

The Expressions of CD44, CD90 and Alpha Fetoprotein Biomarkers in Indonesian Patients with Advanced Liver Disease: an Observational Study

Syifa Mustika, Hengki Wijaya, Bogi Pratomo

Departement of Internal Medicine Faculty of Medicine, Brawijaya University - dr. Saiful Anwar Hospital, Malang, Indonesia.

Corresponding Author:

Syifa Mustika, MD. Gastroenterohepatology Division, Departement of Internal Medicine, Faculty of Medicine, Brawijaya University - dr. Saiful Anwar Hospital. Jalan Jaks Agung Suprpto no. 2, Malang 65112, Indonesia. email: drtika_78@yahoo.com cc drtika_78@ub.ac.id.

ABSTRAK

Latar belakang: meningkatnya kadar alfa fetoprotein (AFP) serum seringkali ditemui pada pasien karsinoma sel hati (KSH) tahap lanjut. Cluster Differentiation 44 (CD44) dan CD90 adalah biomarker sel punca yang diduga merupakan penanda awal KSH dan berhubungan dengan onset dan progresivitas KSH. Hingga kini masih belum ada studi terkait sel punca KSH di Indonesia. Studi ini bertujuan untuk mengevaluasi ekspresi penanda sel punca kanker CD44, CD90 dan kadar AFP pada pasien dengan penyakit hati lanjut. **Metode:** studi observasional dilakukan pada 41 pasien dengan hepatitis B dan/ atau C kronis, sirosis hati, dan KSH di RSUD dr. Saiful Anwar Malang. Ekspresi CD44 dan CD90 dalam darah diukur menggunakan flow cytometri dan kadar AFP serum menggunakan ELISA. Data karakteristik pasien dilakukan analisa statistik bivariat dan multivariat (One way ANOVA, Mann-Whitney, Chi-Square, Kruskal-Wallis). Data CD44, CD90 dan AFP dianalisis menggunakan uji Kruskal Wallis dengan signifikansi $p < 0,05$, serta uji kekuatan diagnostik dengan analisis receiver operating characteristic (ROC). **Hasil:** subjek penelitian ini adalah 16 pasien hepatitis kronis, 15 pasien sirosis hepatis, dan 10 pasien KSH. CD44+CD90+ dan AFP pada ketiga kelompok mempunyai perbedaan signifikan ($p=0.001$; $p=0.000$), khususnya pada hepatitis kronis yang dibandingkan dengan sirosis hepatis ($p=0.002$; $p=0.000$) dan KSH ($p=0.002$; $p=0.000$). Analisa ROC menunjukkan kekuatan diagnostik paling baik pada kombinasi CD44+CD90+ dengan AFP (AUC=0.981; $p=0.000$);. **Kesimpulan:** ekspresi CD44+CD90+ dan kadar AFP serum pada pasien dengan KSH lebih tinggi dibandingkan hepatitis kronis dan sirosis hepatis. Kombinasi kedua parameter ini memiliki kekuatan diagnostik KSH yang paling baik.

Kata kunci: alfa fetoprotein (AFP), biomarker, hepatitis kronis, sirosis hepatis, karsinoma sel hati.

ABSTRACT

Background: increased serum alpha fetoprotein (AFP) levels are often found in patients with advanced hepatocellular carcinoma (HCC). Cluster Differentiation 44 (CD44) and CD90 are stem cell biomarkers that have been assumed as the early HCC markers and associated with onset and progressivity of HCC. The study related to HCC stem cell has not been available in Indonesia. The present study aimed to evaluate the expression of cancer stem cell markers (CD44, CD90) and AFP levels in patients with advanced liver disease. **Methods:** an observational study was conducted in 41 patients with chronic hepatitis B and/or C infection, liver cirrhosis, and HCC at dr. Saiful Anwar General Hospital. CD44 and CD90 expressions were measured with flow cytometry, and AFP serum levels with ELISA. Data on patient characteristics were evaluated using bivariate and multivariate statistical analysis (One-way ANOVA, Mann-Whitney, Chi-Square, Kruskal-Wallis). Data of CD44, CD90 and AFP were analyzed using Kruskal Wallis test with a significance value of $p < 0.05$.

