The cancer stem cell marker CD133 is a predictor of the effectiveness of S1+ pegylated interferon α-2b therapy against advanced hepatocellular carcinoma

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Abstract

Background Combination therapy with the oral fluoropyrimidine anticancer drug S1 and interferon is reportedly effective for the treatment of advanced hepatocellular carcinoma (HCC), but selection criteria for this therapy have not been clarified. In this study, we attempted to identify factors predicting the effectiveness of this combination therapy.

Methods Pathological specimens of HCC were collected before treatment from 31 patients with advanced HCC who underwent S1+ pegylated-interferon (PEG-IFN) α-2b therapy between January 2007 and January 2009. In these pathological specimens, the expression levels of CD133, thymidylate synthase (TS), dihydropyrimidine dehydrogenase (DPD), and interferon-receptor 2 (IFNR2) proteins were determined by Western blot assay. The presence or absence of p53 gene mutations was determined by direct sequencing. The relationships between these protein expression levels and the response rate (RR), progression-free survival (PFS), and overall survival (OS) were evaluated.

Results The CD133 protein expression level was significantly lower in the responder group than in the nonresponder group. Comparing the PFS and OS between high- and low-level CD133 expression groups (n = 13 and 18, respectively) revealed that both parameters were significantly prolonged in the latter group. The expression levels of TS, DPD, and IFNR2 protein and the presence of p53 gene mutations did not correlate with the RR.

Conclusions CD133 was identified as a predictor of the therapeutic effect of S1+ PEG-IFN α-2b therapy against advanced HCC.

Keywords 5-Fluorouracil · Pegylated interferon · CD133 · Cancer stem cell · Hepatocellular carcinoma

Abbreviations

5FU 5-Fluorouracil
DPD Dihydropyrimidine dehydrogenase
HCC Hepatocellular carcinoma
IFNR2 Interferon-receptor 2
NR Nonresponder
OS Overall survival
PD Progressive disease
PEG-IFN Pegylated interferon
PFS Progression-free survival
PR Partial response
RR Response rate
Introduction

Hepatocellular carcinoma (HCC) is one of the most common malignancies in Asia, including Japan [1, 2], and its prevalence has recently been increasing globally [3]. In most patients, the background of HCC is chronic hepatitis or liver cirrhosis due to hepatitis B or hepatitis C infection. Recently, HCC has increasingly been detected at relatively early stages due to the periodic follow-up of chronic liver disease patients and the development of diagnostic imaging modalities. There have been significant improvements in the treatment of patients with early HCC, and the therapeutic results have been markedly improved by site-specific treatments such as transcatheter arterial chemoembolization, percutaneous ethanol injection therapy, microwave coagulation therapy, and radiofrequency ablation, as well as hepatectomy [4–6].

However, when existing HCC is cured radically, new cancers develop due to the underlying chronic liver disorders. Treatments must then be performed alone or in combination each time a new cancer appears. Repeated treatment often leads to portal vein tumor thrombosis or distant metastasis, making standard treatments difficult to perform. Recently, the treatment efficacy and safety of the molecular targeted drug sorafenib (Nexavar; Bayer HealthCare Pharmaceuticals–Onyx Pharmaceuticals, Leverkusen, Germany) have been reported and the results placed it as a first-line drug [7]. Sorafenib is a small molecule that inhibits tumor-cell proliferation and tumor angiogenesis and increases the rate of apoptosis in a wide range of tumor models. It acts by inhibiting the serine-threonine kinases Raf-1 and B-Raf and the receptor tyrosine kinase activity of vascular endothelial growth factor receptors (VEGFRs) 1, 2, and 3 and platelet-derived growth factor receptor β (PDGFR-β). Llovet et al. [7] reported that sorafenib prolonged median survival and the time to progression by nearly 3 months in about 300 patients with advanced HCC. Although different types of molecular targeted drugs have been under development, there is no treatment option at present for the patients who fail to respond to sorafenib. Thus, second-line treatment for advanced HCC needs to be established.

The oral fluoropyrimidine anticancer drug, S1, includes the dihydropyrimidine dehydrogenase (DPD) inhibitor 5-chloro-2,4-dihydroxypyridine as a component, and this component is expected to exhibit a marked anticancer effect by preventing 5-fluorouracil (5FU) degradation [8–10]. Combination therapy with S-1 and interferon (IFN) has recently been attempted in patients with advanced HCC, and relatively satisfactory results have been reported [11–14]. However, the response rate for this therapy is limited, and the outcome deteriorates in patients resistant to it. Therefore, if the effectiveness of this therapy can be predicted in advance, unnecessary adverse effects can be avoided, and other treatments may be attempted.

