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Targeted sequencing of cancer-associated genes in hepatocellular carcinoma using next-generation sequencing

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Abstract. Hepatocellular carcinoma (HCC) is the third leading cause of cancer mortality worldwide. Although the clinical success rate for the treatment of early-stage HCC has improved, the prognosis of advanced HCC remains poor owing to the high recurrence rate and the refractory nature of HCC for various anticancer drugs. A better understanding of the pathogenesis of HCC is therefore critically needed in order to treat HCC, including its genetic alterations. Next-generation sequencing (NGS) has provided an unbiased platform to systematically identify gene mutations and reveal the pathogenesis of various cancers. In the present study, a total of 118 samples (59 liver tissues including cancer and adjacent normal tissues) were sequenced using the AmpliSeq Hotspot Cancer Panel (version 2). The most common somatic mutations identified were tumor protein 53 (*TP53*; 35.6%) and β-catenin 1 (*CTNNB1*; 30.5%), and the most frequent variants of those genes were missense variants. In addition, somatic mutations including those in genes encoding colony-stimulating factor 1 receptor (5.1%), epidermal growth factor receptor (6.8%), *RET* proto-oncogene (3.4%), Erb-B2 receptor tyrosine kinase 4 (*ERBB4*; 1.7%) and serine/threonine kinase 11 (*STK11*, also known as liver kinase B1; 6.8%) were also identified at a low frequency in patients with HCC. A frameshift variant in *STK11*, a splice acceptor variant in *TP53*, a splice region variant in *ERBB4* and a stop-gained variant in *TP53* were also specifically determined. The most abundant alteration was a C:G>T:A transition (50%) and other transversions, i.e., C:G>G:C (19.6%), T:A>C:G (19.6%), C:G>A:T (12.5%), T:A>G:C (12.5%) and T:A>A:T (5.4%). This spectrum pattern differs from that in other solid tumors. *TP53* mutations in the tumors at advanced stages were significantly more frequent compared with those in early-stage tumors. Additionally, age (<70 vs. ≥70 years) was significantly associated with *CTNNB1* mutations. Using NGS, a number of novel gene mutations were identified in HCC, including established mutations and disproved mutations. The results of the present study offer new insight and improved understanding of the etiology and the development of HCC.

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Introduction

As the third leading cause of cancer-associated mortality worldwide, hepatocellular carcinoma (HCC) is one of the most common types of cancer (1). Although the clinical management of early-stage HCC has improved, the prognosis of HCC remains poor owing to its high recurrence rate (2). The prognosis of advanced HCC is particularly poor, due in part to its refractory nature to various anticancer drugs. An improved understanding of the pathogenesis of this type of cancer may contribute to more effective outcomes for the treatment of advanced HCC.

The progression of liver cirrhosis has been demonstrated to be a primary step in the pathogenesis of HCC (3). Chronic infections with hepatitis B (HBV) or hepatitis C (HCV) and other major risk factors, including alcoholic liver diseases, non-alcoholic steatohepatitis, autoimmune hepatitis and primary biliary cirrhosis (4), frequently cause liver inflammation, hepatic damage and subsequently cirrhosis. It has been speculated that the processes of HCC tumorigenesis with cirrhosis include an accumulation of genetic alterations. Han (5) reported that the development of HCC is also associated with genetic aberrations (5). However, the key drivers of the development of HCC remain unclear, and there is a requirement to elucidate the underlying molecular mechanisms (including various gene mutations) in the development of HCC.

Next-generation sequencing (NGS) has provided new paradigms in many fields, including molecular biology,

physiology and medicine, that may be used to disclose the genetic basis of various diseases (6,7). Novel genetic mutations associated with tumorigenesis, tumor progression and metastasis have been identified using NGS, including those in genes encoding isocitrate dehydrogenase 1 (*IDH1*) in glioblastoma multiforme (8) and acute myeloid leukemia (9), chromodomain helicase DNA-binding protein 7 in small cell lung cancers (10), glutamate metabotropic receptor 3, transformation/transcription domain-associated protein, mitogen-activated protein kinase kinase 1/2, mitogen-activated protein kinase kinase kinase 5/9 and phosphatidylinositol 3,4,5-trisphosphate-dependent Rac exchange factor 2 in melanoma (11-15), Notch homolog 1 (*NOTCH1*) in chronic lymphocytic leukemia (16), splicing factor 3B subunit 1 in myelodysplasia (17,18), and chromatin-remodeling proteins such as AT-rich-interaction domain 1A in ovarian, kidney and gastric cancer (19,20).