and diagnostic power was analyzed using receiver operating characteristic (ROC). **Results:** the subjects of our study were 16 patients with chronic hepatitis, 15 patients with liver cirrhosis, and 10 patients with HCC. There was a significant difference regarding CD44+CD90+ and AFP among those three groups ($p=0.001$; $p=0.000$) specifically in chronic hepatitis compared to liver cirrhosis ($p=0.002$; $p=0.000$) and HCC ($p=0.002$; $p=0.000$) respectively. ROC analysis showed the best diagnostic power for the combination of CD44+CD90+ and AFP (AUC=0.981; $p=0.000$). **Conclusion:** there are higher expressions of CD44+CD90+ and serum AFP levels in patients with HCC compared to the other two groups (those with chronic hepatitis and liver cirrhosis). The combination of both parameters has the best diagnostic power of HCC.

Keywords: alpha fetoprotein (AFP), biomarker, chronic hepatitis, liver cirrhosis, hepatocellular carcinoma.

INTRODUCTION

Hepatocellular carcinoma (HCC) is the fifth most frequent cancer in the world and third most common cause of cancer death. The incidence of HCC in Asian countries is directly proportional to increased incidence of chronic hepatitis B and C.¹⁻³ Host factors, viral infection, lifestyle and diet may have impacts on the development of HCC and it may also have contribution on epigenetic changes involved in the development of normal stem cells programming during cellular differentiation. Epigenetic deregulation causes loss of stem cell differentiation during the course of cancer pathogenesis, which further develops into cancer stem cells (CSCs). The development of CSCs is affected by the activation of hepatocarcinogenesis signaling pathway. It is suspected that the progression of advanced liver disease into HCC occurs as a result of the ability of cancer stem cells to self-renew, proliferate and maintain tumor growth.^{4,5}

Regular monitoring of high-risk patients is one of the efforts aimed at early diagnosis of HCC and it has high likelihood of recovery. The regular monitoring consists of ultrasonography and/ or evaluation of tumor markers. Based on the APASL guideline, periodic monitoring of liver cirrhosis patients includes evaluating alpha fetoprotein (AFP) levels and monitoring using abdominal ultrasound every 6 months.³ While the AASLD guideline recommends only abdominal ultrasound for monitoring without evaluating serum AFP levels.^{6,7}

Serum alpha fetoprotein (AFP) levels is the most common diagnostic marker for HCC. AFP levels of greater than 200 ng/mL are highly suggestive of malignancy as they have been found

in 60-70 % of HCC patients. Nevertheless, it should be noted that a single AFP serum levels in regular monitoring has low sensitivity and specificity, and increased levels may not be found in patients with HCC who have tumor diameter of less than 3 cm. The limitations of AFP has lead to the use of ultrasound examination as an additional method for detecting HCC.⁷ The combination of AFP and ultrasound examination may be effective for screening and diagnosis of HCC; however, it is still limited for detection. It has been known that the sensitivity of ultrasonography in the early detection of HCC ranges from 45%; while the combination of ultrasonography and AFP ranges from 63%.⁸ It indicates the need for additional modalities in HCC detection and prognostic assessment.

CSCs may become another alternative HCC diagnosis as they have the capacity for self-renewal, proliferation and maintaining tumor growth.² CSCs can be marked using variety of biomarkers, such as: CD13, CD24, CD44, CD90, CD133, DLK1, and EpCAM. Expressions of these markers are associated with resistance to chemotherapy agents.⁹ Increased CD44 and CD90 expressions are thought to be associated with onset, progression, and early signs of HCC.^{1,2} Nowadays, the newly developed therapy using stem cell for liver disease also carry the risk and the potential to develop into CSCs.¹⁰ Until now, no research has been conducted in Indonesia that studied about HCC stem cells as a potential early diagnostic test that may affect patient outcomes. Our cohort study explored the value of HCC stem cell marker expression in relation to advanced liver disease.

