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Combination Assay of Methylated *HOXA1* with Tumor Markers Shows High Sensitivity for Detection of Early-Stage Hepatocellular Carcinoma

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Keywords
HOXA1 · Alpha-fetoprotein · Des-gamma-carboxy prothrombin · Methylation · Hepatocellular carcinoma

Abstract
Introduction: Patients with hepatitis virus-related hepatocellular carcinoma (viral HCC) are decreasing as hepatitis control improves, but those with non-viral-related HCC (non-viral HCC) are increasing in Japan. No established surveillance system exists for patients with non-viral HCC, so they are often diagnosed at an advanced stage. To address this, we performed this study. ***Methods:*** We collected serum samples from 516 participants (154 healthy subjects, 93 chronic liver disease [CLD] patients without HCC, and 269 HCC patients). Participants were divided into a control group comprising healthy subjects and patients with CLD and an HCC group. We evaluated serum methylated *HOXA1* (m-*HOXA1*) copy numbers using modified combined restriction digital PCR (CORD) assay (1-step CORD assay). We assessed diagnostic performance of m-*HOXA1* compared to HCC tumor markers alpha-fetoprotein (AFP) and des-gamma-carboxy prothrombin (DCP) and created a novel index to improve HCC prediction. ***Results:*** Serum m-*HOXA1* level was significantly higher in each HCC stage group versus the control group. Its sensitivity was 69.1% and specificity was 78.5% for diagnosing HCC. The area under the curve (AUC) of m-*HOXA1* was superior to that of AFP and equal to that of DCP. Multivariate logistic regression analysis revealed independent contributions of m-*HOXA1*, DCP, and AFP, in that order of strength, to diagnose HCC after adjustment for age and sex. We designated the predictive probability of

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HCC based on the regression model as the ASDAm-H1 (Age, Sex, DCP, AFP, and m-*HOXA1*) index. Its diagnostic accuracy was 0.96 by AUC with a sensitivity of 86.2% and specificity of 93.9%. Sensitivity was identical for viral and non-viral HCCs. When limited to early-stage HCC, sensitivity of the ASDAm-H1 index was 76.3%. ***Conclusions:*** We showed distinguished performance of the ASDAm-H1 index to detect viral and non-viral HCC, even at an early stage. This index might have potential as a non-viral HCC surveillance system.

Introduction

Annually, liver cancer is the sixth leading cause of morbidity and the third leading cause of mortality. Each year, approximately 900,000 patients are affected, and approximately 830,000 patients die of this cancer worldwide [1]. Hepatocellular carcinoma (HCC) accounts for approximately 90% of primary liver cancers. The 5-year survival rate of early HCC is 70%, but the median survival duration of advanced-stage HCC is around 1–1.5 years [2]. As the prognosis of patients with advanced HCC is severe, the best way to improve their prognosis is by early detection.

Alpha-fetoprotein (AFP) is widely used in the world for HCC surveillance [3]. In Japan, three tumor markers, AFP, des-gamma-carboxy prothrombin (DCP), and AFP-L3, are recommended as adjunctive diagnostic markers for HCC [4]. Owing to the HCC surveillance system using these three tumor markers and imaging tests, patients with HCC in Japan tend to be diagnosed at an early stage compared with those in other countries [5]. However, as the surveillance system is mainly aimed at virus-related HCC (viral HCC) [4], patients with non-viral-related HCC (non-viral HCC) are often diagnosed at an advanced stage. Thus, a novel surveillance system is required, regardless of the etiology of HCC.

To solve these problems, we previously developed a novel technology of highly sensitive quantification of methylated genes, referred to as the combined restriction digital PCR (CORD) assay [6], and applied it as a liquid biopsy of methylated *SEPT9* to detect HCC [7]. Another candidate biomarker of HCC is methylated *Homeobox A1* (m-*HOXA1*) [8–10]. HOX genes play an important role in apoptosis as well as development, receptor signaling, differentiation, motility, and angiogenesis [11], and hypermethylation of *HOXA1* is observed in HCC tissue [8]. Although m-*HOXA1* seems to be a useful biomarker of HCC, there are no reports of the diagnostic performance of m-*HOXA1* using the CORD assay.

In this study, we developed a modified CORD assay to shorten the conventional CORD assay processing time. Using this modified CORD assay, we evaluated the clinical significance of m-*HOXA1* for detecting HCC. Additionally, we created a novel index consisting of age, sex, tumor markers (DCP, AFP), and m-*HOXA1* to seek improved prediction of HCC, and its diagnostic performance was also evaluated.

Materials and Methods

Serum Samples

This was a prospective study in which 523 participants were enrolled between December 2016 and January 2022 at Yamaguchi University Hospital, St. Hill Hospital, and Ajisu Kyoritsu Hospital in Japan. Seven participants were excluded because of having tumors other than HCC or taking warfarin. Blood samples were collected prior to any medical treatments the participants would undergo. Finally, serum samples from 516 participants were collected. The subjects comprised 154 healthy subjects, 93 patients with chronic liver disease (CLD) without HCC, and 269 patients with HCC (Fig. 1).

The healthy subjects had neither CLD based on results of a medical examination nor a previous history of any cancers. In the patients with CLD, we confirmed by imaging modalities that there was no HCC 3 months before and 6 months after the blood sampling. HCC was diagnosed using pathological findings and/or imaging modalities based on Japanese guidelines [12], and the Barcelona Clinic Liver Cancer (BCLC) staging system was used for HCC tumor staging [13]. This study was conducted in compliance with the ethical principles of the Declaration of Helsinki. The study protocol was approved by the Institutional Review Boards of Yamaguchi University Hospital, St. Hill Hospital, and Ajisu Kyoritsu Hospital (H28-124 and H17-83).

