ORIGINAL ARTICLE

Stem Cell Markers Predict the Response to Sorafenib in Patients with Hepatocellular Carcinoma

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Background/Aims: Sorafenib remains the only approved molecular targeted agent for hepatocellular carcinoma (HCC); however, reliable biomarkers that predict its efficacy are still lacking. The aim of this study was to explore whether cancer stem cell (CSC) markers have a predictive role with regard to the sorafenib response in HCC patients. **Methods:** We enrolled 47 patients with HCC for whom tumor samples obtained before starting sorafenib treatment were available. RNA was extracted from formalin-fixed, paraffin-embedded samples, and real-time polymerase chain reaction was used to quantify mRNA expression of the CSC genes *EpCAM*, *CD13*, *CK8*, *CD24*, *CD44*, *CD90*, *CD133*, *SALL4*, *ALDH1A1*, *ALB*, and *AFP*. **Results:** Of 47 patients, 14.9% and 74.5% had vascular invasion and extrahepatic spread, respectively. Patients with low *CD133* expression tended to have longer progression-free survival (PFS) than those with high *CD133* expression (5.5 months vs 4.0 months), although without statistical significance. The expression levels of other markers were not associated with PFS. When examining markers in combination, patients with high *CD133* and *CD90* expression had shorter PFS rates than those with low expression (2.7 months vs 5.5 months; p=0.04). Patients with low *CD133* and *EpCAM* expression demonstrated better PFS than those with high expression (7.0 months vs 4.2 months; p=0.04). Multivariable analysis indicated that an Eastern Cooperative Oncology Group performance status score of 1 and high *CD133/CD90* expression were significantly associated with shorter PFS. **Conclusions:** Overexpression of the CSC markers *CD133* and *CD90* in HCC was associated with poorer response to sorafenib. These two genes may serve as predictive biomarkers for sorafenib therapy. **(Gut Liver 2019;13:342-348)**

Key Words: Biomarkers; Cancer stem cells; Carcinoma, hepatocellular; Prognosis; Sorafenib

INTRODUCTION

Sorafenib is the first-line treatment option for patients with advanced hepatocellular carcinoma (HCC) who are unlikely to benefit from resection, transplantation, and other locoregional treatments; indeed, two large phase III clinical trials demonstrated a survival advantage with this drug.[1,2] However, responses to sorafenib therapy vary among patients. Given the adverse effects and relatively high costs of sorafenib therapy, the identification of patients more likely to benefit from sorafenib therapy is necessary.

Numerous studies have revealed predictive factors for sorafenib therapy outcomes. The SHARP study, which aimed to assess potentially useful predictive biomarkers, showed that high baseline soluble c-KIT and low hepatocyte growth factor levels were independent predictors of survival in patients receiving sorafenib; however, the predictive trends were not significant.[3] Early alpha-fetoprotein (AFP) expression, a des-γ-carboxyprothrombin response, and the development of hypertension or diarrhea may also predict the therapeutic efficacy of sorafenib therapy.[4-9] Although additional studies exist, the results are inconsistent.

Accumulating evidence suggests that a subset of cancer cells with stem cell properties, referred to as cancer stem cells (CSCs), is capable of self-renewal and differentiation.[10] CSCs are reported to be resistant to chemotherapy and to contribute to tumor persistence and relapse.[11,12] Liver CSCs can be identified through certain cell surface markers, including CD133, EpCAM, CD90, CD44, OV6, CD13, CD24, DLK1, α2δ1, ICAM-1 and CD47.[13] Recent studies have also found that the expression of CSC markers

is associated with HCC prognosis. An *in vitro* study demonstrated that the downregulation of CD44 protected HCC cells from sorafenib-induced apoptosis.[14] In addition, high CD133 expression in HCC tissues was reported to be correlated with a poor response to sorafenib.[15] Therefore, we performed this study to explore the role of CSC markers in predicting the sorafenib response in HCC patients.

