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Soluble Vascular Endothelial Growth Factor Receptor-2 as a Predictive Factor for Progression of Illness in Chronic Liver Diseases and Hepatocellular Carcinoma

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Angiogenesis is generally induced in the process of necro-inflammation and regeneration in chronic liver diseases (CLD). Whereas VEGF is a major humoral factor in relation to neo-vascularization, the receptor, VEGFR-2, is located in hepatocytes and sinusoid endothelial cells. The aim in this study is to investigate the significance of soluble form of VEGFR-2 (sVEGFR-2) in various CLDs.

A cross sectional study was conducted from 2010 to 2013 at Dr. Sardjito Hospital Yogyakarta, Indonesia. 149 patients with chronic hepatitis (CH), liver cirrhosis (LC) or hepatocellular carcinoma (HCC) were enrolled in this study. sVEGFR-2 serum was examined using Quantikine®HS kit human immunoassay. Data were analyses by STATA (P value <0.05). The median of sVEGFR-2 was decreased according to the disease progression (LC: 7014.95 pg/mL; CH: 8805.15 pg/mL; healthy subject: 9785.2 pg/mL). However, sVEGFR-2 in HCC (8043.73 pg/mL) was significantly higher than that in LC (P= 0.0059). Based on AUROC analyses, the clinical cut-off point of sVEGFR-2 with >80% sensitivity was used (CH-LC ≤7236.7, LC-HCC ≥7215). The odds ratio (OR) LC to HCC was 5.87 and CH to LC was 4.63. The significant correlations were showed significantly between sVEGFR-2 with MELD and ALT in LC, and with APRI and FIB-4 in CH. In conclusion, the serum sVEGFR-2 could be used as a predictive factor progressing CH to LC, but not HCC.

Liver cirrhosis (LC) is an end-stage of chronic hepatitis and frequently induced by chronic infection of hepatitis B virus (HBV) and hepatitis C virus (HCV). In addition, hepatocellular carcinoma (HCC) is sometimes generated because HBV and HCV are directly and/or indirectly mediate the mechanism of hepato-carcinogenesis.[1-5] Whereas as many as 240-360 million people are suffering from chronic hepatitis B, 150-170 million people are infected with chronic hepatitis C (CHC).[6-9] Especially in developing countries, the incidence of LC and HCC due to HBV is relatively prevalent.[10]

Chronic infection of hepatitis viruses induce the necro-inflammation of hepatocytes and finally reach to liver cirrhosis. In the chronic hepatitis, the process of neo-angiogenesis is generally induced as a result of the insufficient nutrition and oxygen in the liver tissues. The neo-angiogenesis is also activated in the growth of liver cancer. The vascular endothelial growth factor (VEGF) is the regulator of angiogenesis besides angiopoietin and endostatin.[11,12]

The bond between VEGF and its receptors (VEGFR) will initiate the process of down regulation, trigger the mitogen-activated protein kinase (MAPK) signaling pathway during the angiogenesis, proliferation, and metastasis.[13-15] The increased expression of VEGF in the cirrhotic liver is resulted from the stimulation of fibroblast in the formation of the fibrotic tissues, whereas it is resulted from the invasion and intrahepatic metastasis in liver cancer.[16-18] The expressions of VEGF and VEGFR in the liver tissues generally correlate with the levels in the serum and plasma. The level of the VEGF serum is affected by the platelet, leucocyte, cytokine, extracellular matrix component, fibronectin, fibrin and thrombospodin-1.[19-20]

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However, the analysis of VEGFR-2 expression is usually difficult because the majority of patients refused biopsy and very few HCC patients underwent liver transplantation. Previous research dealt with soluble VEGFR-2 (sVEGFR-2) revealed as a better predictor of therapy response than other seromarkers of angiogenesis.[21-22] They reported that sVEGFR-2 in the serum was correlated with the progression from chronic hepatitis to cirrhosis and HCC. The aim in this study was to identify the difference of the sVEGFR-2 in the serum among CH, LC, and HCC patients and their correlation with the severity of diseases.

