Research Paper

An investigation of the role of gene copy number variations in sorafenib sensitivity in metastatic hepatocellular carcinoma patients

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Abstract

Background: Metastatic hepatocellular carcinoma (HCC) is a highly aggressive tumor with limited treatment options. While sorafenib has recently been shown to provide a survival advantage in patients with advanced HCC, the overall outcomes such as time to progression (TTP) and overall survival (OS) ought to be further improved. To that end, several targeted agents aimed at amplified oncogenes such as *HER2* and *FGFR2* have recently been developed. In this study, we aimed to identify genetic markers in the form of copy number variations (CNVs) that influence clinical outcomes post-sorafenib treatment in advanced HCC patients.

Methods: We surveyed 38 metastatic HCC patients who were treated with sorafenib for the presence of CNVs using the NanoString nCounter assay.

Results: The median TTP and OS for all patients were 2.7 months (95% confidence interval [CI]: 2.0–3.3 months) and 13.4 months (95% CI: 8.4–18.4 months), respectively. Several genes previously implicated in liver cancer were amplified, including *CCND1* (n = 4), *CDKN1A* (n = 2), *KRAS* (n = 2), *MDM2* (n = 1), and *ERBB2* (n = 1). However, we found no correlations between CNVs and survival in our sorafenib-treated patients.

Conclusions: The clinical features and biomarkers that account for sensitivity to sorafenib in HCC are complicated and remain unclear. Further investigation to identify predictive biomarkers and therapeutic strategies, including combining sorafenib with other target agents, are warranted.

Key words: Hepatocellular carcinoma, metastatic disease, copy number variations, sorafenib.

Introduction

Primary tumors of the liver represent the fifth most frequently diagnosed type of cancer worldwide, and are the second most frequent cause of cancer-related death [1]. More than 75% of cases occur in the Asia-Pacific region, and are largely associated with chronic hepatitis B virus infection [2]. Surgical resection leads to a five-year survival rate of 60–70%

for patients with hepatocellular carcinoma (HCC) who present with a solitary tumor and have excellent liver function [3]; however, such resection is only an option for less than 20% of patients [3].

HCC that is diagnosed at an advanced stage or with progression after locoregional therapy has a dismal prognosis owing to the underlying liver

disease and lack of effective treatment options [4]. The systemic treatment that can lead to significant improvement in survival in advanced HCC patients is sorafenib, a multi-kinase inhibitor targeting RAF kinase and receptor tyrosine kinases such as platelet-derived growth factor receptor (PDGFR), vascular endothelial growth factor receptor (VEGFR), and c-KIT (a receptor specific for stem cell factor) [5, 6]. Sorafenib simultaneously inhibits two key functions that are reported to play an important role in the pathogenesis of HCC: proliferation (via the Raf-MEK-ERK pathway) and angiogenesis (via VEGFR and PDGFR) [7]. Despite some encouraging data, sorafenib extends survival by only 2–3 months; moreover, the drug has a low partial response rate (3%) [5, 6]. Therefore, it is important to be able to predict sensitivity to sorafenib in advanced HCC patients before commencing treatment.

The mechanisms accounting for HCC sensitivity to sorafenib are complicated and remain unclear. The IC_{50} values for sorafenib-induced growth inhibition in HCC cell lines *in vitro* vary widely [8]; one explanation for this is the genetic heterogeneity of HCC [9]. To further elucidate the mechanism of hepatocarcinogenesis, it is useful to reconstruct molecular events at both the gene expression and DNA copy number levels. Copy number variation (CNV) is defined as a gain or loss of copies of DNA segments that are larger than 1 kb in length when compared to a reference genome [10]. CNVs can affect gene expression and have been associated with disease susceptibility [11]; hence, several groups have applied recent technologies to identify CNVs and putative driver genes in HCC [12, 13]. Identifying such markers would be applicable to personalized medicine and/or would enhance existing therapeutic strategies by combining sorafenib with other molecular targeting agents.