Sample Preparation and DNA Extraction

Serum samples were stored at −80°C until DNA extraction. After serum samples were thawed, a part of them was used for AFP and DCP measurements. Further, 1.0 mL of each serum sample was used for DNA extraction with Maxwell DNA Purification Kits (Promega, Madison, WI, USA) according to the manufacturer's instructions. DNA was eluted in 60 μL of elution buffer.

AFP and DCP

Serum AFP and DCP levels were measured using an AIA-packCL AFP kit and an AIA-packCL PIVKAII kit on an AIA-CL2400 Fully Automated Chemiluminescent Enzyme Immunoassay System (all, Tosoh, Tokyo, Japan).

*Modified CORD Assay of m-*HOXA1

In the conventional CORD assay, we treated DNA using a 2-step procedure in which the initial treatment of DNA is performed with methylation-sensitive restriction enzymes (HhaI and HpaII) and exonuclease I (ExoI) (all, Thermo Fisher Scientific, Waltham, MA, USA) for 16 h at 37°C followed by additional treatment of DNA with another methylation-sensitive restriction enzyme,

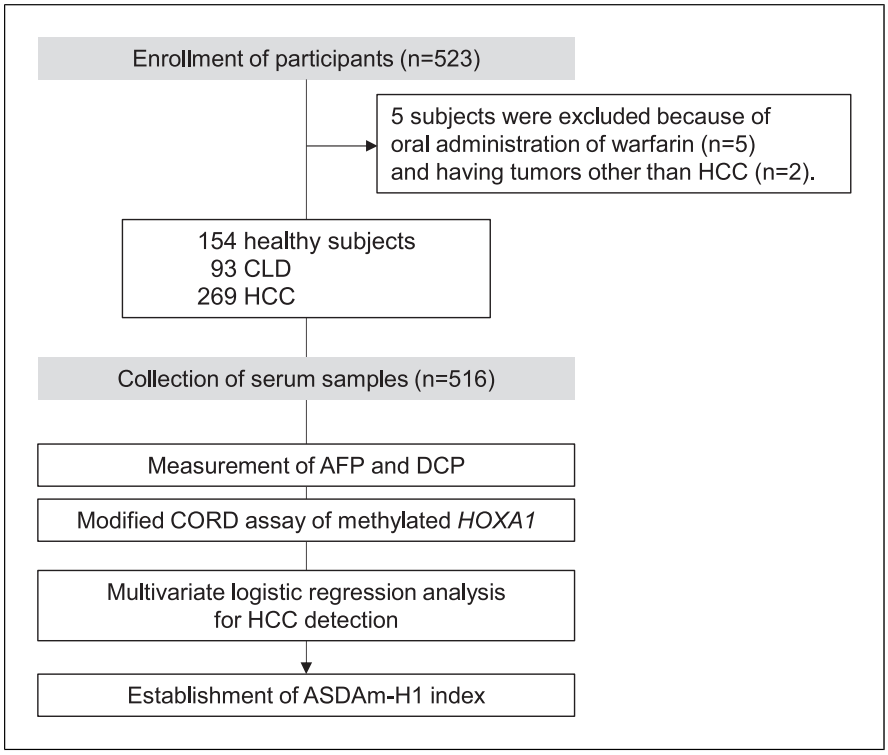

Fig. 1. Study design. AFP, alpha-fetoprotein; CLD, chronic liver disease; CORD, combined restriction digital PCR; DCP, des-gamma-carboxy prothrombin; HCC, hepatocellular carcinoma.

BstUI (New England Biolabs, Hitchin, UK), for 16 h at 60°C (Fig. 2a) [6, 7]. To simplify the procedures and further decrease the time of restriction enzyme reaction, we developed a modified CORD assay, i.e., the 1-step CORD assay, in which DNA is treated simultaneously with all of the enzymes (Fig. 2b). The reaction mixture consisted of 10 μL of extracted DNA, 1 μL of 25 mmol/L $MgCl_2$, 1 μL of 10× AmpliTaq Gold Buffer II (both, Thermo Fisher Scientific), and 1 μL of HhaI (10 U/μL), 1 μL of HpaII (10 U/μL), 1 μL of ExoI (20 U/μL), and 1 μL of BstUI (10 U/μL). The reaction mixture was incubated for 1 h at 37°C, followed by 16 h at 60°C, and final heating for 10 min at 98°C. We performed droplet digital PCR to count the absolute copy numbers of m-*HOXA1*. As m-*HOXA1* had a recognition site of HpaII, when the sites were methylated, m-*HOXA1* would escape digestion by these enzymes and would be amplified by PCR. The PCR reaction solution consisted of 8 μL of enzyme-treated DNA (an amount of DNA equivalent to that in 0.1 mL serum), 1 × ddPCR Supermix for Probes (Bio-Rad, Hercules, CA, USA), 0.25 μmol/L of each primer, and 0.125 μmol/L of a probe in a total volume of 20 μL. The primers and probe of *HOXA1* are forward primer: 5′-CCCATG-GAGGAAGTGAGAAA-3′, reverse primer: 5′-GGGGTATTCC-AGGAAGGAGT-3′, and probe: 5′-FAM-GCACAGTCACGCCGG-MGB-3′. The length of the PCR product is 62 bp [14, 15]. After making droplets with an automated droplet generator (Bio-Rad), PCR was performed using a T100 Thermal Cycler (Bio-Rad) with the following PCR conditions: 10 min at 94°C; 40 cycles of 94°C for 30 s and 56°C for 60 s; and 98°C for 10 min. A QX200 droplet reader (Bio-Rad) was used for measuring fluorescence intensity, and QuantaSoft Analysis Pro Software (Bio-Rad) was used for analysis (Fig. 1). The copy number per mL of serum was determined.

