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Detection of Tumor-Related Biomarkers in Hepatocellular Carcinoma Patients by Sequencing Circulating Cell-Free DNA

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Abstract

In this study, we sequenced paired Cell-Free DNA (cfDNA) and tumor tissue DNA samples from 16 Hepatitis B Virus (HBV)-infected Hepatocellular Carcinoma (HCC) patients to detect tumor-related biomarkers in cfDNA samples. We analyzed a panel of 41 HCC-related target genes and detected 35 mutations among 13 genes in 10 HCC samples as well as 14 mutations among 7 genes in 6 cfDNA samples. The top 6 mutant genes were *TP53* (22.86%), *TERT* (20%), *ALB* (14.29%), *AXIN1* (11.43%), *FLT1* (5.71%) and *ARID1A* (5.71%). The mutation relevance ratios of matched HCC tissues and cfDNA samples were not associated with tumor size or serum α-Fetoprotein (AFP) levels. The *TERT* upstream gene variants, -124C>T (chr5:g.1295228G>A) is the only mutation shared by more than one case. Tumors with *ALB* mutations were associated with more gene mutations than tumors without *ALB* mutations (p=0.021407). Our study demonstrates the feasibility of cfDNA-based sequencing for detecting circulating tumor DNA biomarkers in HCC patients.

Keywords: HCC; cfDNA; Matched; Targeted Sequencing

Introduction

Tumor-related mutations are clinically relevant for early diagnosis, targeted therapy as well as for monitoring tumor heterogeneity, metastasis and drug response [1-3]. Circulating cell-free DNA from liquid biopsies are biomarkers useful for early diagnosis, predicting disease progression, and personalized treatment [4,5]. cfDNA analysis is non-invasive and overcomes the limitations of tissue biopsy and tumor heterogeneity [6]. However, cfDNA-based detection is challenging in HCC because of low circulating cfDNA levels, poorly characterized genetic alterations and a high degree of tumor heterogeneity.

HCC is the third most commonly diagnosed cancer in China and the leading cause of cancer deaths in Chinese males [7]. Most of the HCC cases are associated with liver cirrhosis linked to hepatitis B or hepatitis C infections [7,8]. In China, the infant HBV vaccination program that has been implemented in high-risk areas since 1986 has shown 95% reduction in HCC deaths in the younger population [7,9]. This showed that HBV infections are linked to HCC.

There has been limited use of Next-Generation Sequencing (NGS) technology to investigate HCC. The first sequencing and analysis of the HCC genome in 2011 identified 11,731 tumor-enriched somatic mutations [10]. Moreover, HCV-, HBV- and alcohol-associated HCC have also been analyzed by NGS [11-13]. The COSMIC database (http://cancer.sanger.ac.uk/cosmic/) shows 3187 oncogenic mutations that are linked to HCC. The top five mutated genes in HCC are *TP53* (27%), *TERT* (26%), *CTNNB1* (19%), *AXIN1* (8%), and *ARID2* (7%) [14]. Multiple chromatin regulators, such as *ARID1A*, *ARID1B* and *ARID2* are mutated in nearly 50% of tumors [11], suggesting the crucial role of the *ARID* family in HCC. Moreover, HBV integration into the *TERT* locus confers growth advantage in HCC [11-13].

Peripheral blood cell-free DNA (cfDNA) testing has been successfully used for monitoring minimal residue disease and molecular markers of target therapy in lung and colorectal cancers [15,16]. However, cfDNA based testing has not been fully evaluated in HCC individuals. Recently, several studies reported the effectiveness of sorafenib, a multi-kinase inhibited approved by the Food and Drug Administration, in HCC patients using different potential biomarkers [8,17-19].

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Table 1: Clinicopathological characteristics of the 16 HCC patients.

