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In daily practice, the concept of fiberoptic intubation in the awake patient is not clearly defined. In most cases, the choice of technique is dependent on institutional and personal preferences. Ultimately, such a choice is a compromise between safety, practicability, and acceptance. The technique as shown in the video is a thoroughly documented, well-tested method that has not been changed for many years.[5]

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Since publication of his article, the author reports no further potential conflict of interest.

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Deferoxamine for Advanced Hepatocellular Carcinoma

TO THE EDITOR: We have previously reported that the iron chelator deferoxamine can prevent liver injury as well as the development of preneoplastic lesions in rats,[1,2] and we have proposed the use of deferoxamine as an anticancer drug. The antiproliferative effect of deferoxamine arrests the cell cycle and induces apoptosis.[3] To our knowledge, no clinical study has been performed to evaluate deferoxamine therapy in patients with hepatocellular carcinoma.[4]

Our study involved 10 patients (6 men and 4 women) who had advanced hepatocellular carcinoma and did not have a response to hepatic arterial infusion chemotherapy with anticancer drugs. The average age of the patients was 64 years (range, 43 to 77). Written informed consent was obtained before the study, which was approved by the institutional review board of Yamaguchi University Hospital. Seven patients had hepatitis C virus infection, 2 patients had hepatitis B virus infection, and 1 patient did not have either type of infection. The tumor stages were classified as II, IVA, and IVB (according to the Liver Cancer Study Group of Japan criteria) for 1, 2, and 7 patients, respectively. The Child–Pugh class was A, B, and C for 3, 5, and 2 patients, respectively. (In the Child–Pugh classification of liver disease, class A indicates the least severe disease, class B moderately severe disease, and class C the most severe disease.) The patients received an arterial infusion of deferoxamine (at a dose of 10 to 80 mg per kilogram of body weight) over 24 hours on alternate days, through the injection port.

Deferoxamine was administered an average of 27 times (range, 9 to 78). Two, three, and five patients had a partial response, stable disease, and progressive disease, respectively (according to the Eastern Cooperative Oncology Group criteria). The overall response rate was 20%.

Tumor-marker levels (alpha-fetoprotein, des-γ-carboxyprothrombin, alpha-fetoprotein L3, or all of these levels) decreased in patients with a partial response. In one patient, a massive hepatocellular tumor with lung metastases disappeared with deferoxamine treatment (Fig. 1). The 1-year cumulative survival rate was 20%. Four patients had grade 2 or 3 interstitial pneumonia (according to the Common Terminology Criteria for Adverse Events, version 4.0), and one patient had grade 2 renal dysfunction. However, no grade 4 adverse events were observed.

Sorafenib, a multikinase inhibitor, has recently been established as the standard of care for patients with advanced hepatocellular carcinoma and preserved liver function (Child–Pugh class A) because it increases survival.[5] However, its safety and efficacy for patients with Child–Pugh class B or C disease is still unknown. Deferoxamine may warrant testing in patients with Child–Pugh class B or C hepatocellular carcinoma.

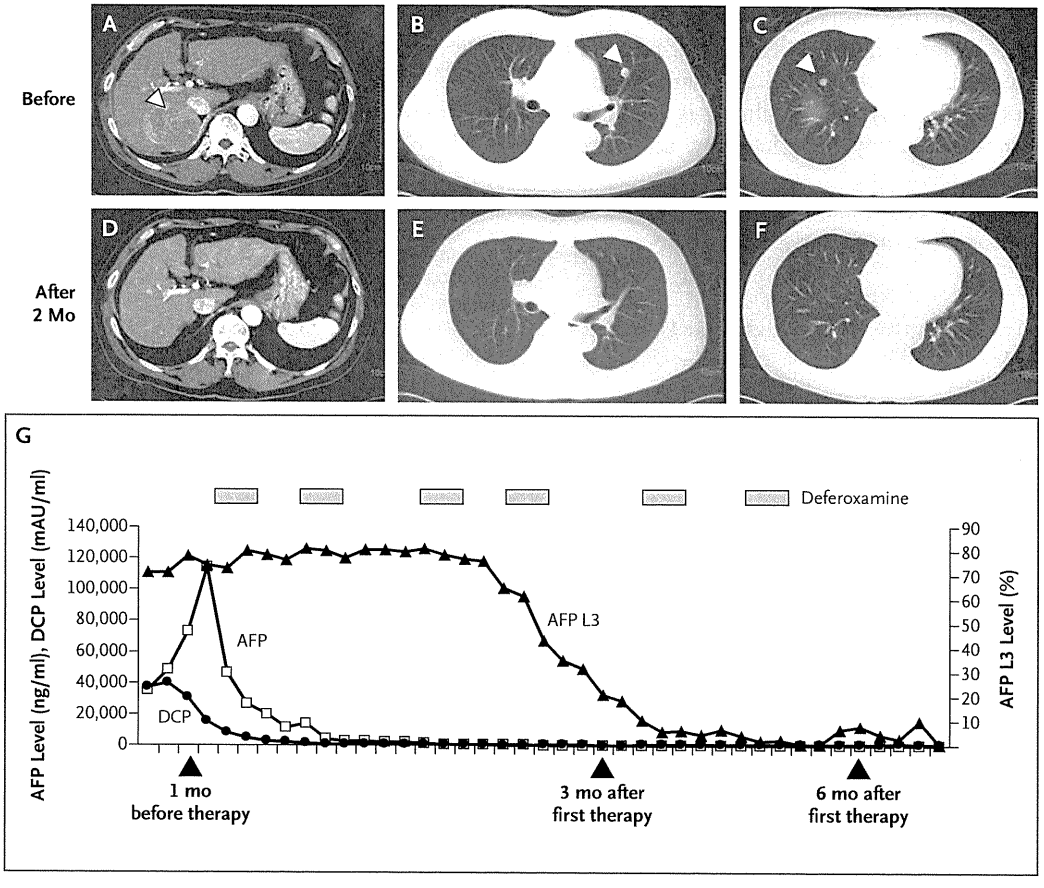

Figure 1. **Deferoxamine Treatment in One Patient.**

A 62-year-old man presented with a massive hepatocellular tumor with multiple small lung metastases (stage IVB). The hepatic reserve function was Child–Pugh class B (a score of 8) before treatment (in the Child–Pugh classification of liver disease, class A [a score of 5 or 6] indicates the least severe disease, class B [7 to 9] moderately severe disease, and class C [10 to 15] the most severe disease). In Panel A, abdominal dynamic computed tomography (CT) before treatment shows a tumor (arrowhead). In Panels B and C, plain chest CT scans obtained before treatment show multiple lung metastases (arrowheads). Two months after arterial infusion of deferoxamine (at a dose of 50 mg per kilogram of body weight per day), the liver tumor and multiple lung metastases were not observed on abdominal dynamic CT (Panel D) and plain chest CT (Panels E and F). As shown in Panel G, after arterial infusion of deferoxamine, the levels of three tumor markers (alpha-fetoprotein [AFP], des-γ-carboxyprothrombin [DCP], and AFP L3) decreased. The patient was considered to have a partial response according to the Eastern Cooperative Oncology Group criteria. The patient survived 20.9 months with deferoxamine treatment.

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Disclosure forms provided by the authors are available with the full text of this letter at NEJM.org.

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Contact Tamara Rios, Rios Associates, 3729 N. Bay Horse Loop, Tucson, AZ 85719; or call (520) 907-3318; or see http://www.medspanish.org.

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Senescence marker protein 30 (SMP30)/regucalcin (RGN) expression decreases with aging, acute liver injuries and tumors in zebrafish

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ABSTRACT

Senescence marker protein 30 (SMP30)/regucalcin (RGN) is known to be related to aging, hepatocyte proliferation and tumorigenesis. However, expression and function of non-mammalian SMP30/RGN is poorly understood. We found that zebrafish SMP30/RGN mRNA expression decreases with aging, partial hepatectomy and thioacetamide-induced acute liver injury. SMP30/RGN expression was also greatly decreased in a zebrafish liver cell line. In addition, we induced liver tumors in adult zebrafish by administering diethylnitrosamine. Decreased expression was observed in foci, hepatocellular carcinomas, cholangiocellular carcinomas and mixed tumors as compared to the surrounding area. We thus showed the importance of SMP30/RGN in liver proliferation and tumorigenesis.

1. Introduction

Senescence marker protein 30 (SMP30) was originally identified by two-dimensional gel electrophoresis as a 34 KDa protein of which expression decreases by up to 40% in the aged rat liver without androgen dependence [1]. But it was subsequently found to be the same as regucalcin (RGN), a calcium-related protein without an EF-hand motif [2]. RGN has been intensively studied by Yamaguchi and Takahashi who reported that RGN regulates intracellular Ca^{2+} homeostasis by activating Ca^{2+} pump activity in the cell membrane [3]. Moreover, SMP30/RGN inhibits Ca^{2+}-dependent protein kinase, protein phosphatase and nitric oxidase synthase, and thereby inhibits cell proliferation [4]. SMP30/RGN is known to exist in a broad range of species from mammals to bacteria, and its expression in mammals is high in hepatic parenchymal cells and the renal cortex [5]. Compared with normal mice, SMP30/RGN knockout mice age more rapidly and have increased fat droplets and lysosomes in their livers. Furthermore, SMP30/RGN was shown to be gluconolactonase, an enzyme indispensable for vitamin C synthesis, and the relationship between vitamin C and aging appears to be important [6,7]. SMP30/RGN mRNA expression is reportedly decreased in chemically-induced tumors as compared to surrounding normal liver tissue, based on in situ hybridization [8]. Microarray analysis showed SMP30/RGN to be one of the down-regulated genes in the GST-P positive area [9]. Although overexpression of SMP30/RGN in hepatoma cells down-regulates oncogenes such as c-myc, Haras and c-src [10], details of how SMP30/RGN takes part in the development and progression of cancer remain unknown. Zebrafish are the simplest vertebrate model because of their low breeding costs and the capacity for high-through-put screening [11,12]. Zebrafish provide a good cancer model because various diethylnitrosamine (DEN)-induced tumors resemble human tumors [13]. There are no reports concerning zebrafish SMP30/RGN except a study on dioxin administration to embryos [14]. Moreover, the expression of SMP30/RGN in mixed tumors and cholangiocellular carcinomas has not previously been reported. In this study, we examined the importance of SMP30/RGN in aging, liver proliferation and liver tumorigenesis using the zebrafish model.

2. Materials and methods

2.1. Animals

Zebrafish were maintained in accordance with the Animal care Guidelines of Yamaguchi University. Fish were kept in tap water in plastic tanks and illuminated with fluorescent lighting set at 16 h light and 8 h dark.