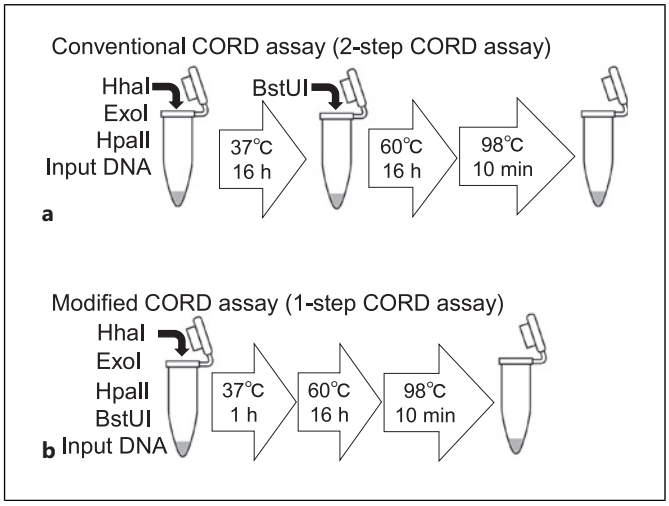

Fig. 2. Combined restriction digital PCR (CORD) assay. **a** The 2-step CORD assay, which was previously reported. DNA was treated in a two-step procedure using three methylation-sensitive restriction enzymes (HhaI, HpaII, and BstUI) and exonuclease I (ExoI). Two methylation-sensitive restriction enzymes (HhaI and HpaII) and ExoI were used in the first step of the procedure, and BstUI was used in the second step of the procedure. **b** The 1-step CORD assay described in this study. All three methylation-sensitive restriction enzymes (HhaI, HpaII, and BstUI) and ExoI were used in one step.

Table 1. Clinical characteristics of the participants

Parameters	Control group		HCC group
	healthy subjects (n = 154)	CLD (n = 93)	HCC (n = 269)
Age in years, median (range)	59 (36–88)	67 (36–86)	75 (39–95)
Sex			
Male	68	42	194
Female	86	51	75
Etiology			
HBV	–	33	37
HCV	–	25	94
HBV+HCV	–	0	2
Non-viral	–	35	136
CLD			
None	–	–	10
Chronic hepatitis	–	62	103
Liver cirrhosis	–	31	156
Severity of liver cirrhosis[1]			
A	–	30	110
B	–	0	43
C	–	1	3
Tumor stage[2]			
0	–	–	29
A	–	–	89
B	–	–	44
C	–	–	100
D	–	–	7
ECOG PS (0/1/2/3/4)	–	–	233/26/6/3/1
Tumor size, mm, median (range)	–	–	29 (0–200)
Tumor number, median (range)	–	–	1 (0–10<)
Vascular invasion, present/absent	–	–	71/198
Extrahepatic spread, present/absent	–	–	29/240

CLD, chronic liver disease; ECOG PS, Eastern Cooperative Oncology Group Performance Status; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; –, not available. [1]Severity of liver cirrhosis was determined using the Child-Pugh scoring system. [2]Tumor stage is based on the Barcelona Clinic Liver Cancer (BCLC) staging system.

Statistical Analysis

The Mann-Whitney U test was used to compare the difference in numerical variables between the two groups. The Kruskal-Wallis test and Dunn's multiple comparison test were used to compare differences in numerical variables between multiple groups. Categorical variables were analyzed using the χ^2 test or Fisher's exact test. The optimal cutoff value was determined by the Youden index method from the receiver operating characteristic (ROC) curve. Multivariate logistic regression analysis was performed to find independent factors that contribute to the diagnosis of HCC (Fig. 1).

Statistical significance was defined as a p value less than 0.05. For statistical analysis, we used GraphPad Prism ver.9 (GraphPad Software, San Diego, CA, USA) and StatFlex ver.7.0 (Artec Corporation, Osaka, Japan).