MATERIALS AND METHODS

1. Patients

A total of 620 consecutive HCC patients were treated with sorafenib at the Center for Liver Cancer, National Cancer Center (Goyang, Korea) between June 2007 and March 2012. Of these patients, 417 were administered sorafenib for 6 weeks or longer. Patients were enrolled in this study if they met the following criteria: (1) they were treated with sorafenib for 6 weeks or longer; (2) pathological specimens extracted before commencement of sorafenib therapy were available; and (3) they discontinued sorafenib because of tumor progression or adverse events. Those who discontinued sorafenib because of economic burdens or other nonmedical problems were excluded. In addition, those who did not provide consent for the use of their specimens for genetic research were excluded. Patients were also excluded due to technical failure of RNA extraction and quantitative real-time polymerase chain reaction (RT-PCR) on their samples. Forty-seven patients were ultimately enrolled in this study (Fig. 1).

The diagnosis of HCC was based on histology and/or clinico-radiological evidence according to the Korean practice guidelines for HCC.[16-18] The noninvasive diagnostic criteria established by these guidelines include the presence of one or more risk factors (i.e., hepatitis B virus, hepatitis C virus, or cirrhosis), typical enhancement on the arterial phase as well as washout in the delayed portal/venous phase in dynamic liver imaging (via methods such as dynamic spiral computed tomography) or contrast-enhanced dynamic magnetic resonance imaging, and/

or an elevated serum AFP level. Sorafenib was administered to patients with vascular invasion or extrahepatic spread and to those whose disease was not amenable to locoregional therapy.

Relevant data on clinical and tumor characteristics were extracted retrospectively from medical records. This study was conducted in accordance with the Declaration of Helsinki and International Conference on Harmonization-Good Clinical Practice. Moreover, this study was approved by the Institutional Review Board at the National Cancer Center (IRB number: NCC2016-0247); the requirement for written informed consent was waived.

2. Outcomes and follow-up

Patients were followed from the date of sorafenib initiation to the date of death or the last follow-up visit. Progression-free survival (PFS) was calculated from the date of sorafenib initiation to the date of first-documented disease progression or the date of death. Overall survival (OS) was calculated from the date of sorafenib initiation to the date of death or the date last seen alive. The response to treatment was assessed every 6 to 8 weeks according to the modified Response Evaluation Criteria in Solid Tumors for HCC.[19]

3. RNA extraction from formalin-fixed paraffin-embedded HCC tissues

Five 4-μm sections were cut from formalin-fixed paraffin-embedded (FFPE) HCC tissues and deparaffinized. Total RNA was extracted using an RNeasy FFPE Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. The RNA concentration was determined using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA).

4. Quantitative RT-PCR

One microgram of extracted RNA was reverse transcribed using a RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific). Quantitative RT-PCR was performed using Taq-
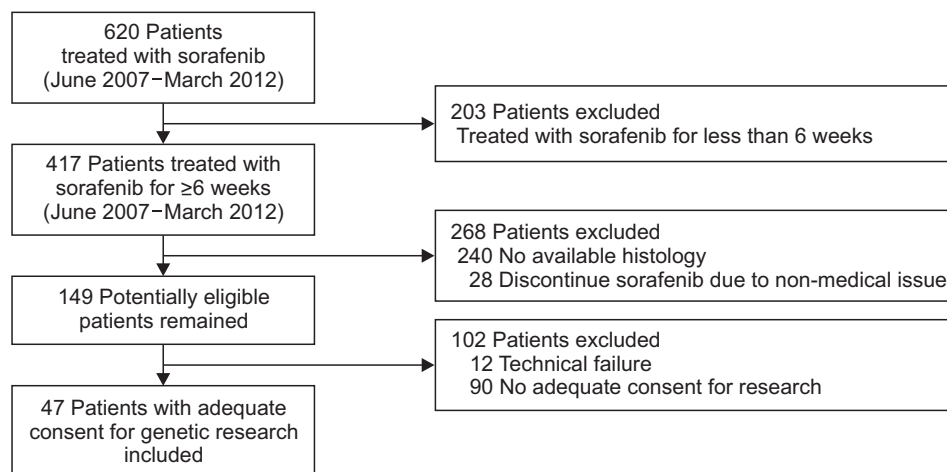