We have noted some candidate proteins which may have a possible relationship with the effect of this therapy. The cancer stem-cell marker CD133 [15–18] can reportedly resist anticancer drugs through an intrinsic drug resistance mechanism [19, 20]. Thymidylate synthase (TS) and DPD are enzymes involved in 5FU metabolism, and many reports have suggested their relationship with the therapeutic effects of 5FU in lung [21] and colon cancers [22]. Furthermore, interferon-receptor 2 (IFNR2) is reported to be the most important of the receptors through which IFN acts directly on HCC [23, 24]. Apoptosis is the primary mechanism of the anticancer effect of anticancer drugs, and p53 is closely involved in apoptosis [25–27].

In the present study, we sought to identify factors predicting the effectiveness of S1+ pegylated-interferon (PEG-IFN) α-2b therapy. HCC tissue samples were collected before the therapy was started, the expression levels of CD133, TS, DPD, and IFNR2 proteins were determined by Western blot analysis, and the presence or absence of p53 gene mutations was examined by direct sequencing. We found that the expression level of CD133 was significantly correlated with the therapeutic effect. Thus, measurement of the CD133 expression level before treatment may facilitate prediction of the therapeutic effect and the avoidance of unnecessary adverse effects.

Subjects, materials, and methods

Patients

Between January 2007 and January 2009, a total of 31 patients with refractory HCC that could not be controlled by standard therapeutic modalities (transcatheter arterial chemoembolization, percutaneous ethanol injection therapy, microwave coagulation therapy, radiofrequency ablation, and hepatectomy) underwent S-1 and PEG-IFN α-2b combination therapy. Patient characteristics are shown in Table 1, with more details shown in Table 2. All pathological specimens of HCC were collected by needle biopsy.

Eligibility criteria

Eligibility criteria for the combination therapy included: (1) advanced HCC that was uncontrollable with standard treatment, or HCC with distant metastasis; (2) age <80 years; (3) an Eastern Cooperative Oncology Group performance status of 0 or 1; (4) Child–Pugh grade A; (5) encephalopathy.
degree 0; (6) leukocyte count >3,000 cells/mm$^3$; hemoglobin level >10 g/dl and platelet count >80,000 cells/mm$^3$; and (7) serum creatinine <1.5 mg/dl, serum aspartate aminotransferase <200 IU/l, serum alanine aminotransferase <200 IU/l, and serum total bilirubin level <3.0 mg/dl. The diagnosis of HCC was made based on the hematoxylin–eosin staining of histopathological specimens in all patients.

Treatment regimen

After the obtaining of informed consent, 31 patients were treated with S-1 (TS1; Taiho Pharmaceutical, Tokyo, Japan) and PEG-IFN α-2b (Pegintron; Schering-Plough, Kenilworth, NJ, USA) combination therapy. S-1 was given orally at a daily dose of 80–120 mg (depending on the body surface area: <1.25 m$^2$: 80 mg, >1.25 to <1.5 m$^2$: 100 mg, <1.5 m$^2$: 120 mg), divided into two equal doses, from days 1 to 28. PEG-IFN α-2b was given subcutaneously at a dose of 50 μg on days 1, 8, 15, and 22. One course consisted of consecutive administration for 28 days followed by a 2-week drug-free interval. The Medical Ethics Committee of Kinki University of Medicine approved the study.

Assessment of response

Responses of HCC patients to the combination therapy were assessed by contrast-enhanced computed tomography after each course. The response was defined according to the Response Evaluation Criteria in Solid Tumours (RECIST). A partial response (PR) was defined as a minimum 30% decrease in the sum of the longest diameters of the target lesions, with the baseline sum of the longest diameters of these lesions as the reference. Progressive disease (PD) was defined as a minimum 20% increase in the sum of the longest diameters of the target lesions. Stable disease (SD) was defined as meeting neither PR nor PD criteria. When the response achieved regarding intrahepatic HCC was different from that for extrahepatic HCC, the poorer one was determined as the achieved response.

Assessment of toxicity

Blood cell counts and biochemical profiles were performed at least once every week. Adverse reactions were assessed using the National Cancer Institute-Common Toxicity Criteria (NCI-CTC, version 3).