Case No.	Sex (M/F)	Age (years)	Complicated	Tumor Size (cm^3)	ALB (g/L)	AFP (μg/L)	TNM Stage	Operation date	TACE	Relapse	Metastasis	Death
1	M	71	B(4)+C	1645.37	34.5	241.3	IIIC	2013-3-19	-	-	-	2017-9-4(C)
2	M	52	B(20)	168.10	44.9	5.7	I	2013-3-19	2013-4-26	-	-	-
3	M	60	B(5)+C	254.856	39.3	>1210	I	2013-2-6	2013-3-19	2016-7-1	-	-
4	M	47	B(10)	142.08	41.5	4.7	I	2013-2-28	2013-4-15	-	-	-
5	M	77	B(L)	1375.89	36.6	603.7	IIIC	2013-3-18	-	-	-	-
6	M	50	B(8)+C	37.44	38	785.1	I	2013-3-26	-	-	-	2013-12-29
7	M	66	B(L)	133.28	35.9	54.7	I	2013-3-4	2013-4-19	2013-7-1	2014-7-1	2014-8-9
8	M	42	B(42)	87.204	41.1	224	I	2013-4-1	-	2013-9-4	2014-12-1	2016-3-3
9	M	51	B(10)	304.92	43.7	1.9	I	2013-2-19	-	-	-	-
10	M	47	B(20)+C	112.48	45.8	236.5	I	2013-2-1	2013-3-15	-	-	-
11	M	59	B(20)+C	602.615	39.9	14.9	II	2013-1-17	2013-3-4	-	-	-
12	M	58	B(30)	74.52	42.2	7.3	I	2013-2-21	2013-3-29	-	-	-
13	M	39	B(20)	52.08	41.1	111.7	IIIB	2013-2-25	2013-3-29	2014-6-13	-	-
14	M	46	B(20)+C	687.96	42.1	112.2	IIIC	2013-3-5	2013-4-12	2013-9-29	2013-8-2	2014-2-8
15	F	32	B(L)	851.904	43.9	653.6	IIIA	2013-3-19	2013-3-26	2013-6-26	2015-8-24	-
16	M	61	B(10)	469.476	34.9	>1210	I	2013-6-6	-	-	-	2017-5-15(H)

Abbreviations: B: Hepatitis B; L: Latent Hepatitis B; C: Cirrhosis: ALB: Albumin; AFP: Alpha Fetoprotein; TACE: Transarterial Chemoembolization; H: Heart Disease

Moreover, several pre-clinical trials are underway for novel targeted therapies against HCC [20]. Therefore, we investigated the scope of cfDNA sequencing to detect gene mutations in 16 paired cfDNA and HCC tumor samples with a panel of 41 target genes.

Methods

Patients and samples

The study was conducted in accordance with the Declaration of Helsinki guidelines and was approved by the Institutional Review Board of Eastern Hepatobiliary Surgery Hospital (EHSH), Second Military Medical University, Shanghai, China. Informed consent was obtained from individual patients. We reviewed the electronic medical records of patients with advanced HCC that were treated at EHSH in 2013. Two pathologists assessed the HCC pathology of the patients independently. The plasma samples were stored at -80°C and the cryopreserved HCC and adjacent non-tumor tissues were obtained from the Bio-Medical Sample Center at EHSH and were prepared as previously reported [42,43]. DNA was extracted from frozen paired HCC and adjacent non-tumor tissues using the QIAamp DNA mini kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. Circulating cfDNA was extracted from plasma using the QIAamp Circulating Nucleic Acid Kit (QIAGEN, Hilden, Germany) with the QIAvac 24 Plus vacuum manifold according to the manufacturer's instructions. The cfDNA concentration was determined using the Qubit fluorometer.

Study design and NGS sequencing

Mutations in 41 HCC-related genes were analyzed in the DNA samples from plasma and HCC tissue samples by HiSeq-2500™ sequencing (Illumina, San Diego, CA, USA). The sequencing data from genome DNA samples from the adjacent non-tumor tissue was used to verify if the mutations were somatic genetic alterations. The panel of 41 HCC-related genes (Accu-Act HCC, Accuragen, Inc.) included hotspot regions of clinical significance (Table 7). This panel spanned about 190 kb of the human genome and covered nearly 70% HCC cases in TCGA database.