METHODS

Our cross-sectional study was conducted between 2016 and 2017 at dr. Saiful Anwar General Hospital Malang, Indonesia. Subjects were 41 patients with chronic hepatitis B and/or C infection, either with or without liver cirrhosis and HCC. Subjects were divided into 3 groups: a chronic hepatitis group, a liver cirrhosis group and a HCC group based on a series of physical examinations and investigations. The investigations included complete blood count, liver function, physiological hemostasis as well as HBsAg and anti HCV serological tests, AFP, abdominal ultrasound and abdominal CT scan whenever necessary. Diagnosis of hepatitis B and/or C was made if HBsAg and/or anti HCV was reactive. Liver cirrhosis was defined if there was a decreased in synthetic liver function and detoxification capability, which was also marked by portal hypertension, portal vein and collateral circulation. HCC was established when serum AFP levels were ≥ 200 ng/ml and nodules were present in the liver on ultrasound examination or abdominal CT scan. Inclusion criteria were: age >20 years, patients were diagnosed with chronic hepatitis B and/or C infection without or with liver cirrhosis or HCC. The exclusion criteria were had a malignancy other than HCC or metastasis in the liver, and pregnancy.

Venous blood samples (10 mL) were obtained from each subject and then were collected in heparinized tubes. The first 5 cc of venous blood samples were used for examining CD44 and CD90 expressions, which was performed at the Physiology Laboratory of Faculty of Medicine, University of Brawijaya Malang. The examination included the isolation of peripheral blood mononuclear cells, which was followed by staining process using PE anti-human CD44 produced by Bio-Legend and FITC anti-human CD90 produced by Bio-Legend of Gamma Scientific Biolab Ltd., and the reading was performed using flow cytometer by BD FACS Calibur. The remaining 5 cc of venous blood sample were sent for serum AFP test using ELISA method to Prodia Laboratory in Malang. The normal range of serum AFP levels is < 20 ng/mL.¹¹ CD44+ expression in peripheral blood of healthy subject was $46.1 \pm 13.4\%$;¹² while

CD90+ expression in cultured mononuclear cells of healthy subjects was 0.48% .¹³

Data on subject characteristics were presented as mean (SD) and percentage. One way ANOVA was performed when there was normal data distribution; while Kruskal Wallis test was used when the distribution was not normal. Chi Square tests were used for both nominal scale parameters. Mann Whitney test was performed when the criteria for Chi Square test was not fulfilled. Data was considered significant when $p < 0.05$.

CSCs and AFP data were presented as mean and interval quartile range (IQR) since there was a wide range of data. Kruskal Wallis test was used since the distribution of data was not normal and the data was considered significant when $p < 0.05$. The test was then followed by the post-hoc Mann Whitney test.

ROC curve analysis was performed for CSCs and AFP parameters. Parameters with a significant value $p < 0.05$ were evaluated further to measure the cut-off value using the Youden's index and their sensitivities and specificities were determined.

This study has been approved by the Ethics Committee of dr. Saiful Anwar Hospital with a reference number 400/79/K.3/302/2017.

RESULTS

Sixteen patients had chronic hepatitis, 15 had liver cirrhosis, and 10 had HCC. All of them had no history of alcohol consumption. Other characteristics are shown in **Table 1**.

Comparison of Stem Cell Marker Expression on Blood Mononuclear Cells and Serum AFP Levels in Patients with Advanced Liver Disease

Expression of CD44+CD90+ was significantly different among groups ($p=0.001$); however, the expression of CD44+CD90- and CD44-CD90+ were not.

The mean of CD44+CD90+ expression had a tendency to increase with disease progressivity. We found a significant difference when we compared the chronic hepatitis group and the liver cirrhosis group ($p=0.002$) as well as when we compared the hepatitis group and the HCC group ($p=0.002$). In contrast, there was no significant difference between the cirrhosis group

and the HCC group ($p=0.657$).

A comparative analysis of serum AFP levels using a cut off of 200 ng/mL among chronic hepatitis group, liver cirrhosis and HCC revealed significant difference ($p=0.000$). Post hoc analysis showed that there was significant difference between chronic hepatitis group compared to liver cirrhosis ($p=0.000$), as well as in the hepatitis group compared to the HCC group ($p=0.000$). But there was no significant difference between the cirrhosis group and HCC

($p=0.185$). Data of CSCs expressions and serum AFP levels are shown in **Table 2**.

