of tumor cells by lymphocytes *in vivo* for individual immunotherapy approaches [19].

In this study, we analyzed the efficacy of systemic treatment for patients with recurrent liver cancer after radical resection. However, some limitations of this study should be acknowledged. This was a non-interventional, prospective cohort study with a small sample size, which may have introduced an element of bias. In particular, the effect of potential confounding factors, especially in the selection of treatment, cannot be ruled out. Future studies should aim to overcome the above shortcomings in order to obtain more definitive evidence.

CONCLUSION

To conclude, HBV integration is not only a risk factor for the occurrence and development of HBV, but also a risk factor for postoperative recurrence in patients with HBV HCC. HBV integration events significantly shortened the survival period of patients. These patients are potential candidates for active intervention in the early postoperative period.

DATA AVAILABILITY

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding authors.

COMPETING INTERESTS

All authors have no conflicts of interest to declare.

ETHICS APPROVAL

The studies involving human participants were reviewed and approved by the ethics committee of Nanjing Jinling hospital. Institutional research ethics committee approval number: 2020DZSKTZX-016.

AUTHOR CONTRIBUTIONS

L-XF and Z-YJ conceived and designed the study. C-C and L-HY performed the literature search, S-AF generated the figures and tables. L-ZX wrote the manuscript. Q-WS, Z-J and X-ZJ collected and analyzed the data, and critically reviewed the

manuscript. L-LH supervised the study and reviewed the manuscript.

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