To that end, we aimed to identify genetic markers that predict clinical outcomes of sorafenib treatment in advanced HCC patients. We hypothesized that CNVs are found in unique oncogenes and tumor suppressors; hence, we analyzed clinical features of 38 advanced HCC patients who were treated with sorafenib and profiled their tumor specimens for any existing CNVs.

Materials and Methods

Study Design and Samples

HCC formalin-fixed paraffin-embedded samples from 38 patients were collected for CNV detection on the NanoString nCounter platform (NanoString Technologies, Seattle, WA, USA). All cases used in the present study were retrieved during an 8-year period

(2005–2012) from Samsung Medical Center. Clinical information including age, sex, etiology, Eastern Cooperative Oncology Group (ECOG) performance status, Barcelona Clinic Liver Cancer (BCLC) staging system, Child-Pugh class, macroscopic vascular invasion, extrahepatic spread, alpha-fetoprotein levels, and previous treatment data were extracted from hospital records. This study was approved by the Institutional Review Board of Samsung Medical Center.

Genomic DNA isolation

Tissue from the needle biopsy was disrupted and homogenized in Buffer RLT plus, Qiagen AllPrep DNA/RNA MiniKit, using the Bullet Blender™ (Next Advance, Inc.). Specifically, tissue was transferred to a microcentrifuge tube containing 600 µL of Buffer RLT Plus and stainless steel beads. The tissue was homogenized in the Bullet Blender at room temperature. The sample was then centrifuged at full speed and the lysate transferred to a Qiagen AllPrep DNA spin column. Genomic DNA purification was conducted as directed by the AllPrep DNA/RNA Mini Handbook (Qiagen). DNA was quantified using the Nanodrop spectrophotometer, and its quality was accessed using the 260/280 nm and 260/230 nm absorbance ratios method.

NanoString nCounter assay

For CNV detection, a panel of 21 gene probes including *AURKA*, *CCND1*, *CCNE1*, *CDK4*, *CDK6*, *CDKN1A*, *CDKN2A*, *EGFR*, *ERBB2*, *ERBB3*, *FGFR1*, *FGFR2*, *IGF1R*, *KLF5*, *KRAS*, *MDM2*, *MET, MITF*, *MYC*, *PIK3CA*, and *TNIK* were designed using NanoString nCounter technology and subsequently analyzed on the NanoString nCounter platform [14]. Three probes were designed for each gene. Each assay contained six positive dsDNA control probes, eight negative control probes, and 10 invariant control probes (INVs) designed for autosomal genomic regions predicted not to contain common CNVs.

The NanoString nCounter assay was performed according to NanoString's standard protocol. Briefly, 600 ng of fragmented genomic DNA per assay was hybridized with the capture and reporter probes in 30 µL total volume and incubated at 65°C for a minimum of 16 h. The target and probe complexes were washed and immobilized in the cartridge. Genomic DNA was fragmented into small pieces (200–800 bp) and denatured to produce single strands. The custom CNV CodeSet was then hybridized to the fragmented denatured DNA sample in a single multiplexed reaction (up to 800 genomic loci per CodeSet). Hybridized DNA-CodeSet complexes were purified using the fully automated nCounter prep station, and

reporters were counted using the nCounter digital analyzer. The data were normalized to the INVs and to positive and negative controls in each hybridization reaction. Finally, data analysis was performed using the nSolver software.

The copy number was determined by averaging the copy numbers measured by three probes per region. Based on the manufacturer's protocol, the gene was considered to be a single copy if the average copy number was below 1.4, two copies if between 1.5 and 2.4, and three copies if between 2.5 and 3.4.

Copy number gain using real-time quantitative PCR

Based on the CNV results of the NanoString nCounter assay, quantitative PCR (qPCR) analysis was performed on the three most frequent genes using commercially available, predesigned TaqMan Copy Number Assays (Fig. 1). To allow a comparison between both approaches, we selected qPCR probes focused on the immediate region of the NanoString's probes. The reaction mixture contained 2 mL genomic DNA template, 10 mL of Taqman universal PCR master mixture (Applied Biosystems, Foster City, CA, USA), and 0.2 mM of each primer.