Western blot analysis

To prepare tissue lysate, HCC tissue was homogenized with Celllytic-MT Mammalian Tissue Lysis/Extraction reagent (Sigma-Aldrich, St. Louis, MO, USA) along with 2% sodium dodecyl sulfate (SDS) and the protease inhibitor, Complete$^\text{TM}$ (Roche Diagnostics, Mannheim, Germany), and centrifuged. Equal protein amounts (8 μg) of tissue lysates were electrophoresed through a reducing SDS polyacrylamide gel and electroblotted onto a polyvinylidene difluoride (PVDF) membrane. The membrane was blocked and incubated with polyclonal IgG for TS (1/500; Taiho Pharmaceutical, Tokyo, Japan), DPD (1/3,000; Taiho Pharmaceutical), IFN-α/βR (1/500; Otsuka Pharmaceutical., Tokyo, Japan), CD133 (1/1,000; Cell Signaling Technology, Danvers, MA, USA), and β-actin (1/2,000; Sigma-Aldrich) in Can Get Signal$^\text{TM}$ immunostain solution (TOYOBO, Osaka, Japan). For CD133 detection, lysis of the human colon cancer cells WiDr and DLD1 (positive and negative controls, respectively) was examined. Protein levels were detected using horseradish peroxidase (HRP)-linked secondary antibodies and the ECL-plus System (GE Healthcare, Buckinghamshire, UK).

To evaluate the signal intensity, the obtained Western blot image data were quantified using Image J software (NIH, Bethesda, MD, USA).

Immunohistochemistry

We performed immunohistochemical analysis of paraffin-embedded sections of HCC. Immunohistochemical staining was carried out with antibodies raised against CD133 (1:100), and visualized using the Dako LSAB System-HRP.
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<th>HBs-Ag</th>
<th>HCV-Ab</th>
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<th>Vascular invasion</th>
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*a* Poorly differentiated  
*b* Moderately differentiated
(Dako, Carpinteria, CA, USA). Sections were counter-stained with hematoxylin.

**Determination of p53 sequence**

Total RNA extracted from HCC tissue using TRIZOL (Invitrogen, Carlsbad, CA, USA) was reverse-transcribed employing the Takara RNA PCR kit (AMV) Ver.3 (Takara, Tokyo, Japan). p53 was amplified using the forward primer 5’-GAGCCGAGTCAGATCCTA-3’ and the reverse primer 5’-CAGTCTGAGTCAGGCGCTTC-3’, and nested polymerase chain reaction (PCR) was performed using the primers 5’-CCCCTCTGAGTCAGGAAACA-3’ and 5’-TTATGGCAGGGAGGTAGACTG-3’. The PCR product was purified and sequenced using BigDye terminator version 3.1 cycle sequencing on an ABI 3100 DNA sequencer (Applied Biosystems, Foster City, CA, USA).

**Statistical analysis**

Differences between groups were examined for significance using the Mann–Whitney U-test and Fishers exact test where appropriate. Multivariate analysis was performed by using a logistic regression model. Cumulative survival and progression-free survival (PFS) curves were constructed using the Kaplan–Meier method and compared using the log-rank test. All the analyses described above were performed using the SPSS program (version 11.5; SPSS, Chicago, IL, USA).

**Results**

**Response**

Complete and partial responses were achieved in 0 (0%) and 10 (32.3%) of the 31 patients, respectively. The overall response rate was 32.3%. Stable disease (SD) was noted in 3 patients (9.7%), and the disease control rate (complete response + partial response + SD) was 41.9%. Progressive disease (PD) was noted in 17 patients (54.8%). One patient was excluded from the assessment of response, because the patient died of HCC rupture 17 days after the start of treatment and the computed tomography could not be performed.

Progression-free survival rate and survival assessment

The median PFS was 1.6 months (95% confidence interval [CI] 1.5–1.7 months). The cumulative PFS rates at 6, 12, and 18 months were 38, 19, and 9%, respectively.

All enrolled patients were also included in a survival assessment. Twelve patients were still alive at the end of the observation period (median 8.2 months, range 2–25.4 months), while 19 patients had died. The causes of death were tumor progression (n = 18) and infectious lung disease (n = 1). The median survival time was 5.3 months (95% CI 1.7–9.0 months). The cumulative survival rates at 6, 12, 18, and 24 months were 44, 35, 35, and 17%, respectively.

**Relationship of CD133, TS, DPD, and IFNR2 protein expression levels in HCC with the anticancer effect of the combined therapy**

The expression levels of CD133, TS, DPD, and IFNR2 proteins, which were candidate predictive factors for the therapeutic effect, were studied by Western blotting in all specimens. Figure 1 shows the results in five samples from the PR group and four samples from the PD group. The expression level of CD133 was low in the PR group but high in the PD group. However, no marked differences were noted in the expression levels of TS, DPD, or IFNR2 between the two groups.