2.2. Cell culture

An adult zebrafish liver cell line (ATCC CRL-2643) was cultured in ZFL medium as described by Ghosh et al. [15].

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2.3. DEN exposure

One-year old fish were exposed to 200 ppm DEN for 2 months. DEN solutions were changed every week to compensate for degradation. Following exposure, the fish were maintained in tap water without DEN for 4 months.

2.4. Quantitative RT-PCR

Total RNA was isolated with TRIzol (Invitrogen), and treated with Turbo DNase (Ambion). RT-PCR was performed by utilizing the step one plus real time PCR system and Fast SYBR Green Master Mix (Applied Biosystems). Specific primers for SMP30/RGN (5′-ACT ATG ACA TCC AAA CTG GAG GA-3′ and 5′-CTT CTG TGT CTA TGC ACA TAC CG-3′) were used. Elongation factor 1-alpha was used as an internal control.

2.5. Tissue collection and histology

Fish were killed and opened from the anal vent to the gills. The entire body was fixed with 4% paraformaldehyde in 0.1 M phosphate buffer (PB). The liver was dissected, dehydrated in alcohol and embedded in paraffin according to routine procedures. Serial sections were cut at a thickness of 3–5 μm. Staining was performed using hematoxylin and eosin (HE).

2.6. Immunohistochemistry (IHC)

Antibody for SMP30/RGN was obtained from Shima Laboratories (Tokyo, Japan). SMP30/RGN was immunohistochemically assessed using the avidin–biotin–peroxidase complex method, as described previously [16].

2.7. Western blot analysis

Samples were prepared by the same methods as previously reported [17]. The blots were incubated for 1 h at room temperature with primary antibodies against SMP30/RGN (Shima), and β-actin (Sigma) in blocking buffer. After being washed, the blots were incubated for 1 h at room temperature with secondary antibodies. Reactive bands were identified using an enhanced chemiluminescence kit (Amersham Biosciences) and autoradiography according to the manufacturer's instructions.

2.8. Partial hepatectomy

Liver regeneration was induced by partial hepatectomy. The ventral lobes of zebrafish livers were removed by the methods reported by Sadler et al. [18].

2.9. Thioacetamide (TAA) treatment

Six-month-old female zebrafish were injected with 300 mg/kg body TAA intraperitoneally. Two days after injection, the livers were collected and used for further examinations.

2.10. Statistical analysis

All data are expressed as means ± S.D. One way ANOVA followed by the Dunnett post hoc multiple comparison test was performed to assess the statistical significance of differences in SMP30/RGN expression with aging. The Kruskal–Wallis test followed by the Steel method was performed to assess the statistical significance of SMP30/RGN expression changes caused by partial hepatectomy. Student's *t*-test was performed to assess the results of other examinations. *P* values less than 0.05 were considered to be significant.

3. Results

3.1. Expression of SMP30/RGN in normal zebrafish tissues

SMP30/RGN is reportedly expressed in the livers and kidneys of mammals. We examined SMP30/RGN expression in various zebrafish tissues by Western blotting methods. The 35 KDa form was observed in livers, intestine and kidneys (Fig. 1A). We then examined immunohistochemical expression patterns and found that SMP30/RGN was expressed in hepatic parenchymal cells and the renal cortex of adult zebrafish (Fig. 1B).

3.2. SMP30/RGN expression changes with aging

Mammalian SMP30/RGN expression is known to decrease with aging. We examined SMP30/RGN expression changes in the liver with aging. We used 3-month-old, 6-month-old, 1-year-old and 3-year-old zebrafish. Body lengths were 19.6 ± 1.9, 22.9 ± 1.9, 28.7 ± 1.5, and 30.3 ± 3.2 mm (Fig. 2A), weights.7 ± 8.3, 295.8 ± 84.7, 493.0 ± 108.3, and 721.3 ± 259.3 mg (Fig. 2B), body mass index (BMI) 0.034 ± 0.002, 0.055 ± 0.009, 0.063 ± 0.008, and 0.076 ± 0.010 g/cm^2 (Fig. 2C), respectively. Three-year old zebrafish had spinal curvature which is a feature of advanced age (Fig. 2D). Fold changes in SMP30/RGN mRNA expression by quantitative RT-PCR were 1.00 ± 0.41,.0 0.43 ± 0.25, 0.41 ± 0.27, and 0.17 ± 0.05, respectively (Fig. 2E). SMP30/RGN expression thus decreased significantly with aging.

3.3. SMP30/RGN expression changes during regeneration after partial hepatectomy

We isolated total RNA from partially hepatectomized livers and regenerating livers after partial hepatectomy, and examined SMP30/RGN mRNA expressions by quantitative PCR with EF1a expression as an internal control. Expression of SMP30/RGN mRNA was significantly decreased to 50.0% ± 13.6% and 50.9% ± 10.4% 1

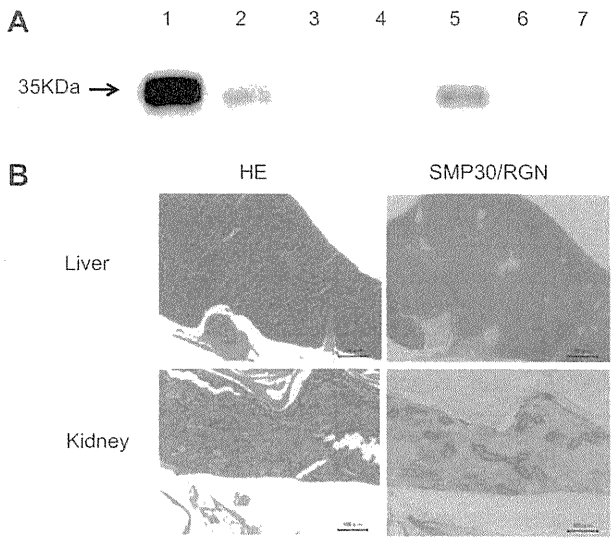

Fig. 1. SMP30/RGN expressions in various tissues in zebrafish. (A) SMP30/RGN expressions in various tissues. 1, liver; 2, intestine; 3, muscle; 4, brain; 5, kidney; 6, heart; 7, testicle. (B) IHC staining of zebrafish liver and kidney with polyclonal antibody for SMP30/RGN. Left: HE staining, right: anti-SMP30/RGN antibody, bar: 100 μm.

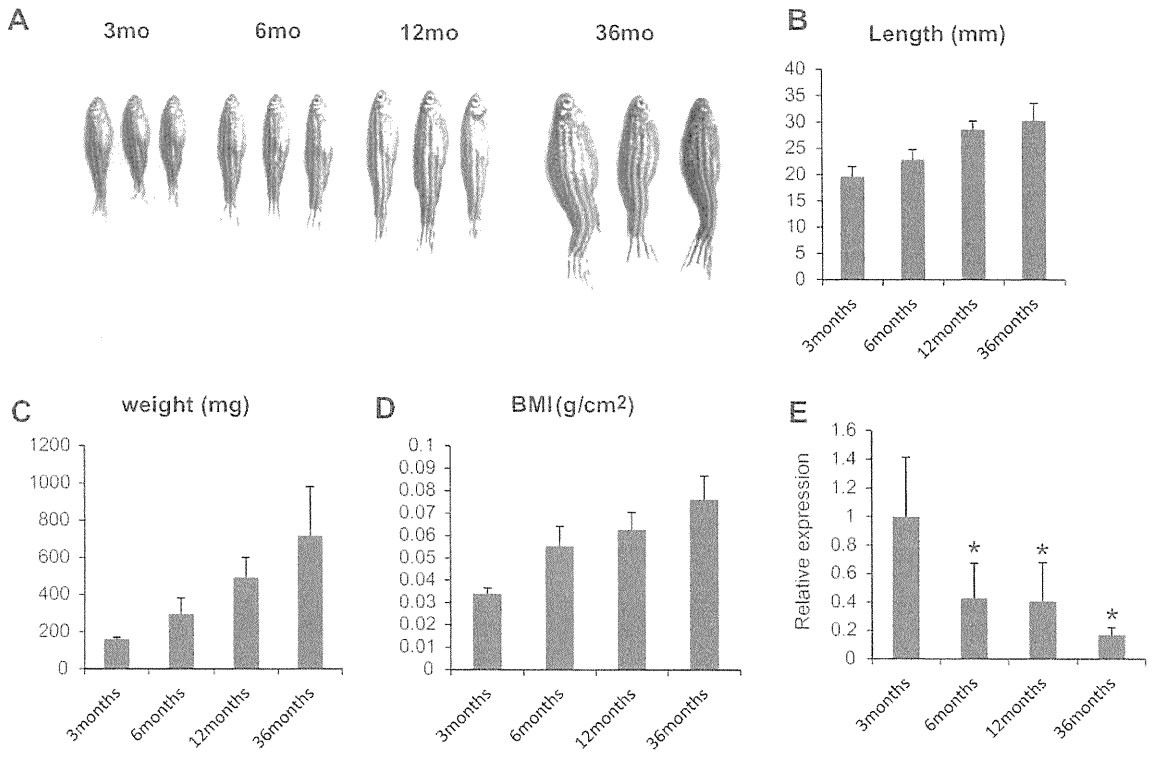

Fig. 2. Change of SMP30/RGN expression with aging. (A) Whole images of zebrafish at 3, 6, 12 and 36 months of age. (B) Body length (cm). (C) body weight (mg). (D) BMI (g/cm^2). Data are expressed as means ± S.D. (E) Expressions of SMP30/RGN mRNA changed with aging, EF1a is used as an internal control. Statistical analysis was performed by one-way ANOVA followed by Dunnett post hoc multiple comparison test, *indicates $P < 0.01$ between 3 months and the groups at 6, 12 and 36 months.

and 2 days after partial hepatectomy, respectively (Fig. 3A). No expression changes were observed 3 and 7 days after partial hepatectomy.

3.4. SMP30/RGN expression changes in acute liver injury induced by TAA administration

We examined expression of SMP30/RGN mRNA in the liver 48 h after intraperitoneal administration of TAA, a hepatotoxic agent, by quantitative RT-PCR. SMP30/RGN mRNA was significantly decreased in TAA-treated livers. Levels of SMP30/RGN expression in control and TAA livers were 1.00 ± 0.08 and 0.58 ± 0.09, respectively (Fig. 3B). The number of PCNA positive cells was increased in TAA-treated liver by IHC (Fig. 3C and D).