To accurately detect copy number gain (CNG), we analyzed three different regions of the *CCND1*, *KRAS*, and *MDM2* genes (Supplementary Table 1). CNG was determined using the following qPCR

sequence: 2 min at 50 ˚C, denaturation at 95 ˚C for 10 min, followed by 40 cycles of 95 ˚C for 15 s and 60 ˚C for 1 min using relative quantification in a 7900 HT fast real-time PCR system in quadruplicate. A RNaseP assay kit (Applied Biosystems) was used as a control. After amplification, the resulting threshold cycle values for the copy number and reference assay were imported into the CopyCaller Software (Applied Biosystems) for post-PCR data analysis as previously described. CNG and the number of copies were determined based on concordance of the results with one or more of the three regions of each probe.

Statistical analysis

Overall survival (OS) was measured from the start of sorafenib administration until the date of death from any cause. The time to progression (TTP) was defined from the start of sorafenib administration until the first documented event of progression (according to RECIST v1.1). Survival curves were constructed using the Kaplan-Meier method for all 38 patients. The associations between clinical features and each of the dichotomized groups were analyzed by the chi-square test or Fisher's exact test. Two-sided P values of less than 0.05 were considered significant. All analyses were performed using SPSS for Windows, version 12.0.
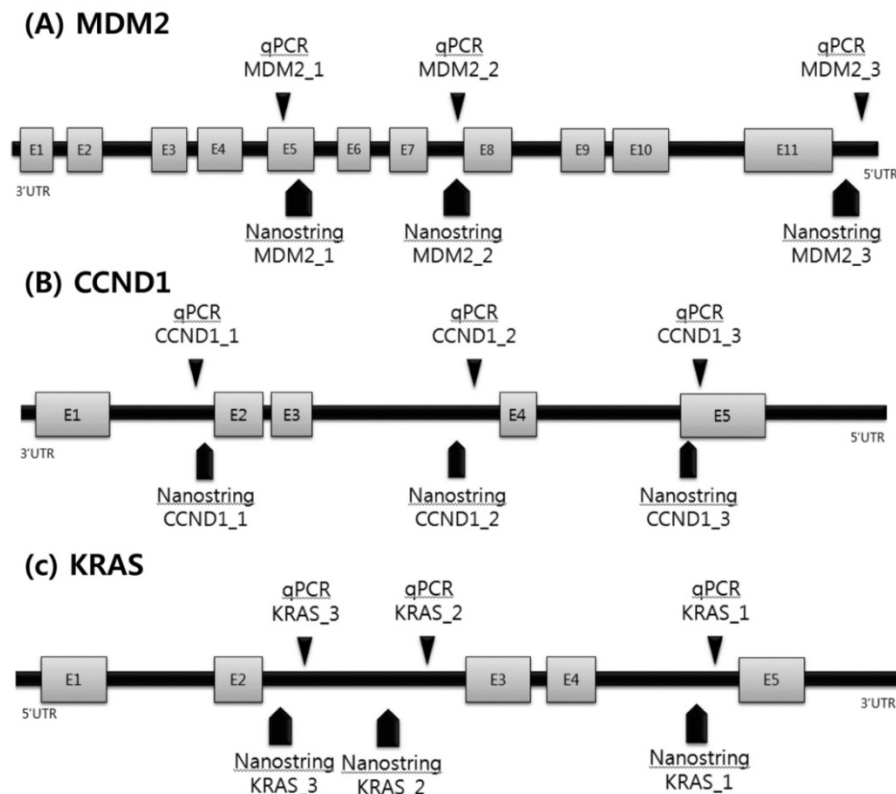

Figure 1. Scheme of validation of copy number variations using qPCR.