Historically, the discovery of somatic mutations in various types of cancer has been unexpected due to conventional methods based on direct sequencing. Direct sequencing has a major limitation regarding the identification of new somatic mutations because of its candidate gene-based methodology. Conversely, NGS has provided an unbiased platform to systematically discover gene mutations and reveal the pathogenesis of various types of cancer.

In the present study, 50 genes associated with the development of various types of cancer were targeted, and the association between the genetic mutations and the clinical characteristics of HCC patients was investigated using an NGS platform.

Materials and methods

Patients. The present study involved 57 patients (48 males, 11 females; mean age, 69.1±10.1) who had undergone surgery for HCC at Kagawa University Hospital (Miki, Japan) between January 2001 and March 2013. Written informed consent was provided by all patients and the present study was conducted according to the Ethical Guidelines for Medical and Health Research approved by the Ministry of Health, Labour and Welfare of Japan.

Tissue samples. Cancerous and adjacent non-cancerous tissues were collected macroscopically (3-5 mm thick sections) and immediately frozen in liquid nitrogen following surgery. Tissues were stored at -80˚C until DNA extraction.

Next-generation sequencing. Genomic DNA was extracted from tissue samples using the PureLink Genomic DNA Mini kit (Thermo Fisher Scientific, Inc., Waltham, MA, USA), according to the manufacturer's protocol. For library construction, 10 ng DNA was amplified using the AmpliSeq Cancer hotspot panel (version 2; Thermo Fisher Scientific, Inc.) and Ion AmpliSeq HiFi Master Mix (Ion AmpliSeq Library kit 2.0, Thermo Fisher Scientific, Inc.). An amplicon library was thus generated for sequencing 2,850 hotspot mutations in 50 genes: *ABL1*, *AKT1*, *ALK*, *APC*, *ATM*, *BRAF*, *CDH1*, *CDKN2A*, *CSF1R*, *CTNNB1*, *EGFR*, *ERBB2*, *ERBB4*, *EZH2*, *FBXW7*, *FGFR1*, *FGFR2*, *FGFR3*, *FLT3*, *GNA11*, *GNAQ*, *GNAS*, *HNF1A*, *HRAS*, *IDH1*, *IDH2*, *JAK2*, *JAK3*, *KDR*, *KIT*, *KRAS*, *MET*, *MLH1*, *MPL*, *NOTCH1*, *NPM1*, *NRAS*, *PDGFRA*, *PIK3CA*, *PTEN*, *PTPN11*, *RB1*, *RET*, *SMAD4*, *SMARCB1*, *SMO*, *SRC*, *STK11*, *TP53* and *VHL*.

The amplicons were then digested, barcoded and amplified with the Ion AmpliSeq Library kit 2.0 and Ion Xpress barcode adapters kit (Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. The library was then quantified using the High Sensitivity DNA kit for the Agilent 2100 Bioanalyzer (Agilent Technologies, Inc., Santa Clara, CA, USA). A total of 8 pM of each library was multiplexed and clonally amplified on Ionsphere particles (ISPs) by emulsion polymerase chain reaction (PCR) performed using the Ion One Touch 2 instrument with the Ion PGM template OT2 200 kit (Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol.

Quality control was performed using the Ionsphere quality control kit (Thermo Fisher Scientific, Inc.) to ensure that between 10 and 30% of template-positive ISPs were generated in the emulsion PCR. Finally, the template ISPs were enriched, loaded onto an Ion 318 chip and sequenced using a PGM sequencer with the Ion PGM sequencing 200 kit (version 2), according to the manufacturer's protocol.

Data analysis. The raw data were aligned to Human Genome version 19 (hg19) using Torrent Suite software (version 3.6.2; Thermo Fisher Scientific, Inc.). The coverage analysis was performed using the Coverage Analysis plugin (version 3.6; Thermo Fisher Scientific, Inc.). Cases for which the quality was <20% and/or the average base coverage was <500X reads and/or the frequency was <10% were considered non-informative. Mutations were detected using the Variant Caller plugin (version 3.6; Thermo Fisher Scientific, Inc.). Each mutation was verified using the Integrative Genome Viewer (IGV) from the Broad Institute (www.broadinstitute.org) (21).