Standard NGS method was used to analyze genomic DNA from tumor and adjacent non-tumor tissues as previously described [4]. Briefly, NGS library was constructed with the KAPA sequencing library construction kit (Kapa Biosystems, Boston, MA, USA) using 100 ng of fragmented genomic DNA. Then, the genomic DNA NGS library was captured by the Accu-Act panel (AccuraGen Inc., Shanghai, China) and sequenced with 100 bp paired-end runs on an Illumina HiSeq 2500 system (Illumina, San Diego, CA, USA). The average coverage depth for all probes was at least 500X.

Firefly NGS assay (Accu-Act) based on circularized cfDNA technology (AccuraGen Inc., Shanghai, China) was performed on cfDNA samples as previously described [4]. NGS libraries were sequenced on an Illumina HiSeq 2500 (Illumina, San Diego, CA, USA). The unique sequencing reads were determined by using the AccuraGen proprietary algorithm. The average coverage depth for all probes in plasma was approximately 7,000X.

Germline and somatic variants were identified with the AccuraGen's NGS pipeline [4]. The sequencing data from the cfDNA and tumor genomic DNA was cross-checked with the germline variants from adjacent non-tumor tissue DNA to identify somatic mutations. The allele frequency was calculated by comparing the number of unique reads in the variants to the total number of sequencing reads that map to the position of the variant.

Statistical analysis

All statistical analyses were carried out using the SPSS 19.0 statistical software package (SPSS, Chicago, IL, USA). Data was analyzed by two-tailed t-tests. $P<0.05$ was considered statistically significant.

Results

Patient characteristics

We analyzed 16 HCC patients (15 male and 1 female) that were diagnosed with Child-Pugh classification a disease in 2013 and underwent surgical resection at EHSH, Shanghai, China.

Table 2: Mutation list from 16 HCC patients.

