

Original Paper

Gene Signature for Sorafenib Susceptibility in Hepatocellular Carcinoma: Different Approach with a Predictive Biomarker

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Keywords

Sorafenib · Biomarker · Gene signature · Hepatocellular carcinoma

Abstract

Background/Aim: Uniform treatment of hepatocellular carcinoma (HCC) with molecular targeted drugs (e.g., sorafenib) results in a poor overall tumor response when tumor subtyping is absent. Patient stratification based on actionable gene expression is a method that can potentially improve the effectiveness of these drugs. Here we aimed to identify the clinical applica-

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tion of actionable genes in predicting response to sorafenib. **Methods:** Through quantitative real-time reverse transcription PCR, we analyzed the expression levels of seven actionable genes (*VEGFR2*, *PDGFRB*, *c-KIT*, *c-RAF*, *EGFR*, *mTOR*, and *FGFR1*) in tumors versus noncancerous tissues from 220 HCC patients treated with sorafenib. Our analysis found that 9 responders did not have unique clinical features compared to nonresponders. A receiver operating characteristic curve evaluated the predictive performance of the treatment benefit score (TBS) calculated from the actionable genes. **Results:** The responders had significantly higher TBS values than the nonresponders. With an area under the curve of 0.779, a TBS combining *mTOR* with *VEGFR2*, *c-KIT*, and *c-RAF* was the most significant predictor of response to sorafenib. When used alone, sorafenib had a 0.7–3% response rate among HCC patients, but when stratifying the patients with actionable genes, the tumor response rate rose to 15.6%. Furthermore, actionable gene expression is significantly correlated with tumor response. **Conclusions:** Our findings on patient stratification based on actionable molecular subtyping potentially provide a therapeutic strategy for improving sorafenib's effectiveness in treating HCC.

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Introduction

Hepatocellular carcinoma (HCC) is a lethal malignancy that is notoriously resistant to chemotherapy [1], leading to a search for alternative treatment strategies. Among these, molecular targeted drugs are designed to antagonize “oncogenic-actionable genes” that are specifically altered in tumors [2]. Actionable molecules have been successfully applied to patient stratification in various cancers [3]. Lung cancer can be classified into 18 subtypes based on specific genetic aberrations (e.g., overexpression and mutations) [4], while melanoma has 11 subtypes associated with targeted treatment [5]. Unfortunately, the highly heterogeneous HCC lacks molecular predictors of treatment response. Although many molecular targeted agents have been tested in HCC, only sorafenib and lenvatinib have been approved as first-line agents for advanced stages of this cancer [6–8]. The lack of a second-line option after lenvatinib [9], however, means that sorafenib is the recommended standard treatment for advanced HCC [9], despite its poor therapeutic response (0.7–3%) [6, 7]. New therapeutic strategies are necessary to improve sorafenib's efficacy in treating HCC.

Currently, there is no clinically applicable biomarker for predicting HCC response to sorafenib [10], although *VEGFA* [11] and *FGF3/4* [12] are more frequently amplified in sorafenib responders. Previously, we investigated the mRNA expression of five actionable genes (*VEGFR2*, *PDGFRB*, *EGFR*, *mTOR*, and *FGFR1*) in HCC tumors and surrounding noncancerous tissues, and then used the results to stratify HCC patients. Additionally, we found evidence suggesting that *c-RAF* and *PDGFRB* expression could predict sorafenib susceptibility [13].

In this study, we aimed to confirm whether actionable gene expression can predict response to sorafenib in HCC tissues, and if so, identify those with the best diagnostic performance. We compared the mRNA expression of seven actionable genes in responder and nonresponder tumor tissues from 220 HCC patients treated with sorafenib. We then calculated and compared treatment benefit scores (TBS) from relative mRNA levels per gene.

Subjects and Methods

Patients and Tissue Samples

Patients with histologically confirmed HCC were enrolled if they met the following criteria: (1) age ≥ 20 years; (2) diagnosed with unresectable advanced HCC; (3) Eastern Cooperative Oncology Group performance status ≤ 2 ; (4) Child-Pugh class A; and (5) receiving sorafenib as a palliative first-line systemic

treatment. Individuals were excluded if they required combination therapy (including chemotherapy, radiotherapy, hepatic arterial chemoembolization, and radiofrequency ablation) or possessed severe, uncontrolled medical conditions. In total, 390 patients were enrolled from 7 medical institutions; all provided written informed consent. Sorafenib treatment occurred during 2014–2018. Inoperable patients were subjected to ultrasound-guided needle biopsy before sorafenib treatment. For patients experiencing recurrence within 3 months after surgical resection with curative intent, needle biopsy was omitted. Instead, tumor tissues frozen at the time of resection were used for analysis.