Comparison between Diagnostics Power of Cancer Stem Cell and AFP

ROC test was generated to analyze the diagnostic power of CSCs and AFP. Results are shown in **Figure 1-5**. We found that CD44+CD90- and CD44-CD90+ did not have significant p value and AUC value was low ($p=0.903$, $AUC=0.487$ and $p=0.249$, $AUC=0.377$) (**Figure 1 and Figure 2**); while CD44+CD90+ and AFP

Table 1. Subject characteristics

Clinical Characteristics	Advanced Liver Disease			p [^]
	Chronic Hepatitis (n=16)	Liver Cirrhosis (n=15)	Hepatocellular Carcinoma (n=10)	
Age, mean (SD)	42,68 (14.84)	52,2 (9.39)	60,10 (10.51)	0.003*
Sex				
- Male	10 (62.5%)	14 (93.33)	9 (90.0)	0.262#
- Female	6 (37.5%)	1 (6.67)	1 (10.0)	
Antiviral Hepatitis Therapy				
- Yes	14 (87.5%)	5 (33.33)	0 (0.0)	0.000†
- No	2 (12.5%)	10 (66.67)	10 (100)	
Length of therapy (weeks), mean (SD)	62.81 (62.41)	14.93 (31.8)	0	0.000‡
Smoking Status, n (%)				
- Not smoking	10 (62.5)	7 (46.67)	4 (40.0)	
- Mild (4.29 sticks/day)	2 (12.5)	2 (13.33)	1 (10.0)	0.223‡
- Moderate (12 sticks/days)	4 (25.0)	6 (40.0)	2 (20.0)	
- Heavy (32 sticks/day)	0 (0.0)	0 (0.0)	3 (30.0)	
Duration of smoking (years), mean (SD)	8.25 (12.90)	14.07 (15.93)	21.8 (20.58)	0.204‡
Body Mass Index (kg/m ²), n (%)				
- Underweight	1 (6.3)	1 (6.7)	6 (60.0)	
- Normal	6 (37.5)	7 (46.7)	3 (30.0)	
- Overweight	3 (18.8)	0 (0.0)	1 (10.0)	0.009‡
- Obesity 1	5 (31.3)	6 (40.0)	0 (0.0)	
- Obesity 2	1 (6.3)	1 (6.7)	0 (0.0)	
Viral Hepatitis Infection Type, n (%)				
- Hepatitis B	11 (68.8)	11 (73.3)	10 (100.0)	
- Hepatitis C	5 (31.3)	3 (20.0)	0 (0.0)	0.161‡
- Hepatitis B and C	0 (0.0)	1 (6.7)	0 (0.0)	
AST (U/L), mean (SD)	63.25 (37.68)	73 (54.27)	143.2 (108.32)	0.023‡
ALT (U/L), mean (SD)	60.94 (39.66)	69.13 (72.97)	54.8 (48.17)	0.671‡
Albumin serum (g/dL), mean (SD)	3.9 (0.10)	3.09 (0.22)	3.07 (0.26)	0.002*
Child Turcotte Pugh Score, n (%)				
- A		8 (53.3)	3 (30.0)	
- B		6 (40)	5 (50.0)	0.201#
- C		1 (6.7)	2 (20.0)	

[^] Significant with $p<0.05$; * One-way ANOVA test; † Chi-square test; ‡ Kruskal-Wallis test; # Mann-Whitney test.

Table 2. CSCs expressions and serum AFP levels in patients with advanced liver disease

Blood Mononuclear Cell Marker Expression	Chronic Hepatitis (n=16)	Liver Cirrhosis (n=15)	Hepatocellular Carcinoma (n=10)	p [^]
CD44+CD90- (% gated), median (IQR)	9.55 (0.038-1.04)**	22.37 (0.48-28.78)	8.68 (0.26-17.67)	0.09 ‡
CD44-CD90+ (% gated), median (IQR)	11.86 (5.97-22.04)	7.32 (2.34-10.32)	5.45 (1.85-6.36)	0.225 ‡
CD44+CD90+ (% gated), median (IQR)	0.73 (0.28-1.19)	8.29 (1.38-6.92)**	10.35 (1.77-10.72)	0.001 ‡
Serum α-fetoprotein, median (IQR)	7.25 (1.47-8.20)	27.00 (3.20-30.00)	13088.55 (216.25-23914.00)	0.000 ‡
- ≤ 200 ng/mL, n (%)	16 (100.0)	15 (100.0)	3 (30.0)	
- > 200 ng/mL, n (%)	0 (0.0)	0 (0.0)	7 (70.0)	