Statistical analysis. Fisher's exact test (two-sided) was performed to analyze the association between *TP53*, *CTNNB1* and *SMARCB1* mutations, and clinicopathological parameters using Prism 6 software (version 6.02; GraphPad Software, Inc., La Jolla, CA, USA). $P<0.05$ was considered to indicate a statistically significant difference.

Results

Study population. As presented in Table I, 48 males and 11 females were characterized. Of these, 26 were <70 years old and 33 were ≥70 years old. In total, 25 of the HCV-positive patients and 14 of the HBV-positive patients were included, and 20 non-viral hepatitis patients were also included in the study population. A total of 24 patients had liver cirrhosis, and 35 patients had normal or chronic hepatitis. In addition, 47 of the well- or moderately differentiated patients with HCC and 12 of the poorly differentiated patients with HCC were examined. A total of 28 stage I/II patients and 31 stage III/IV patients (TNM classification) were characterized in the present study (Table I). *TP53* mutations were identified to be associated with age and TNM stages, and *CTNNB1* mutation was associated with viral infection (Table I).

Mutation profiling by NGS. A total of 118 samples (59 liver tissues including both cancer and adjacent normal tissues) were

Table I. Association between clinicopathological features and representative genetic mutations in hepatocellular carcinoma.

		TP53			*CTNNB1*			*SMARCB1*		
Characteristic	n	Wild-type	Mutant	P-value	Wild-type	Mutant	P-value	Wild-type	Mutant	P-value
Sex				0.513			0.077			0.573
Male	48	24	24		28	20		43	5	
Female	11	7	4		10	1		11	0	
Age, years				0.019			0.102			0.372
<70	26	9	17		20	6		25	1	
≥70	33	22	11		18	15		29	4	
Viral infection				0.228			0.006			0.952
HCV	25	16	9		14	11		23	2	
HBV	14	5	9		14	0		13	1	
NBNC	20	10	10		10	10		18	2	
Fibrosis stage				0.597			0.18			0.309
F0, F1, F2, F3	35	17	18		20	15		30	5	
F4	24	14	10		18	6		24	0	
Histological grade				0.999			0.182			0.573
WD/MD	47	25	22		28	19		42	5	
PD	12	6	6		10	2		12	0	
TNM				0.037			0.576			0.639
I/II	28	16	12		17	11		26	2	25
III/IV	31	9	22		21	10		28	3	

TP53, tumor protein 53; *CTNNB1*, β-catenin 1; *SMARCB1*, SWI/SNF-associated matrix-associated actin-dependent regulator of chromatin subfamily B member 1; HCV, hepatitis C virus; HBV, hepatitis B virus; NBNC, non-B, non-C hepatocellular cancer; WD, well-defined; MD, moderately defined; PD, poorly defined; TNM, tumor-node-metastasis.

sequenced for the AmpliSeq Hotspot Cancer panel (version 2). In order to determine the appropriate variants, variations were retrieved that were present only in the cancerous region of each patient and absent from the normal portion of the same individual.

In total, 14 of the 50 genes (28%) revealed any mutation, and the number of missense variants was the highest in several variants (Fig. 1), including synonymous variant, intron variant and frameshift variant (Fig. 2). The most common somatic mutations identified were in genes *TP53* (35.6%) and *CTNNB1* (30.5%), and the most frequent variants of those genes were missense variants (Fig. 3).

Somatic mutations in *CSF1R* (5.1%), *EGFR* (6.8%), *RET* (5.1%) and *STK11* (6.1%) genes were also identified in the HCC patients, although these genes exhibited various types of variant at low frequency (Fig. 3). Frameshift variant in *STK11*, splice acceptor variant in *TP53*, splice region variant in *ERBB4* and stop-gained variant in *TP53* were specifically determined, and these variants may alter the transcription of those genes.

The mutation spectrum revealed C:G>T:A transitions (50%), which were the most abundant alteration (22), and other transversions including C:G>G:C (19.6%), T:A>C:G (19.6%), C:G>A:T (12.5%), T:A>G:C (12.5%) and T:A>A:T (5.4%) (Fig. 4). This spectrum pattern differs from that in other solid tumors (22).

Clinicopathological features and genetic mutations. The p53 pathway was identified to be the most frequently altered in HCC in the present study. The presence of *TP53*-inactivating mutation (35.6%) and *CDKN2A* mutations (1.7%) revealed the significance of the p53 pathway during the development of HCC. Indeed, *TP53* mutations in the tumors at advanced stages were significantly more frequent than those in the tumors at early stages (P=0.037; Table I).