Complete clinical information was available for all cases. Radiologic analyses (computed tomography [CT] and magnetic resonance imaging [MRI]) evaluated tumor response to sorafenib, following the modified Response Evaluation Criteria in Solid Tumors for HCC [14]. Immediately after needle biopsy, HCC tissue samples were snap-frozen in liquid nitrogen and stored at -80°C . Patient staging information was obtained from CT or MR images, and conventional TNM (Tumor, Node, and Metastasis) classification (American Joint Committee on Cancer, 7th edition), along with BCLC (Barcelona Clinic Liver Cancer) staging, was used.

Measurement of Clinical Outcomes

The primary endpoint was tumor response to sorafenib, assessed 3 and 6 months after drug administration.

RNA Extraction and cDNA Synthesis

Published methods were used for RNA extraction and cDNA synthesis [13]. Total RNA was extracted from both tumor and surrounding noncancerous frozen tissues using the RNeasy Mini Kit (Qiagen, Hilden, Germany) with DNase I treatment (Qiagen). Total RNA integrity was verified using a Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA, USA). Next, 4 μg of RNA was incubated with 2 μL of 10 μM oligo(dT)18 primer (GenoTech, Daejeon, South Korea) at 70°C for 7 min, before being cooled on ice for 5 min. Reverse transcriptase enzyme mix was added to the annealed total RNA sample, and the reaction was incubated for 90 min at 42°C . Reverse transcriptase was then heat-inactivated at 80°C for 10 min. Diethylpyrocarbonate-treated water was added to bring the final volume of the cDNA samples to 400 μL .

Quantitative Real-Time PCR

Quantitative real-time PCR was performed as described previously [13], using an ABI PRISM 7900HT instrument (Applied Biosystems, Foster City, CA, USA). The total reaction volume was 10 μL . The thermocycling conditions were as follows: 95°C for 10 min, followed by 45 cycles of 95°C for 15 s and 60°C for 1 min. The primer and probe sequences were designed in Primer Express 3.0 (Applied Biosystems); all probes were labeled with TAMRA at the 3' end and FAM at the 5' end. The target genes were *mTOR*, *VEGFR2*, *PDGFRB*, *FGFR1*, *c-KIT*, *EGFR*, and *c-RAF*. The internal control was the average expression of five reference genes (β_2 -microglobulin [*B2M*], glyceraldehyde 3-phosphate dehydrogenase [*GAPDH*], hydroxymethylbilane synthase [*HMBS*], hypoxanthine phosphoribosyltransferase 1 [*HPRT1*], and succinate dehydrogenase complex, subunit A, flavoprotein variant [*SDHA*]). The $2^{-\Delta\text{Ct}}$ method was used to determine target gene expression levels.

Treatment Benefit Score

The TBS is the summation of all \log_2 -transformed target gene expression multiplied by its corresponding regression coefficient, as follows: $\text{TBS} = (0.118910 \times mTOR) + (0.138561 \times VEGFR2) + (0.258877 \times c-KIT) + (0.147012 \times c-RAF)$. The regression coefficients of each gene were calculated by logistic regression analysis with the R package (version 3.3.3; R Development Core Team; <https://www.r-project.org/>).

Statistical Analysis

Receiver operating characteristic curve analysis was used to determine the accuracy of the threshold values separating tumor responders and nonresponders using the TBS. Relationships between tumor response and clinicopathologic variables or gene expression were evaluated using either χ^2 tests or Fisher's exact tests. Gene expression data were \log_2 transformed and tested for normality with the Shapiro-Wilk test. As the data did not meet the normality assumptions, significant differences between responders and nonresponders were evaluated using the Mann-Whitney U test. Every tested gene from the 220 patients was analyzed using logistic regression to understand the relationships between response to sorafenib treatment, TBS classification, and clinicopathologic variables. Significance was set at $p < 0.05$ (two-tailed). All statistics were performed in R version 3.3.3.