[^] Significant with p<0.05; ‡ Kruskal-Wallis test; ** Mean value was higher than Q3

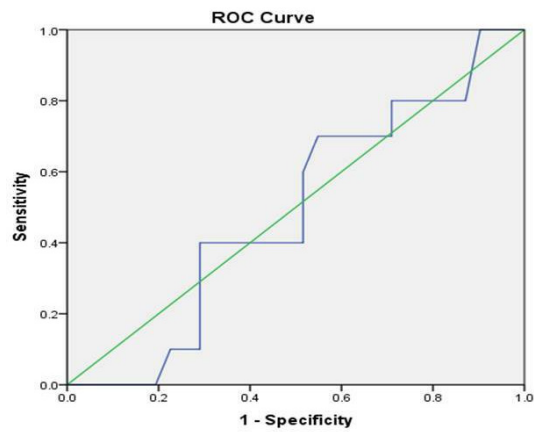


Figure 1. ROC curve of CD44+CD90- (p=0.903, AUC=0.487)

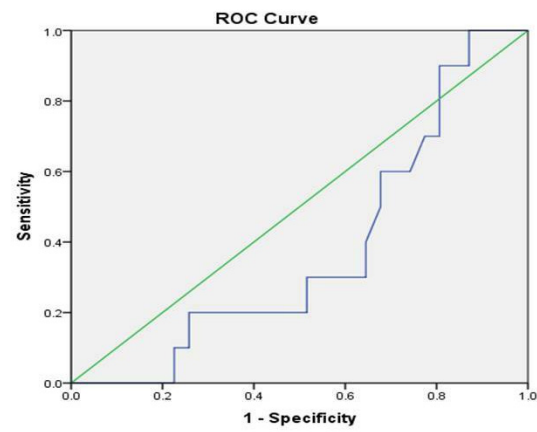


Figure 2. ROC curve of CD44-CD90+ (p=0.249, AUC=0.377)

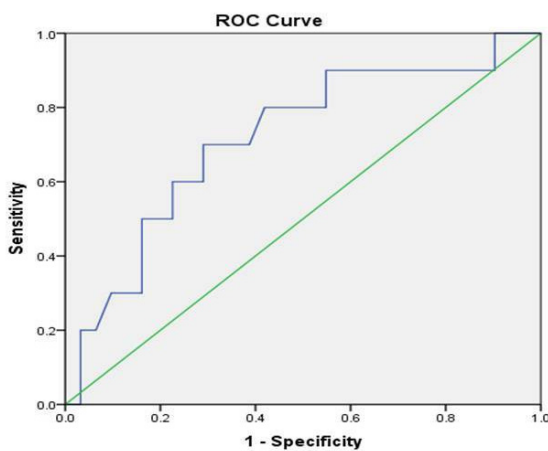


Figure 3. ROC curve of CD44+CD90+ (p=0.042, AUC=0.716)

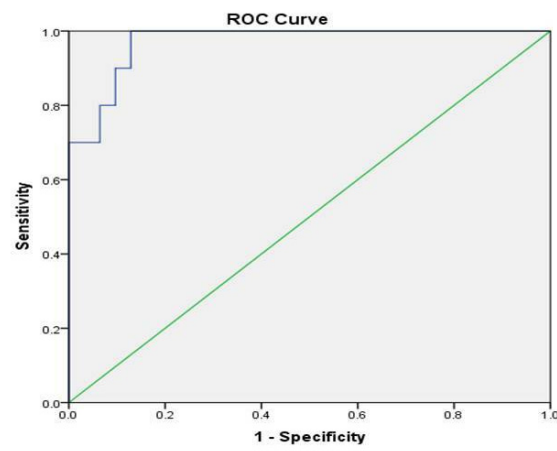


Figure 4. ROC curve of AFP (p=0.000, AUC=0.971)