Case	Mutation	cfDNA AF	tDNA AF	ClinVar	COSMIC	Mutation type	Impact
1	*ARID1A* K1129Ter, chr1:g.27097796A>T	0.7559%	41.8072%	-	-	stop_gained	HIGH
	AXIN1 Q235HfsTer12, chr16:g.396455_396471delCCTGGATTTCGGTCTGG	0.1666%	52.8428%	-	-	frameshift	HIGH
	AXIN1 Q233H, chr16:g.396474C>G	0.4615%	59.1121%	-	-	missense	MODERATE
	AXIN1 F231=, chr16:g.396480A>G	0.6186%	59.8698%	-	-	synonymous	LOW
	TP53, chr17:g.7577498C>A	-	42.4698%	-	-	splice_donor	HIGH
	TP53 P85LfsTer38, chr17:g.7579436delG	-	40.0244%	-	-	frameshift	HIGH
	ALB 5_prime_UTR_variant, chr4:g.74270031A>G	0.1397%	42.5925%	-	-	5_prime_UTR	MODIFIER
	TERT upstream_gene_variant, chr5:g.1295228G>A	-	54.1035%	-	-	upstream_gene	MODIFIER
2	*ARID2* intron_variant chr12:g.46230488dupT	-	30.9392%	-	-	intron	MODIFIER
	TP53 R273L, chr17:g.7577120C>A	-	45.6486%	rs28934576/lp	COSM10779/p	missense	MODERATE
	ALB V78A, chr4:g.74272441T>C	-	30.7901%	-	-	missense	MODERATE
	ALB splice_acceptor_variant chr4:g.74285222_74285223delAG	-	31.677%	-	-	splice_acceptor	HIGH
	TERT P343A, chr5:g.1293974G>C	1.0239%	77.6855%	-	-	missense	MODERATE
	HGF Y167N, chr7:g.81381562A>T	-	34.327%	-	-	missense	MODERATE
	CDKN2A D74V, chr9:g.21971137T>A	-	29.8726%	-	COSM13546/P	missense	MODERATE
3	*TP53* L194R, chr17:g.7578268A>C	0.1927%	78.9189%	rs1057519998	COSM44571/p	missense	MODERATE
	ALB A585_K588del, chr4:g.74285325_74285336delCTGACGATAAGG	-	73.7288%	-	-	inframe	MODERATE
	TERT upstream_gene_variant chr5:g.1295228G>A	0.885%	51.98%	-	-	upstream_gene	MODIFIER
4	*ARID1A* M841IfsTer31, chr1:g.27089566dupT	-	91.0867%	-	-	frameshift	HIGH
	TP53 R213L, chr17:g.7578211C>A	2.2082%	88.1527%	rs587778720	COSM43650	missense	MODERATE
	CTNNB1 Y604F, chr3:g.41277847A>T	0.5626%	17.2583%	-	-	missense	MODERATE
	TERT upstream_gene_variant chr5:g.1295250G>A	-	61.5545%	-	-	upstream_gene	MODIFIER
5	*TERT* upstream_gene_variant chr5:g.1295228G>A	5.2288%	27.9707%	-	-	upstream_gene	MODIFIER
6	*AXIN1* splice_donor_variant, chr16:g.396147C>T	0.4975%	19.5364%	-	-	splice_donor	HIGH
	TP53 R249S, chr17:g.7577534C>A	0.4552%	14.2857%	rs28934571	COSM10817	missense	MODERATE
	*BRAF 3*_prime_UTR_variant, chr7:g.140425328C>A	0.2375%	15.8027%	-	-	3_prime_UTR	MODIFIER
7	*TP53* R280G, chr17:g.7577100T>C	-	89.4197%	rs753660142	COSM11123/p	missense	MODERATE
8	*TP53* Y103S, chr17:g.7579379T>G	-	15.0234%	-	-	missense	MODERATE
9	*FGFR2* Y813F, chr10:g.123239402T>A	-	18.5915%	-	-	missense	MODERATE
	FLT1 intron_variant, chr13:g.28961898A>T	-	21.1111%	-	-	intron	MODIFIER
	FLT1 intron_variant, chr13:g.28963690G>T	-	20.4166%	-	-	intron	MODIFIER
	KDR splice_region_variant&intron_variant, chr4:g.55961132dupA	-	18.552%	-	-	splice_region & intron	LOW
	ALB F73HfsTer65, chr4:g.74272425_74272434delTTTGCAAAAA	-	13.1884%	-	-	frameshift	HIGH
	TERT L810P, chr5:g.1271273A>G	-	16.134%	-	-	missense	MODERATE
10	*TERT* upstream_gene_variant chr5:g.1295228G>A	-	27.7214%	-	-	upstream_gene	MODIFIER
11	-	-	-	-	-	-	-
12	-	-	-	-	-	-	-
13	-	-	-	-	-	-	-
14	-	-	-	-	-	-	-
15	-	-	-	-	-	-	-
16	-	-	-	-	-	-	-

Abbreviations: AF: Allele Frequencies; tDNA: Tumor DNA; cfDNA: Circulating Free DNA

Thirteen of these sixteen patients were hepatitis B-infected for 4 to 42 years, whereas, 3 patients were diagnosed with latent hepatitis B based on HBV immunological testing. Six of these patients showed complications due to liver cirrhosis, whereas, ten patients experienced Transcatheter Arterial Chemoembolization (TACE). Of the six patients that experienced relapse, four progressed to metastatic disease. As shown in Table 1, six patients died by September 30; 4 died from HCC, 1 succumbed to liver cirrhosis and 1 died from heart disease.

Table 3: Tumor size and mutation relevance ratio.

Large size group				Small size group			
Case No.	Tumor size (cm^3)	tDNA mutation No.	cfDNA mutation No.	Case No.	Tumor size (cm^3)	tDNA Mutation No.	cfDNA Mutation No.
15	851.904	0	0	2	168.1	7	1
1	1645.37	8	5	10	112.48	1	0
5	1375.89	1	1	12	74.52	0	0
11	602.615	0	0	7	133.28	1	1
9	304.92	6	0	4	142.08	4	2
14	687.96	0	0	8	87.204	1	0
16	469.476	0	0	13	52.08	0	0
3	254.856	3	2	6	37.44	3	3
Mean ± SD	774.124 ± 499.526	2.250 ± 3.151	1.000 ± 1.773	Mean ± SD	100.898 ± 45.807	2.125 ± 2.416	0.875 ± 1.126

P-value (tDNA Mutation No.): 0.930397838
P-value (cfDNA Mutation No.): 0.869149176

Table 4: Liver cirrhosis in HCC and mutation relevance ratio.

