Combined analysis of AFP and HCCR-1 as an useful serological marker for small hepatocellular carcinoma: A prospective cohort study

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Abstract. Hepatocellular carcinoma (HCC) is one of the most frequent malignant tumors in the world. The only serological marker widely used for the diagnosis of HCC is alpha-fetoprotein (AFP). Despite that AFP is widely used for the diagnosis of HCC, it has a limit as a serological marker due to its low sensitivity and specificity. The human cervical cancer proto-oncogene 1 (HCCR-1) was previously reported as a new biomarker for HCC. To further evaluate the HCCR-1 as a biomarker for HCC, we conducted the prospective cohort study. We evaluated the significance of simultaneous measurement of 2 tumor markers in the diagnosis of HCC in China, Japan and Korea. Two markers for HCC, AFP and HCCR-1, were measured in the sera obtained from 1,338 patients at the time of initial diagnosis of HCC. Of the 1338 HCC patients, 616 (46%) and 686 (51.3%) were sero-positive for AFP and HCCR-1, respectively. The positive rate for HCC was increased up to 74.1% in combined use of AFP and HCCR-1. Many cases (54%) for AFP-negative HCC were positive for HCCR-1 and vice versa. More importantly, the diagnostic rate for small HCC (< 2 cm) was significantly improved in the combined analysis of AFP and HCCR-1 to 56.9% although it was only 40.1% and 23.4% in the single analysis of HCCR-1 and AFP, respectively. Our result suggests that the HCCR-1 could be an useful biomarker for HCC while the diagnostic rate could be significantly improved in the combined use of HCCR-1 and AFP.

Keywords: Hepatocellular carcinoma, biomarker, AFP, HCCR-1

Abbreviations

HCC,	hepatocellular carcinoma
CH,	chronic hepatitis
LC,	liver cirrhosis

HCCR1,	human cervical cancer oncogene 1
AFP,	alpha fetoprotein
AFPL3,	sugar chain of *Lens culinaris*
DCP,	des-γ-carboxy prothrombin

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1. Introduction

Hepatocellular carcinoma (HCC) is the fifth most common cancer in the world and the third leading cause

of cancer-related death [1]. HCC is the second and the third most common causes of cancer death in China and Korea, respectively [1,2]. Currently, the only available tools for HCC surveillance are testing for serum AFP level and hepatic ultrasonography [3,4]. However, AFP level has poor sensitivity and specificity for HCC [3] and ultrasonography is operator dependent and limited in its ability to discriminate HCC from such non-neoplastic lesions as regenerative nodules [4]. Newer methods for the early detection of HCC are needed in the form of either biomarkers or radiological tests. Although serum AFP level is an useful tumor marker for the detection and monitoring of HCC, the false-negative rate with AFP level alone may be as high as 40% for patients with early stage HCC [5]. Even in patients with advanced HCC, the AFP levels may remain normal in 15%-30% of the patients. Other HCC markers, such as des-γ-carboxy prothrombin (DCP), alkaline phosphatase isoenzyme-I, and tissue polypeptide specific antigen, have been developed to improve the sensitivity, specificity, early detection, and prediction of prognosis; but the overall results have been unsatisfactory [6]. In recent years, clinically useful tumor markers for HCC diagnosis have included AFP, a fucosylated variant of the AFP glycoprotein which has a high affinity to the sugar chain of *Lens culinaris* (AFPL3), and DCP.

Several laboratories have recently reported that HCCR-1 is expressed by a large proportion of HCCs, but is undetectable in normal hepatocytes and non-malignant liver disease [2,7–10]. Elevated level of HCCR-1 has been shown to be associated with an increase in the risk of developing HCC [7]. We identified a new oncogene associated with human cervical cancer that is also overexpressed in various human tumors including liver cancer [7,11,12]. The functional roles of these oncogenes in tumorigenesis is manifested as a negative regulator of the p53 tumor suppressor [11,12]. HCCR-1 is a 42 kDa protein which is overexpressed in various human tumors including liver cancer [7,11, 12]. Northern and Western blot analyses and immunohistochemical studies indicate that the HCCR-1 mRNA and protein was overexpressed in tumorous compared with non-tumorous cirrhotic tissue of HCC [7]. These results indicate that *HCCR-1* probably represents a new oncogene that is related to liver tumorigenesis, functioning as a negative regulator of the p53 tumor suppressor [11,12]. Moreover, the expression of HCCR-1 is modulated by the phosphatidylinositol 3-kinase/Akt pathway which is involved in the tumorigenesis by regulating cell cycle progression and survival [13].