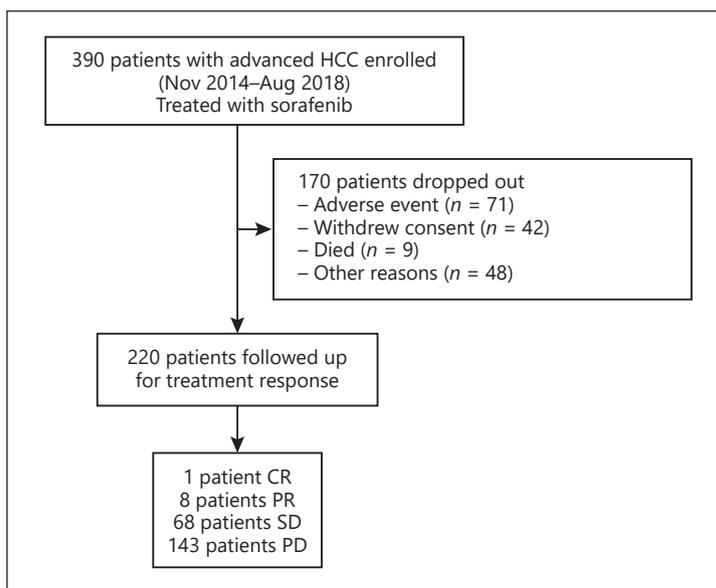


Fig. 1. Flowchart of treatment enrollment and follow-up. HCC, hepatocellular carcinoma; CR, complete response; PR, partial response; SD, stable disease; PD, progressive disease.

Results

Clinicopathologic Characteristics of the Sorafenib Responders

Of the 390 sorafenib-treated patients, 220 were retained for follow-up treatments. The remainder dropped out due to adverse events ($n = 71$), withdrawal of consent ($n = 42$), death ($n = 9$), or other reasons ($n = 48$) (Fig. 1).

The results of the CT and MRI scans revealed 1 patient with a complete response, 8 with a partial response, 68 with stable disease, and the remaining 143 with progressive disease. The overall tumor response rate was 4.1%. Representative tumors presented a dramatic disappearance of nodules in the lung and liver after sorafenib treatment (Fig. 2). An analysis of clinicopathologic features associated with response to sorafenib revealed variable α -fetoprotein levels among responders, ranging from 1 to 14,046 ng/mL (Table 1). A comparison between responders and nonresponders did not reveal any clinical features unique to responders (Table 2).

Comparison of Actionable Gene Expression between Responders and Nonresponders

In a comparison across responders and nonresponders to sorafenib, we found that five genes ($mTOR$: $p = 0.309$; $PDGFRB$: $p = 0.119$; $c-KIT$: $p = 0.014$; $c-RAF$: $p = 0.002$; and $EGFR$: $p = 0.137$) had a higher mean expression in the responders than in the nonresponders (Fig. 3a, c–f). However, the mean $VEGFR2$ ($p = 0.224$) and $FGFR1$ ($p = 0.475$) expression was lower in the responders than in the nonresponders (Fig. 3b, g).

Comparing TBS between Responders and Nonresponders

We found that combinations of $mTOR$, $VEGFR2$, $c-KIT$, and $c-RAF$ were most effective in discriminating responders from nonresponders. Compared with the TBS of a single gene ($mTOR$, $VEGFR2$, $c-KIT$, or $c-RAF$), the TBS of $mTOR/VEGFR2$ and $c-KIT/c-RAF$ combinations differed significantly between the responders and the nonresponders (Fig. 4a). The mean TBS of $mTOR/VEGFR2$ and $c-KIT/c-RAF$ was -10.110464 in the nonresponders and -7.805714 in the responders, respectively ($p < 0.001$).

Table 1. Clinicopathologic features of sorafenib responders

Patient No.	Age, years	Sex	Viral status	TNM stage (AJCC 7th ed.)	BCLC stage	AFP, ng/mL	Metastasis	Tumor response
1	60	F	HBV	IV	C	1.6	X	PR
2	46	F	HBV	IV	C	489.7	O	PR
3	52	F	HBV	IV	B	6,519	O	PR
4	52	M	HBV	III	C	14,046.0	X	PR
5	56	M	HBV	IV	B	10.1	O	PR
6	38	M	HBV	III	C	7.6	X	PR
7	43	M	HBV	IV	C	617.0	O	PR
8	56	M	HBV	IV	C	3	X	CR
9	62	M	HBV	IV	C	3.9	X	PR

AJCC, American Joint Committee on Cancer; BCLC, Barcelona Clinic Liver Cancer; AFP, α -fetoprotein; HBV, hepatitis B virus; CR, complete response; PR, partial response.