3.5. SMP30/RGN expression in zebrafish liver cell line

In order to examine expression of SMP30/RGN in proliferating cells, we used a zebrafish liver cell line (ZFL) (Fig. 4A). ZFL cells were derived from normal adult zebrafish liver, and synthesize and release several proteins into the culture medium, including a 70 kDa protein recognized by anti-bovine serum albumin IgG [15]. SMP30/RGN protein expression was significantly decreased, while PCNA expression was increased in ZFL cells as compared to normal liver extracts (Fig. 4B).

3.6. Induction of liver tumors by DEN administration

We maintained adult zebrafish with 200 ppm DEN solution for 2 months with weekly solution changes, and then kept them in tap water without DEN for 4 months to allow liver tumor development. We obtained some zebrafish with bulging abdomens (Fig. 4C). We then dissected these zebrafish and confirmed liver enlargement (Fig. 4D and D′).

3.7. SMP30/RGN expression in zebrafish liver tumors

We examined SMP30/RGN expressions in various liver tumors by IHC. Decreased SMP30/RGN expression and increased PCNA expression were observed in foci as compared to surrounding normal liver tissues (Fig. 4F and G). Decreased expression was observed in hepatocellular carcinomas (HCC) (Fig. 4I), which are characterized by complete loss of normal tubular architecture and compression of the peripheral area on HE staining (Fig. 4H). Increased PCNA-positive staining in the nuclear was also seen in HCC (Fig. 4J). We noted lower expressions in cholangiocellular carcinomas, which have highly developed ductal structures (Fig. 4K–M). We also recognized decreased SMP30/RGN expression in mixed cell tumors, which have irregular cords of small hepatocytes and small ductules of biliary epithelial cells (Fig. 4N–P). We checked all 18 DEN-treated zebrafish, and found three to have foci, six adenoma/HCC, three cholangiocellular carcinomas and four for mixed type tumors. We examined SMP30/RGN expression in each tumor by IHC, and found decreases in three of the four foci (75%), 15 of the 17 adenoma/HCC (88.2%), all three cholangiocellular carcinomas (100%) and all six mixed tumors (100%) (Table 1).

4. Discussion

SMP30 was originally reported to be a protein which is greatly reducing with aging [1]. Although the existence of SMP30/RGN was observed in various organisms, there are few reports concerning non-mammalian SMP30/RGN. Detailed SMP30 examination was reported for abalone, a marine invertebrate [19], but detailed

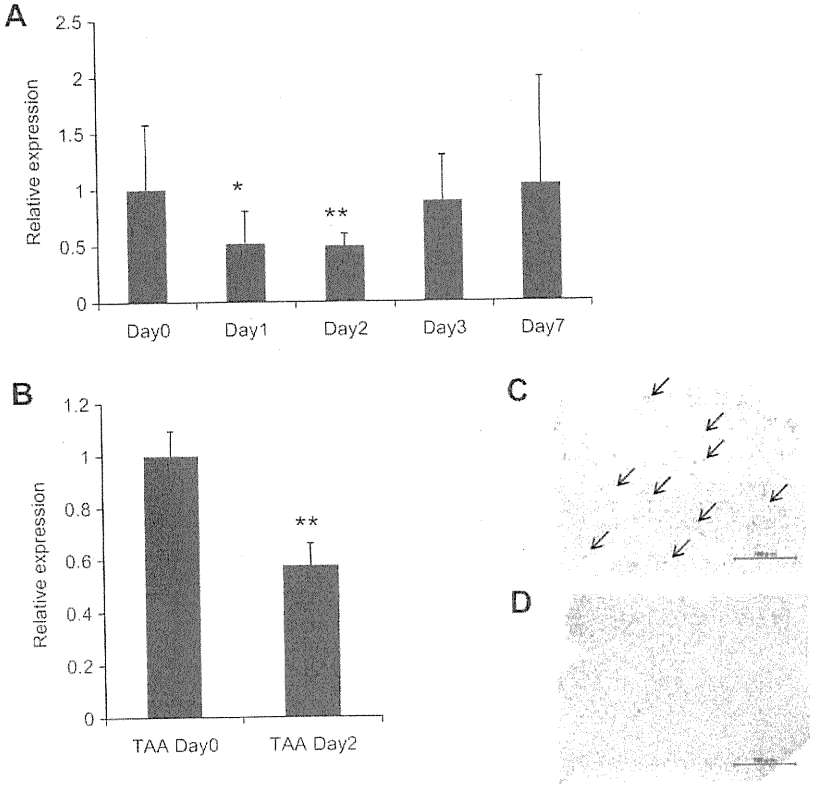

Fig. 3. SMP30/RGN mRNA expression in liver regeneration after partial hepatectomy and liver injury after TAA injection. (A) Expressions of SMP30/RGN mRNA after partial hepatectomy * and ** indicate $P < 0.05$ and 0.01 between control (Day 0) and partial hepatectomy group (Days 1–3 and 7). (B) SMP30/RGN mRNA expression changed in TAA-treated livers. (C) PCNA expression in TAA-treated liver, bar: 100 μm. Arrow indicated PCNA-positive cells. (D) PCNA expression incontrol liver. Bar: 100 μm.

examinations of zebrafish SMP30/RGN are lacking. We examined SMP30/RGN in zebrafish which is a good model for tumorigenesis and high-through-put screening is possible.

Zebrafish are becoming a new aging model [20]. Being different from mammals, teleosts keep growing in adulthood. In this study, 3-year-old zebrafish had 1.6 times the body length of a 6-month zebrafish. We examined whether SMP30/RGN mRNA expression decreases in the growing liver. We found SMP30/RGN mRNA expression to be significantly decreased with aging (Fig. 2E). SMP30/RGN has antioxidant properties that protect cells from oxidative stress, and overexpression of SMP30/RGN in a liver cell line contributed to a marked decrease in reactive oxygen species (ROS) formation as well as decreased lipid peroxidation, superoxide dismutase activity and glutathione levels [21]. Down-regulation of SMP30/RGN during the aging process may increase ROS formation. Moreover, calcium metabolism is important for understanding the aging process because it is related to electrolyte balance, calculus formation and osteoporosis. From the viewpoint of calcium metabolism, calbindin-D, a calcium binding protein, shows similar age-associated decreases in the brains of rats and humans. There is a possibility that the age-associated decrease in SMP30/RGN mRNA expression is related to calcium homeostasis, which deteriorates with aging.

SMP30/RGN mRNA expression is reportedly decreased 12 h after 70% partial hepatectomy, and then increased 2–3 days thereafter [22]. Zebrafish SMP30/RGN mRNA expression is decreased 1–2 days after partial hepatectomy. Overexpression of SMP30/RGN in hepatoma cells suppresses the expression of c-src but induces p53 and Rb expressions [23]. It is reasonable that SMP30/RGN expression is suppressed within 2 days after partial hepatectomy, when hepatocytic proliferation activity is high. However, in contrast to the mouse SMP30/RGN expression data, we found no increase in expression after partial hepatectomy. Partial hepatectomy is achieved in zebrafish by removing about 30% of the total liver, and the PCNA index peaks 48 h thereafter [18]. On the other hand, mouse partial hepatectomy requires that more than 70% of the liver be removed. There is a possibility that the zebrafish SMP30/RGN expression pattern differs from that in mice due to the smaller amount of liver removed. On the other hand, there was reportedly no increase in expression after partial hepatectomy in the mouse. A microarray analysis revealed that SMP30/RGN expression was decreased 12 h after partial hepatectomy, but there were no expression changes 1–2 days after partial hepatectomy [24]. The difference in expressions and the role of SMP30/RGN after partial hepatectomy require further investigation.

TAA is a centrilobular hepatotoxic agent which is metabolized by CYP2E1. Mice receiving a single dose of TAA are used as an acute liver injury model [25]. In this study, SMP30/RGN mRNA expression was decreased 48 h after TAA administration. TAA administration caused centrilobular necrosis and there was a subsequent regenerative response. As after partial hepatectomy, SMP30/RGN expression diminished when the liver was in a proliferative state.

Changes in SMP30/RGN expression in some tumor types such as breast cancer, prostate cancer and HCC were reported [26]. At the transcriptional level, SMP30/RGN mRNA was profiled as being a down-regulated gene in rat GST-P positive DEN-induced lesions as compared to the surrounding area [9]. A clinical proteomic study showed SMP30/RGN expression to be decreased in tumors [27].

In this study, we found expression of SMP30/RGN protein to be decreased in a zebrafish liver cell line (Fig. 4B) and in DEN-induced