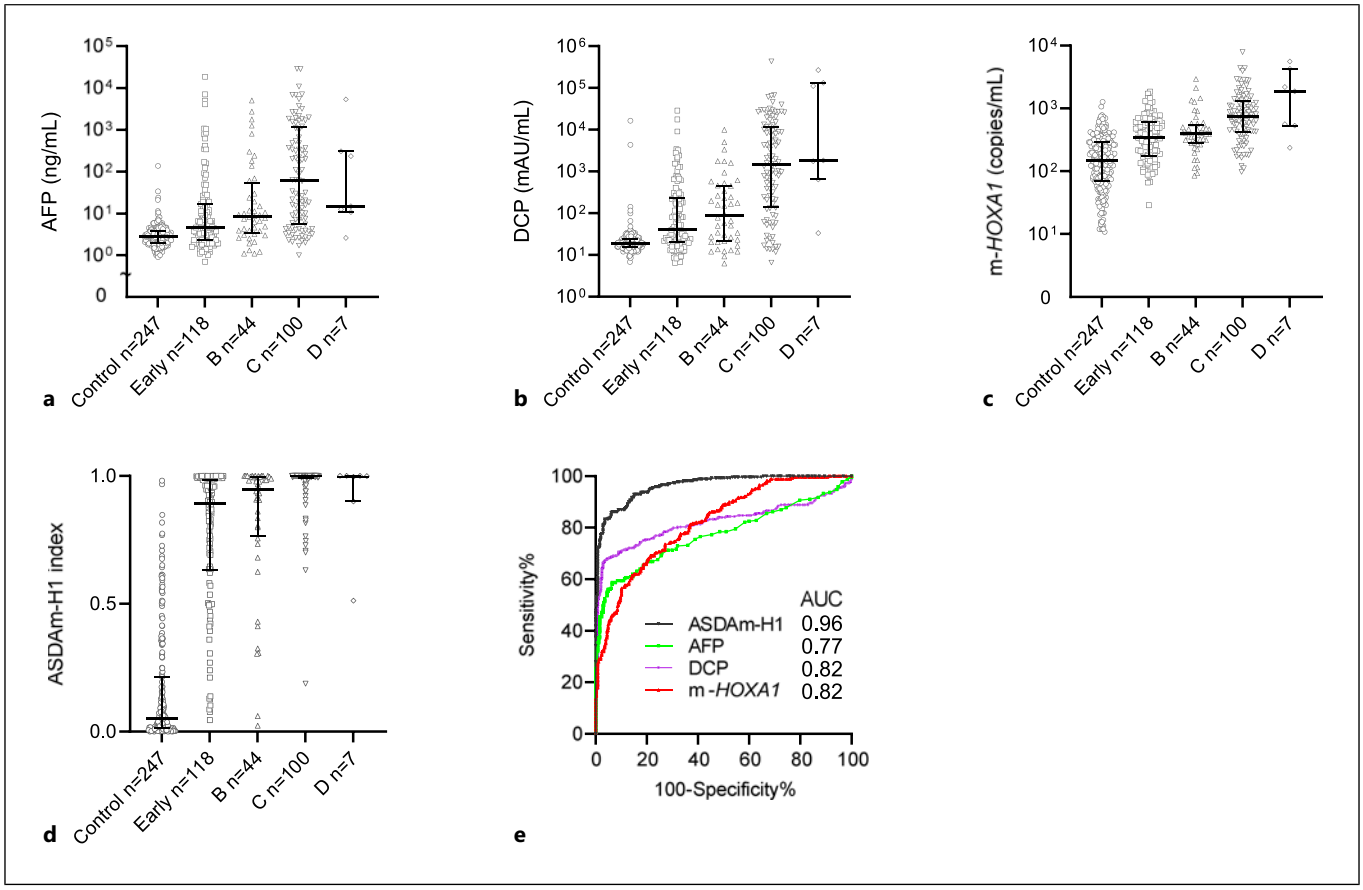

Fig. 3. a AFP assay. The median values of AFP were 2.8, 4.7, 8.4, 60.3, and 14.9 ng/mL in the control group, the early-stage hepatocellular carcinoma (HCC) group, and the BCLC stages B, C, and D HCC groups, respectively. **b** DCP assay. The median values of DCP were 19.0, 41.5, 87.9, 1,513.4, and 1,825.1 ng/mL in the control group, the early-stage HCC group, and the BCLC stages B, C, and D HCC groups, respectively. **c** Methylated *HOXA1* (m-*HOXA1*)assay. The median copy numbers were 146.4, 350.3, 391.3, 739.4, and 1,864.7 copies/mL in the control group, the early-stage HCC group, and the BCLC stages B, C, and D HCC groups, respectively. **d** ASDAm-H1 index. The median values of the ASDAm-H1 index were 0.05, 0.89, 0.95, 1.00, and 1.00 in the control group, the early-stage HCC group, and the BCLC stages B, C, and D HCC groups, respectively. **e** Area under the curve (AUC) of m-*HOXA1*, AFP, DCP, and ASDAm-H1 index for overall HCC detection. The AUC for the ASDAm-H1 index was 0.96, compared with 0.77 for AFP ($p < 0.001$), 0.82 for DCP ($p < 0.001$), and 0.82 for m-*HOXA1* ($p < 0.001$). AFP, alpha-fetoprotein; DCP, des-gamma-carboxy prothrombin.

Results

Subject Characteristics

Table 1 shows the clinical characteristics of the participants. We categorized the patients as having hepatitis B virus (HBV)-related CLD or HCC, hepatitis C virus (HCV)-related CLD or HCC, HBV and HCV-related CLD or HCC, and non-viral CLD or HCC. HBV-CLD or HCC was defined as positivity for hepatitis B surface antigen (HBs Ag), and HCV-CLD or HCC was defined as positivity for antibody to HCV (HCV Ab). HBV+HCV-CLD or HCC was defined as positivity for both HBs Ag and HCV Ab, whereas non-viral CLD or HCC was defined as negativity for both. The non-viral etiologies of the background liver diseases were categorized as follows: alcoholic liver disease (ALD), autoimmune hepatitis (AIH), primary biliary cholangitis (PBC), non-alcoholic fatty liver disease (NAFLD), and unknown. For the diagnosis of ALD, we used the previously reported criteria for alcohol consumption of ≥60 g/day for more than 5 years [16]. NAFLD was defined as having fatty liver and alcohol consumption of <30 g/day in males and <20 g/day in females [17].