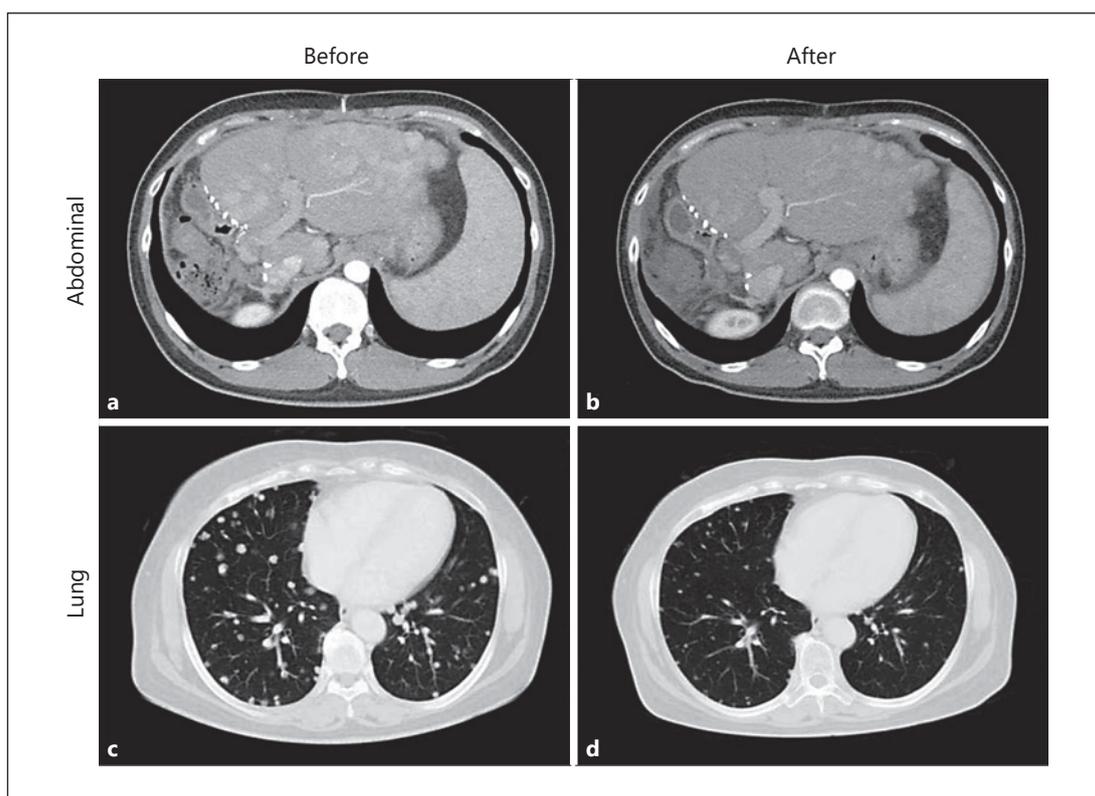


Fig. 2. Abdominal (a, b) and lung (c, d) images from representative responders. CT images taken before (a, c) and after (b, d) sorafenib treatment, showing changes in multiple nodular lesions spread in the organs.

Performance of Gene Classifiers in Predicting Tumor Response to Sorafenib

Multiple actionable genes were differentially expressed in tumor tissues between responders and nonresponders. We performed a receiver operating characteristic curve analysis of genes in combination; the number of genes combined was from 1 to 7. Among all the gene combinations, 9 of the classifiers revealed over 70% in sensitivity and specificity.

Table 2. Baseline characteristics and comparison of clinical factors between responders and nonresponders

Clinicopathologic parameters	Responders (n = 9)	Nonresponders (n = 211)	p value ¹
Age		(-1)	0.7384
<55 years	3	88	
≥55 years	6	122	
Gender		(-1)	0.4148
Male	6	165	
Female	3	45	
HBV		(-1)	0.3627
Absent	0	37	
Present	9	173	
HCV		(-2)	1
Absent	9	200	
Present	0	9	
Tumor TNM stage (AJCC 7th ed.)		(-6)	0.7607
I	0	0	
II	0	10	
III	2	29	
IV	7	166	
BCLC stage		(-2)	0.4223
A	0	3	
B	2	23	
C	7	181	
D	0	2	
AFP		(-14)	0.7363
<100 ng/mL	5	91	
≥100 ng/mL	4	106	
Tumor response			
Complete response	1	0	
Partial response	8	0	
Stable disease	0	68	
Progressive disease	0	143	

HBV, hepatitis B virus; HCV, hepatitis C virus; TNM, Tumor, Node, and Metastasis classification; AJCC, American Joint Committee on Cancer; BCLC, Barcelona Clinic Liver Cancer; AFP, α-fetoprotein.
¹ p values were calculated using Fisher's exact test.