HCC patients with cirrhosis			HCC patients without cirrhosis		
Case No.	tDNA mutation No.	cfDNA mutation No.	Case No.	tDNA mutation No.	cfDNA mutation No.
1	8	5	2	7	1
3	3	2	4	4	2
6	3	3	5	1	1
10	1	0	7	1	1
11	0	0	8	1	0
14	0	0	9	6	0
			12	0	0
			13	0	0
			15	0	0
			16	0	0
Mean ± SD	2.500 ± 3.017	1.667 ± 2.066	Mean ± SD	2.000 ± 2.667	0.500 ± 0.707

P-value (tDNA Mutation No.): 0.744825513
P-value (cfDNA Mutation No.): 0.231915251

Molecular profiling

Ten out of 16 (62.5%) HCC tissue specimens showed at least one mutation. Overall, we detected 35 mutations distributed among 13 genes. Six cfDNA samples also showed at least one mutation, which were also found in the corresponding HCC tumor specimens. The top 6 mutant genes included *TP53* (22.86%), *TERT* (20%), *ALB* (14.29%), *AXIN1* (11.43%), *FLT1* (5.71%) and *ARID1A* (5.71%). As shown in Table 2, the *TERT* upstream gene variant (chr5: g. 1295228G>A) was reported in more than one patient.

Eleven of the 35 mutations were reported in the ClinVar and/or COSMIC databases (https://www.ncbi.nlm.nih.gov/clinvar/, http://cancer.sanger.ac.uk/cosmic/) (Table 2). These 35 mutations were classified as modifiers (10/35), moderate (15/35), high (8/35) and low (2/35) impact mutations. The high impact variants included stop gained, frame shift, splice donor and splice acceptor mutations. The modifier variants included 5'-UTR, 3'-UTR, intronal and upstream gene mutations. The moderate variants were all missense mutations. Low-impact variants showed mutations in the splice region and introns (Table 2).

Statistical analysis

The differences between mutation relevance ratios of different tumor tissues (relative to tumor size) were evaluated by t-test. The patients were divided into 2 groups based on tumor size. The large tumor size group included cases 15, 1, 5, 11, 9, 14, 16 and 3, which ranged from 254.856 m^3 (case 3) to 1645.371 m^3 (case 1). We detected 22 mutations from 4 cases (2.250 ± 3.151 mutations/case). The smaller size group included cases 2, 10, 12, 7, 4, 8, 13 and 6, which ranged from 37.44 m^3 (case 6) to 168.1 m^3 (case 2). We detected 17 mutations from 5 cases (2.125 ± 2.416 mutations/case). The tumor size difference was nearly 6 times between the larger and the smaller groups (p=0.001198). However, the mutation relevance ratio between the 2 groups was similar (p=0.930198). Moreover, the mutation relevance ratio between the cfDNA of the two groups was also similar (p=0.869149; Table 3).

We detected mutations in the tumor tissues of 4 out of 6 HCC patients with liver cirrhosis. Moreover, 3 out of 4 tumor samples with mutations showed the corresponding mutation in the cfDNA. We detected mutations in the tumor tissues of 6 out of 10 patients without cirrhosis and 4 of these 6 patients showed the corresponding mutation in the cfDNA. However, the mutation relevance ratios in both the tumor DNA (p=0.744826>0.05) and cfDNA (p=0.231915>0.05) were not significant (Table 4). Moreover, there was no association between the mutations and serum AFP levels (Table 5).

We detected 5 *ALB* mutations in 4 tumor samples. However, the plasma *ALB* concentration was similar for patients with or without *ALB* mutations (data not shown). Tumors with *ALB* mutations were

Table 5: AFP and mutation relevance ratio.