We divided the subjects into two groups: a control group and an HCC group. The control group comprised 154 healthy subjects (62.3%) and 93 patients with CLD without HCC (37.7%) (110 males [44.5%], median age: 63 years). In the control group with CLD, the number of

Table 2. Statistical differences of each marker between two groups

Group	Control	Early	B	C	D
a AFP					
Control	–	<0.001	<0.001	<0.001	0.003
Early	<0.001	–	0.521	<0.001	0.334
B	<0.001	0.521	–	0.115	1.000
C	<0.001	<0.001	0.115	–	1.000
D	0.003	0.334	1.000	1.000	–
b DCP					
Control	–	<0.001	<0.001	<0.001	<0.001
Early	<0.001	–	1.000	<0.001	0.035
B	<0.001	1.000	–	0.002	0.154
C	<0.001	<0.001	0.002	–	1.000
D	<0.001	0.035	0.154	1.000	–
c m-*HOXA1*					
Control	–	<0.001	<0.001	<0.001	<0.001
Early	<0.001	–	1.000	<0.001	0.166
B	<0.001	1.000	–	0.033	0.542
C	<0.001	<0.001	0.033	–	1.000
D	<0.001	0.166	0.542	1.000	–
d ASDAm-H1					
Control	–	<0.001	<0.001	<0.001	<0.001
Early	<0.001	–	1.000	<0.001	1.000
B	<0.001	1.000	–	0.032	1.000
C	<0.001	<0.001	0.032	–	1.000
D	<0.001	1.000	1.000	1.000	–

Early, B, C, and D indicate Barcelona Clinic Liver Cancer stages. The *p* values shown represent the significant differences between two groups. –, not applicable.

patients with virus-related CLD was 58 (62.4%) including 33 with HBV and 25 with HCV. The 35 (37.6%) patients with non-viral-related CLD included 13 with NAFLD (37.1%), 7 with ALD (20.0%), 7 with PBC (20.0%), 6 with AIH (17.1%), and 2 with unknown (5.7%) (online suppl. Fig. 1a; for all online suppl. material, see https://doi.org/10.1159/000536211). In the HCC group, which comprised 269 patients (pathological diagnosis: 136 [50.6%]), 194 (72.1%) were male with a median age of 75 years. In this group, 133 (49.4%) patients had viral HCC consisting of HBV ($n = 37$), HCV ($n = 94$), and HBV+HCV ($n = 2$), whereas 136 (50.6%) patients had non-viral HCC including 56 with ALD (41.2%), 34 with NAFLD (25.0%), 1 with PBC (0.7%), 1 with AIH (0.7%), and 44 with unknown type (32.4%) (online suppl. Fig. 1b). The numbers of HCC patients with stage 0, A, B, C, and D were 29 (10.8%), 89 (33.1%), 44 (16.4%), 100 (37.2%), and 7 (2.6%), respectively. Early-stage HCC was defined as BCLC-stage 0/A, and the number of patients with early-stage HCC was 118 (43.9%). There were significant differences in sex and age between the control group and the HCC group ($p < 0.001$).

Alpha-Fetoprotein

The median values of AFP were 2.8, 4.7, 8.4, 60.3, and 14.9 ng/mL in the control group, the early-stage HCC group, and the stages B, C, and D HCC groups, respectively (Fig. 3a). Table 2 shows the statistical differences in AFP between two groups. The area under the curve (AUC) by ROC analysis was 0.77 to discriminate between the overall HCC group and the control group (Fig. 3e). At the cutoff value of 20 ng/mL as recommended by the American Association for the Study of Liver Diseases (AASLD) for HCC screening [3], the sensitivity was 37.5% for all-stage HCC, and the specificity was 98.4% (Table 3a, c).

Table 3. Diagnostic performance for HCC detection

a Sensitivity for all-stage HCC detection						
Biomarkers	Cutoff	Sensitivity, %				
		overall HCC (*n* = 269)	non-viral HCC (*n* = 136)	viral HCC (*n* = 133)	HBV-HCC (*n* = 37)	HCV-HCC (*n* = 94)
AFP	≥20	37.5	36.0	39.1	43.2	38.3
DCP	≥40	66.5	69.9	63.2	73.0	60.6
m-*HOXA1*	≥310	69.1	72.1	66.2	70.3	66.0
ASDAm-H1	≥0.62	86.2	87.5	85.0	83.8	85.1
b Sensitivity for early-stage HCC detection						
Biomarkers	Cutoff	Sensitivity, %				
		overall HCC (*n* = 118)	non-viral HCC (*n* = 50)	viral HCC (*n* = 68)	HBV-HCC (*n* = 17)	HCV-HCC (*n* = 49)
AFP	≥20	22.9	14.0	29.4	23.5	32.7
DCP	≥40	50.9	50.0	51.5	70.6	44.9
m-*HOXA1*	≥310	56.8	58.0	55.9	58.8	57.1
ASDAm-H1	≥0.62	76.3	78.0	75.0	70.6	75.5
c Specificity for all-stage HCC detection						
Biomarkers	Cutoff	Specificity, %				
		control (*n* = 247)	healthy subjects (*n* = 154)	CLD (*n* = 93)		
AFP	≥20	98.4	100.0	95.7		
DCP	≥40	96.8	98.1	95.7		
m-*HOXA1*	≥310	78.5	85.1	67.7		
ASDAm-H1	≥0.62	93.9	96.8	89.2		

Early-stage HCC is defined as Barcelona Clinic Liver Cancer (BCLC) stage 0/A. AFP, alpha-fetoprotein; CLD, chronic liver disease; DCP, des-gamma-carboxy prothrombin; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HCV, hepatitis C virus.

Next, we divided HCC into two groups according to the etiology: the non-viral HCC group and viral HCC group. The sensitivity was 36.0% for non-viral HCC and 39.1% for viral HCC (Table 3a). In addition, focusing on early-stage HCC, the sensitivity was 14.0% for non-viral early-stage HCC and 29.4% for viral early-stage HCC (Table 3b), suggesting the insufficient sensitivity of AFP, especially for non-viral early-stage HCC.