The performance of the best classifier (combinations of *mTOR*, *VEGFR2*, *c-KIT*, and *c-RAF*) was at the threshold value at -8.6237; the prediction sensitivity in separating responders from nonresponders was 77.8% (95% CI: 40.0–97.2), while the specificity was 82.0% (95% CI: 76.1–86.9) (Fig. 4c). Additionally, the area under the curve was 0.779 (95% CI: 0.724–0.833), which was statistically significant ($p < 0.001$; Table 3).

TBS-Based Classifiers and Clinicopathologic Characteristics Both Predict Response to Sorafenib Treatment

Logistic regression demonstrated that TBS-based classifiers stemming from *mTOR*, *VEGFR2*, *c-KIT*, and *c-RAF* combinations significantly predicted tumor response to sorafenib treatment ($p < 0.001$; Table 4). Logistic regression also showed that none of the measured clinicopathologic variables (age: $p = 0.611$; sex: $p = 0.404$; hepatitis B virus: $p = 0.993$; hepatitis C virus: $p = 0.994$; TNM stage: $p = 0.812$; BCLC stage: $p = 0.399$; and α-fetoprotein levels: $p = 0.373$) significantly predicted response to sorafenib (Table 4).

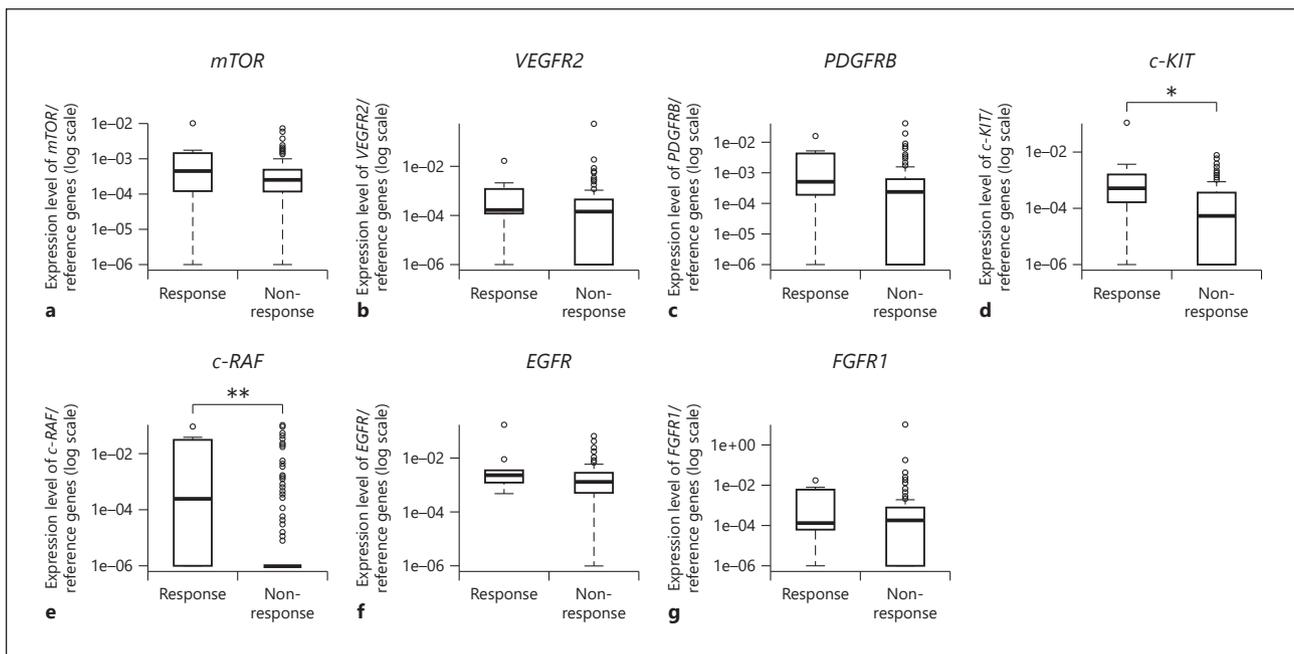


Fig. 3. Actionable gene expression in responders versus nonresponders. Relative expression of seven actionable genes in 9 responders and 211 nonresponders. * $p < 0.05$, ** $p < 0.01$. **a** *mTOR*. **b** *VEGFR2*. **c** *PDGFRB*. **d** *c-KIT*. **e** *c-RAF*. **f** *EGFR*. **g** *FGFR1*.