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┌─────────────────────────────┐
│ 620 Patients                │
│ treated with sorafenib      │
│ (June 2007−March 2012)      │
└─────────────────────────────┘
              │         ┌──────────────────────────────────────────┐
              │────────▶│ 203 Patients excluded                    │
              │         │   Treated with sorafenib for less than 6 weeks │
              ▼         └──────────────────────────────────────────┘
┌─────────────────────────────┐
│ 417 Patients treated with   │
│ sorafenib for ≥6 weeks      │
│ (June 2007−March 2012)      │
└─────────────────────────────┘
              │         ┌──────────────────────────────────────────┐
              │────────▶│ 268 Patients excluded                    │
              │         │   240 No available histology             │
              ▼         │    28 Discontinue sorafenib due to non-medical issue │
┌─────────────────────────────┐ └────────────────────────────────┘
│ 149 Potentially eligible    │
│ patients remained           │
└─────────────────────────────┘
              │         ┌──────────────────────────────────────────┐
              │────────▶│ 102 Patients excluded                    │
              │         │    12 Technical failure                  │
              ▼         │    90 No adequate consent for research   │
┌─────────────────────────────┐ └────────────────────────────────┘
│ 47 Patients with adequate   │
│ consent for genetic research│
│ included                    │
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Fig. 1. Patient flow diagram.

Man® gene expression master mix and the following TaqMan® gene expression probes: EpCAM (Hs00158980_m1), CK8/KRT8 (Hs01670053_m1), ALB (Hs00910225_m1), AFP (Hs01040598_ m1), GAPDH (Hs00266705_g1), CD13/ANPEP (Hs00174265_ m1), CD24 (Hs03044178_g1), CD44 (Hs01075856_m1), CD90/ THY1 (Hs00174816_m1), CD133/PROM1 (Hs01009250_m1), SALL4 (Hs00360675_m1), and ALDH1A1 (Hs00167445_m1) (Applied Biosystems, Foster City, CA, USA). The delta delta CT ($\Delta\Delta$CT) method was used to analyze the RT-PCR results. A QuantStudio^{TM} 7 Flex Real-Time PCR Systems instrument (Applied Biosystems) was used for RT-PCR. Relative gene expression was calculated using the $2^{-\Delta\Delta CT}$ method.

5. Statistical analysis

All patients who met the eligibility criteria at baseline were included in the analyses. Continuous variables are expressed as medians and interquartile ranges. The total number of patients for each parameter varied because some data points were not available for every patient. Survival probabilities were estimated by the Kaplan-Meier method and compared by the log-rank test. Differences in survival were tested by using the log-rank test. Univariate and multivariable analyses were performed using the Cox proportional hazards model to identify significant variables related to PFS. Variables that were significant in the univariate analysis ($p \leq 0.05$) were subjected to multivariable analysis using the backward elimination method. All statistical analyses in this study were performed using STATA software version 12.0 (StataCorp LP, College Station, TX, USA). All reported p-values are two-sided; p-values <0.05 were considered statistically significant.

RESULTS

1. Patient characteristics

Forty-seven patients were eligible for this study (Fig. 1). The median age was 55, and the predominant cause of liver disease was hepatitis B virus (63.6%), as shown in Table 1. Three patients had combined HCC and cholangiocarcinoma. Vascular invasion was present in seven patients (14.9%); extrahepatic spread, in 35 (74.5%). Sixteen patients (34.0%) had extrahepatic spread without viable intrahepatic lesions. The median duration of sorafenib administration was 123 days (interquartile range, 75.5 to 168.5). Forty patients (85.1%) showed progressive disease with sorafenib therapy. The median PFS was 4.9 months (95% confidence interval [CI], 3.7 to 6.2 months) and the median OS was 9.7 months (95% CI, 6.4 to 13.0 months).