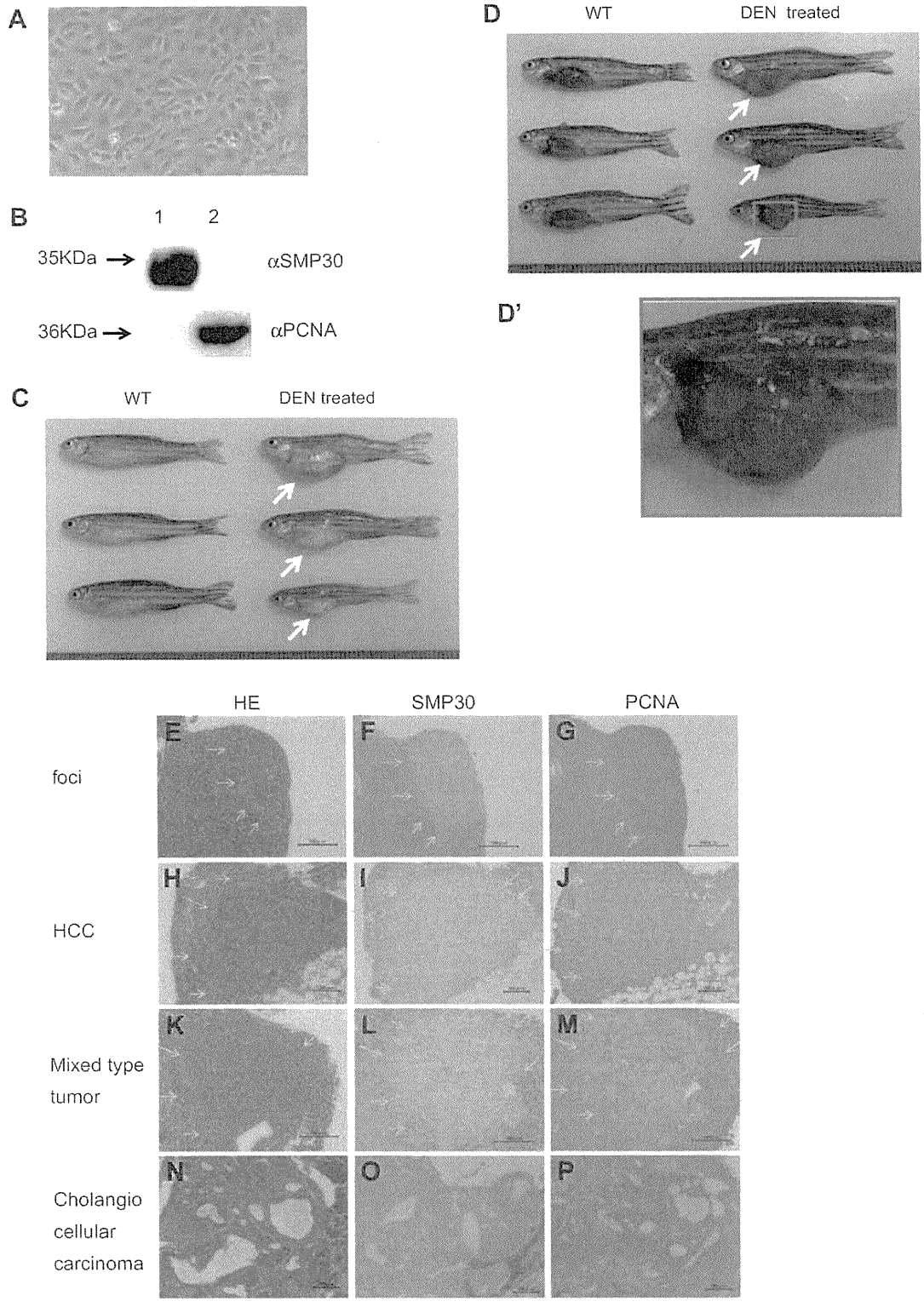

Fig. 4. SMP30/RGN expression in zebrafish liver tumor. (A) Photomicrograph of ZFL. (B) Expressions of SMP30/RGN and PCNA in normal liver and zebrafish liver cell line. (C) Whole image of DEN-treated zebrafish: arrows indicate bulging abdomen. (D) Image from DEN-treated zebrafish: arrows indicate liver tumor. (D') High-power field of swollen liver. (E–P) IHC analyses of various liver tumors. Left: HE staining, middle: anti-SMP30/RGN antibody, right: anti-PCNA antibody, bar: 100 μm. (E–G) foci, (H–J) HCC, (K–M) mixed type tumor, (N–P) cholangiocellular carcinoma. Arrows indicate boundary of tumor and surrounding area.

foci and adenoma/HCC, as compared to surrounding tissues (Fig. 4E–J). Furthermore, SMP30/RGN expression was decreased in mixed tumors and cholangiocellular carcinomas. This is, to our knowledge, the first report of SMP30/RGN expressions in these

Table 1
Expression of SMP30/RGN in zebrafish tumors induced by DEN treatment.

	Number of fish with tumors/ total number of fish examined	Total number of tumors	Number of tumors with decreased SMP30 expression	Ratio of SMP30 decreased tumors (%)
Foci	3/18	4	3	75
Adenoma/HCC	6/18	17	15	88
Mixed tumor	4/18	6	6	100
Cholangiocellular carcinoma	3/18	3	3	100

two tumors. Mixed tumors are a rare type of primary hepatic tumor thought to originate from a single site, but with two possible endpoints of differentiation. Cholangiocellular carcinoma is an adenocarcinoma and the actual cell origin is unknown, although it has been suggested to arise from pluripotent hepatic stem cells. Overexpression of SMP30/RGN in HepG2 suppresses cell proliferation and expressions of oncogenes, while increasing the expressions of tumor suppressor genes [28]. In addition, the frequency of antibody against SMP30/RGN is significantly higher in well-differentiated than in poorly-differentiated HCC [26]. We thus speculate that reduced SMP30/RGN expression enhances cell proliferation and is ultimately related to tumor progression. SMP30/RGN expression reduction was observed at a high frequency in tumors (Table 1). SMP30/RGN would thus be a good marker for liver various tumors.

In conclusion, we demonstrated the importance of SMP30/RGN in aging, partial hepatectomy, acute liver injury and liver tumorigenesis. SMP30/RGN has multiple functions in aging, proliferation and tumorigenesis, but physiological roles of SMP30/RGN have yet to be fully elucidated. Detailed investigations of the functions of SMP30/RGN are needed.

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Efficient detection of hepatocellular carcinoma by a hybrid blood test of epigenetic and classical protein markers

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ABSTRACT

Background: There are few blood tests for an efficient detection of hepatocellular carcinoma (HCC) associated with hepatitis C virus (HCV) infection.
Methods: The abilities of quantitative analyses of 7 genes hypermethylation in serum DNA, α-fetoprotein (AFP) and prothrombin-induced vitamin K absence II (PIVKA-II), and various combinations to detect HCC were evaluated in a training cohort of 164 HCV-infected patients (108 HCCs; 56 non-HCCs). An optimal hybrid detector, built using data for 2 methylated genes (*SPINT2* and *SRD5A2*), AFP, and PIVKA-II, achieved the most satisfactory ability to detect HCC in the training cohort. We evaluated the ability of the optimal hybrid detector to detect HCC in an independent validation cohort of 258 consecutive HCV-infected patients (112 HCCs; 146 non-HCCs) who were newly enrolled in 4 distinct institutes.
Results: In the validation cohort of 258 patients, accuracy, sensitivity, and specificity of the hybrid detector for detection of HCC were 81.4%, 73.2%, and 87.7%, respectively. Notably, even when detecting HCC ≤ 2 cm in diameter, the hybrid detector maintained markedly high abilities (84.6% accuracy, 72.2% sensitivity, 87.7% specificity). Youden's index (sensitivity + specificity − 1) for HCC ≤ 2 cm was 0.60, vastly much superior to the 0.39 for AFP at a cut-off value of 20 ng/ml and the 0.28 for PIVKA-II at a cut-off value of 40 mAU/ml.
Conclusions: These results show that the optimal hybrid blood detector can detect HCV-related HCC more accurately.

1. Introduction

For the last decade, evidence has been accumulating in various countries that hepatocellular carcinoma (HCC) is increasing [1–4]. This phenomenon can be explained partly by endemic infection with hepatitis C virus (HCV), one of the major etiological agents for development of HCC [5,6]. Despite the recent advent of treatment, HCC detected after the onset of symptoms shows a dismal prognosis (5-year survival, <10%) [5], indicating an urgent need for efficient detection systems to identify small, asymptomatic HCV-related HCC.

Current methods for diagnosis and screening of HCC include physical examination, various imaging techniques including ultrasonography (US), and measurements of serum α-fetoprotein (AFP) in certain risky populations, such as HCV-infected patients with liver cirrhosis (LC) [4,7]. AFP measurement for the detection of small HCCs (diameter ≤ 2 cm) has been questioned due to the low sensitivity and unstable cut-off values among studies or institutes [8]. The detection ability of US depends on examiner expertise, degree of patient obesity, presence of LC, and size of the liver tumor [9].

Epigenetic inactivation of transcription by aberrant methylation of CpG islands is a fundamental contributor to carcinogenesis [10].

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Several genes reportedly undergo hypermethylation in the process of hepatocarcinogenesis [11–13]. Some studies have revealed the presence of circulating methylated genes in the bloodstream of HCC patients, but none has been applied to daily clinical use as a diagnostic tool [14,15].

In a genome-wide search using DNA array data, our recent study used a quantitative methylation-specific PCR (qMSP) technique to identify 2 unique genes (*BASP1* and *SRD5A2*) for which promoter methylation is specific for small HCC associated with HCV infection [16]. Moreover, we found that 5 known genes (*APC*, *RASSF1A*, *SPINT2*, *CCND2* and *CFTR*) were exclusively methylated in early HCC tissues [17].

Taken together, these prompted us to develop a serological parameter for the efficient detection of HCC associated with HCV. The present study therefore quantified levels of the 7 methylated marker genes [16,17], and classical tumor markers AFP and prothrombin-induced vitamin K absence II (PIVKA-II) in the blood of HCV-infected patients.

2. Materials and methods

2.1. Patients of the training cohort

In the present study, we utilized a training-validation approach [18,19] in which a hybrid detector was built *in silico* on the basis of information from only a training cohort, then the ability of this detector to identify HCC was evaluated in an independent validation cohort at multiple institutions (Fig. 1). Written informed consent was obtained from all patients. The study protocol was undertaken according to the REMARK criteria (http://www.cancerdiagnosis.nci.nih.gov/assessment/progress/remark.htm), and was approved by the Institutional Review Board for the Use of Human Subjects at Yamaguchi University School of Medicine and Review Boards for the Use of Human Subjects at another 3 institutes defined below.

Our training cohort (Table 1) included 164 patients positive for HCV antibody, all of whom were treated at Yamaguchi University Hospital between May 1998 and April 2006, and were subjected to analyses of AFP and PIVKA-II, routine radiography, US, computed tomography (CT), magnetic resonance imaging (MRI), and, if necessary, hepatic angiography, dynamic CT, or dynamic MRI before and after treatment. On the basis of those imaging techniques, 108 of the 164 patients were diagnosed with HCC. Subsequently, 95 of these 108 patients (88.0%) bearing HCC underwent hepatic surgery or biopsy; and all tumors from the 95 patients were pathologically confirmed as HCC. Moreover, none of the 108 HCC patients showed any other malignancies at enrollment. We confirmed that none of the remaining 56 patients developed HCC during the follow-up period of >2 years. On the basis of these findings, we classified the 108 patients with HCC and the remaining 56 patients into HCC and non-HCC groups, respectively (Table 1). Using the results of imaging techniques and pathological examinations, we judged that 79 of the 164 patients (48.2%) had liver cirrhosis (LC). As summarized in Table 1, we used the tumor-node-metastasis (TNM) staging system as revised by the Liver Cancer Study Group of Japan (LCSGJ) [20]. The present study defined HCC ≤ 2 cm in diameter as "small HCC".