In this study, we undertook the following aims: 1) To investigate the diagnostic efficiency of HCCR-1 in HCC, 2) To investigate its usefulness in the early diagnosis for small HCC, 3) To compare with AFP test, 4) To test for the combined analysis of HCC with AFP and HCCR-1 especially in small HCCs.

2. Methods and materials

2.1. Patients and study design

1380 subjects were recruited during the study period from September 2003 to August 2007 from 4 clinical sites (Catholic University, Korea; Seoul National University, Korea; Nanjing University, China and Sanyo Chemical, Japan) (Table 1). The cohort consisted of 1006 males (76.1%) with an average age of 54.4 years and 332 females (23.9%) with an average age of 56.6 years (Table 2). Blood samples from Catholic University, Korea and Seoul National University, Korea were frozen for analysis and shipped to our institution. On the contrary, blood samples from Nanjing University, China and Sanyo Chemical, Japan were analyzed at their own laboratory and sent us the results. Clinical data were collected for serum chemistries, imaging (CT, MRI and/or ultrasound), presenting symptoms, medications/interventions/therapeutics, and other medical informations including the presence of hepatitis (Table 2).

The enrollment criteria are as follows. Inclusion criteria are 1) patients were confirmed to have HBV or HCV infection at study onset, 2) HCC diagnosis histologically confirmed by the examination of liver specimens obtained by percutaneous biopsies, 3) pathological grading was contextually assessed in all the patients according to Edmonson's classification, and 4) patient's informed consent to provide blood samples for AFP and HCCR-1 determination. Exclusion criteria are 1) incomplete medical records, 2) misdiagnosis, 3) follow-up visit cannot be achieved within 30 months period, and 4) previous use of immunomodulators such as interferon. The clinical study consisted of consecutive patients who met both inclusion and exclusion criteria. Because time is irrelevant to our determining the HCC progression, we cannot state success criteria. Rather, we simply measure the HCCR-1 value at the time of visit.

At the end of the study, all evaluable subjects were subdivided into 4 categories by the site investigators based on biopsy, explanted liver histology, imaging

Table 1
Patient profiles of HCC regarding clinical site information

Clinical site information

Site	# Subjects	% Total	Study period
Catholic Univ. Hospital, Seoul, Korea	686	50	9/1/2003–5/30/2006
Seoul National Univ. Hospital, Seoul, Korea	72	6	10/1/2004–12/30/2005
Nanjing Univ. Hospital, Nanjing, China	380	29	3/1/2004–8/1/2006
Sanyo Chemical, Japan	200	15	7/1/2006–8/1/2007
Total	1338	100	

Site distribution

Site	Group 1 HCC	Group 2 CH/LC *to* HCC	Group 4 No HCC	Group 3 Suspicious	Total
Catholic, Korea	325	169	183	9	686
Seoul, Korea	65	5	0	2	72
Nanjing, China	370	0	0	10	380
Sanyo, Japan	200	0	0	0	200
Total	960	174	183	21	1338

Table 2
Patient profiles of HCC regarding gender distribution, age distribution, hepatitis status and HCCR-1 values

Gender distribution		Group 1 HCC	Group 2 CH/LC $\rightarrow$ HCC	Group 4 No HCC	Group 3 Suspicious	Total
Male	N	725	131	133	17	1006
	%	75.5	75.3	72.7	81.0	76.1
Female	N	235	43	50	4	332
	%	24.5	24.7	27.3	19.0	23.9
Total	N	960	174	183	21	1338