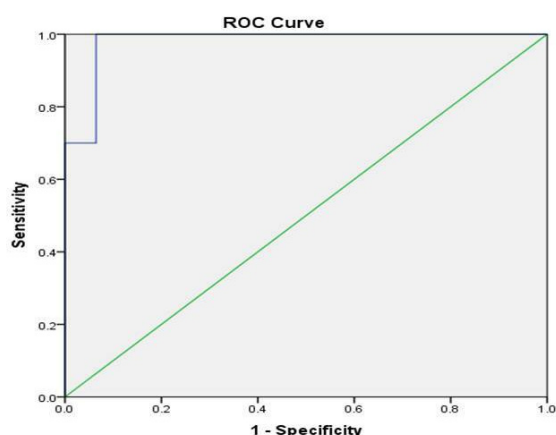


Figure 5. ROC curve of CD44+CD90+ with AFP ($p=0.000$, AUC=0.981)

had significant p value in addition to moderate and high value of AUC ($p=0.042$, AUC=0.716 and $p=0.000$, AUC=0.971) (**Figure 3** and **Figure 4**). The results were analyzed further to compare the diagnostic power of CD44+CD90+ only (**Figure 3**), AFP only (**Figure 4**) and combination of both (**Figure 5**). Our study showed that the combination of CD44+CD90+ with AFP had significant results with very high diagnostic power ($p=0.000$, AUC=0.981). Data was further evaluated to measure the cut-off value using the Youden's index; moreover, the sensitivity and specificity were determined. Detailed results are shown in **Table 3**.

DISCUSSION

Comparison of Cancer Stem Cells and Serum AFP levels in Patients With Advanced Liver Disease

There was no significant difference of CD44+CD90- expression among the chronic hepatitis, liver cirrhosis and HCC groups. Naor et al in their previous study showed that single expression of CD44+ between cancer and control groups did not significantly differ.¹⁴ Jaggupilli et

al also showed that single expression of CD44+ is not a marker for carcinogenesis since the CD44+ is expressed in almost all normal cells as well as cancer cells.¹⁵ A recent study showed that CD44-CD90+ expression among patients with chronic hepatitis, liver cirrhosis and HCC did not differ significantly. Sun et al. demonstrated that progenitor cells or normal adult hepatic stem cells also express CD90+.¹⁶ However, Yang et al explained that blood samples of HCC patients with normal serum AFP levels expressing CD45-CD90+ still indicates the presence of cancer stem cells.¹⁷

Our study demonstrated that there were significantly different expressions of CD44+CD90+ among the HCC, chronic hepatitis and liver cirrhosis groups. Romano et al showed that CD44+ expression along with other CSCs markers could better determine HCC phenotype of stem cell surface; moreover, they also showed that double expression of CD44 and CD90 defined more aggressive phenotype of HCC.¹⁸ Yang et al¹¹ conducted a multiparametric analysis and found that most HCC cells that have CD90+ expression also have CD44+ expression. Sun-et al.¹⁶ suggested that cells with CD44+CD90+ expressions have more aggressive behavior than CD44-CD90+ cells and have higher levels of tumorigenicity and ability to form metastatic lesions in those with immunodeficiency conditions.

Our results regarding serum AFP levels in HCC group, which was compared to the serum levels in chronic hepatitis and liver cirrhosis groups, was substantially different from the results in a study conducted by Marrero et al that showed increased serum AFP levels in HCC patients.¹⁹ AFP serum levels < 400 ng/mL indicates a low sensitivity in detection of HCC, which may explain that a third of HCC patients may be undetected and have subclinical

Table 3. ROC curve analysis and cut off value of CD44+CD90+, AFP, and combination of CD44+CD90+ and AFP