2. Association of CSC marker expression with response to sorafenib

All specimens were obtained by surgical resection. When evaluated according to the Edmonson-Steiner grading system, 27 and nine specimens showed grade III and IV carcinoma,

respectively. Expression of the CSC markers EpCAM, CD13, CD24, CD44, CD90, CD133, CK8, SALL4, ALDH1A1, ALB, and AFP was assessed in 47 patients using quantitative RT-PCR (Supplementary Fig. 1). For each marker, the median expression level was chosen as the cutoff value for separating the high-expression group from the low-expression group.

Patients with high CD133 expression (CD133-high) showed shorter PFS than those with low expression (CD133-low); however, the difference was not statistically significant (4.0 months vs 5.5 months, respectively; hazard ratio [HR], 1.97) (Table 2, Fig. 2).

To further investigate the utility of CSC markers as predictive factors, we examined combinations of CD133 plus other markers with respect to PFS. Patients with a CD133-high/CD90-high status demonstrated shorter PFS than those without high expression of both CD133 and CD90 (2.7 months vs 5.5 months, respectively; HR, 2.35; p=0.04) (Supplementary Table 1, Fig. 3A). However, patients with a CD133-low/EpCAM-low status had longer PFS than those without low expression of both CD133 and EpCAM (7.0 months vs 4.2 months, respectively; HR, 0.31;

Table 1. Baseline Characteristics of the Study Population (n=47)

| Characteristic	Value
Clinical	
Age, yr	55 (49–63)
Male sex	38 (80.9)
Etiology	
HBV/HCV/alcoholic/others	34 (72.3)/2 (4.3)/4 (8.5)/7 (14.9)
Performance status	
ECOG 0/ECOG 1	29 (61.7)/18 (38.3)
Cirrhosis	28 (59.6)
Child-Turcotte-Pugh class	
A/B	42 (89.4)/5 (10.6)
MELD score	9.1 (7.8–10.4)
Duration of sorafenib treatment, day	123 (75.5–168.5)
Tumor characteristics	
Pathology	
HCC	44 (93.6)
Combined HCC/CCC	3 (6.4)
BCLC stage	
A/B/C	2 (4.3)/9 (19.1)/36 (76.6)
Vascular invasion	7 (14.9)
Extrahepatic spread	35 (74.5)
Extrahepatic spread only	16 (34.0)
AFP, ng/mL	23.6 (4.7–186.7)

Data are presented as the median (interquartile range) or number (%). HBV, hepatitis B virus; HCV, hepatitis C virus; ECOG, Eastern Cooperative Oncology Group; MELD, Model for End-Stage Liver Disease; HCC, hepatocellular carcinoma; CCC, cholangiocarcinoma; BCLC, Barcelona Clinic Liver Cancer; AFP, alpha-fetoprotein.

Table 2. Progression-Free Survival According to Cancer Stem Cell Marker Expression

CSC marker		Progression-free survival (mo)	HR	95% CI	p-value
EpCAM	Low	4.2 (1.3–7.2)	1		
	High	4.7 (3.6–5.7)	1.10	0.58–2.10	0.77
CD13	Low	4.2 (2.5–6.0)	1		
	High	4.9 (4.1–5.8)	1.09	0.60–1.98	0.77
CD24	Low	4.2 (3.1–5.3)	1		
	High	4.5 (1.1–7.9)	0.96	0.45–2.05	0.91
CD44	Low	4.0 (2.6–5.4)	1		
	High	4.7 (2.9–6.4)	1.15	0.57–2.34	0.69
CD90	Low	5.4 (3.4–7.4)	1		
	High	4.5 (2.4–6.5)	1.07	0.59–1.94	0.82
CD133	Low	5.5 (3.9–7.0)	1		
	High	4.0 (1.7–6.3)	1.97	0.91–4.30	0.09
CK8	Low	2.6 (0.5–4.7)	1		
	High	4.5 (2.4–6.5)	0.70	0.24–2.08	0.52
SALL4	Low	3.9 (1.0–6.9)	1		
	High	5.4 (3.4–7.4)	0.73	0.38–1.41	0.35
ALDH1A1	Low	4.9 (3.5–6.4)	1		
	High	4.7 (3.2–6.1)	0.96	0.53–1.74	0.89
Albumin	Low	5.4 (3.2–7.6)	1		
	High	4.7 (3.4–5.9)	1.15	0.62–2.11	0.66
AFP	Low	4.9 (3.0–7.3)	1		
	High	4.1 (3.0–5.2)	1.32	0.69–2.54	0.41