Des-Gamma-Carboxy Prothrombin

The median values of DCP were 19.0, 41.5, 87.9, 1,513.4, and 1,825.1 mAU/mL in the control group, the early-stage HCC group, and the stages B, C, and D HCC groups, respectively (Fig. 3b). The statistical differences in DCP between two groups are shown in Table 2. The AUC by ROC analysis was 0.82 to discriminate between the overall HCC group and the control group (Fig. 3e). When 40 mAU/mL was set as the cutoff value [18], the sensitivity for overall HCC was 66.5% with a specificity of 96.8% (Table 3a, c). The sensitivity was 69.9% and 63.2% for non-viral HCC and viral HCC, respectively (Table 3a). Focusing on early-stage HCC, the sensitivity was 50.0% for non-viral early-stage HCC and 51.5% for viral early-stage HCC (Table 3b).

Methylated HOXA1

Using serial dilutions of EpiScope Methylated HCT116 gDNA (Takara Bio, Shiga, Japan), we first confirmed the coincidence of m-*HOXA1* copy numbers between the 1-step CORD assay and the conventional CORD assay (r = 1.00; Fig. 4). Then, we measured serum m-*HOXA1* copy numbers using the 1-step CORD assay. The median copy numbers of m-*HOXA1* were 146.4, 350.3, 391.3, 739.4,

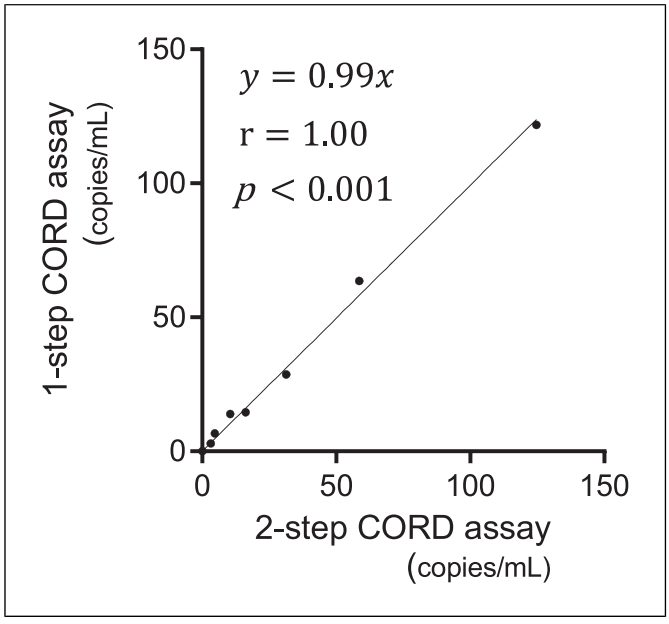

Fig. 4. Correlation of m-*HOXA1* copy numbers between the 1-step combined restriction digital PCR (CORD) assay and the 2-step CORD assay using control DNA of serial dilution.

and 1,864.7 copies/mL in the control group, the early-stage HCC group, and the stages B, C, and D HCC groups, respectively (Fig. 3c). The statistical differences in the m-*HOXA1* copy numbers between the two groups are shown in Table 2. According to ROC analysis, the AUC was 0.82 for overall HCC (Fig. 3e). The optimal cutoff value by the Youden index was 310 copies/mL, resulting in a sensitivity of 69.1% and specificity of 78.5% for overall HCC (Table 3a, c). There were significant differences in tumor stage, vascular invasion, and tumor size between <310 copies/mL of m-*HOXA1* and ≥310 copies/mL (online suppl. Table 1): the rate of positivity of m-*HOXA1* increased with HCC progression. Focusing on the etiology of HCC, the sensitivity was 72.1% for non-viral HCC and 66.2% for viral HCC (Table 3a). In addition, the sensitivity was 56.8% for early-stage HCC including 58.0% for non-viral early-stage HCC and 55.9% for viral early-stage HCC (Table 3b).

Prediction of HCC by Combining Three Diagnostic Markers

To seek improved prediction of HCC, we performed multivariate logistic regression analysis by combining the three diagnostic markers of AFP, DCP, and m-*HOXA1* after adjustment for differences in age and sex between the HCC and control groups. Because all three markers showed highly skewed distributions, they were logarithmically transformed before the analyses. As a result, m-*HOXA1,* AFP, and DCP were revealed as mutually independent factors, in that order of strength (*z* value), contributing to the diagnosis of HCC (Table 4). From the regression model, the predictive probability of HCC can be formulated as shown below. We designated this formulation as the ASDAm-H1 (Age, Sex, DCP, AFP, and m-*HOXA1*) index (Fig. 1).

$$\text{ASDAm-H1index} = \frac{1}{1+e^{\{-(-21.19-0.95\,(Sex)+0.13\,(Age)+1.02\,(Log\_AFP)+0.82\,(Log\_DCP)+1.46\,(Log\_m-HOXA1))\}}}$$