The Wnt/β-catenin pathway was identified as the second most frequently altered pathway in HCC. This was evident by the presence of *CTNNB1* (30.5%) and *APC* (1.7%) mutations. Although no association between the *CTNNB1* mutations and clinicopathological features, including sex, viral infection, fibrosis stage, histological grade and TNM classification was identified to be significant in the patients with HCC, age (<70 and ≥70 years) was identified to be significantly associated with *CTNNB1* mutations (P=0.019; Table I).

Discussion

The accumulation of genetic alterations is required during the development of HCC. Although advances in investigative techniques have exposed genetic deviations in HCC, including genetic mutations in *CTNNB1* and *TP53* (5), the overall picture of genetic alterations during the tumorigenesis of HCC remains unclear.

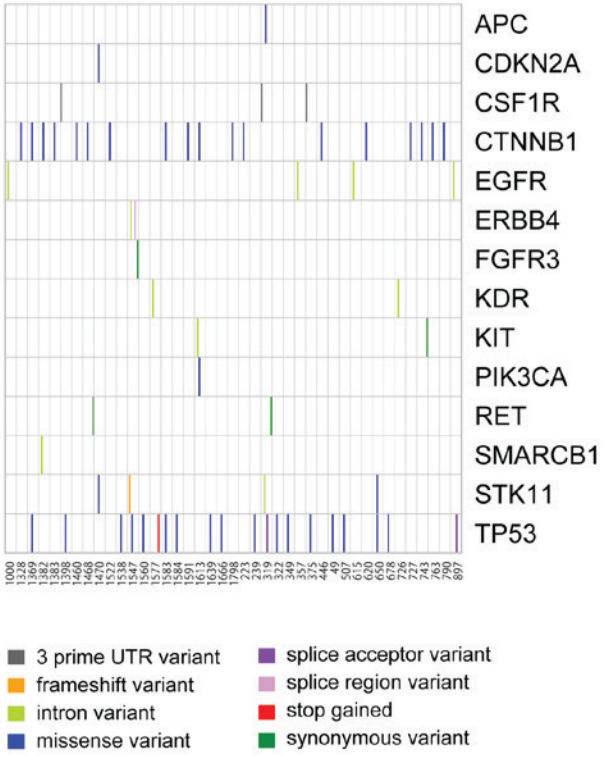

Figure 1. Overview of somatic mutations of genes associated with hepatocellular carcinoma. The heat map indicates genes (rows) and sample numbers (columns) with 3' UTR variants (gray), frameshift variants (orange), intron variants (light green), missense variants (blue), splice acceptor variants (purple), splice region variants (pink), stop-gained variants (red) and synonymous variants (green). UTR, untranslated region.

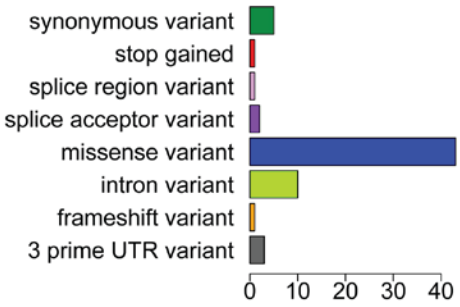

Figure 2. Numbers of various somatic mutations of genes associated with HCC. In total, 8 types of variant including 3' UTR variants (gray), frameshift variants (orange), intron variants (light green), missense variants (blue), splice acceptor variants (purple), splice region variants (pink), stop-gained variants (red) and synonymous variants (green) were classified by next-generation sequencing in HCC. HCC, hepatocellular carcinoma; UTR, untranslated region.

The oncogene *CTNNB1* has been identified as one of the most commonly mutated genes in HCC (23). Overall, *CTNNB1* is mutated in nearly 30% of HCC, and the mutation frequency alters by different etiologies. In the present study, no *CTNNB1* mutation was observed in the HBV-associated HCC, although *CTNNB1* mutations were observed in 44 and 50% of the HCV-associated HCC and non-virus-associated HCC, respectively. Guichard *et al* (24) identified that the frequency of *CTNNB1* mutation is only 11% in HBV-associated HCC compared with 40% in HCC of other etiologies (24). These results support those of the present study that the *CTNNB1* mutation was less predominant in HBV-associated hepatocarcinogenesis.