Data are presented as the median (95% CI).
CSC, cancer stem cell; HR, hazard ratio; CI, confidence interval; AFP, alpha-fetoprotein.
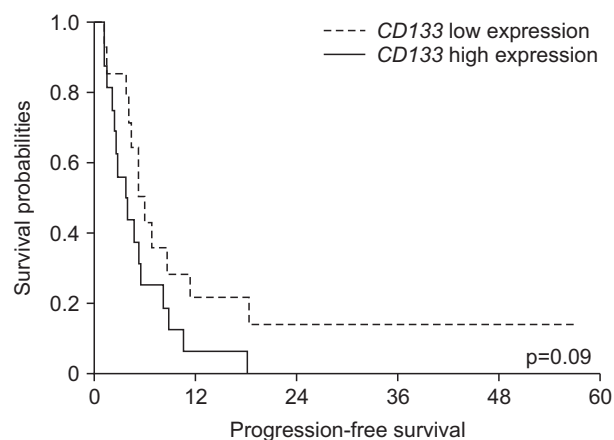
Fig. 2. Progression-free survival in hepatocellular carcinoma patients receiving sorafenib according to *CD133* expression.

Table 3. Univariate and Multivariable Analysis for Progression-Free Survival

Variable	HR (95% CI)	p-value
Univariate analysis		
ECOG 1	1.86 (0.997–3.45)	0.05
Child-Turcotte-Pugh class B	1.62 (0.63-4.20)	0.32
Vascular invasion	2.93 (1.26–6.83)	0.01
Extrahepatic spread	1.07 (0.54–2.09)	0.85
Extrahepatic spread only	0.65 (0.34–1.24)	0.19
Multivariable analysis		
ECOG 1	3.08 (1.27–7.47)	0.01
CD133-high/*CD90*-high	2.97 (1.24–7.07)	0.01

HR, hazard ratio; CI, confidence interval; ECOG, Eastern Cooperative Oncology Group.

p=0.04) (Supplementary Table 1, Fig. 3B).

Univariate analysis revealed that among the clinical features tested, Eastern Cooperative Oncology Group (ECOG) performance status and vascular invasion were associated with PFS (Table 3). Among the variables selected for multivariable analysis (ECOG performance status, vascular invasion, *CD133*-high/*CD90*-high status, and *CD133*-low/*EpCAM*-low status), an ECOG performance status of 1 and a *CD133*-high/*CD90*-high status were significantly associated with shorter PFS (Table 3).