Age distribution		Group 1 HCC	Group 2 CH/LC→HCC	Group 4 No HCC	Group 3 Suspicious	Total
Male	N	725	131	133	17	1006
	Average $\pm$ SD	55.2 $\pm$ 6.6	52.4 $\pm$ 5	51.7 $\pm$ 4.9	54.2 $\pm$ 3.1	54.4 $\pm$ 6.3
	Range	41–71	41–64	41–63	50–59	41–71
Female	N	235	43	50	4	332
	Average $\pm$ SD	58.6 $\pm$ 4.9	51.8 $\pm$ 5.6	50.8 $\pm$ 4.9	55.0 $\pm$ 0.0	56.5 $\pm$ 6.0
	Range	46–70	40–63	41–63	55–55	40–71
Total	N	960	174	183	21	1338
	Average+SD	56.0 $\pm$ 6.4	52.2 $\pm$ 5.2	51.5 $\pm$ 4.9	54.3 $\pm$ 2.8	54.9 $\pm$ 6.3
	Range	41–71	40–64	41–63	50–59	40–71

Hepatitis status	Group 1 HCC	Group 2 CH/LC→HCC	Group 4 No HCC	Group 3 Suspicious	Total
HBV+	741 (77.2%)	134 (77.0%)	137 (74.9%)	15 (71.4%)	1027 (76.8%)
HCV+	186 (19.4%)	28 (16.1%)	33 (18.0%)	5 (23.8%)	252 (18.8%)
HBV+/HCV+	33 (3.4%)	12 (6.9%)	13 (7.1%)	1 (4.8%)	59 (4.4%)
Total	960	174	183	21	1338

Distribution of HCCR-1 values (ng/ml)	Group 1 HCC	Group 2 CH/LC→HCC	Group 4 No HCC	Group 3 Suspicious
# subject	960	174	183	21
Average	2362	4022	83	1241
Median	9	4277	7	8
SD	3161.63	2778.19	277.06	2384.62
P value vs. Group 4	$P < 0.0001$	$P = 0.0001$		$P < 0.024$

Wilcoxon rank sum test.

interpretation of tumor load, etc.: HCC at study onset (Group 1), developed confirmed HCC during study (Group 2), suspected of possible HCC (Group 3) and no HCC (Group 4) (Tables 1 and 2).

Because we planned 30 month to be the period for follow up of patients for the development of HCC, subjects who had 30 months between the first HCCR-1 positive result and end of study were included which resulted in excluding 42 subjects from the Group 4. Here, 30 months duration doesn't imply the clinical relevance but it is decision duration to observe patients for HCC development. The final number of subjects was 1338.

None of the subjects had other malignancies or active pulmonary disease. All subjects and controls were subjected to the analysis with individual consent for the study. The use of blood samples was approved by the ethics committee of each 4 institution.

2.2. Detection of HCCR-1 in serum by sandwich ELISA

ELISA used in this study is an enzymatically amplified "two-step" sandwich-type immunoassay. The HCCR-1 levels were measured using a sandwich ELISA. The standard curve was constructed with serial dilutions of HCCR-1$_{167-360}$, ranging from 20 to 320 ng/ml. By using 20, 40, 80, 160, and 320 ng/ml of HCCR-1$_{167-360}$ protein, a calibration curve was prepared.

In the assay, standards, controls and unknown serum samples are incubated in microtitration wells which have been coated with anti-HCCR-1 monoclonal antibody (mAb) 54115. After incubation and washing, the wells are treated with another anti-HCCR-1 detection polyclonal antibody labeled with the enzyme horseradish peroxidase (HRP). The reference ranges were obtained by receiver operating characteristics (ROC) curve analyses: 10 ng/ml was used as a cutoff level for HCC. To determine the serum HCCR-1 cutoff value, ROC analysis was performed using the study population composed of another new group of 880 patients with HCC as a positive control and 380 patients with cirrhosis or chronic hepatitis as a negative control. ROC curve analysis gave a cut-off value of 10 ng/ml in which sensitivity and specificity were 48.5% and 93.4%, respectively. With this cut-off value, we then conducted a prospective study for another 1338 patients to evaluate the significance of simultaneous measurement of 2 tumor markers in the diagnosis of HCC (Supplementary Table 1 and Supplementary Fig. 1).