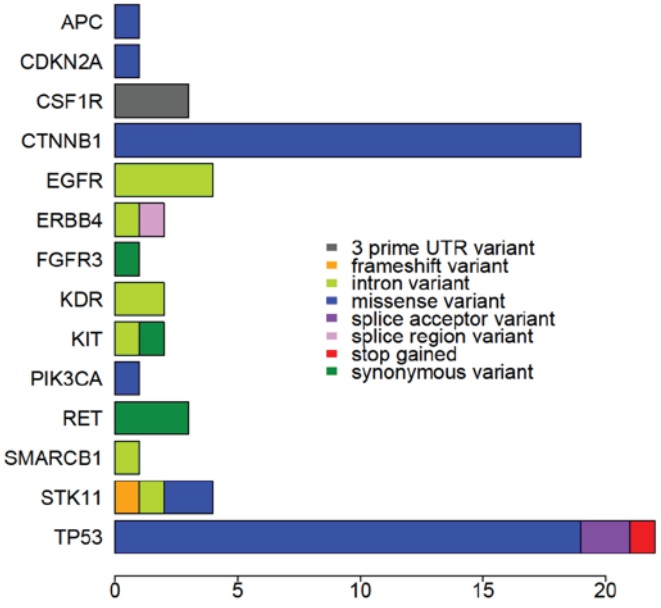

Figure 3. Genes having at least one somatic mutation determined in hepatocellular carcinoma. UTR, untranslated region.

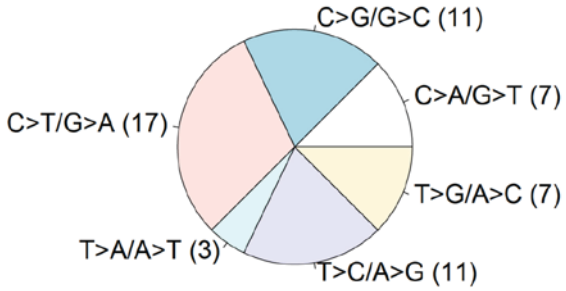

Figure 4. Profiles of somatic substitution patterns of the 59 hepatocellular carcinoma genomes. The number of events is indicated within parentheses.

It is also well known that *TP53*, which is a tumor suppressor, is frequently mutated and inactivated in HCC (24). Interestingly, in contrast with the *CTNNB1* mutation, a *TP53* mutation was detected in 64% of the HBV-associated HCC in the present study, although *TP53* mutations were detected in 36 and 50% of the HCV-associated HCC and non-virus-associated HCC. This result was confirmed by independent studies reporting that *TP53* occurs more frequently in HBV-associated HCC with a frequency between 30 and 40% compared with nearly 20% in HCV-associated HCC (24,25). In addition, *TP53* mutation was exclusive from *CTNNB1* mutation in HBV-associated HCC (24). This result indicates that *TP53* mutation may be a key factor in HBV-associated HCC.

Single point somatic mutations contain both a transition and a transversion. Various types of mutation spectrum have been found in various tumor with different rates of transitions and transversions (22). In the present study, C:G>T:A transition (50%), C:G>G:C transversion (19.6%), T:A>C:G transition (19.6%), C:G>A:T transversion (12.5%), T:A>G:C (12.5%) transversion, and T:A>A:T transversion (5.4%) were detected in the HCC. A number of studies have demonstrated

that somatic mutation patterns are significantly distinct from the expected spectrum (24,26-28). In HCC, the C:G>T:A transition, T:A>C:G transition and C:G>A:T transversion are common mutations; however, in the present study, the C:G>G:C transversion was observed at a high frequency. This suggests that the C:G>G:C transversion may be associated with the etiology of HCC.

The use of NGS in the present study also revealed mutations associated with HCC that had not been clearly determined, including *STK11*, *CSF1R* and *RET*. In those mutations, *STK11* mutations were critical, since 75% of those mutations were frameshift variants and missense variants. A previous genetic analysis of the *STK11* gene demonstrated that this mutation may serve a role in tumor progression in a subset of HCC, protecting from p53-dependent apoptosis (29). Huang *et al* (30) identified that decreased expression of *STK11* is associated with poor prognosis. These results indicate that *STK11*, which has a lower mutation frequency, may be critical for a subset of HCC.

In conclusion, the use of NGS in the present study identified a number of novel gene mutations in HCC, including established mutations and disproved mutations. These results provide new insight into and improved understanding of the etiology, and the development, of HCC. Further investigations including whole exome sequencing are required to fully elucidate genetic mutations in HCC.

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