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RESEARCH ARTICLE

Profile of HBV Integration in the Plasma DNA of Hepatocellular Carcinoma Patients

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Abstract: ***Background*:** Hepatitis B Viral (HBV) infection is one of the major causes of Hepatocellular Carcinoma (HCC). Mounting evidence had provided that the HBV integration might be a critical contributor of HCC carcinogenesis.

***Objective and Methods*:** To explore the profile of HBV integration in the plasma DNA, the method of next-generation sequencing, HBV capture and bioinformatics had been employed to screen for HBV integration sites in the plasma samples.

***Results*:** In the initial experiment, a total of 87 breakpoints were detected in the 20 plasma samples. The distribution of breakpoints showed that there was significant enrichment of breakpoints in the region of intron. Furthermore, the HBV breakpoints were prone to occur in the region of X protein (1,700-2,000bp) in the plasma samples. The pathway analysis had revealed that the HBV integrations sites were specifically enriched in the cancer pathway.

***Conclusion*:** Altogether, our results had provided direct evidence for the HBV integration in plasma DNA, and they might be potentially useful for future HCC prognosis and diagnosis.

Keywords: Hepatocellular carcinoma, Plasma, HBV integration, Cell free DNA, HBV genome, Breakpoints.

1. INTRODUCTION

HBV infection is an epidemic in Asia, Africa, Southern Europe and Latin America, and HBV consists of at least eight genotypes (A-H) [1]. Several factors have been known to be related to higher HCC risk among HBV carriers: demographic (male gender, older age, ethnicity), genetic (family history of HCC), viral (high viral load, viral genotype, duration of infection, co-infection with HCV, HIV or HDV), and environmental (exposure to aflatoxin, alcohol abuse or cigarette smoking) [2, 3]. Generally, HBV is the main causative agent in the high incidence HCC areas, while HCV is the major etiological factor related to HCC in low incidence HCC areas [2, 4-6]. In addition, HDV chronic infection has been found to be associated with a worsening of HBV infection. Usually, it increased the risk of liver decompensation and Hepatocellular Carcinoma (HCC) occurrence [7]. Furthermore, HBV DNA and HBeAg were detected less frequently in anti-HD-positive than in anti-HD-negative subjects among patients with severe liver disease [8]. These findings indicated that HBV infection was closely related to HDV infection, although the mechanism kept unclear.

Among HBV genotypes, HBV/B and HBV/C are more restricted to east/south-east Asia [9]. HBV infection of these two types has been known as a leading cause for chronic hepatitis, cirrhosis, and hepatocellular carcinoma (HCC) in China [10]. The integration events of HBV/B and HBV/C are also frequent to be detected in the cancer tissues of HCC patients.

Massive Parallel Sequencing (MPS) technology has provided an efficient mean to detect HBV integration through whole genome. Jiang *et al.* investigated the effect of HBV integration by whole genome sequencing. They found that HBV integration could trigger the copy number variation of genome and induce the abnormal expression [11]. Sung *et al.* had surveyed the hotspot genes of HBV integration through the whole genome and determined that there was a significant relationship between HBV integration and survival time [12]. Furthermore, the researcher found that the viral-human chimeric transcript may trigger the Wnt signaling pathways

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and had close relation with the development and progression of liver cancer [13]. Based on the above studies, it suggested that HBV integration is an important event in the process of tumorigenesis. HBV integration can not only cause genetic damage and chromosomal instability but also cause disorder to the host gene expression [14]. In addition, expression of viral proteins such as X protein and S antigen may further induce the tumorigenesis [13]. Indeed, the first descriptions of HBV integration events were based on primary HCC tissues and HCC-derived cell lines, prompting suggestions that HBV integration events might be causative in tumorigenesis [15, 16]. Hitherto, the reported mechanisms include (1) HBV integration mediated insertional mutagenesis of HCC-associated genes; (2) induction of chromosomal instability by HBV integration; (3) the expression of HBV genes from the HBV integration. However, the mechanism of HBV-induced HCC carcinogenesis still remains unclear so far [17].

However, the samples in these studies were collected *via* invasive procedures. In order to facilitate the clinical utilization, HBV integration events in plasma were urgently needed to be surveyed. The characteristic of HBV integration in the plasma might promote the clinical utilization potential of HBV integration.

A high throughput virus integration detection (HIVID) approach was adopted to investigate the HBV integration sites [18]. Overall, there were 15 samples with HBV integration among 20 plasma samples and 87 breakpoints were found in the 15 plasma samples. Furthermore, we determine the characteristic of HBV integration sites in the plasma samples. HBV integration was prone to the region of INTRON. Pathway analysis indicated that the HBV integration events were enriched in the pathway of cancer. Altogether, our results provided evidence for the HBV integration in these plasma samples and explored the characteristic of HBV integration in the plasma, which might be useful for HCC prognosis and diagnosis.