MATERIALS AND METHODS

Study subjects

A cross-sectional study was conducted from 2010 to 2013 at the Dr. Sardjito General Hospital and polyclinic in Yogyakarta, Indonesia. 149 patients including 39 CH, 39 LC and 46 HCC were enrolled in this study. Patients with positive for HBs antigen (HBsAg) or anti-HCV antibody were diagnosed as chronic hepatitis (CH). LC was diagnosed by clinical symptoms, laboratory findings and imaging study. HCC was diagnosed by imaging study (ultrasonography and/or computed tomography) and histologically confirmed by fine needle biopsy. In cases without histological confirmation, HCC was diagnosed as a hypervascular lesion over 2cm by two imaging examinations using ultrasonography and CT scanning, otherwise one imaging examination and increased level of alpha fetoprotein (AFP) over 400ng/ml. All subjects were aged >18, clinically stable. As for the in-patients by gastrointestinal bleeding, they were included in this study after stopped bleeding more than one week. The exclusion criteria were subjects with severe sepsis, comorbid with other diseases such as chronic kidney disease, heart failure, obstructive lung disease and non-liver malignancy. 27 healthy controls were also enrolled and examined about serological variables. All of subjects read and signed the informed consent before enrolled in this study.

This study protocol was approved by the Ethic Commission of Medical and Health Research of the Faculty of Medicine, Gadjah Mada University based on the ethical principles outlined in the Declaration of Helsinki 2008. This study protocol was given a permission letter from the Director of Dr. Sardjito General Hospital, Yogyakarta, Indonesia.

Measurement of sVEGFR-2 and clinical characteristics

The serum sVEGF-R2 level was examined using ELISA (Quantikine® HS kit, human immunoassay R&D System, Minneapolis, USA). To evaluate the severity of disease, fibrosis-4 (FIB-4) and aspartate aminotransferase to platelet ratio index (APRI) score were examined among CH patients, platelet count, Child Pugh Turcotte (CPT) score, and modified end state liver disease (MELD) score were evaluated on LC patients, and Cancer of the Liver Italian Program (CLIP) score, AFP, and Okuda stage were evaluated on HCC subjects

Statistical Analyses

The data were analyzed using STATA 11.0 (Stata Corp, Texas USA). Unpaired *t* tests and analyses of variance were used to determine the differences in variables displaying normal distribution. The Mann-Whitney *U* test and Kruskal Wallis test were used for variables that did not display a normal distribution. Chi^2 and Fisher exact tests were used for the nominal variables. Spearman's ρ coefficient test was counted to find out the correlation between variables. The area under receiver operating characteristic (AUROC) curves were used for calculating the cut-off value of serum sVEGFR-2. The >80% sensitivity of the curve was used for the clinical cut-off point due to clinical screening. Estimation of prediction factor used 2x2-table test for determining the Odds ratio (OR). $P<0.05$ value and 95% confidence intervals (CI) were used to evaluate the significance.

RESULTS

Clinical characteristics of study subjects

The baseline characteristics in this study were summarized in Table I. There was no difference of age and sex among four groups. The prevalence of male was much higher than that of female in all groups. HBV infection was etiologically most frequent in all groups. Subjects without HBV and HCV infections were only detected in LC and HCC groups.

Table I. Base line characteristic

Variables	HCC (n=46)	LC (n=38)	CH (n=38)	Healthy (n=27)	*P*
Age (year)	52.4±12.8	54.7±11.7	48.3±14.0	47.9±12.1	0.468[$]
Gender (M/F)	32/14	26/12	18/20	15/12	0.139[*]
Etiology (n)					
B/C/B+C/non B&C	26/5/0/15	19/8/1/10	25/13/0/0	-	0.483[†]
PLT (10^3/mm^3)	241.5(60;359)	98(40;307)	173(23;373)	-	0.0001[#]
AST (IU/mL)	129(22;737)	56(14;206)	60.5(18;486)	-	0.0001[#]
ALT (IU/mL)	54(17;284)	33(11;261)	63.5(14.2;881)	-	0.0007[#]
CPT (A/B/C)	17/22/7	10/22/7	-	-	0.536[*]
MELD score	-	11(6;46)	-	-	-
APRI score	-	-	0.94(0.36;13.17)	-	-
FIB-4 score	-	-	1.87(0.64;28,09)	-	-
AFP (n)					
<400/ ≥400 (ng/mL)	15/31	-	-	-	-