Results

Patient characteristics

The characteristics of the study patients at the initiation of sorafenib treatment are summarized in Table 1. The median age was 56 years (range, 35–85 years). The most common etiology for HCC was hepatitis B (74%); 5% of the patients had hepatitis C and the remainder had non-B non-C HCC. Patients had advanced-stage HCC (BCLC stage C: 95%) with preserved liver function (Child-Pugh Class A: 95%) and good ECOG performance status (0: 66%). At baseline, nine patients (24%) had macroscopic vascular invasion and 34 (90%) had extrahepatic spread, with the most common extrahepatic sites being the lymph nodes and lung. Before sorafenib treatment, patients received liver resection (79%), locoregional therapy (transarterial chemoembolization [TACE], 71%; radiofrequency ablation, 40%; radiotherapy, 50%; metastasectomy, 34%; and systemic cytotoxic chemotherapy, 16%).

Table 1. Baseline patient characteristics (n=38).

Variable	No. (%)
Median age, years (range)	56 (35-85)
Male, n (%)	33 (87)
Cause of disease, n (%)	
Hepatitis B	28 (74)
Hepatitis C	2 (5)
Unknown	8 (21)
ECOG performance status, n (%)	
0	25 (66)
1	12 (32)
2	1 (2)
BCLC stage, n (%)	
B	2 (5)
C	36 (95)
Macroscopic vascular invasion, n (%)	9 (24)
Extrahepatic spread, n (%)	34 (90)
Extrahepatic sites, n (%)	
Lung	22 (58)
Lymph node	8 (21)
Child-Pugh class, n (%)	
A	36 (95)
B	2 (5)
AFP ≥ 200ng/mL, n (%)	13 (34)
Previous therapy, n (%)	
Liver resection	30 (79)
Locoregional therapy	
Transarterial chemoembolization	27 (71)
Radiofrequency ablation	15 (40)
Radiotherapy	19 (50)
Metastatectomy	13 (34)
Systemic chemotherapy	6 (16)

ECOG, Eastern Cooperative Oncology Group; BCLC, Barcelona Clinic Liver Cancer; AFP, alpha-fetoprotein.

CNV landscape in HCC

We surveyed CNVs of 21 genes, and identified amplifications in *CCND1* (n = 4), *CDKN1A* (n = 2), *KRAS* (n = 2), *MDM2* (n = 1), and *ERBB2* (n = 1) (Table 2). Because of the small number of patients, further survival analysis was not performed. Of the five genes with amplifications identified by the nCounter assay, we further validated those of *CCND1*, *MDM2*, and *KRAS* using conventional qPCR (Table 3); this confirmed amplification in all four *CCND1* samples and the MDM2 sample. However, *KRAS* amplification results were not consistent between the nCounter assay and qPCR in one of the two patients; the other did not have a sufficient amount of DNA for further analysis by qPCR.

Table 2. The frequency of copy number variations in 38 patients by Nanostring nCounter analysis.

Gene	Localization	Case no.	Total CNVs frequency no. (%)
CCND1	chr11:69166461-69173877	38	4 (11)
CDKN1A	chr6:36760296-36761391	38	2 (5)
KRAS	chr12:25261832-25285922	38	1 (3)
ERBB2	chr17:35112859-35135347	38	1 (3)
MDM2	chr12:67498111-67518304	38	1 (3)

CNVs, copy number variations.

Table 3. Copy number variations validation by qPCR.