2.3. Serum AFP concentration

The AFP level was measured by a 2-step sandwich immunoradiometric assay using a commercially available kit (IRMA AFP MONOCLONAL, BECKMAN COULTER Inc., Fullerton, CA, USA). To determine positive responses, we used the value of 11 ng/ml as the cutoff for AFP as instructed by the manufacturer.

2.4. Statistical analysis

To determine the positive responses, we used 11 ng/ml as the cutoff value for AFP and 10 ng/ml for HCCR-1 which was derived from ROC curve analysis from another new 880 patients of HCC as a positive control and 380 patients of liver cirrhosis or chronic hepatitis as a negative control. Serum levels of HCCR-1 were presented as the mean $\pm$ SD and compared using Wilcoxon rank sum test and Kruskal-Wallis test. Positive response rates of AFP and HCCR-1 were presented in terms of marginal proportions and compared by McNemar test for all patients and in each stage of clinicopathologic factors, respectively. A two-tailed significance level of 5% and SAS (version 9.1) were used for all statistical analyses.

3. Results

3.1. Determination of serum cutoff values

To determine the cutoff value of HCCR-1, we performed ROC curve analysis using another new 880 patients of HCC and 380 patients of liver cirrhosis or chronic hepatitis. None of the patients had other malignancies or active pulmonary disease. The mean values of serum HCCR-1 in 380 cirrhotic or chronic hepatitis patients and 880 HCC patients were 9.5 $\pm$ 14.4 and 2436.3 $\pm$ 3300.9 ng/ml, respectively (Table 3). There was a statistically significant difference between the mean values of HCCR-1 in HCC patients and liver cirrhosis patients ($P < 0.001$).

ROC curve for liver cirrhosis and HCC patients was obtained and sensitivities, specificities, and total accuracies for five cutoff values were presented (Supplementary Table 1 and Supplementary Fig. 1). We chose the value of 10 ng/ml as the cutoff for diagnostic evaluation since the specificity is over 90% at that point although the sensitivity was relatively low. Hereafter, the cutoff value for AFP and HCCR-1 will be 11 ng/ml and 10 ng/ml, respectively.

Table 3
The values of serum HCCR-1 in 380 cirrhotic or chronic hepatitis patients and 880 HCC patients for the serum cutoff determination

Group	N	Mean	SD	Range	Median
HCC*	880	2436.3	3300.9	5–11401	9
Liver cirrhosis or chronic hepatitis	380	9.5	14.4	3–93	6
Total	1260	1704.4	2974.7	3–11401	8

*$P < 0.001$ by Wilcoxon rank sum test.

Table 4
Diagnostic results of HCCR-1 compared with AFP among 1338 patients

		AFP +	AFP −	Total
HCCR-1	+	281	405	686 (51.3%)*
	−	335	317	652
	Total	616 (46%)		1338 (100%)

*$P = 0.011$ by McNemar test.

Table 5
AFP and HCCR-1 levels related to tumor sizes in HCC patients

HCC marker	Tumor size	Positive rate
AFP	HCC*	46% (616/1338)
	Large HCC ($\geqslant$ 2 cm)	66.8% (466/697)
	Small HCC† ($<$ 2 cm)	23.4% (150/641)
HCCR-1	HCC	51.3% (686/1338)
	Large HCC ($\geqslant$ 2 cm)	61.5% (429/697)
	Small HCC ($<$ 2 cm)	40.1% (257/641)
Combined (AFP + HCCR-1)	HCC	74.1% (990/1338)
	Large HCC ($\geqslant$ 2 cm)	89.7% (625/697)
	Small HCC ($<$ 2 cm)	56.9% (365/641)

*HCC: AFP vs. HCCR-1 ($p = 0.011$ by McNemar test); AFP alone vs. Combined ($P < 0.001$ by McNemar test); HCCR-1 alone vs. Combined ($P < 0.001$ by McNemar test).
†Small HCC: AFP vs. Combined ($P < 0.001$); HCCR-1 vs. Combined ($P < 0.001$).