[$]Anova test; [#]Kruskal Wallis test; [*]Chi[2] test; [†]Fisher exact test; Significant *P*<0.05;PLT: platelet; AST: aspartate aminotransferase; ALT: alanine aminotransferase; CPT: Child Pugh Turcotte; MELD: Model of End Stage Liver Disease; APRI: AST to platelet ratio index; FIB4: Fibrosis-4; AFP: alpha feto protein; LC: liver cirrhotic; HCC: Hepatocellular carcinoma; CH: chronic hepatitis

Comparison of soluble VEGFR-2 in the serum

There were significant differences of the median of serum sVEGFR-2 level between HCC, LC, CH and healthy subjects (P=0.0001). The median of serum sVEGFR-2 in HCC (8043.73; min 3458.2; max 28062.3), LC (7014.95; min 3722.3; max 11439.5) and CH (8805.15; min 5892.5; max 15807.2) were lower than those in healthy subjects (9785.2; min 6456.8; max 13506.2). However, the median of sVEGFR-2 serum in HCC was lower than that in CH (P= 0.239) but significantly higher than that in LC (table II and figure 1).

Table II. Difference of sVEGFR-2 serum in clinical severity

		sVEGFR-2 serum (pg/mL)				*P*
		n	Median	Min; max	Mean±SD	
Diagnosis						0.0001[*]
HCC		46	8043.73	3458.2 ; 28062.3		
LC		38	7014.95	3722.3 ; 11439.5		
CH		38	8805.15	5892.5 ; 15807.2		
Healthy		27	9785.2	6456.8 ; 13506.2		
CPT (LC)						0.969[#]
A		10			6774.93±1306.59	
B		21			7090.05±1448.03	
C		7			8413.57±1509.04	
Staging (HCC)						
CLIP	0-2	13	8257.20	5400,7 ; 11274,3		0.864[$]
	3-6	33	7938.60	3458,2 ; 28062,3		
BCLC	C	30	7938.60	4945 ; 21322.4		0.601[$]
	D	36	8210.30	3458.2 ; 28062.3		
AFP	<400 ng/mL	15	7762.10	5400.7 ; 14738.2		0.170[$]
	≥400ng/mL	31	8148.90	3458.2 ; 28062.3		

[*)]Kruskal Wallis test; [#)]Anova test; [$)]Mann Whitney test; significant *P*<0.05; LC: liver cirrhotic; HCC: Hepatocellular carcinoma; CH: chronic hepatitis; CLIP: Cancer of the Liver Italian Program; BCLC: Barcelona Clinic Liver Cancer Group; CPT: Child Pugh Turcotte; AFP: alpha feto protein

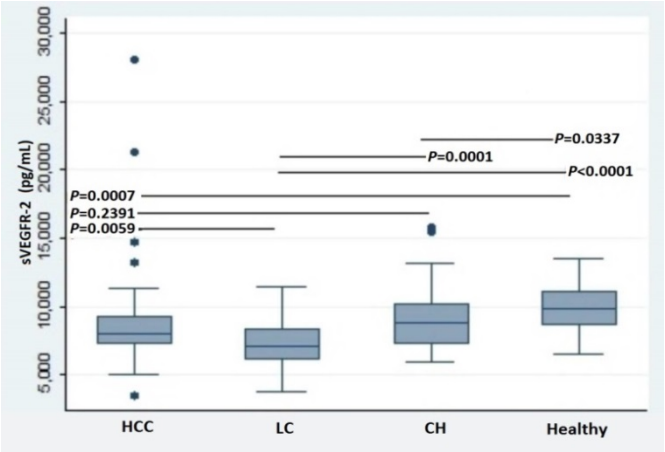

Figure 1.
Based on statistical analyses (Kruskal-Wallis test and Mann Whitney test), there were significant difference level of soluble vascular endothelial growth factor receptor 2 (sVEGFR-2) between hepatocellular carcinoma (HCC), liver cirrhosis (LC) and chronic hepatitis (CH) compared to healthy (P <0.05). However, there was showed no difference level of sVEGFR-2 serum in HCC compared to CH (P >0.05)