Sample	CNVs by Nanostring	Gene_1 CN Predicted	Gene_2 CN Predicted	Gene_3 CN Predicted	Result
CCND1					
#4	181	2	178	7	concordant
#6	40	15	22	12	concordant
#18	8	ND	7	ND	concordant
#25	44	ND	47	ND	concordant
Negative sample	3	0	3	0	
MDM2					
#11	46	2	0	9	concordant
Negative sample	2	2	2	2	
KRAS					
#11	9	2	1	0	disconcordant
#20	90	ND	ND	ND	failed
Negative sample	2	2	2	2	
Normal control		2	2	2	

Correlation between copy number variation and response to sorafenib

Among 34 patients who were evaluable for response, three (9%) achieved partial response, 10 (26%) had stable disease, and 21 (55%) had progressive disease per RECIST v1.1. Of 38 patients, 4 (11%) had to discontinue sorafenib owing to intolerance or toxicities. The median TTP and OS for

the patients were 2.7 months (95% confidence interval [CI]: 2.0–3.3 months) and 13.4 months (95% CI: 8.4–18.4 months), respectively (Fig. 2). No significant correlations were found between clinical features and TTP as well as OS (Supplementary Table 2). At the time of progression, 13 patients received experimental therapy; these included sunitinib (2 patients), everolimus (1 patient), and double-blind randomized placebo-controlled trials of molecular targeted agents such as brivanib, ramucirumab, regorafenib, and E7080 in the context of clinical trials (10 patients).

Of the three responders to sorafenib, two did not have any CNVs detected by the 21-gene NanoString panel. The first of these three patients was a 61-year-old man who was an HBsAg carrier with multiple lung metastases. The patient had an exceptional response to sorafenib, with a persistent reduction in the volumes of multiple lung metastases over 5 years (Fig. 3a). The second responder was an 84-year-old non-B non-C HCC patient with multiple metastases who responded to sorafenib for 30 months (Fig. 3b). This patient did not have any significant amplification in the 21-gene panel. The third patient, a 47-year-old man who was an HBsAg carrier with multiple lung metastases, responded to sorafenib but progressed after 9 months. This patient had *KRAS* and *MDM2* amplifications in his tumor specimen.

Additionally, patient #15 was diagnosed with hepatitis B-related HCC at the age of 31 years and underwent liver sectionectomy. Shortly afterward, the patient had recurrent disease in the liver and was treated with TACE multiple times. The patient then received sorafenib for unresectable recurrent HCC but progressed after two months; this patient's tumor specimen harbored *CDKN1A* and *ERBB2* amplifications.

Discussion

Recently, gene amplifications in various tumors have been highlighted as potential therapeutic targets.

Hence, screening for amplified genes using high-throughput technology has become an important diagnostic tool in cancer medicine. In our previous study of gastric cancer specimens, we tested a CNV panel comprising 21 candidate genes for which targeted drugs are available [15]. We demonstrated that the NanoString nCounter assay had a concordance rate of 83.4% for *HER2* amplification when compared to conventional immunohistochemistry and fluorescent in situ hybridization methods. In this study, we surveyed HCC specimens with a 21-gene CNV panel to identify potential amplifications in HCC.

Four patients in this study (11%) showed significant CNVs of *CCND1*. In the TCGA cohort (n = 370), 27 (7.3%) HCC patients were identified to have CCND1 amplification (Fig. 4). This gene, which is located on chromosome 11q13, is amplified in about 4% of HCCs [16]. *CCND1* encodes cyclin D1, which interacts with the cyclin-dependent kinases CDK4 and CDK6, resulting in the inactivation of the tumor suppressor *RB1* and progression of the cell cycle [17]. Amplification of *CCND1* may therefore lead to increased proliferation; in fact, a recent study revealed that lentivirus-mediated shRNA targeting of cyclin D1 inhibits the proliferation of a gastric cancer cell line [18].