Table 3. Classifiers of tumor response to sorafenib

Rank	Gene combination	Combination	Sensitivity, %	Specificity, %	AUC	Accuracy	PPV	NPV
1	<i>mTOR_VEGFR2_c-KIT_c-RAF</i>	4	77.78	81.99	0.779	81.82	15.56	98.86
2	<i>mTOR_c-KIT_c-RAF</i>	3	77.78	81.52	0.778	81.36	15.22	98.85
3	<i>mTOR_VEGFR2_c-KIT_c-RAF_FGFR1</i>	5	77.78	81.52	0.770	81.36	15.22	98.85
4	<i>VEGFR2_c-KIT_c-RAF</i>	3	77.78	80.57	0.781	80.45	14.58	98.84
5	<i>mTOR_c-KIT_c-RAF_FGFR1</i>	4	77.78	80.09	0.764	80.00	14.29	98.83
6	<i>c-KIT_c-RAF</i>	2	77.78	77.73	0.791	77.73	12.96	98.80
7	<i>c-KIT_c-RAF_FGFR1</i>	3	77.78	77.73	0.769	77.73	12.96	98.80
8	<i>VEGFR2_c-KIT_c-RAF_FGFR1</i>	4	77.78	77.25	0.765	77.27	12.73	98.79
9	<i>mTOR_VEGFR2_c-KIT_c-RAF_EGFR</i>	5	77.78	73.46	0.769	73.46	11.11	98.73
	<i>mTOR</i>	1	44.44	87.68	0.600	85.91	13.33	97.37
	<i>VEGFR2</i>	1	88.89	36.02	0.618	38.18	5.59	98.70
	<i>c-KIT</i>	1	88.89	56.87	0.731	58.18	8.08	99.17
	<i>c-RAF</i>	1	55.56	86.73	0.706	85.45	15.15	97.86
	<i>mTOR_VEGFR2_PDGFRB_c-KIT_EGFR_FGFR1</i>	6	55.56	82.94	0.730	81.82	12.20	97.77
	<i>mTOR_VEGFR2_PDGFRB_c-KIT_c-KIT_EGFR_FGFR1</i>	7	88.89	58.29	0.747	59.55	8.33	99.19

AUC, area under the curve; PPV, positive predictive value; NPV, negative predictive value; *mTOR*, mammalian target of rapamycin; *VEGFR2*, vascular endothelial growth factor receptor 2; *c-KIT*, KIT proto-oncogene receptor tyrosine kinase; *c-RAF*, Raf-1 proto-oncogene serine/threonine kinase; *FGFR1*, fibroblast growth factor receptor 1; *EGFR*, epidermal growth factor receptor; *PDGFRB*, platelet-derived growth factor receptor beta.