2.2. Patients of the validation cohort

Our validation cohort comprised 262 consecutive HCV-infected patients (Table 1) who were enrolled in 4 distinct institutes between

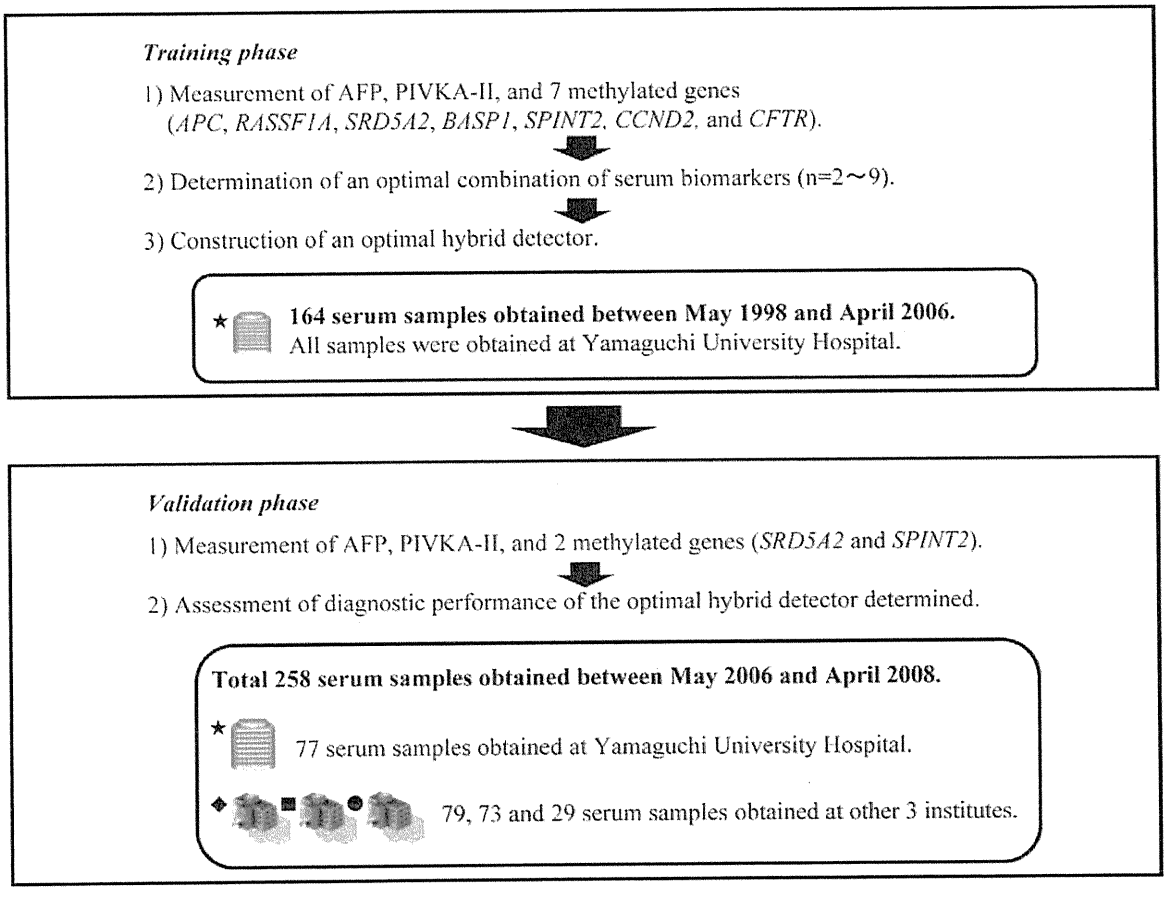

Fig. 1. Overview of the Training-Validation approach used for construction and evaluation of the hybrid detector for hepatocellular carcinoma.

Table 1
Patient characteristics in training and validation cohorts.

	HCC patients			Non-HCC patients		
	Training cohort (n = 108) (%)	Validation cohort (n = 112) (%)		Training cohort (n = 56) (%)	Validation cohort (n = 146) (%)	
Sex			$P = 0.004^a$			$P = 0.062^a$
Male	83 (76.8)	66 (58.9)		30 (53.6)	57 (39.0)	
Female	25 (23.2)	46 (41.1)		26 (46.4)	89 (61.0)	
Age (years) (mean ± SD)	66.6 ± 7.9	70.4 ± 8.0	$P < 0.0001^b$	64.6 ± 7.8	64.6 ± 10.3	$P = 0.985^b$
Serum ALT (U/L) (mean ± SD)	62.2 ± 65.4	55.9 ± 36.9	$P = 0.376^b$	49.1 ± 34.0	51.0 ± 39.3	$P = 0.749^b$
Platelet ($10,000/mm^3$) (mean ± SD)	12.3 ± 5.8	10.3 ± 5.4	$P = 0.008^b$	14.5 ± 7.7	11.9 ± 6.1	$P = 0.012^b$
Non-cancerous liver			$P = 0.028^a$			$P < 0.0001^a$
Chronic hepatitis	43 (39.8)	29 (25.9)		42 (75.0)	68 (46.6)	
Cirrhosis	65 (60.2)	83 (74.1)		14 (25.0)	78 (53.4)	
α feto-protein			$P = 0.618^a$			$P = 0.041^a$
<20 ng/ml	46 (42.6)	44 (39.3)		48 (85.7)	105 (71.9)	
≥20 ng/ml	62 (57.4)	68 (60.7)		8 (14.3)	41 (28.1)	
PIVKA-II			$P = 0.207^a$			$P = 0.088^a$
<40 mAU/ml	42 (38.9)	59 (52.7)		49 (87.5)	138 (94.5)	
≥40 mAU/ml	66 (61.1)	53 (47.3)		7 (12.5)	8 (5.5)	
Tumor size			$P = 0.006^a$			
≤2.0 cm	22 (20.4)	36 (32.1)				
2.1–5.0 cm	62 (57.5)	67 (59.8)				
>5.0 cm	24 (22.1)	9 (8.1)				
Primary lesion			$P = 0.992^a$			
Single	52 (48.1)	54 (48.2)				
Multiple	56 (51.9)	58 (51.8)				
Histological grading			$P = 0.900^c$			
G1	21 (22.1)	12 (23.5)				
G2	63 (66.3)	32 (62.7)				
G3–G4	11 (11.6)	7 (13.8)				
Stage			$P = 0.077^a$			
I	12 (11.1)	21 (18.7)				
II	42 (38.9)	32 (28.6)				
III	36 (33.3)	30 (26.8)				
IVA + IVB	18 (16.7)	29 (25.9)				

PIVKA-II, Prothrombin Induced Vitamin K Absence II.
[a] Chi-square test.
[b] Student's *t* test.
[c] Fisher exact test.

May 2006 and April 2008. Out of the 262 patients, 1 was excluded due to daily intake of warfarin, which may affect serum levels of PIVKA-II, and 3 were excluded because of small amounts of extracted cell-free DNA (cfDNA). Among the remaining 258 patients, 77 were treated at Yamaguchi University hospital, 73 at Shimonoseki Kohsei Hospital, 79 at Sapporo-Kosei General Hospital, and 29 at Kurume University Hospital. The detection program for HCC in individual institutes was performed according to the nationwide follow-up survey conducted by the LCSGJ [20] and/or the guidelines of the American Association for the Study of Liver Diseases (AASLD) [4]. On the basis of findings from multiple imaging modalities (US, CT, MRI, hepatic angiography, dynamic CT, and dynamic MRI), hepatologists from the individual institutes diagnosed 112 of the 258 patients (43.4%) as HCC. Among the 112 HCC patients, 52 were diagnosed at Yamaguchi University Hospital, 23 at Shimonoseki Kohsei Hospital, 24 at Sapporo-Kosei General Hospital, and 13 at Kurume University Hospital. Hepatic surgery or biopsy was subsequently performed for 51 of the 112 HCC patients (45.5%). All tumors, including 15 tumors ≤2 cm in diameter, from the 51 patients were pathologically confirmed as HCC, indicating the justification of our detection programs for HCC. Our follow-up program did not detect HCCs in any of the 146 patients initially defined as without HCC for 6 months after enrollment. Collectively, we categorized the 112 patients with HCC and the remaining 146 patients as HCC and non-HCC groups, respectively, in the validation cohort (Table 1).

2.3. Extraction and quantification of DNA in sera

Blood samples were collected from patients before treatment to measure methylated marker genes, AFP, PIVKA-II, alanine aminotransferase (ALT) and platelet count. We set a cut-off value of 20 ng/ml for AFP and a cut-off value of 40 mAU/ml for PIVKA-II for the discrimination of HCC, as these values have been shown to offer the highest diagnostic ability for HCV-related HCC and have been used most frequently in clinical practice [8,21]. As a source for methylation analysis, cfDNA was extracted from 1 ml of sera using a DNA Extractor SP Kit for Serum and Plasma (Wako Pure Chemical Industries, Osaka, Japan) according to the instructions from the manufacturer, and was quantified as described previously [22].

2.4. Measurement of methylated gene fragments circulating in sera

We performed qMSP assays for 2 novel methylated genes (*SRD5A2* and *BASP1*) and 5 other genes (*APC*, *RASSF1A*, *SPINT2*, *CCND2*, and *CFTR*), as described previously [16,17] (For gene selection, see supplementary material). In the training phase (Fig. 1), methylated forms of the 7 genes in patient sera were measured and calculated as methylated DNA amount in serum (picograms per 1 ml of serum). In the validation phase (Fig. 1), methylated forms of only *SRD5A2* and *SPINT2* in sera of patients were measured and calculated.

2.5. Development and evaluation of the hybrid detector

We used the Fisher linear classifier (FLC) [19] to construct a hybrid detector *in silico* where "HCC" and "non-HCC" are defined as groups A and B, respectively.

In FLC, the score is defined by

$$T(x) = f_A(x) - f_B(x)$$

where

$$f_A(x) = \frac{1}{2}(x-\hat{\mu}_A)^T\left[P(A)\hat{\Sigma}_A + P(B)\hat{\Sigma}_B\right]^{-1}(x-\hat{\mu}_A) + C(A).$$

$\hat{\mu}_A$ and $\hat{\Sigma}_A$ in $f_A(x)$ are the sample mean vector and sample covariance matrix for Group A, respectively, and $P(A)$ is a prior probability for Group A. $C(A)$–$C(B)$ in $T(x)$ is called Cut off. The value of Cut off can be optimized by minimizing the error rate estimated on the training samples. Then, FLC assigns a given x to be classified to Group A (i.e., HCC) if $T(x)<0$. FLC assigns a given x to be classified to Group B (i.e., non-HCC) if $T(x)>0$.

We input data for n markers ($n=2$-9) from the 164 training samples into FLC and evaluated the ability of constructed individual FLCs to detect HCC in the 164 training samples. Mean detection ability (i.e., sensitivity and accuracy) of top-10 combinations was maximal when the FLC was built using 4 markers (Fig. 2A). We next plotted specificity, sensitivity and diagnostic accuracy of each top-ranked combination of n markers ($n=2$–9). Likewise, a 4-marker combination (*SRD5A2*, *SPINT2*, AFP and PIVKA-II) achieved the highest sensitivity and accuracy among combinations of n markers (Fig. 2B).