2. MATERIALS AND METHODS

2.1. Sample Collection and Plasma DNA Extraction

We obtained the plasma and tumor tissue samples from the Affiliated Hospital of Jining Medical University, Jining, China, and patients had been diagnosed with HCC. All patients signed the written informed consent form, and the study had been approved by the Ethics Review Committee in the Jining Medical University. Plasma was stored at 80°C and DNA was extracted from a 0.5-ml plasma aliquot with DNA Blood Midi Kit (Qiagen, Germany) according to the manufacturer's instructions and stored at -20°C before further analysis. The inclusion criteria for this study included: (i) HBV-positive HCCs (HBV-B type) (ii) obtained from consenting patients and (iii) all patients were HCV-negative and HIV-negative (iv) negative for autoimmune hepatitis and metabolic and/or genetic disorders, such as Wilson's disease, hemochromatosis.

2.2. HBV Fragments Enrichment and Sequencing

The construction of sequencing library strictly followed the standard instructions provided by Illumina. The cell free DNA purified, and their ends were blunted, "A" tailed, ligated to adaptors and then PCR. The DNA libraries were quantified using Bioanalyser 2100 (Agilent Technologies, Santa Clara, CA, USA). The hybridization procedures were carried out following MyGenostics's GenCapTM Target Enrichment Protocol (GenCapTM Enrichment, MyGenostics, USA). The DNA libraries were hybridized with HBV probes at 65°C for 24 hours and subsequently subjected to washes to remove unbound. The eluted fragments were amplified by 12 PCR cycles in order to generate the sequencing library. Each library was further quantified and proceeded to 101 cycles of paired-end index sequencing in the Illumina HiSeq 2000 sequencer according to the manufacturer's official instruction.

2.3. Breakpoints Detection and Annotation of HBV Integration Sites

Deploying an algorithm established by our team previously [18], the low-quality reads, duplication reads and adaptor contaminations were removed. Subsequently, the filtered clean reads were mapped to both the human (NCBI build 37, HG19) and the HBV genomes. The chimeric reads that partially aligned to the human genome and partially aligned to the HBV genome were remained as the reads of our interest. The selected chimeric reads were then subjected to paired-end reads assembly, which helped to reconstruct fragment sequences, and additionally increased the efficacy to locate the precise position of the breakpoints. The PE-assembled reads were re-mapped to the human and the HBV genome using BWA [19]. The HBV integration breakpoints were annotated using ANNOVAR [20].

3. RESULTS

3.1. The Distribution of Breakpoints in the Human Genome

Plasma samples of 20 HCC patients were obtained in order to investigate the HBV integration sites (Table **1**). The DNA of plasma was processed according to our innovative HIVID approach [18]. Among the samples, 15 samples showed positive for HBV integration, altogether 87 integration sites were determined (Table **2**). Among all the integration sites, 46 of them were in the intron region, and 24 were in the intergenic region (Table **3**). Therefore, it seems that the breakpoints were more prone to be enriched in the intron region (Fig. **1a**; Intron observed ratio=0.53; Intron random ratio=0.34; Chisquare Test $P<0.01$).

3.2. The Distribution of Breakpoints in the HBV Genome

The distribution of breakpoints in the HBV genome was then analysed. It was also revealed that the breakpoints from plasma samples were enriched in the region of 1700-2000 bp of the HBV genome specifically (Fig. **1b**).

3.3. The Pathway Analysis of Breakpoints

Using DAVID pathway enrichment software, the genes integrated by HBV were analysed [21, 22], and the results indicated that cancer pathway was particularly targeted ($P<0.01$). These five genes *CTNNA2, EGFR, MITF, STK36* and *RALA* were located in the cancer pathway.

Table 1. Demographic and clinicopathologic characteristics of 20 HCC patients.