The median values of the ASDAm-H1 index were 0.05, 0.89, 0.95, 1.00, and 1.00 for the control group, the early-stage HCC group, and the stages B, C, and D HCC groups, respectively (Fig. 3d). Table 2 shows the statistical differences in the ASDAm-H1 index between the two groups. The ASDAm-H1 index showed the best diagnostic accuracy with an AUC of 0.96 for overall HCC as compared to 0.77 by AFP alone, 0.82 by DCP alone, and 0.82 by m-*HOXA1* alone (Fig. 3e). Setting an optimal cutoff value at 0.62 by the Youden index, the sensitivity of the ASDAm-H1 index for HCC was 86.2% with a specificity of 93.9%, and the sensitivity for early-stage HCC was 76.3% (Table 3a, c). Notably, the ASDAm-H1 index could also differentiate between healthy subjects and patients with CLD ($p = 0.016$, online suppl. Tables 2, 3). In addition, upon analyzing stages 0 and A separately, the sensitivity of the ASDAm-H1 index reached 62.1% for stage 0 and 80.9% for stage A, showing higher sensitivity than both AFP and DCP (online suppl. Table 4). When we divided the HCC groups into the non-viral HCC group and viral HCC group, the sensitivity was 87.5% for non-viral HCC and 85.0% for viral HCC (Table 3a). We also performed subgroup analysis based on each type of viral HCC. Consequently, the ASDAm-H1 index showed almost equal diagnostic performance regardless of the causative virus (HBV-HCC, 83.8%; HCV-HCC, 85.1%) (Table 3a). The sensitivity was 78.0% for non-viral early-stage HCC and 75.0% for viral early-stage HCC (Table 3b).

Discussion

In the conventional CORD assay, we had performed a two-step procedure that spanned 32 h of total incubation time (Fig. 2a). In the present study, we developed a modified CORD assay (the 1-step CORD assay) to save labor and reduce incubation time. We improved the enzyme reactions from the 2-step treatment to a 1-step

Table 4. Logistic regression analysis for overall HCC

Variables	β	SE (β)	*z* value	*p* value	OR (95% CI)
Intercept	−21.19	2.22			
Sex (female)	−0.95	0.34	−2.79	0.005	0.39 (0.20–0.75)
Age, years	0.13	0.02	7.08	<0.001	1.14 (1.10–1.18)
Log_AFP	1.02	0.19	5.44	<0.001	2.79 (1.93–4.03)
Log_DCP	0.82	0.15	5.50	<0.001	2.27 (1.70–3.04)
Log_m-*HOXA1*	1.46	0.22	6.80	<0.001	4.32 (2.83–6.58)

Male is the reference for sex. CI, confidence interval; HCC, hepatocellular carcinoma; Log_AFP, logarithmically transformed AFP; Log_DCP, logarithmically transformed DCP; Log_m-*HOXA1*, logarithmically transformed methylated *HOXA1*; OR, odds ratio.

treatment in which DNA is treated simultaneously with the three methylation-sensitive restriction enzymes and ExoI. We also modified the incubation time to reduce it from 32 h to 17 h (Fig. 2b). Thus, the modified CORD assay could reduce the processing time from approximately 3 days to about 1 day. After we confirmed coincidence of the m-*HOXA1* levels between the 1-step CORD assay and the conventional CORD assay (Fig. 4), we assessed clinical performance of the serum m-*HOXA1* level using the 1-step CORD assay.

The serum m-*HOXA1* level was significantly higher in each stage group of HCC than in the control group and had a sensitivity and specificity of 69.1% and 78.5%, respectively, for diagnosing HCC. Its sensitivity for early-stage HCC was 58.0% and 55.9% in non-viral HCC and viral HCC, respectively, with no significant difference in the etiology of HCC. The AUC of m-*HOXA1* was superior to that of AFP and equal to that of DCP (Fig. 3e). However, the specificity of 78.5% by m-*HOXA1* was lower than that of AFP (98.4%) and DCP (96.8%). Furthermore, it has been reported that DNA hypermethylation is associated with liver fibrosis [19], and we previously confirmed with the CORD assay that methylated *SEPT9* levels were detected in non-tumor tissue samples [7]. For the above reasons, serum m-*HOXA1* levels were significantly increased in CLD patients compared to healthy subjects (median: healthy subjects, 106.2 copies/mL; CLD, 234.0 copies/mL; $p < 0.001$; online suppl. Tables 2 and 3), and the specificity for patients with CLD was further reduced to 67.7% (Table 3c). Therefore, sole use of m-*HOXA1* appeared to be insufficient for diagnosing HCC. Thus, we performed a multivariable logistic regression analysis and found m-*HOXA1*, DCP, and AFP to be independent contributors, in that order of strength, to the diagnosis of HCC after adjustment for age and sex.

Consequently, we created the ASDAm-H1 index. The AUC of the ASDAm-H1 index was superior to that of each biomarker with a sensitivity of 86.2% and a specificity of 93.9% (Fig. 3e; Table 3a, c). In addition, when focusing on early-stage HCC, the sensitivity was considerably high at 76.3% and, moreover, was 78.0% for non-viral early-stage HCC and 75.0% for viral early-stage HCC (Table 3b). This is the first report to show the clinical usefulness of the ASDAm-H1 index for detecting early-stage HCC, regardless of the etiology of HCC.

There are other scoring systems for diagnosing HCC [10, 20, 21]. One is the multitarget blood test (mt-HBT), which uses sex and logarithmic copy values for methylated DNA genes (*HOXA1*, *TSYL5*, and *B3GALT6*) and log-transformed AFP [10]. The mt-HBT had a sensitivity of 71–84% for diagnosing viral early-stage HCC with a specificity of 88–90% and a sensitivity of 71–80% for non-viral early-stage HCC with a specificity of 84–87%. In the present study, the ASDAm-H1 index had a sensitivity of 75.0% for viral early-stage HCC and 78.0% for non-viral early-stage HCC with a specificity of 93.9% (Table 3b, c), thus showing a similar sensitivity with a greater specificity as compared to the mt-HBT. However, we admit that the compositions of the control groups are quite different between the two studies, which could affect specificity. In the mt-HBT study, the great majority of the subjects in the control group were patients with liver cirrhosis (93.2%, 605/649), whereas in the present study, the control group was comprised of healthy subjects (154/247, 62.3%) and patients with chronic hepatitis (62/247, 25.1%) and liver cirrhosis (31/247, 12.6%). In our study, when control subjects were limited to patients with CLD, we still found a considerably high specificity of 89.2% (Table 3c). Thus, the ASDAm-H1 index may have similar diagnostic performance as compared to mt-HBT.