Although our study included a small number of patients, tumors from two of three sorafenib responders did not harbor any CNVs. These two patients responded to sorafenib for >30 months and 5 years, respectively. In contrast, one patient with a partial response to sorafenib progressed after nine months; this patient's tumor harbored amplifications in *KRAS* and *MDM2*. *KRAS* amplification in lung cancer has been reported to increase expression levels of p21, suggesting a dysregulated cell cycle [19]. In endometrial cancers, a subset of patients whose tumors had amplifications of the 12p12.1 region harboring *KRAS* had poorer prognoses [20].
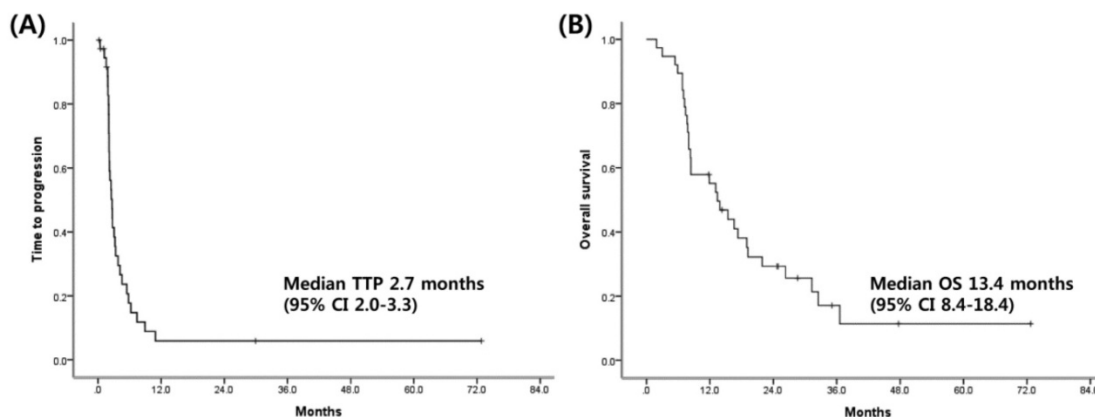

Figure 2. Kaplan-Meier curves for Time to progression (A), overall survival (B).
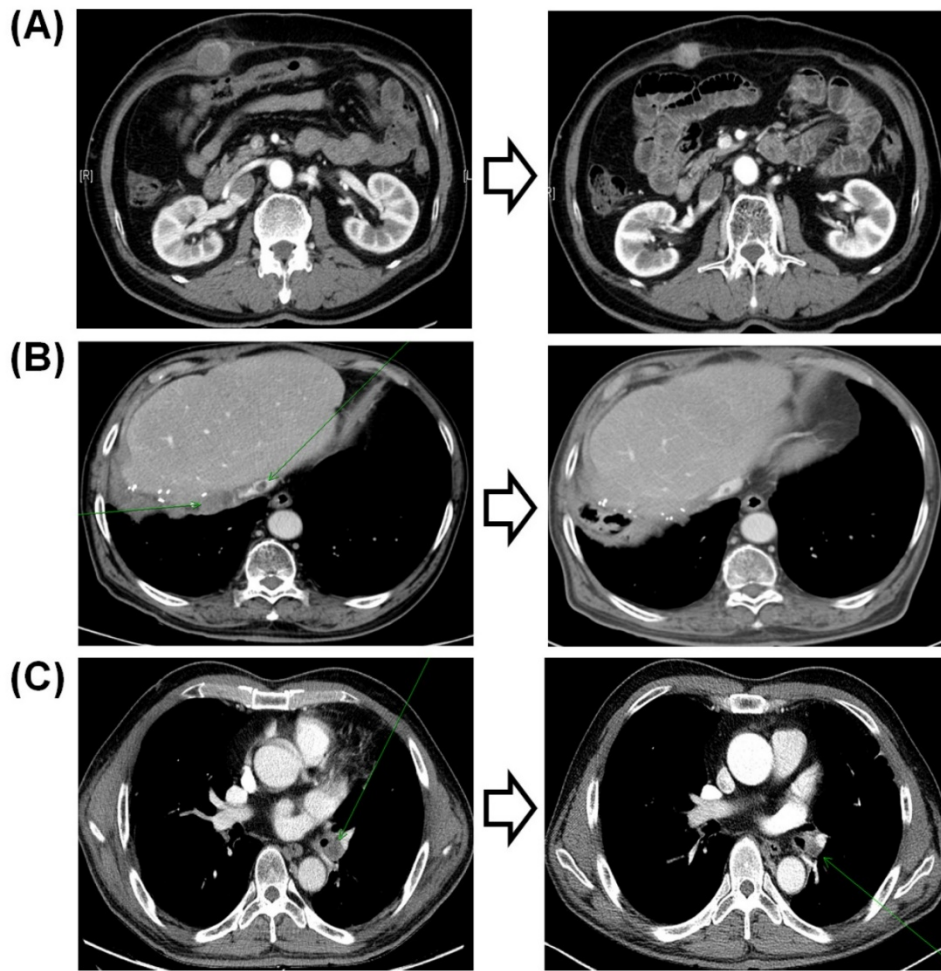
Figure 3. Computed tomography findings of the three responders to sorafenib.
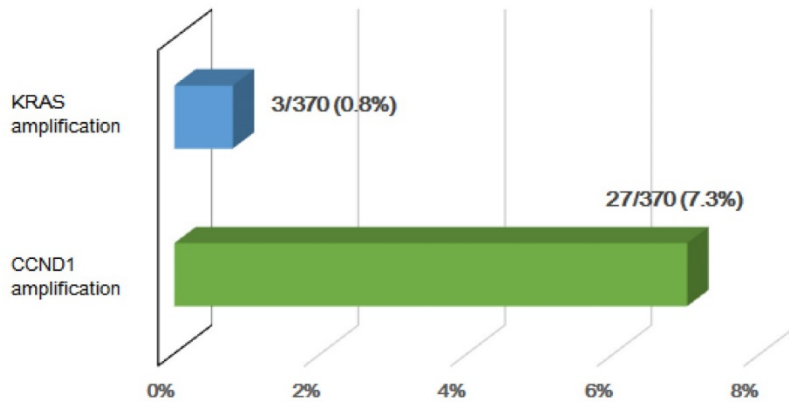