Next, the relationships of the CD133, TS, DPD, and IFNR2 protein expression levels with the antitumor effect were evaluated. To compare the protein expression levels among specimens, the relative expression level was calculated by dividing the intensity of each signal on Western blotting by the signal intensity of actin, which is an internal control. The CD133 protein expression level was significantly lower in the responder group (median 0.05) than in the nonresponder group (median 0.58) group (p = 0.005) (Fig. 2a). In contrast, the TS, DPD, and IFNR2 protein expression levels showed no significant differences between the responder and nonresponder groups (Fig. 2b–d).

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**Fig. 1** Expression of CD133, thymidylate synthase (TS), dihydropyrimidine dehydrogenase (DPD), and interferon receptor 2 (IFNR2) as possible factors predicting the therapeutic effect in hepatocellular carcinoma (HCC). Results of Western blotting in typical cases in the partial response (PR) and progressive disease (PD) groups. For CD133, negative (DLD1) and positive (WiDr) controls were used.
The expression of CD133 was also studied in liver cancer tissues of all specimens using immunohistochemistry. Figure 3 presents specimens that showed intense staining for CD133. The presence or absence of an immunohistological signal was correlated with the CD133 protein expression level determined by Western blotting. Comparison of the patient background and candidate factors predicting treatment effects with the final outcome (results of univariate analysis and multivariate analysis with a logistic regression model) showed a significantly higher CD133 level ($p < 0.01$) in the nonresponder (NR) than the responder group and a slightly higher TS level ($p = 0.11$) in the responder group (Table 3). Using CD133 and TS showing $p < 0.15$ on univariate analysis, multivariate analysis with a logistic regression model was performed. This analysis revealed that only CD133 was a significant factor (odds ratio 0.076, 95% CI 0.007–0.88, $p = 0.039$) (Table 4). Thus, irrespective of the TS level, CD133 was identified as an independent factor predicting the treatment effects.

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The positive predictive value for nonresponders was 100% in patients whose CD133 expression level exceeded 0.4. Thus, we classified patients into high- and low-CD133 expression groups with a cutoff level of 0.4 (Fig. 2a). Thirteen patients were classified into the high-CD133 expression group and 18 patients into the low-CD133 expression group.

The relationship between the CD133 protein expression level and PFS is shown in Fig. 4. The median PFS was 1.6 months (95% CI 1.5–1.6 months) in the high-CD133 expression group and 7.2 months (95% CI 2–12.3 months) in the low-CD133 expression group. The log-rank test using the Kaplan–Meier method showed that the PFS was significantly prolonged in the low-level compared to the high-level CD133 expression group ($p = 0.036$).

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The relationship between the CD133 protein expression level and overall survival (OS) is shown in Fig. 5. The median survival time was 3.1 months (95% CI 1.4–4.8 months) in the high-CD133 expression group, but 8.1 months (95% CI 0–19.3 months) in the low-CD133 expression group. The log-rank test using the Kaplan–Meier
The method showed that, in the low-level CD133 expression group, the OS was significantly prolonged compared to that in the high-level group \((p = 0.022)\).

Relationship between p53 mutations and the anticancer effect

Mutant p53 was observed in 3 of the 31 patients. The response rate was 32.1% in the wild-type and 33.3% in the mutant specimens, with no significant difference. The disease control rate was 39.3% in the wild-type and 66.7% in the mutant specimens, with no significant difference.

![Fig. 4 Progression-free survival of patients who received combination therapy with S-1 and pegylated interferon (PEG-IFN) α-2b, stratified according to the CD133 expression level. Patients were divided into high- and low-CD133 expression groups, with a cutoff value of 0.4 (Fig. 2a). Thirteen and 18 patients belonged to the high- and low-expression groups, respectively.](image)
Toxicity

NCI-CTC grade 3 leukocytopenia, neutropenia, anemia, and thrombocytopenia were observed in 2 (6%), 2 (6%), 1 (3%), and 3 (10%) of the 31 patients, respectively. Grade 3 anorexia, stomatitis, rash, and fatigue were observed in 1 (3%), 1 (3%), 1 (3%), and 2 (6%) of the 31 patients, respectively. All adverse effects were alleviated when the treatment was discontinued, leading to no cases of mortality.

Discussion

Here we sought to identify factors predicting the therapeutic effect of S1 + PEG-IFN α-2b therapy in patients with advanced HCC. We collected pathological samples of HCC from all registered patients and studied proteins considered to be related to the therapeutic effect. The expression level of CD133 was significantly correlated with the therapeutic effect, but the expression levels of TS, DPD, and IFNR2, and the presence or absence of p53 mutations were not.