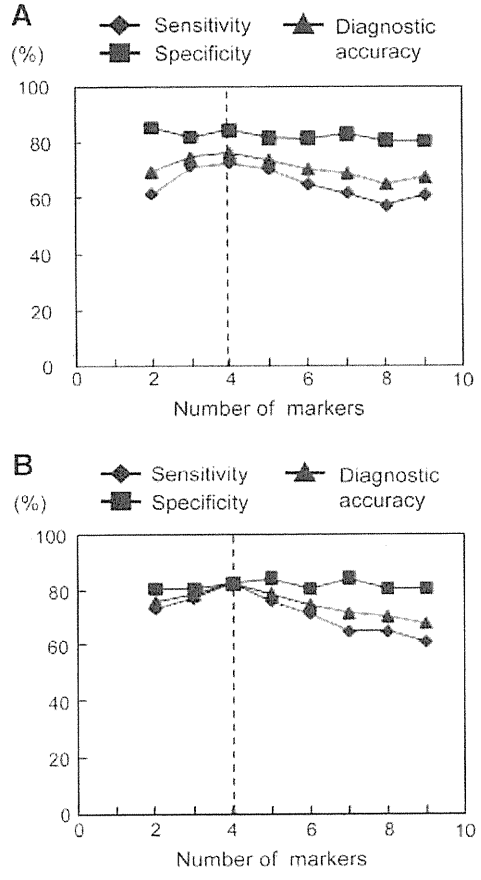

Fig. 2. Performances of markers in the training cohort. We input data for n markers ($n=2$–9) of the 164 training samples into Fisher linear classifier (FLC). This procedure was repeated for all combinations (from 9 C2 to 9 C9) of n markers and performances of the constructed individual FLCs were computed. Mean specificity, sensitivity and accuracy of the top 10 combinations of 2–7 markers and 9 combinations of 8 markers, and specificity, sensitivity and diagnostic accuracy for all 9 markers were plotted (A). Sensitivity and diagnostic accuracy were greater as the number of markers increased to 4; however, sensitivity and accuracy obtained using more than 4 markers were rather inferior to those obtained with 4 markers. We next plotted specificity, sensitivity and accuracy of each top-ranked combination of n markers ($n=2$–9) (B). A 4-marker combination of *SRD5A2*, *SPINT2*, AFP and PIVKA-II achieved the highest sensitivity and accuracy among combinations of n markers.

Collectively, using the optimal combination of 4 markers (*SRD5A2*, *SPINT2*, AFP and PIVKA-II), score was defined by

$$\begin{aligned} T(x) &= f_A(x) - f_B(x) \\ &= -136.28 \times (SRD5A2) - 1.78 \times (SPINT2) - 1.07 \times (\mathrm{AFP}) - 1.99 \\ &\quad \times (\mathrm{PIVKA-II}) + 131. \end{aligned}$$

where sample mean vectors and sample covariance matrices were estimated using the 164 training samples. Our hybrid detector classified samples as HCC or non-HCC for values of $T(x)<0$ and $T(x)>0$, respectively.

2.6. Perfectly blinded assessment of the validation cohort

To evaluate detection ability of the optimal hybrid detector established in the training cohort, we recruited another 258 patients with chronic HCV infection as the validation cohort. These patients were consecutively enrolled at each institute to maintain the independence of patient selection. In the present study, information regarding sample characteristics in the validation cohort was perfectly blinded for analysts of serum markers (TMo, TM, and NK) and bioinformaticians (YH and YF), who constructed a hybrid detector *in silico*.

2.7. Statistical analysis

The χ^2 test, Student's t test and Mann–Whitney U test were used to evaluate differences in tumor and patient characteristics between training and validation cohorts. Receiver operating characteristic (ROC) curve analysis was performed using SPSS for Windows version 11.0 J software (SPSS, Chicago, IL). Values of $P<0.05$ were considered significant.

3. Results

3.1. Patient characteristics

Significant differences in age and sex of HCC patients were seen between the training and validation cohorts ($P=0.004$ and $P<0.0001$, respectively; Table 1). HCC patients in the validation cohort showed significantly fewer platelets, higher frequency of coexisting LC, and smaller tumors compared to the training cohort ($P=0.008$, $P=0.028$ and $P=0.006$, respectively; Table 1). Non-HCC patients in the validation cohort showed significantly fewer platelets in peripheral blood, higher frequency of coexisting LC, and higher AFP levels than patients in the training cohort ($P=0.012$, $P<0.0001$, and $P=0.041$, respectively; Table 1).

3.2. Training phase

Among the 9 markers tested (Table 2), *SPINT2* and *SRD5A2* displayed high specificities (98.2% and 92.9%) but low sensitivities (35.2% and 8.3%) for HCC detection. *RASSF1A* for HCC detection had the highest sensitivity (83.3%), but showed a low specificity of 58.9%. No markers showed a Youden's index (sensitivity + specificity − 1) >0.6 for HCC detection in our training cohort, suggesting limitations to the single use of each marker. To improve this low detection ability, we attempted to build a hybrid detector system by combining data from several markers. We calculated all combinations of markers *in silico* and found that an optimal hybrid detector built using a 4-marker combination (*SRD5A2*, *SPINT2*, AFP and PIVKA-II) achieved the highest sensitivity, specificity and accuracy (82.4%, 82.1% and 82.3%, respectively) in the training cohort among all combinations of markers (Fig. 2B). This optimal hybrid detector showed a higher Youden's index (0.65) than any of the 9 markers tested (Table 2). We also

Table 2
Sensitivity, specificity, and accuracy of 9 biomarkers and the hybrid system for diagnosis of HCC or small HCC in the training cohort.

	Sensitivity (%)	Specificity (%)	Accuracy (%)	Youden's index
Methylation markers (cut-off value)				
BASP1 (0.2 pg per 1-ml serum)	62.0	78.6	71.2	0.41
CCND2 (0.2 pg per 1-ml serum)	64.8	42.9	60.3	0.08
APC (0.2 pg per 1-ml serum)	17.6	78.6	40.4	−0
SPINT2[a] (0.2 pg per 1-ml serum)	35.2	98.2	59.6	0.33
SRD5A2[a] (0.2 pg per 1-ml serum)	8.3	92.9	39.1	0.01
CFTR (0.2 pg per 1-ml serum)	56.5	83.9	69.2	0.40
RASSF1A (0.2 pg per 1-ml serum)	83.3	58.9	72.4	0.42
Classical protein markers				
AFP[a] (20 ng/ml)	57.4	85.7	67.1	0.43
PIVKA-II[a] (40 mAU/ml)	60.2	89.3	70.1	0.50
Four-marker combination (cut-off value)				
Optimal hybrid system (0)	82.4	82.1	82.3	0.65

[a] Four markers used in the optimal hybrid system.

examined the methylation levels of the 7 methylated genes in the three groups consisting of patients who underwent previously or undergo currently therapies of interferon (IFN) combined with ribavirin, and patients who had no therapies of IFN combined with ribavirin. No significant differences in the methylation levels were found between ribavirin and non-rivavirin therapies (data not shown).

3.3. Validation phase

The ability of the optimal hybrid detector to detect HCC was evaluated using 258 sera from 258 HCV-infected patients in the validation cohort. Notably, sensitivity of PIVKA-II for HCC detection decreased from 60.2% in the training cohort to 51.8% in the validation cohort (Fig. 3A). The specificity of AFP for HCC detection decreased from 85.7% in the training cohort to 71.9% in the validation cohort (Fig. 3B). By contrast, the optimal hybrid detector maintained high sensitivity (73.2%), specificity (87.7%), and accuracy (81.4%) for HCC detection in the validation cohort (Fig. 3A–C). The positive predictive value and negative predictive value for HCC detection were 82.2% and 80.8%, respectively. Even for the detection of small HCC in the validation cohort, the optimal hybrid detector showed high sensitivity (72.2%), specificity (87.7%), and accuracy (84.6%) (Fig. 3A–C). As a result, the optimal hybrid detector for detection of HCC and/or small HCC maintained a Youden's index ≥ 0.6 throughout both training and validation cohorts (Fig. 3D). The optimal hybrid detector also judged all of 4 healthy peoples as non-HCC (data not shown).

The present study arbitrarily determined cut-off values of AFP and PIVKA-II, and directly applied these values to the validation cohort. We therefore had to compare the ability of the optimal hybrid detector with the maximal abilities of AFP and PIVKA-II alone in the validation samples. For this purpose, ROC curve analysis for the detection of HCC was performed for the validation cohort. AFP and PIVKA-II alone had areas under the ROC curve of 0.739 (95% confidence interval (CI), 0.678-0.799) and 0.794 (95% CI, 0.736-0.853), respectively, for HCC detection (Fig. 4). The optimal hybrid detector had a more global area under the ROC curve of 0.868 (95% CI, 0.822-0.913) compared to AFP and PIVKA-II, indicating that ability of the optimal hybrid detector was superior to the maximal abilities of AFP and PIVKA-II alone for detecting HCC in the validation cohort.

As summarized in Table 3, *SPINT2* and *SRD5A2* showed the highest accuracy in detecting non-HCC patients with chronic hepatitis or cirrhosis. AFP was most robust in detecting small HCC and PIVKA-II was most robust in detecting HCC >2 cm in diameter. Apparently, the optimal hybrid detector possessed all of individual merits of the 2 methylated markers, AFP and PIVKA-II.

In the present study, the cost per each test of AFP, PIVKA-2, SRD5A2 and SPINT2 was $4, $17.6, $11.7 and $10.6, respectively. In the validation group, the specificity and diagnostic accuracy of AFP alone and the hybrid detector were 71.9% and 67.0%, and 87.7% and 81.4%, respectively (Fig. 3). Thus, AFP test plus $40 resulted in an increase of 15.8% and 14.4% of specificity and diagnostic accuracy, respectively. The areas under ROC curves of AFP alone and the hybrid detector were 0.739 and 0.868, respectively (Fig. 4). AFP test plus $40 resulted in an increase of 0.129 of the area.

In diagnosing HCC, the performance of the combined blood test of *SPINT2*, *SRD5A2*, AFP and PIVKA-2 was superior to that of the methylation test of 3 genes (*RASSF1*, *CCND2* and *SPINT2*) in HCC tissue developed in our previous study [17].