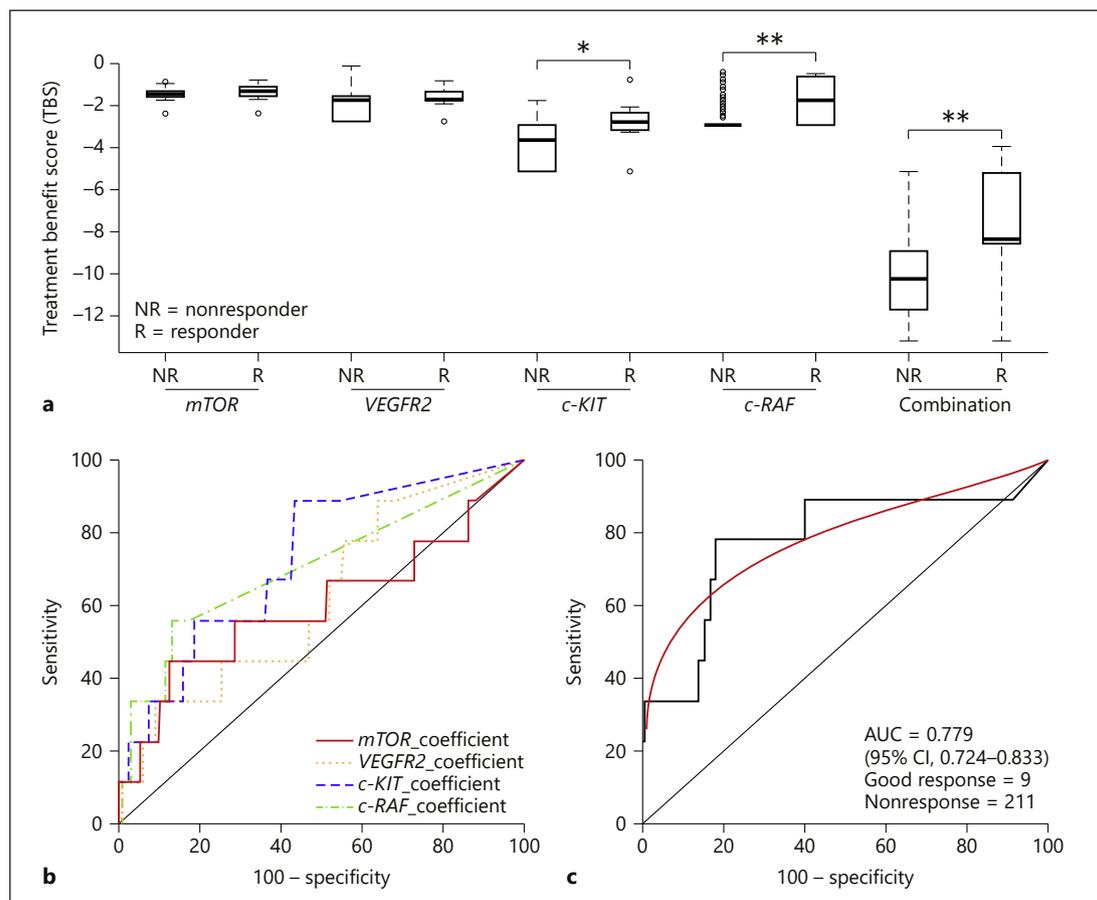


Fig. 4. Treatment benefit score (TBS) of seven actionable genes in responders versus nonresponders. **a** Relative TBS of seven actionable genes compared across 9 responders and 211 nonresponders. Scores were calculated as individual values for *mTOR*, *VEGFR2*, *c-KIT*, and *c-RAF*, as well as paired values from *mTOR* with *VEGFR2* and *c-KIT* with *c-RAF*. The thick line in each box is the median. Circles extend to the most extreme data point, no more than 1.5 times the interquartile range. * $p < 0.05$, ** $p < 0.01$. **b, c** Receiver operating characteristic curve analysis to predict the response to sorafenib (TBS) per gene (**b**) and per pair (**c**). **c** *mTOR*_VEGFR2_ *c-KIT*_ *c-RAF*.

Table 4. Logistic regression analysis of tumor response to sorafenib and variables

Variables	Odds ratio	95% CI	<i>p</i> value
<i>mTOR</i> _VEGFR2_ <i>c-KIT</i> _ <i>c-RAF</i> (low vs. high)	15.93	3.18–79.73	0.002
Age (<55 vs. ≥55 years)	1.44	0.35–5.93	0.611
Gender (male vs. female)	1.83	0.44–7.62	0.404
HBV (absence vs. presence)	Inf.	0.00–Inf.	0.993
HCV (absence vs. presence)	0.00	0.00–Inf.	0.994
TNM stage (II–III vs. IV)	0.82	0.16–4.11	0.812
BCLC stage (AB vs. CD)	0.50	0.10–2.52	0.399
AFP level (<100 vs. ≥100 ng/mL)	0.52	0.12–2.21	0.373

CI, confidence interval; *mTOR*, mammalian target of rapamycin; *VEGFR2*, vascular endothelial growth factor receptor 2; *c-KIT*, KIT proto-oncogene receptor tyrosine kinase; *c-RAF*, Raf-1 proto-oncogene serine/threonine kinase; HBV, hepatitis B virus; HCV, hepatitis C virus; TNM, Tumor, Node, and Metastasis classification; AJCC, American Joint Committee on Cancer; BCLC, Barcelona Clinic Liver Cancer; AFP, α-fetoprotein.