HCC patients with cirrhosis			HCC patients without cirrhosis		
Case No.	**tDNA mutation No.**	**cfDNA mutation No.**	**Case No.**	**tDNA mutation No.**	**cfDNA mutation No.**
1	8	5	2	7	1
3	3	2	4	4	2
6	3	3	5	1	1
10	1	0	7	1	1
11	0	0	8	1	0
14	0	0	9	6	0
			12	0	0
			13	0	0
			15	0	0
			16	0	0
Mean ± SD	2.500 ± 3.017	1.667 ± 2.066	Mean ± SD	2.000 ± 2.667	0.500 ± 0.707

P-value (tDNA Mutation No.): 0.744825513
P-value (cfDNA Mutation No.): 0.231915251

Table 6: *ALB* mutations and mutation relevance ratio.

HCC patients with *ALB* mutations			HCC patients without *ALB* mutations		
Case No.	**tDNA mutation No.**	**cfDNA mutation No.**	**Case No.**	**tDNA mutation No.**	**cfDNA mutation No.**
1	8	5	4	4	2
2	7	1	5	1	1
3	3	2	6	3	3
9	6	0	7	1	0
			8	1	0
			10	1	0
Mean ± SD	6.000 ± 2.160	2.000 ± 2.160	Mean ± SD	1.833 ± 1.329	1.000 ± 1.265

P-value (tDNA mutation No.): 0.021407136
P-value (cfDNA mutation No.): 0.446618403

associated with additional gene mutations than tumors without *ALB* mutations (p=0.021407). However, the differences were not significant between the two groups in the corresponding cfDNA samples (p=0.446618; Table 6). The leading mutant genes such as *TP53* and *TERT* were not associated with the mutation relevance ratios (data not shown).

Discussion

To assess the mutation relevance ratio in HCC cfDNA samples, we analyzed 41 HCC-related mutation hotspot genes that span about 190 kb (Table 7) and cover about 70% of HCC cases in The Cancer Genome Atlas (TCGA) database (https://cancergenome.nih.gov/cancersselected/LiverHepatocellularCarcinoma). To ensure that the mutations in circulating cfDNA were representative of the HCC, we analyzed genomic DNA from HCC and adjacent non-tumor tissues from the same patients with the same panel. Moreover, we used cryopreserved tumor tissues to avoid the bias in Formalin-Fixed Paraffin-Embedded (FFPE) tumor sections. We achieved 500X sequencing depth with our approach, which enabled identification of 1% mutations in the HCC tumors.

We detected 35 mutations in 13 genes from 10 out of 16 (62.5%) HCC samples analyzed. This mutation ratio was lower than the 70% we expected. This was probably because (1) the patients in our study were also diagnosed with hepatitis B-associated HCC and were different from those in the TCGA database and (2) the small sample size may have introduced random bias. Therefore, in future,

Table 7: List of 41 genes in Accu-Act HCC.

ACVR2A	*CDKN2A*	*FGFR3*	*KMT2B*	*TEK*
ALB	*CTNNB1*	*FLT1*	*KMT2D*	*TERT*
ARID1A	*EGFR*	*HGF*	*MET*	*TP53*
ARID2	*EPHA4*	*IL6R*	*MTOR*	*VCAM1*
AXIN1	*ERBB2*	*JAK1*	*NFE2L2*	*VEGFA*
BRAF	*FGF19*	*JAK3*	*PDGFRB*	
CCNE1	*FGF3*	*KDR*	*RB1*	
CDK14	*FGF4*	*KEAP1*	*RPL22*	
CDKN1A	*FGFR2*	*KIT*	*RPS6KA3*	

HBV-related HCC sample testing needs to be integrated into the test panel to resolve this issue. The Allele Frequencies (AF) values of all identified mutations were more than 10% (13.19%~91.09%) (Table 2); this suggested that 10% might be a suitable cutoff value in HCC.