Figure 4. The percentage of KRAS and CCND1 amplification in the TCGA HCC cohort (n = 370).

Of 38 HCC patients, two (5.2%) had *MDM2* amplification; in a previous study, *MDM2* amplification was detected in approximately 44% of patients with HCC [21]. MDM2 prevents the activity of the tumor suppressor p53; therefore, amplification of *MDM2* may be oncogenic [22] and may predict sensitivity to MDM2 inhibitors. However, currently available evidence is inconclusive [23].

Of note, one patient who rapidly progressed following sorafenib had significant CNVs of *ERBB2*. The overexpression of the *HER-2/neu* oncogene has been implicated in the development and modulation of many other cancers; however, whether *HER-2/neu* overexpression plays a similar role in HCC has not been determined [24, 25]. Although phase II trials for lapatinib in HCC revealed a marginal benefit to only a

subgroup of patients (in whom predictive markers had not yet been characterized) [26, 27], its role in the treatment of HCC remains to be elucidated [28].

Our study had several limitations. First, the small study population from a single center makes it difficult to draw definitive conclusions concerning the genetic markers that predict clinical outcomes of sorafenib treatment in advanced HCC patients. Second, NanoString probes were designed for only 21 genes. Because of the limited number of CNV gene probes, the discovery of additional CNVs that could impact the outcomes of sorafenib treatment was restricted. Furthermore, high-throughput sequencing and integrative informatics should be needed to uncover new CNVs.

In conclusion, our study did not reveal any candidate predictive markers for sorafenib sensitivity, as there was no correlation between any CNVs and survival; this indicates that any such markers may be of uncertain value. Well-designed prospective clinical studies are required to determine the exact roles of these markers in predicting the response to sorafenib in HCC. Additionally, more preclinical studies are required to clarify the roles of currently known biomarkers in their respective cancer-promoting signal transduction pathways.

Supplementary Material

Supplementary tables.
http://www.jcancer.org/v08p0730s1.pdf

Competing Interests

The authors have declared that no competing interest exists.

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