Table 5. Response rates based on gene classifier

Classifier	Criterion	Responders	Nonresponders	Response rate, %
<i>mTOR_VEGFR2_c-KIT_c-RAF</i>	>−8.6237	7	38	15.6
	≤−8.6237	2	173	1.16

mTOR, mammalian target of rapamycin; *VEGFR2*, vascular endothelial growth factor receptor 2; *c-KIT*, KIT proto-oncogene receptor tyrosine kinase; *c-RAF*, Raf-1 proto-oncogene serine/threonine kinase.

Improving the Predictability of TBS-Based Classifiers

Thus far, our findings have indicated that TBS can distinguish HCC patients with a complete response or partial response from those with stable disease or progressive disease, indicating that TBS-based patient selection should improve sorafenib’s efficacy compared with the lack of any classification. When we examined 45 HCC patients with TBS >−8.6237 for *mTOR*, *VEGFR2*, *c-KIT*, and *c-RAF* combinations, we found 7 responders (response rate 15.6%; Table 5). The correlation between TBS and tumor responses to sorafenib was significant ($p < 0.001$).

Discussion

In this study, we found that TBS based on actionable gene expression successfully predict HCC tumor response to sorafenib. When the TBS was used for patient selection, the rate of response to sorafenib increased from 4.1 to 15.6%. Our findings suggest that patient stratification based on actionable molecular subtyping is a useful therapeutic strategy for improving sorafenib’s effectiveness in HCC, supporting our previous research [13].

An increased expression of actionable molecules (*PDGFRB* and *c-KIT*) in tumor cell lines may elevate tumor sensitivity to molecular targeted drugs. Based on the previous study, we investigated the mRNA expression of five actionable genes (*VEGFR2*, *PDGFRB*, *EGFR*, *mTOR*, and *FGFR1*) and two additional genes (*c-KIT* and *c-RAF*) in HCC tumors and surrounding noncancerous tissues (online suppl. Fig. 1, 2; for all online suppl. material, see www.karger.com/doi/10.1159/000504548). Then we conducted a seven-actionable-gene-based study. Here, we observed a significantly higher mean expression of three actionable genes (*PDGFRB*, *c-KIT*, and *c-RAF*) in responders than in nonresponders. This outcome is consistent with the fact that these three genes are direct sorafenib targets and associated with an enhanced response to sorafenib treatment [15]. However, *mTOR* and *EGFR* confer resistance to sorafenib through *Akt* activation [16, 17], and, indeed, their expression did not differ significantly between responders and nonresponders, although the mean values were slightly higher in the responders (Fig. 3). Overall, our results suggest that mRNA levels of individual actionable genes are insufficient for predicting therapeutic response to sorafenib.

Although individual actionable genes were not useful biomarkers, our results demonstrated that the TBS of gene combinations were significantly higher in responders (Fig. 4a), with *mTOR/VEGFR2* and *c-KIT/c-RAF* achieving 77.8% sensitivity and 82.0% specificity (Fig. 4c; Table 3). These data are in line with the observation that multiple biomarkers usually outperform single biomarkers in diagnosis.

Furthermore, we demonstrated that adding *VEGFR2*, *c-KIT*, and *c-RAF* to the *mTOR* classifier could maximize predictions of response to sorafenib and thus improve treatment response rates. However, although the TBS raised the rates of response to sorafenib treatment from 4.1 to 15.6%, a rate of 15.6% is still low in absolute terms. We believe that this low

overall response rate is largely due to the small number of responders and heterogeneity in HCC patients (in contrast to the uniformity of HCC cell lines). Nevertheless, TBS based on actionable gene combinations exhibited a promising level of diagnostic performance in predicting response to sorafenib.

Given that sorafenib is the standard therapy for advanced HCC, excluding potential nonresponders might be the best approach to maximizing the drug's benefits. We therefore recommend against sorafenib treatment for patients below our suggested threshold values from our *mTOR/VEGFR2* and *c-KIT/c-RAF* classifiers.

In conclusion, we have demonstrated the potential of actionable gene expression in predicting clinical responses to sorafenib treatment in HCC. Future research should aim to replicate these results using a larger gene pool. Nonetheless, our findings contribute to efforts aimed at improving diagnostic approaches that personalize sorafenib treatment of HCC.

Acknowledgements

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Statement of Ethics

The protocol was approved by the Institutional Review Boards of Ajou University Medical Center, Asan Medical Center, Gachon University Gil Medical Center, Korea University Medical Center, and Catholic University Medical Center. All patients provided written informed consent. The Declaration of Helsinki and Good Clinical Practice guidelines were followed.

Disclosure Statement

The authors have no conflicts of interest to declare.

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