The 16 HCC patients enrolled in this study were all HBV-infected. Persistent HBV infection results in viral genome mutations and integration of the viral genome into the human genome, which promotes HCC [7,8]. The prolonged expression of the HBV regulatory protein HBx and/or preS/S viral envelope proteins deregulate cellular transcription and proliferation and promote hepatocyte transformation [21]. Moreover, HBV mutations such as G1896A, A1762T, G1764A, and A1762T/G1764A are linked to increased risk of HCC [22]. The overall HBV integration frequency

is much higher in males than in females, which also correlates with higher prevalence of HCC in males than in females [23,24]. Our results suggest that HBV infection is an important causative factor of HCC in China. Therefore, HCC treatment may benefit from anti-viral therapies [25]. However, our target panel did not include HBV-related primers to assay HBV integration index. We detected 7 *TERT* gene mutations, 5 of which were located in the up-stream, including chr5:1295228G>A (COSM1716558) 4 times and chr5:1295250G>A (COSM1716559) once. These 2 mutations are located in the *TERT* promoter and may be linked to upregulation of *TERT* activity. This further shows that HBV infection may play an integral role in HCC tumorigenesis [26,27].

In the COSMIC database, the leading mutations of HCC were *TP53* (27%), *TERT* (26%), *CTNNB1* (19%), *AXIN1* (8%) and *ARID2* (7%) [14]. In a recent study of HBV-associated HCC, the top 6 mutations were *TERT* (65 %), *TP53* (38 %), *CTNNB1* (35 %) and *AXIN1* (2 %), *PTEN* (2 %) and *CDKN2A* (2 %) [24]. In this study, the top mutated genes were *TP53* (22.86%), *TERT* (20%), *ALB* (14.29%), *AXIN1* (11.43%), *FLT1* (5.71%) and *ARID1A* (5.71%). The differences among the studies suggest the heterogeneous nature of HCC, although methodological differences can't be ruled out. Mutations in *ALB* and *FLT1* genes were in the top 6 in our study, but were not highlighted in other studies. On the other hand, we only observed 2.85% of *CTNNB1* mutations (1/35), whereas, *CTNNB1* was the top 5 mutated gene in other studies.

We observed at least one mutation in 10 out of the 16 tumor tissues that were analyzed. The low mutation relevance ratio was not associated with tumor size, cirrhosis, and AFP status (Tables 3-5), consistent with reports by Liao et al. and Huang et al. [28,29]. Moreover, the larger tumors did not increase mutations in cfDNA (Tables 2 and 3). Theoretically, the cfDNA mutation ratio should be higher in the larger tumors than in smaller tumors. Therefore, wider genomic coverage and larger sample size is necessary in future studies to resolve this issue.

Liver is rich in degradative enzymes for a number of substrates, including DNA. Liver cirrhosis damages liver functions and we observed that 6 out of 16 HCC patients were associated with cirrhosis. Moreover, the mutation relevance ratio was higher in 4 of the 6 HCC cases with cirrhosis, although, it was statistically insignificant. 14 mutations of cfDNA from 6 cases were isolated, and all of them could find the corresponding origins from 35 mutations of 10 tumor tissues. The transmissivity was 40% (14/35), it is a little lower than a recent report. Not all tissue variants could be confirmed in the matched plasma, up to 57% of the tumor variants were reflected in matched cfDNA [15]. One reason for lower number of mutations in the cfDNA could be the low cfDNA (~5 ng) input used in the assay. In spite of this drawback, our study suggests that cfDNA testing can be used to test liver pathology.

HBV infection induces inflammation and oxidative stress, which promote both cirrhosis and HCC [30]. In our study, 6/16 HCC cases were associated with liver cirrhosis, which is a risk factor for HCC. Integration of HBV into the human genome results in persistent expression of *HBX* and preS1/S2 viral proteins, which destabilize the human genome and accumulate harmful mutations that transform hepatocytes [24]. We detected no HBV integration in our assays. However, because HBV integrates randomly into the human genome [24], future studies should employ specific HBV primers and achieve larger genomic coverage.