Variables	**Mean ± SD / n (%)**
Age, years	49.5±7.2
Sex	
Female	5 (25%)
Male	15 (75%)
Diagnosis	
Primary HCC	20 (100%)
HCC Differentiation (Edmondson-Steiner)	
II	2 (10%)
III	18 (90%)
Tumor diameter, cm	6.2±4.3
Microvascular invasion	
Absence	15 (75%)
Presence	5 (25%)
ALB, g/L	43.9±5.1
TBIL, μmol/L	16.1±5.7
PT, seconds	11.9±1.1
AFP, μg/L	579.8±564.8
HBV type	
HBV_B	20 (100%)
HBV DNA levels, IU/mL	417825.9±856030.9
HBeAg	
Positive	6 (30%)
Negative	14 (70%)
Anti-HBe status	
Positive	17 (85%)
Negative	3 (15%)
HBsAg status	
Positive	20 (100%)
Anti-HBs status	
Negative	20 (100%)

4. DISCUSSION

HBV integration had previously been demonstrated having a close association with the tumorigenesis of HCC. In the studies led by Sung *et al.,* the authors had identified several genes preferentially integrated by HBV [12]. In this study, our team had analysed the HBV integration sites in 20 plasma samples of HCC patients. The results suggested that the genes in the cancer pathway were particularly targeted by HBV integration. In recent years, the use of plasma DNA sample in clinical diagnosis has become increasingly important [23], this is due to the presence of circulating DNA originated from the degenerating tumor cells [24]. According to the established data, one of the major sources of plasma or serum DNA may be from the apoptotic cells [25], though the entire mechanism of DNA being released into circulating blood still remains to be thoroughly investigated.

Generally, there was a significant portion of the cf-DNA from the tumor tissues. Many researchers have identified that

Table 2. Data production of samples. Breakpoint number in human genome and coverage of HBV genome were shown.

Library	Total Bases	Q20	HBV Type	HBV Coverage	Breakpoint Number
B001	5.42G	88.42;79.57	B	99%	3
B002	5.01G	86.90;78.08	B	98%	4
B0074	5.22G	85.49;80.51	B	97%	7
B0068	5.31G	86.97;79.88	B	99%	2
B0064	5.40G	87.38;81.57	B	99%	3
B0061	5.81G	86.08;78.67	B	100%	9
B0049	5.12G	85.49;80.55	B	99%	5
B0037	5.16G	88.97;81.86	B	96%	6
B0032	5.20G	86.55;81.97	B	97%	5
B0075	5.25G	87.89;79.72	B	98%	7
B007	5.86G	83.50;81.52	B	99%	5
B0034	5.27G	87.87;80.78	B	97%	8
B0021	5.68G	86.78;82.47	B	99%	5
B0012	5.09G	88.77;79.88	B	98%	6
B0010	5.50G	84.52;81.63	B	99%	12
B0016	5.91G	85.87;79.25	B	96%	0
B0011	5.53G	89.67;81.98	B	98%	0
B008	5.57G	86.98;78.66	B	99%	0
B009	5.61G	85.59;80.79	B	99%	0
B005	5.55G	86.49;79.74	B	99%	0

Table 3. The breakpoints of plasma samples. The breakpoints detected in the plasma samples.

Sample	Chr	Position	Support	Element	Gene
B0074	chr4	8977829	4	intergenic	LOC650293/USP17L10
B0074	chr8	103211319	2	downstream	RRM2B
B0074	chr8	30611415	2	intronic	UBXN8
B0074	chr8	103119207	2	intronic	NCALD
B0074	chr4	125111322	2	intergenic	LINC01091/ANKRD50
B0074	chr4	65178643	2	intronic	TECRL
B0074	chr20	47656089	2	promoter	CSE1L
B0068	chr4	118968708	2	intronic	NDST3
B0068	chr17	21903348	2	promoter	FLJ36000
B0064	chr11	65986345	8	intronic	PACS1
B0064	chr1	249121201	7	promoter	SH3BP5L
B0064	chr15	34708490	4	intergenic	GOLGA8A

(Table 3) contd….