The advantage of the 1-step CORD assay is that it requires only a small amount of serum (1.0 mL). In contrast, bisulfite-based methylation assays including the mt-HBT use a large amount of plasma (5.0–6.0 mL) [10]

because approximately 90% of the DNA is lost during its treatment with bisulfite [22]. In addition, the ASDAm-H1 index uses only one methylation marker, whereas mt-HBT uses three methylation markers. Thus, we assume that determination of the ASDAm-H1 index would save labor and cost less.

Another similar index, the GALAD score, uses sex, age, AFP isoform L3 (AFP-L3), log-transformed AFP, and log-transformed DCP [20, 21]. Chalasani et al. showed that the AUC of the mt-HBT including m-*HOXA1* was the same as or greater than that of the GALAD score [10]. As the GALAD score is not available in Japan because simultaneous measurement of all three tumor markers (AFP, DCP, AFP-L3) is not permitted under the public health insurance, we could not compare diagnostic performance between the GALAD score and the ASDAm-H1 index. This issue will be addressed in the future. Recently, the ASAP score, which excludes AFP-L3 used in the GALAD score, was evaluated in a multicenter study of patients with HBV-HCC in China [23], and its score showed better diagnostic performance for HCV-HCC than did the GALAD score [24]. We analyzed the diagnostic performance between the ASDAm-H1 index and the ASAP score in the present cohort. As shown in online Supplementary Figure 2, the ASDAm-H1 showed significant accuracy with an AUC of 0.96 for overall HCC compared with that of 0.93 by the ASAP score ($p < 0.001$). At the cutoff value of 0.63, which was calculated based on a previous report [23], the sensitivity of the ASAP score was 69.5% for overall HCC and 55.1% for early-stage HCC. Setting an optimal cutoff value of the score for the present cohort at 0.58 by the Youden index, the sensitivities for overall HCC and for early-stage HCC were 71.4% and 57.6%, respectively. Therefore, the ASAP score showed lower sensitivity than the ASDAm-H1 (online suppl. Table 5).

This study has several limitations. First, patients were collected from a single center with a limited sample size. Second, a validation study is yet to be completed. To address these issues, we have been performing a prospective multicenter study with a larger sample size. A part of the results of the preliminary validation study is shown in online Supplementary Figure 3 in which the AUC of 0.96 was the same as that in the present study to discriminate between the control group and the HCC group. In parallel, we have been performing a follow-up survey to investigate whether the ASDAm-H1 index can be used as a predictive marker both of HCC recurrence after complete remission and of cancer development for patients with CLD. Third, the best formula and the best cutoff value for detecting early-stage HCC remain unsettled. Although we set the cutoff value of the ASDAm-H1 index for overall HCC detection using the Youden index, the optimal cutoff value should be determined that balances benefits and harm [25]. Therefore, further examination will be required to confirm the best cutoff value. Finally, m-*HOXA1* is not specific to HCC. Indeed, hypermethylation of *HOXA1* is observed in the tissue of bile duct cancer, breast cancer, lung cancer, gastric cancer, and thyroid cancer [26], and serum m-*HOXA1* is increased in pancreatic cancer [14, 15]. As few studies using serum and plasma samples have reported the diagnostic performance of m-*HOXA1* for detecting various types of cancers, further studies will be required to address the extent of cancer types by liquid biopsy of m-*HOXA1*.

In conclusion, we showed the distinguished performance of the ASDAm-H1 index to detect HCC of both viral and non-viral types even at an early stage. This index might have potential as a non-viral HCC surveillance system.

Statement of Ethics

This study was conducted in accordance with the guidelines of the Declaration of Helsinki and was approved by the Institutional Review Board of Yamaguchi University Hospital (H28-124 and H17-83). Written informed consent was obtained from all participants.

Conflict of Interest Statement

T. Yamasaki received grant support from Eiken Chemical Co., Ltd. All other authors declare no conflicts of interest.

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Author Contributions

Yuki Kunimune, Yutaka Suehiro, and Takahiro Yamasaki designed the study. Data analysis was performed by Yuki Kunimune, Naoko Okayama, Mitsuaki Nishioka, and Yutaka Suehiro. Data curation was performed by Yuki Kunimune, Issei Saeki, Yurika Yamauchi, Norikazu Tanabe, Toshihiko Matsumoto, Shingo Higaki, Ikuei Fujii, and Chieko Suzuki. Statistical analysis was performed by Yuki Kunimune, Yutaka Suehiro, Takahiro

Yamasaki, and Kiyoshi Ichihara. Yuki Kunimune, Yutaka Suehiro, Issei Saeki, and Takahiro Yamasaki wrote the original draft of the manuscript. Hiroaki Nagano, Isao Sakaida, and Taro Takami reviewed and edited the manuscript. All authors read and approved the final manuscript.

Data Availability Statement

All data relevant to the study are included in this article and its supplementary files. Further inquiries can be directed to the corresponding author.

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