3. Survival outcome according to CSC marker expression

The median OS of patients with high expression of both *CD133* and *CD90* was 9.4 months (95% CI, 1.1 to 17.8 months), shorter than the 14.0 months (95% CI, 7.1 to 20.8 months) in
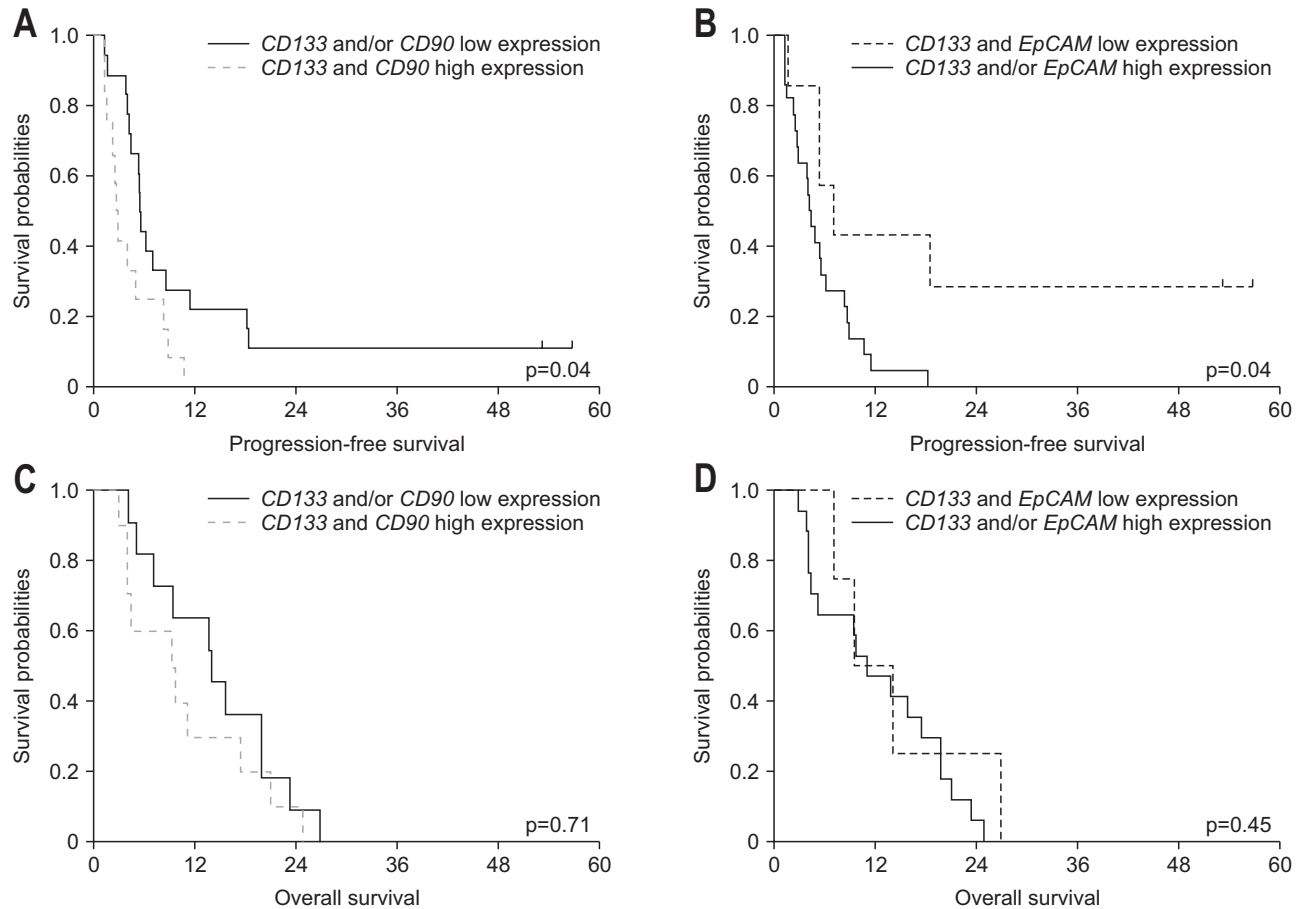
Fig. 3. Survival outcome in hepatocellular carcinoma patients receiving sorafenib according to *CD133*, *CD90*, and *EpCAM* expression. (A) Progression-free survival according to *CD133* and *CD90* expression. (B) Progression-free survival according to *CD133* and *EpCAM* expression. (C) Overall survival according to *CD133* and *CD90* expression. (D) Overall survival according to *CD133* and *EpCAM* expression.

of those who did not have high expression for both *CD133* and *CD90* (p=0.71; Fig. 3C). The median OS of patients with low expression of both *CD133* and *EpCAM* was 11.1 months (95% CI, 5.2 to 16.9 months), longer than the 9.4 months (95% CI, 2.6 to 16.2 months) in those who did not have low expression for both *CD133* and *EpCAM* (p=0.45; Fig.3D). However, these differences were not statistically significant.