Test	Cut off value	AUC	SE	Sensitivity	Specificity	P value	95 % CI
CD44+CD90+	1.77% gated	0.716	0.095			0.042	0.529 - 0.903
AFP	144.3 ng/mL	0.971	0.022	70%	100%	0.000	0.927 - 1.000
CD44+CD90+ and AFP	1.77% gated and or 144.3 ng/mL	0.981	0.017	100%	93.55%	0.000	0.946 - 1.000

AUC: Area Under Curve; SE: Standard Error; CI: Confidence Interval

diagnosis.⁶ Thus, AFP serum levels are not associated with HCC prognosis such as tumor size, stage, or disease progression.¹⁸

Comparison of the Diagnostics Power of Cancer Stem Cell with AFP

Our study showed the smallest size of AUC for CD44+CD90+ biomarker and the greatest size for the combination of CD44+CD90+ and AFP levels which indicates that the combined biomarkers have the highest value sensitivity and specificity. The cut off value of CD44+CD90+ was 1.77% gated, which was based on the Youden's index. Such range was also found in the liver cirrhosis group, but not in the hepatitis group. There was a wide range of values for CD44+CD90+ measurement and the sensitivity was as low as the sensitivity of AFP levels; however, the specificity was lower than the specificity of AFP levels. It might occur due to less homogeneous samples and small sample size.

In our study, we found a great size of AUC, which was 0.971 for AFP levels as a biomarker with a sensitivity of 70% and an optimal cut-off value of 144.6 ng/mL. An earlier study suggested a range of cut off value of AFP serum level for diagnosis of HCC starting from 20 ng/mL with a sensitivity of 62% and specificity of 90.2%.²⁰ Another study demonstrated that the cut off value of AFP serum levels for diagnosis of HCC was 20.45 ng/mL with 73% sensitivity and 92% specificity.²¹ These findings support the results of our study and we can say that although AFP has still been widely used as a diagnostic tool of HCC, but it is not a sensitive test. Increased AFP serum levels are often found in HCC with large tumors, spread to biliary system, massive and diffuse and presence of portal venous thrombosis.¹⁸ It suggests that AFP as a single tools for early diagnosis of HCC is poor and therefore, we require a combination of alternative agents such as a cancer stem cells biomarkers.

The combination of serum AFP levels and CD44+CD90+ expressions appears to be the tests that have the best sensitivity and specificity. Moreover, we can say that it is the best diagnostic parameter compared to AFP levels only or CD44+CD90+ expression only. Liu et al. conducted a meta-analysis of studies

regarding the relationship between cancer stem cell expression and differentiation of liver cell carcinoma. The study found that there was a relationship between CD44, CD90, CD133, EpCAM cancer stem cells with high AFP values in patients with liver cell carcinoma (OR 1.63, 95% CI=1.36-1.95, $p < 0.00001$).²² Stem cells cancer has heterogeneity and complexity related to biological structure and its nature. Studies using liver cancer stem cell markers in liver cell carcinoma with the same cell pathway, will provide comprehensive screening of liver cancer stem cell images.²³ Therefore, the expressions of CD44+CD90+, which show significant different results among population of patients with chronic hepatitis, liver cirrhosis, and liver cell carcinoma, may serve as the biomarkers for diagnosis, prediction, prognosis and indicators of progression of liver disease, particularly when it is used in combination with AFP levels. Patients with chronic hepatitis, hepatic cirrhosis, and liver cell carcinoma who have CD44+CD90+ cancer stem cell expression will be at risk of developing progressive liver disease.

Our study was limited by the small numbers of samples. Moreover, another limitation to our study is that we only performed a single examination of CD44 and CD90 expression. We could not evaluate the role of cancer stem cell markers as a prognostic factor of HCC yet. We hope that results of our cohort study may also enlighten our colleagues to have better understanding about cancer stem cell marker as potential prognostic tool for advanced liver disease.

CONCLUSION

There are higher expressions of CD44+CD90+ cancer stem cells (CSCs) markers and serum AFP levels in patients with HCC compared to those with chronic hepatitis and liver cirrhosis. The combination of CD44+CD90+ expressions and AFP has the best diagnostic power of HCC.

ACKNOWLEDGMENTS

We would like to express our gratitude for the support we have received from Faculty of Medicine of Brawijaya University on our project in the hepatology stem cell workgroups.

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