3.2. Diagnostic results

To evaluate whether HCCR-1 could be used as a biomarker for HCC, the serum HCCR-1 level was examined in HCC patients and its association with clinico-pathologic characteristics such as tumor size and AFP levels was determined (Tables 2 and 5). Diagnostic results of HCCR-1 compared with AFP are summarized in Table 4. Of 1338 patients, 616 (46%) were sero-positive for AFP, and 686 (51.3%) were sero-positive for HCCR-1 (Table 4). There was a statistical significance between diagnostic efficiencies of AFP (46%) and HCCR-1 (51.3%) ($P = 0.011$ by McNemar test) (Table 4) although the diagnostic rate was significantly different in combined analysis of AFP and HCCR-1 for HCC ($P < 0.001$) (Table 5). The positive rate in collaborative use of AFP and HCCR-1 was increased up to 74.1% (990 out of 1338 HCC) (Table 5). The

cases negative both for AFP and HCCR-1 were 317 (25.9%). Many cases for AFP-negative HCC were positive for HCCR-1 and vice versa, indicating that the expression of HCCR-1 is not associated with that of AFP in many HCC. The positive rate for HCCR-1 was 56.1% (405 out of 722) in AFP-negative HCC and the positive rate for AFP was 51.4% (335 out of 652) in HCCR-1-negative HCC (Table 4).

As a consequence, the serological level of at least one of the two markers was elevated in a majority of HCC patients. These results suggest that the diagnostic rate can be significantly improved through the simultaneous measurement of both HCCR-1 and AFP. Therefore, HCCR-1 could be an useful tumor marker when used together with AFP for diagnosis of HCC.

3.3. Relationship of HCCR-1 levels with gender, age, hepatitis and countries

There was no statistical significance in HCCR-1 levels between genders ($P = 0.3742$), ages ($P = 0.9412$), and hepatitis B, C, or both ($P = 0.5290$). In addition, there was no significant difference in HCCR-1 level between countries ($P = 0.2202$). No correlation was found between serum levels of HCCR-1 and liver functions (AST, ALT, GGT, alkaline phosphatase, albumin, and bilirubin levels in serum, data not shown).

3.4. Relationship of HCCR-1 levels to tumor size

Comparison of diagnostic values of total serum AFP and HCCR-1 for HCC is shown in Table 5. Among 697 large HCC ($\geqslant$ 2 cm) excluding 641 small HCC out of total 1338 HCC, the positive rate of HCCR-1 and AFP was 61.5% and 66.8%, respectively (Table 5). This shows that the diagnostic rate of HCCR-1 is significantly different from that of AFP in large HCC ($\geqslant$ 2 cm) ($P = 0.011$ by McNemar test).

Importantly, however, the diagnostic rate of HCCR-1 (40.1%) was significantly higher than that of AFP (23.4%) in small HCC ($P < 0.001$) (Table 5). This suggests that the diagnostic value of HCCR-1 becomes more evident in small HCC.

The combined analysis of both AFP and HCCR-1 for HCC was performed and it demonstrates that the positive rate for HCC is increased up to 74.1% in HCC. In particular, the positive rate for small HCC is increased up to 56.9%. This increase was statistically significant compared to the single analysis of AFP or HCCR-1 alone (Table 5). Therefore, the combined analysis of AFP and HCCR-1 improved the diagnostic rate of HCC both in large and small HCC ($P < 0.001$) (Table 5). This suggests that the simultaneous detection of AFP and HCCR-1 should be effective particularly for the early diagnosis of small HCC.