DISCUSSION

Many studies have suggested that CSCs are responsible for resistance to various chemotherapeutics in cancers;[20] however, the underlying mechanisms by which CSCs elicit chemoresistance remain largely unknown. One hypothesis for the chemoresistance mechanism is that CSCs are located in niches where they are shielded from drugs or can regulate the tumor microenvironment and exposure through drug efflux. Other theories are that CSCs can inactivate drugs in the cytoplasm or that they possess a robust DNA damage response and DNA repair mechanisms that involve the upregulation of prosurvival or antiapoptotic signaling pathways.[20] Active drug efflux is mediated by several ATP-binding cassette transporters such as multidrug resistance pro-

tein 1 (MDR1/ABCB1), multidrug resistance-associated protein 1 (MRP1/ABCC1), and breast cancer resistance protein (BCRP/ABCG2).[20,21]

In the present study, we showed that *CD133* expression was related to the response to sorafenib; however, the association was not statistically significant for *CD133* alone. However, a *CD133*-high/*CD90*-high status was negatively associated but a *CD133*-low/*EpCAM*-low status was positively associated with the sorafenib response. Among the clinical factors and CSC markers investigated, an ECOG performance status of 1 and a *CD133*-high/*CD90*-high status were significantly associated with a poor response to sorafenib. *CD133*-high/*CD90*-high and *CD133*-low/*EpCAM*-low statuses also tended to be associated with OS; however, neither association was statistically significant.

CD133 was originally identified as a member of the prominin family of pentaspan transmembrane glycoproteins (5 transmembrane domains) and is also known as prominin-1.[22] *CD133* is usually recognized as a surface antigen on hematopoietic progenitor cells; however, its biological function is largely unknown. *CD133* is also regarded as a CSC marker in various cancers such as brain, colon, non-small cell lung, laryngeal, and liver cancers.[23-27] In HCC, high expression of *CD133* has been correlated

with increased tumor grade, advanced tumor stage, poor OS, and a high recurrence rate.[28,29] Moreover, *CD133* has been implicated in chemoresistance and radioresistance in glioblastoma, lung cancer, colon cancer, and HCC.[30-34] Ma *et al*.[33] showed that *CD133*+ HCC cells were more resistant than *CD133*– HCC cells to chemotherapeutic agents such as doxorubicin and 5-fluorouracil; this resistance was mediated by the preferential activation of the Akt/PKB and Bcl-2-mediated cell survival pathways.

CD90 encodes a glycophosphatidylinositol-anchored cell surface protein that is involved in cell-cell and cell-matrix interactions and is also known as THY1.[35] This protein is usually expressed on T cells, neurons, endothelial cells, mesenchymal stem cells, hematopoietic stem cells, and fibroblasts.[36] *CD90* is upregulated in cancers such as esophageal cancer, gastric cancer, and high-grade glioma[37-39] and is associated with poor differentiation or high pathological grade in HCC.[40,41] In addition, *CD90* expression was associated with recurrence and predicted poor prognosis in HCC patients who underwent surgical resection.[41,42] Moreover, *CD90*-positive esophageal cancer cells were chemoresistant to 5-fluorouracil and Taxotere.[37] *CD90*-positive HCC cells are reportedly resistant to doxorubicin, and *BCRP/ABCG2* expression can contribute to this chemoresistance.[43,44]

This study has some limitations. First, this study was a retrospective study in a cohort of 47 patients; the small sample size may have biased the results. Second, CSC markers were evaluated in pathological specimens, although blood-based biomarkers are usually preferred over tissue-based biomarkers because blood is more convenient and less invasive to obtain than tissue. In fact, pathological specimens were not available for some of the HCC patients since HCC can be diagnosed via noninvasive methods. Although liver biopsies have been considered confirmatory tests in small or atypical tumors, they have increasingly been considered a prerequisite for companion diagnostics in recent biomarker-driven clinical trials.[45] Third, we were unable to explore the mechanisms linking high expression of *CD133/CD90* with the response to sorafenib. However, the expression of the CD133 or CD90 proteins may promote sorafenib resistance through the regulation of survival and apoptosis signaling pathways as well as through active drug efflux, as the results of previous studies suggest.[33,43,44]

Our results demonstrated that the level of *CD133* and *CD90* expression was associated with the clinical response to sorafenib. Such CSC markers may therefore be useful predictors of sorafenib therapy outcomes; further investigations of the implications of CSCs in HCC are warranted.

CONFLICTS OF INTEREST

No potential conflict of interest relevant to this article was reported.

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