Sample	Chr	Position	Support	Element	Gene
B0061	chr18	22570340	7	intergenic	RP11-449D8.1/ZNF521
B0061	chr6	169471301	4	intergenic	SMOC2/THBS2
B0061	chr3	110721179	4	intergenic	LINC01205/PVRL3-AS1
B0061	chr7	74141512	3	ncRNA_intronic	LOC101926943
B0061	chr21	39816650	3	intronic	ERG
B0061	chr4	86519608	2	intronic	ARHGAP24
B0061	chr2	45757229	2	intronic	SRBD1
B0061	chr1	225407594	2	intronic	DNAH14
B0061	chr1	10649629	2	intronic	PEX14
B0049	chr7	55273463	4	UTR3	EGFR
B0049	chr4	92355373	3	intronic	CCSER1
B0049	chr6	31003961	2	downstream	MUC22
B0049	chr4	31901689	2	intergenic	PCDH7
B0049	chr3	49089016	2	intronic	QRICH1
B0037	chrX	112015468	2	downstream	AMOT
B0037	chr2	28331047	2	intronic	BRE
B0037	chr17	33939459	2	intronic	AP2B1
B0037	chr14	100715229	2	intronic	YY1
B0037	chr10	57261124	2	intergenic	PCDH15/MTRNR2L5
B0037	chr1	38451027	2	intronic	SF3A3
B0032	chr17	21906061	4	ncRNA_intronic	FLJ36000
B0032	chr17	21906355	3	ncRNA_intronic	FLJ36000
B0032	chr11	191802	3	promoter	LOC653486
B0032	chr4	49649534	2	intergenic	CWH43
B0032	chr17	21903348	2	promoter	FLJ36000
B0075	chr8	30611415	3	intronic	UBXN8
B0075	chr5	7349039	3	intergenic	LOC442132/ADCY2
B0075	chr3	73158614	3	intergenic	PPP4R2/PDZRN3
B0075	chr8	124890891	2	intronic	FER1L6
B0075	chr4	100009507	2	intronic	ADH5
B0075	chr18	22570340	2	intergenic	RP11-449D8.1/ZNF521
B0075	chr12	27136775	2	intronic	TM7SF3
B007	chr10	89954886	6	intergenic	PTEN/RNLS
B007	chr10	89954930	4	intergenic	PTEN/RNLS
B007	chr11	47814540	3	intronic	NUP160
B007	chr7	72974855	2	promoter	BCL7B
B007	chr18	42356727	2	intronic	SETBP1

(Table 3) contd….

Sample	Chr	Position	Support	Element	Gene
B0034	chr12	30265504	5	intergenic	TMTC1/IPO8
B0034	chr2	119052347	4	intergenic	INSIG2/LOC101927709
B0034	chr15	67917548	4	intronic	MAP2K5
B0034	chr12	33057564	3	promoter	PKP2
B0034	chr9	98598152	2	ncRNA_intronic	LINC00476
B0034	chr6	32546766	2	UTR3	HLA-DRB1
B0034	chr6	32546685	2	UTR3	HLA-DRB1
B0034	chr4	42527080	2	intronic	ATP8A1
B0021	chr7	39673979	9	intronic	RALA
B0021	chr7	129485990	2	intronic	UBE2H
B0021	chr7	40234460	2	intronic	SUGCT
B0021	chr17	43211456	2	intronic	ACBD4
B0021	chr17	43211378	2	intronic	ACBD4
B0012	chr5	58123101	3	intronic	RAB3C
B0012	chr2	80380053	3	intronic	CTNNA2
B0012	chr17	39084514	3	exonic	KRT23
B0012	chr1	173786892	3	intronic	CENPL
B0012	chrX	61684765	2	intergenic	SPIN4
B0012	chr7	158778486	2	intergenic	WDR60/LINC00689
B0010	chr4	184204577	3	intronic	WWC2
B0010	chr3	69838919	3	intronic	MITF
B0010	chr17	7267199	3	downstream	TMEM95
B0010	chr16	1723993	3	exonic	CRAMP1L
B0010	chr14	69887597	3	intronic	SLC39A9
B0010	chr10	56134248	3	intronic	PCDH15
B0010	chr1	111605674	3	intergenic	LRIF1/DRAM2
B0010	chr9	20278555	2	intergenic	SLC24A2/MLLT3
B0010	chr9	68429933	2	ncRNA_intronic	LOC642236
B0010	chr9	6881037	2	intronic	KDM4C
B0010	chr8	88368708	2	intronic	CNBD1
B0010	chr8	124052577	2	intronic	DERL1
B002	chr5	52674905	2	intergenic	LOC257396/FST
B002	chr3	42691176	2	promoter	ZBTB47
B002	chr12	38638139	4	intergenic	ALG10B
B002	chr2	28693259	2	intergenic	FOSL2/PLB1
B001	chr2	219543519	2	intronic	STK36
B001	chr2	219543501	4	intronic	STK36
B001	chr18	11550976	11	intergenic	PIEZO2/SLC35G4