CD133 is a glycoprotein with five transmembrane regions and is a known blood stem-cell marker [28]. It also reportedly acts as a leukemia [15], brain [16], and colon cancer [17, 18] stem-cell marker. The characteristics of cancer stem cells include an ability to proliferate (self-replication capacity) and to differentiate into several cell types with different functions (multidifferentiation capacity), as well as a tumorigenic capacity, which was verified as tumor reproducibility in an experiment involving tumor implantation in an animal model [29–31]. In HCC, CD133-positive cells reportedly possess each of these cancer stem-cell characteristics. In 2007, Ma et al. [32] showed that 65–95% of cells in multiple HCC cell lines were CD133-positive, and Suetugu et al. [33] reported that the HCC cell line Huh7 expressed CD133. In addition, Song et al. [34], who evaluated 60 patients with HCC, reported both significantly longer postoperative recurrence-free survival and total survival periods in a group with a low compared to that with a high CD133 level. There have been a few such reports on CD133 as a marker of postoperative recurrence.

In the present study, the CD133 protein expression level was significantly lower in the responder group than that in the nonresponder group. HCC showing high-level CD133 expression was resistant to the combination therapy used in this study. Several studies have suggested that the most cancer stem cells exist in the G0 phase, and a reduced cell cycle velocity is involved in the drug resistance of cancer stem cells [35]. Furthermore, these cells are resistant to reactive oxygen-induced DNA damage because of their high radical-scavenging activities [36]. Furthermore, the drug resistance mechanism of cancer stem cells may also involve ATP binding cassette (ABC) transporters [16, 37]. The anti-apoptotic factors Akt/PKB and Bcl-2 are also activated in CD133-positive liver cancer cells by 5FU administration [38]. Because the anticancer effect of S1 + PEG-IFN α-2b therapy is primarily derived from apoptosis, the activation of Akt/PKB and Bcl-2 is considered to be directly related to the resistance to this therapy.

TS is a rate-regulating enzyme involved in the synthesis of deoxythymidine monophosphate, which is indispensable for DNA synthesis. Therefore, the anticancer effect of 5FU decreases when the TS expression level in the tumor is high, because the drug cannot sufficiently inhibit the enzyme [39]. In the present study, the TS expression level did not correlate with the therapeutic effect. Oie et al. [40] reported that TS expression was suppressed by IFN administration in all the HCC-derived cell lines they examined. Although we did not evaluate the TS expression level after IFN administration, the absence of a correlation between the TS expression level and the therapeutic effect may have been due to the inhibition of TS by IFN.

DPD is a 5FU-degrading enzyme present primarily in the liver. 5FU efficiency increases with low DPD expression in tumor cells [41]. In our study, no correlation between the DPD expression level and therapeutic effect was noted. This was an expected result, because S1 contains a DPD inhibitor.

IFNR, and particularly IFNR2, is considered to be the most important IFN-binding unit for IFN activity. IFNR2 is reportedly expressed in 61–77% of HCCs [42, 43], and its anticancer effect increases with its level of expression. In our study, IFNR2 expression did not correlate with the therapeutic effect. When multiple HCC cell lines were
treated with IFN-α in vitro, a relationship between IFNAR2 expression and the anticancer effect was demonstrated [23]. Therefore, IFNAR2 is undoubtedly important in the direct anticancer effect of IFN. However, indirect anticancer effects of IFN, such as the activation of natural killer cells and cytotoxic lymphocytes, must also be considered in regard to in vivo treatment [44–46]. Such indirect actions may be primarily responsible for the anticancer effects of IFN in some patients. Regardless of these findings, the IFNAR2 expression level is not considered useful for the prediction of the therapeutic effect.

p53 is a typical tumor suppressor gene that may arrest the cell cycle and induce DNA repair or promote apoptosis, depending on the degree of DNA damage [47]. However, the mutation of p53 at some sites causes loss of its original function, allowing the initiation of tumor growth and acceleration of tumor proliferation [48]. In our study, no correlation was noted between the presence or absence of p53 mutations and the therapeutic effect. This may have been because there were only three patients with mutant p53, or because the mutations occurred at sites that do not affect p53 function.

In conclusion, S1+ PEG-IFN α-2b therapy can be a second-line treatment option for patients with advanced HCC, especially in those that show low CD133 expression. Further evaluation with a prospective randomized trial is necessary to confirm the results of the present study.

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Conflict of interest None.

References