4. Discussion

To determine the possibility of whether HCCR-1 can be used as a biomarker for HCC, the serum HCCR-1 level was examined in HCC patients. Of the 1338 HCC patients, 46% was sero-positive for AFP, and 51.3% was sero-positive for HCCR-1. The positive rate in collaborative use of AFP and HCCR-1 was increased up to 74.1%. Elevated HCCR-1 levels were not associated with elevated AFP levels in most cases and the diagnostic efficiency for HCC was similar between AFP and HCCR-1. In other words, many cases for AFP-negative HCC were positive for HCCR-1 and *vice versa*. The positive rate for HCCR-1 was 56.1% in AFP-negative HCCs and the positive rate for AFP was 51.4% in HCCR-1-negative HCCs. As a consequence, the serological level of at least one of the two markers was elevated in a majority of HCC patients.

In small HCC (< 2 cm), the positive rate of AFP and HCCR-1 was 23.4% and 40.1%, respectively. The positive rate either for AFP or HCCR-1 was increased up to 56.9% in small HCC. Therefore the combined analysis of AFP and HCCR-1 improved the diagnostic rate in small HCC. The diagnostic rate of HCCR-1 in small HCC was significantly higher than that of AFP. Our results indicate that the HCCR-1 assay has an advantage over the AFP assay in that it is positive more frequently in patients with early liver cancer.

Worldwide, HCC is the 5th most common cancer and the 3rd most important cause of cancer mortality, with a 5-year survival rate of merely 7% [14,15]. Early detection enables us to provide the patient with the most optimal therapy [16,17]. Despite the fact that serum AFP is still the golden standard amongst diagnostic markers for HCC, its diagnostic value is more and more questioned, due to poor sensitivity and specificity of the assay [18]. Previous studies have demonstrated that the sensitivity and specificity of AFP for HCC diagnosis are 41–65 and 80–94%, respectively, with a cut-off value of 20 ng/ml [19]. Nevertheless, AFP remains the most commonly used marker in screening and surveillance programs of HCC to date. Besides its diagnostic value, high AFP level has also been shown to adversely affect the prognosis of patients with HCC treated by hepatic resection or nonsurgical treatment [20,21]. When serum levels are markedly raised (> 400 ng/ml), as occurs in about 50% of symptomatic cases, it is particularly useful for both diagnosis of HCC and monitoring of treatment response [22,23]. However, AFP is secreted in only about 70% of HCC, and hence both false-negative and false-positive rates are high with the use of AFP as the serological marker for detection of HCC [2].

Although the *HCCR-1* oncogene was originally identified in human cervical cancer, its serum levels were found to be elevated in patients with HCC but not with cervical cancer, implying its close relationship with hepatocarcinogenesis [7]. Furthermore, various studies demonstrated that HCCR-1 could be used as a serological test for the diagnosis of patients with HCC [2,7–10]. Although we previously developed HCCR-1 indirect ELISA assay for serological test [7], the development of sandwich ELISA assay was essential to resolve the specificity issue. To achieve this goal, we developed HCCR-1 sandwich ELISA assay in this study to improve the serological assay using HCCR-1 for HCC. The HCCR-1 sandwich ELISA assay revealed that elevated HCCR-1 levels are associated with an increase in the risk of developing HCC.

In conclusion, diagnostic rate of HCC can be improved through the simultaneous measurement of both HCCR-1 and AFP. HCCR-1 could be used as a supplementary to AFP in the diagnosis of HCC, but each of them has no satisfactory accuracy in detecting HCC or prefiguring the prognosis when used alone. Given the high heterogeneity of HCC, it is currently thought that an optimal serological test for HCC will be based on the simultaneous measurement of two or three highly specific serological markers. Combining the information from AFP and HCCR-1 increases the diagnostic rate over AFP alone in particular in small HCC and may discover the recurrence of HCC at earlier period. Our results indicate that the HCCR-1 assay has an advantage over the AFP assay since its level is increased according to disease progression and it is positive more frequently in patients with early liver cancer.

Acknowledgements

Supplemental material

To obtain the supplemental Table and Figure, please contact the corresponding author Prof. Jin Woo Kim: jinwoo@catholic.ac.kr.

Conflict of interest

The authors declare that they have no competing interests.

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