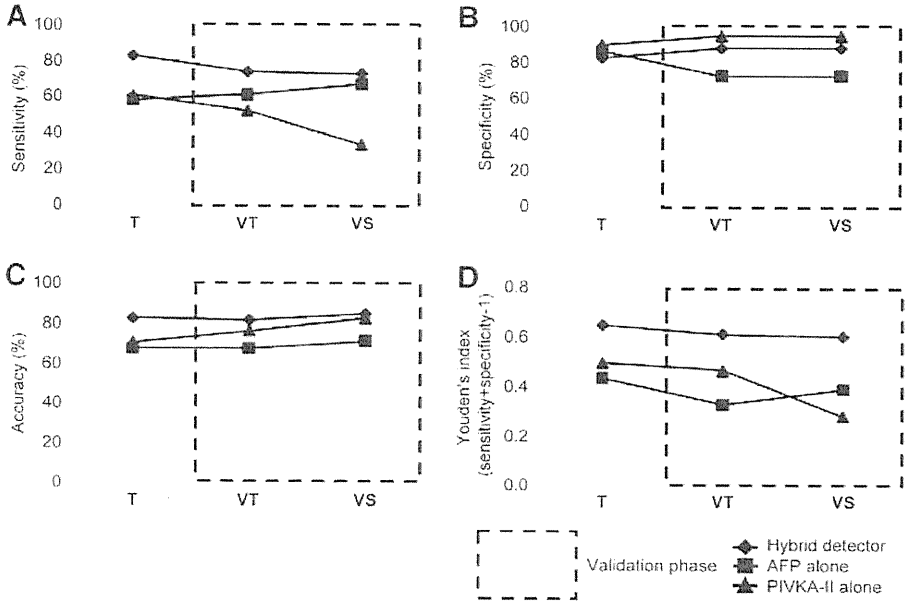

Fig. 3. Performances of the Optimal Hybrid detector (diamond), AFP (square), and PIVKA-II (triangle) in the validation cohort. The optimal hybrid detector showed the most robust performances for detection of HCC (A-D). T, training cohort of 108 HCC patients and 56 HCV carriers without HCC used for comparison with data from the validation cohort; VT, validation cohort of all 112 HCC patients and 146 HCV carriers without HCC; VS, validation cohort of 36 small HCC patients and 146 HCV carriers without HCC.

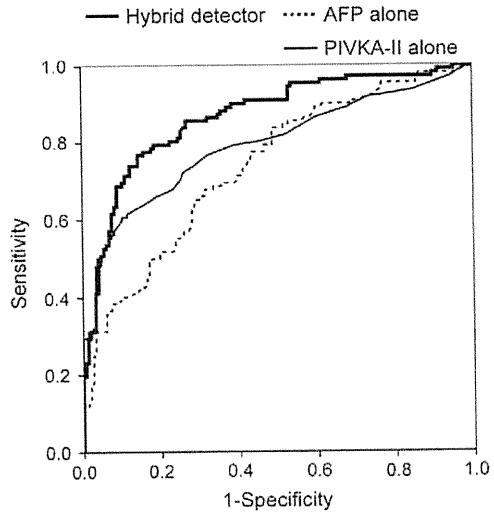

Fig. 4. Receiver operating characteristic curve analysis of the optimal hybrid detector, AFP, and PIVKA-II for the validation cohort.

4. Discussion

Many studies have evaluated AFP and PIVKA-II as detection tools for HCC, particularly small HCC. To the best of our knowledge, among studies using >100 samples, one study [23] showed a maximum sensitivity of 54.8%, but a specificity of only 49.1%, while another study [24] showed a maximum specificity of 71.0%, but a sensitivity of 25.0% in the ability of AFP to detect small HCC at a cut-off value of 20 ng/ml. A recent work by Marrero and colleagues showed that the optimal AFP cut-off value for diagnosis of HCC was 10.9 ng/ml leading to a sensitivity of 70% and a specificity of 82% [25]. However, the performance decreased to a sensitivity of 66% in diagnosing early HCC [25]. Another study showed that an AFP elevation (optimal cut-off value of 16 ng/ml) was indicative of HCC in non-infected patients, but not in HCV-infected patients [26]. For PIVKA-II, most studies with more than 100 samples showed sensitivities <40% for the detection of small HCC, with one study [27] reaching 53.5% sensitivity. Thus, reliance on the classical tumor markers AFP and PIVKA-II for the detection of HCC thus remains unsatisfactory, particularly given the low diagnostic powers and unstable cut-off values used between institutes [4,5,28]. To address these issues, we carefully conducted a multi-institutional study with multiple parameters, designed to develop a hybrid detector with more stable performance by searching for all combinations of marker candidates including methylated markers, as demonstrated previously by our laboratory [19]. The present study was also intended to minimize selection bias by using data collected consecutively only from HCV-infected patients [18,29]. We thus successfully developed a hybrid detector that accurately detected HCV-related HCC, particularly HCC ≤ 2 cm in diameter, in a perfectly blinded manner in a multi-institutional large cohort.

Since the disclosure of epigenetic regulation in key genes, many studies [30–32] have shown the clinical efficacy of measuring promoter hypermethylation in various specimens such as tumor tissue, feces, and urine for determining the diagnosis and prognosis of cancer patients. Most studies measuring methylated DNA in the bloodstream of HCC patients have reported positive results, but almost all have been far from the setting of daily clinical use because of the insufficient performance due to the single use of a methylated marker gene [13–15,33]. We have provided herein the first evidence that a hybrid of methylation and classical protein markers has high potential for detecting HCV-related HCC in a blinded setting, opening new avenues toward the daily clinical application of methylated genes as tumor markers.

SPINT2 encodes hepatocyte growth factor (HGF) activator inhibitor type 2 (HAI-2) (http/www.ncbi.nlm.nih.gov/gene/10653), which regulates HGF activity. Epigenetic inactivation of *SPINT2* reportedly causes loss of tumor suppressor activity in renal cancer cells [34] and this gene is frequently hypermethylated in human HCC [12]. Consistent with those findings, our recent study [17] showed that *SPINT2* was frequently methylated in small HCC tissues, but unmethylated in non-HCC liver tissues, promising a high specificity for methylation patterns of *SPINT2* circulating in the bloodstream. *SRD5A2* encodes an enzyme that converts testosterone to the more active androgen dihydrotestosterone. Several polymorphisms in *SRD5A2* gene have been implicated as risk factors for prostate cancer [35]; however, how these polymorphisms act in the pathogenesis of HCC remains unclear.

We found that *RASSF1A*, *BASP1*, and *CCND2* offered more robust diagnostic performances than *SPINT2* and *SRD5A2* in the training phase. However, our *in silico* procedure predominantly selected the latter 2 genes for the optimal hybrid detector (Table 2). This result was consistent with our previous work [19,36], in which the diagnostic power of a detector built using several markers was independent of the ranking for diagnostic power of individual markers when combination was considered. In the validation phase, *SPINT2* and *SRD5A2* were very robust in detecting non-HCC patients, expectedly complementing the low detection ability of AFP and PIVKA-II (Table 3). Methylated *SPINT2* was also detectable in sera from 2 HCC cases negative for both AFP and PIVKA-II. This complementary effect is attributable to the absence of correlations between serum concentrations of AFP and PIVKA-II and those of methylated *SPINT2* and *SRD5A2* (data not shown). In addition to these independent expression patterns, our successful results might be partly attributable to a harmony of genetic features of *SPINT2* and *SRD5A2* and proteomic features of AFP and PIVKA-II. These features might maximize the synergistic power of the 4 markers.

The diagnostic accuracy of any test is related to the frequency of the underlying disease in the population being studied [4]. In the present study, many differences were seen between patient characteristics in the training and validation cohorts. In particular, the validation cohort included a significantly larger number of small HCCs than the training cohort ($P = 0.006$; 36/112 vs. 22/108). This sample heterogeneity indeed resulted in decreased sensitivity of PIVKA-II alone and decreased specificity of AFP alone (Fig. 3A, B) for detecting small HCC in the validation cohort. The sensitivity and specificity of any test are inversely related. As a result, most studies have reported a Youden's index <0.5 for the diagnosis of small HCC. In contrast, our

Table 3
Diagnostic accuracy of markers and disease progression in the validation cohort.

Markers (cut-off value)	CH (%)	LC (%)	HCC ≤ 2 cm (%)	HCC (2.1–5 cm) (%)	HCC > 5 cm (%)	Total accuracy (%)
Optimal hybrid system (0)	65/68 (95.5)	63/78 (80.8)	26/36 (72.2)	48/67 (71.6)	8/9 (88.9)	210/258 (81.4)
SPINT2 (0.2 pg per 1-ml serum)	68/68 (100)	78/78 (100)	1/36 (2.78)	15/67 (22.4)	2/9 (22.2)	164/258 (63.6)
SRD5A2 (0.2 pg per 1-ml serum)	68/68 (100)	76/78 (97.4)	2/36 (5.56)	1/67 (1.50)	1/9 (11.1)	148/258 (57.4)
AFP (20 ng/ml)	60/68 (88.2)	45/78 (57.7)	24/36 (66.7)	37/67 (55.2)	7/9 (77.8)	173/258 (67.0)
PIVKA-II (40 mAU/ml)	67/68 (98.5)	71/78 (91.0)	12/36 (33.3)	40/67 (59.7)	7/9 (77.8)	197/258 (76.3)

CH, chronic hepatitis; LC, liver cirrhosis without HCC.
AFP, α-feto protein; PIVKA-II, prothrombin induced vitamin K Absence II.

hybrid detector showed markedly high performance (72.2% sensitivity, 87.7% specificity, 84.6% accuracy) and a Youden's index of approximately 0.6 for the detection of small HCC. The high accuracy of our hybrid detector in the present blinded, multi-institutional setting is thus fascinating from the perspective of screening for heterogeneous samples within or among various institutes.

We found that AFP test plus \$40 resulted in increases of 15.8% and 14.4% of specificity and diagnostic accuracy, respectively. However, the cost-effectiveness of the hybrid detector in surveillance setting remains unclear; further studies are needed to clarify whether the hybrid detector we built could serve as a non-invasive and easy-to-use tool in surveillance programs for HCV-related HCC in the near future.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at doi:10.1016/j.cca.2010.09.028.