TP53 is one of the most commonly mutated genes in most human tumors including HCC [24,31]. We identified 8 mutations of *TP53* in our study. Among these, *TP53* R249S (case 6) is related to poor prognosis of HCC [32]. We also identified *CDN2A* D74V mutant, which is part of the *TP53* signaling pathway [24]. Several clinical trials (NCT02999893; NCT02935907) are underway for drugs targeting *TP53* and related pathway genes to improve the prognosis of *TP53* mutant tumors [33,34].

Deregulated *TERT* activity as a result of mutations improves tumor cell survival [26,35]. *TERT* promoter is a hot spot for HBV-induced mutations, and -124G>A (chr5:g.1295228G>A) and −146G>A (chr5:g.1295250G>A) mutations are reported in 42.4% of Asian HCC patients [26]. These two mutations are in the vicinity of *TP53* binding sites (chr5: g. 1295221 and chr5: g. 1295255) (http://www.sabiosciences.com/chipqpcrsearch.php?gene=*TERT*&species_id=0&factor=p53&ninfo=n&ngene=n&nfactor=n). This suggests that these *TERT* promoter mutations diminish tumor suppressor activity of *TP53*. We identified 5 *TERT* promoter mutations in the HCC tumor samples (Table 2). These mutations were also identified from 2 cfDNA samples. This suggests that these *TERT* promoter mutations are potential biomarkers for HCC screening and targeted therapy.

WNT/β-catenin pathway is another commonly deregulated signaling pathway in HCC [36]. We identified 4 *AXIN1* mutations from 2 cases and 1 *CTNNB1* mutation in the third case. *AXIN* and *CTNNB1* are two key integral players of the *WNT* signaling pathway [37]. Loss of function mutations in the *AXIN1* gene plays a critical role in several tumors [36]. Aberrant activity of *CTNNB1*, which is an intracellular signal transducer in the *WNT* signaling pathway, is associated with HCC [37]. It is plausible that *TERT* promoter mutations increase the *WNT* pathway activity *via AXIN1* and/or *CTNNB1* mutations [38,39]. Preclinical studies on targeted *CTNNB1* therapy are currently underway and probably will play a critical role in future HCC therapy [40].

We also identified 5 mutations in the *PI3K-AKT-mTOR* pathway genes. We identified the *HGF* Y167N mutation (AF=34.33%) in case 2 and *FGFR2* Y813F, 2 *FLT1* intronal variants and a KDR splice region and intronal variants from case 9 (Table 2). Since many drugs targeting the *PI3K/AKT/mTOR* pathway are already in use, these mutations suggest use of related drugs in targeted HCC therapy.

We also identified secondary *ALB* mutations (14.29%) in HCC samples, and it was similar to a recent study, which observed *ALB* mutations in 13% of tumors [31]. Albumin is synthesized in the liver and comprises about one-half of the blood serum proteins. Tumor cells with *ALB* inactivating mutations are selected probably because they efficiently divert energy into cancer-relevant metabolic pathways [41]. We identified five *ALB* mutations in our study, namely, *ALB* 5_prime_UTR_variant (chr4:g.74270031A>G), A585_K588del (chr4:g.74285325_74285336delCTGACGATAAGG), V78A (chr4:g.74272441T>C), splice acceptor variant (chr4:g.74285222_74285223delAG), and F73HfsTer65 (chr4:g.74272425_74272434delTTTGCAAAAA). Several of these were loss-of-function mutations. Moreover, as shown in Table 6, the number of cases with *ALB* mutations (6.000 ± 2.160) were more than cases without *ALB* mutations (1.833 ± 1.329; p=0.021407), suggesting hypermutation.

Compliance with Ethics Guidelines

All procedures followed were in accordance with the ethical

standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2000 (5). Informed consent was obtained from all patients for being included in the study.

Authors Contribution

Heping Hu conceived the project, designed the experiments and supervised the study; Xiaoyan Li, Liping Lei, Peng Wang and Guofang Liu collected the clinical data; Hao Wang, Nan He and Huabang Zhou performed the experiments and analyzed the data; Hao Wang wrote the manuscript; Heping Hu helped correct and review the manuscript; All the authors have read this manuscript and approved the manuscript.

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