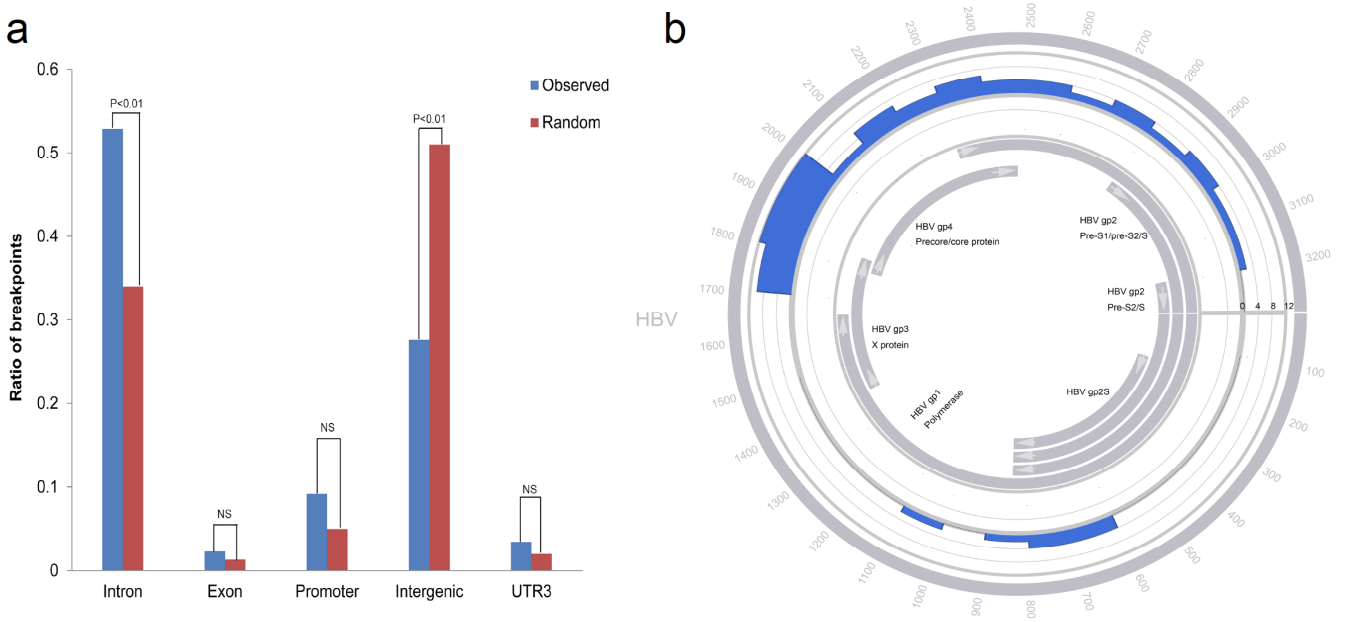

Fig. (1). The distribution of breakpoints in gene elements and HBV genome. (**a**) Observed represented the observed ratio of breakpoints; Random represented the random ratio of breakpoints. NS represented no significant. (**b**) Histograms were constructed for 100-bp intervals. HBV genes with different functions are marked. The number of breakpoints located in the HBV genome was shown.

common genetic alterations exist in both tumor tissue and paired plasma samples [26]. The level and characteristic of cf-DNA in human plasma have been affected by the dynamic balance between cellular DNA release and DNA degradation. Thus, stability of distinct cf-DNA forms [27, 28], activity of blood nucleases [29], adsorption of cf-DNA on blood cells [30, 31], as well as degradation of cf-DNA by phagocytes should also be considered as factors regulating the characteristic and level of cf-DNA in cancer patients [32].

Moreover, it is curious to find a number of breakpoints located in the intergenic regions. In recent years, an increasing trend of research interests had drawn to resolve the usefulness of breakpoints located in the intergenic region. For instances, MYC activation was driven by an upstream integration of HPV-18 genome [33]; β-catenin transactivity could be modulated by HBV integration in Long Interspersed Nuclear Element (LINE) [13]. Thus the importance and significance of HBV integration sites in the intergenic region remain elusive.

The distribution of breakpoints in the HBV genome was also investigated. It was revealed that the breakpoints were particularly enriched in the region of HBV X and core genes, which is in line with the previous findings by others and also our group.

CONCLUSION

Our study had adopted an effective method to seek HBV integration sites in the plasma samples. The results provided evidence for HBV integration in the plasma samples, which could be potentially useful for future HCC prognosis and diagnosis.

AUTHORS' CONTRIBUTIONS

WYL, QSK, and CXF conceived and designed the experiments. QH, MHT, YWQ and APZ performed the experiments. YHS and QSK analyzed the data and HQ, CXF, WYL wrote the paper.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

The study has been approved by the Ethics Review Committee of Jining Medical University (No. 2018KY003), Jining, China.

HUMAN AND ANIMAL RIGHTS

No animals were used in this study, Reported experiments on humans were in accordance with the ethical standards of the committee responsible for human experimentation (institutional national), and with the *Helsinki Declaration* of 1975, as revised in 2008 (http://www.wma.net/en/20activities/ 10ethics/10helsinki/).

CONSENT FOR PUBLICATION

Informed consent was obtained from all individual participants included in the study.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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