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ORIGINAL ARTICLE—LIVER, PANCREAS, AND BILIARY TRACT

Evaluation of molecular targeted cancer drug by changes in tumor marker doubling times

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Abstract
Background We evaluated the usefulness of tumor marker doubling time (DT) as an efficacy indicator of a molecular targeted anticancer agent.
Methods Twenty-five patients with advanced hepatocellular carcinoma (HCC) received TSU-68, a multiple tyrosine kinase inhibitor. Exponential increase in HCC-specific tumor marker levels (alpha-fetoprotein or des-gamma-carboxyprothrombin) was seen in 15 of them prior to TSU-68 administration. The relationship between tumor marker DT and tumor volume DT was evaluated. Next, tumor marker DT in the first 8 weeks of TSU-68 administration was compared with tumor marker DT before treatment. Efficacy evaluation based on changes in tumor marker DT was compared with Response Evaluation Criteria In Solid Tumors (RECIST).
Results Tumor marker DT and tumor volume DT were almost identical ($r^2 = 0.94$, $P < 0.001$) in each patient before TSU-68 administration. Efficacy evaluation based on changes in tumor marker DT on TSU-68 administration was in accordance with RECIST in 12/15 cases. Discordance was observed in three cases, for which RECIST indicated disease progression in spite of elongated tumor marker DT. Those cases showed substantial tumor necrosis without volume shrinkage or appearance of new lesions in spite of apparent effects on target lesions.
Conclusions Serum tumor marker DT can be used to evaluate viable tumor burden irrespective of the presence of tumor necrosis which can compromise radiographic evaluation. This approach may be applicable to the evaluation of responses to chemotherapy, particularly to cytostatic agents (ClinicalTrials.gov number, NCT00784290).

Keywords Doubling time · RECIST · AFP · PIVKA-II · HCC · TSU-68

Abbreviations

AFP	Alpha-fetoprotein
CEA	Carcinoembryonic antigen
CR	Complete response
CT	Computed tomography
DCP	Des-gamma-carboxyprothrombin
DT	Doubling time
FGFR	Fibroblast growth factor receptor
HCC	Hepatocellular carcinoma
PD	Progressive disease
PDGFR	Platelet-derived growth factor receptor
PR	Partial response
PSA	Prostate-specific antigen
RECIST	Response Evaluation Criteria In Solid Tumors
SD	Stable disease
TACE	Transcatheter arterial chemoembolization
VEGFR-2	Vascular endothelial growth factor receptor-2

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Introduction

Phase II trials of chemotherapeutic agents for solid tumors usually adopt an objective tumor response as the primary endpoint, the rationale being that the tumor response will be a surrogate for the effects of a particular agent on survival outcomes [1–4]. In evaluating a tumor response to a cancer drug, the Response Evaluation Criteria In Solid Tumors (RECIST) guidelines are usually adopted. However, the total tumor volume thus determined is not necessarily proportional to the number of viable tumor cells, e.g., in cases of massive tumor necrosis without tumor shrinkage [5–9].

With the progress in molecular targeted cancer drugs, concerns about the appropriate design of clinical trials of such agents have emerged [10, 11]. In contrast to conventional cytotoxic agents, molecular targeted agents often show cytostatic effects, i.e., a slowing of tumor growth. The effects of such agents upon the tumor growth rate may be better evaluated not by the changes in tumor burden but by the rate of changes for which RECIST may not be particularly suitable.

Most solid malignant tumors show an exponentially increasing volume in the natural course of their growth. The tumor volume doubling time (DT) is the parameter that defines the speed of the increase. Serum levels of several tumor markers, including prostate-specific antigen (PSA), carcinoembryonic antigen (CEA), and alpha-fetoprotein (AFP), have been reported to correlate with tumor volume in an individual patient [12–15]. The rate of changes in tumor volume may be calculated on the basis of repeated measurements of the serum tumor marker levels. The DT of serum PSA levels has also been proposed as a biological parameter that can be used to predict the prognosis of prostate cancer, and PSA determination has now become an integral part of the disease management [16–18].

The aim of the present study was to elucidate the usefulness of tumor marker DT to evaluate the efficacy of TSU-68 against hepatocellular carcinoma (HCC). TSU-68 is an orally administered, small-molecule inhibitor of multiple receptor tyrosine kinases, vascular endothelial growth factor receptor-2 (VEGFR-2), platelet-derived growth factor receptor (PDGFR), and fibroblast growth factor receptor (FGFR) [19]. As a potent antiangiogenic agent, TSU-68 is expected to be effective against HCC [20], and a phase I/II study has been recently conducted in Japan [21]. In that clinical trial, the serum levels of AFP and des-gamma-carboxyprothrombin (DCP) were also scheduled to be periodically determined. Although the effect was assessed by radiologic examinations, the effect of TSU-68 may be more accurately evaluated by changes in tumor growth speed based on specific tumor marker levels [22–26].

Methods

Clinical trial

This study was conducted according to the ethical guidelines for epidemiologic research designed by the Ministry of Education, Culture, Sports, Science and Technology and Ministry of Health, Labour and Welfare of Japan. The study design was approved by the institutional review board of the University of Tokyo Hospital.

An open-label phase I/II trial of TSU-68 for the treatment of HCC was performed between September 2003 and February 2007 at three institutions in Japan [21]. Twenty-five of the participating patients were enrolled from the University of Tokyo Hospital. In the present study, clinical data for these 25 patients, including analyses conducted before and after the trial, were further evaluated. Briefly, histologically confirmed HCC patients without indication or response to resection, ablation, or transcatheter arterial chemoembolization (TACE) were deemed eligible. The eligibility criteria also included a World Health Organization (WHO) performance status of 2 or better, a life expectancy of not less than 90 days, and a liver function of Child–Pugh class A or B. Patients were not eligible if they had received ablation, TACE, chemotherapy, or irradiation within 4 weeks, or surgery within 6 weeks, of the commencement of the trial (washout phase).

The phase I study began with a 400 mg bid oral dose of TSU-68. Because of dose-limiting toxicities, however, this was reduced to 200 mg bid in the subsequent phase II study. At the end of each 4-week cycle, dynamic contrast-enhanced computed tomography (CT) consisting of early and late arterial, and portal venous phases was performed, and contiguous transverse sections with a thickness of 5 mm were obtained. Responses were assessed on the basis on the RECIST evaluations in predetermined target lesions. The serum levels of HCC-specific tumor markers, AFP and DCP, were scheduled to be determined every 2 weeks. TSU-68 administration was discontinued when progressive disease (PD) was observed by RECIST.

Patients

Among the patients who had participated in the aforementioned trial, those who met the following criteria were included in the present study: (1) tumor growth prior to TSU-68 administration could be evaluated with two CT examinations performed 1–3 months before the trial and upon enrollment; (2) serum tumor marker levels could be determined at least three times during the washout phase, and a linear regression of the logarithmic transformation of marker levels over time showed an r^2 greater than

0.80; and (3) TSU-68 had been administered for at least 4 weeks.

Radiological evaluation of tumor volume

Radiological evaluations were performed according to RECIST guidelines version 1.0 [27]. Not more than 10 lesions, including intrahepatic tumors and extrahepatic metastases, were selected as target lesions prior to TSU-68 administration. In addition to RECIST, we also in our present analyses estimated the volume of each target lesion as a sphere taking the average of its major and minor axes as the diameter [28], and thereby calculating the radiological tumor volume DT as

$$\mathrm{DT} = \log(2) \times \frac{t_2 - t_1}{\log(V_2) - \log(V_1)}$$

where V_1 and V_2 are the volumes at times t_1 and t_2 [29].

Tumor markers

The HCC-specific tumor markers, AFP and DCP, were measured every 2 weeks for each patient registered in the trial. The serum AFP levels were measured via an enzyme immunoassay (ST AIA-PACK AFP, Tosoh, Tokyo, Japan) and DCP was measured using a chemiluminescent enzyme immunoassay (LUMIPULSE PIVKA-II, Eisai, Tokyo, Japan). These markers were also assayed after the termination of TSU-68 treatment, usually with a longer interval.

Tumor marker doubling time

In the present analyses, we assumed that the serum levels of tumor marker are proportional to the viable tumor volume with a fixed coefficient intrinsic to an individual case, when the tumor was producing the marker in question. Independently of the coefficient, the DT values can be calculated from two data points as

$$\mathrm{DT} = \log(2) \times \frac{t_2 - t_1}{\log(C_2) - \log(C_1)}$$

where C_1 and C_2 are the serum concentrations of tumor marker at times t_1 and t_2.

When data were available at more than two points, we first performed linear regression analysis of log-transformed tumor marker levels over time to determine the slope, and the DT was then calculated as

$$\mathrm{DT} = \frac{\log(2)}{\mathrm{slope}}.$$

Note in this case that the DT becomes negative when the tumor marker levels decrease following treatment.

Tumor volume and tumor marker levels during the washout phase

The total volume of target lesions was measured via two CT examinations during the washout phase: one at 4–12 weeks before and another immediately prior to the commencement of TSU-68 treatment. The tumor volume DT was then calculated as described above. The tumor marker DT was also calculated, and the relationship between the two sets of DT values was analyzed.

Changes in the DTs during TSU-68 treatment

The serum tumor marker DT during the first 8 weeks of TSU-68 administration was similarly calculated and compared with the DT measured before the drug therapy. If TSU-68 administration had been effective, the DT should be elongated, or yield a negative value. The evaluation of drug responses based on tumor marker DT was then compared with that by the RECIST method.

Tumor marker DT after the cessation of TSU-68 treatment

When a patient was observed without any anticancer treatment for more than 4 weeks after the cessation of TSU-68 treatment and tumor marker levels were determined more than once during this period, tumor marker DTs after the cessation of TSU-68 were similarly calculated and compared with those measured at 4 weeks and immediately before the cessation of treatment. The study design we used for estimating tumor marker DT before, during, and after TSU-68 administration is summarized in Fig. 1.

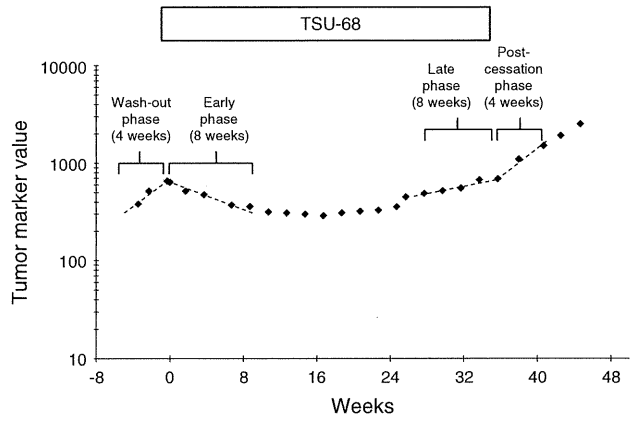

Fig. 1 Linear regression representation of the log tumor marker levels over time where DT = log(2)/slope. The slope can be calculated using a least-squares regression or two log-transformed tumor marker values: $\mathrm{DT} = \log(2) \times (t_2 - t_1)/[\log(\mathrm{TM2}) - \log(\mathrm{TM1})]$, where t = time and TM = tumor marker level