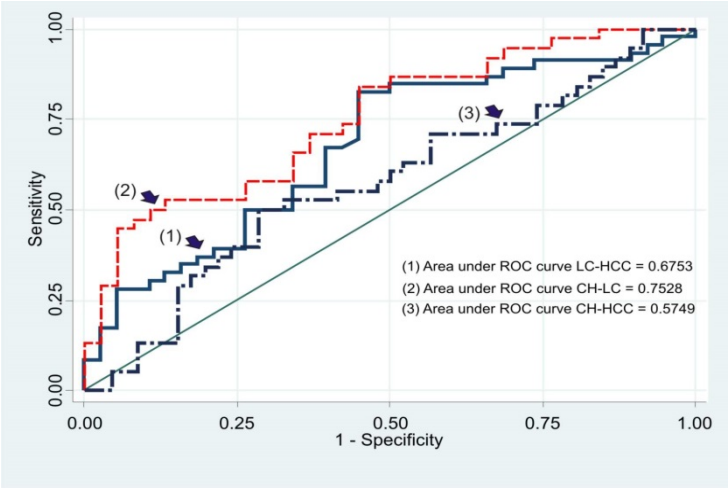

Figure 2.
The area under receiver operating characteristic (AUROC) curve analyses of serum sVEGFR-2 showed mild discrimination of sVEGFR-2 value in chronic hepatitis (CH) to liver cirrhosis (LC)(75.28%); weak discrimination in liver cirrhosis (LC) to hepatocellular carcinoma (HCC)(67.53%); and weak discrimination in chronic hepatitis (CH) to hepatocellular carcinoma (HCC) (57.49%). The clinical cut off points of serum sVEGFR-2 were chosen using >80% sensitivity due to screening aims.

Using the receiver operating characteristic (ROC) curve and area under curve (AUC) (figure 2 and table III), the clinical cut-off point of sVEGFR-2 serum was chosen with >80% sensitivity due to screening aimed. The cut-off values of serum sVEGFR-2 were LC to HCC= 7215 pg/mL; CH to HCC= 7236.7 pg/mL; and CH to LC= 7236.7 pg/mL. The odds ratio (OR) value were: LC to HCC= 5.87 (95%CI 1.969-18.224); CH to HCC= 1.23 (95% CI 0.370-4.290) and CH to LC= 4.63 (95% CI 1.528-14.621).

Tabel III. The Odds Ratio analyses based on cut-off value serum sVEGFR-2

		HCC	LC	OR	95% CI
Cut-off value serum sVEGFR-2 (pg/mL)	≥7215	38	17	5.87	1.969-18.224
	<7215	8	21		
		HCC	CH	OR	95% CI
	≤7236.7	10	7	1.23	0.370-4.290
	>7236.7	36	31		
		LC	CH	OR	95% CI
	≤7236.7	21	8	4.63	1.529-14.621
	>7236.7	17	30		

HCC: hepaatocellular carcinoma; LC: liver cirrhosis; CH: chronic hepatitis; OR: Odds ratio; CI: convidence interval

Association between sVEGFR-2 level and variables

There were no differences of levels of sVEGFR-2 from severity categories of LC and HCC (Table 2). The positive correlation between sVEGFR-2 with MELD score (ρ= 0.46; P= 0.004), CPT score (ρ= 0.35; P= 0.029) and ALT (ρ= 0.33; P= 0.049) were shown in LC subjects (figure 3). And the negative correlation between

sVEGFR-2 with APRI score (ρ= -0.37; *P*= 0.042) and FIB4 score (ρ= -0.36; *P*= 0.049) were shown in CH subjects (figure 4).

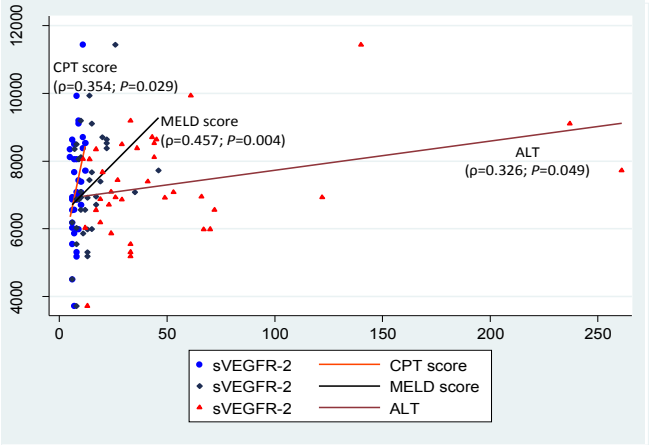

Figure 3.
Increasing of sVEGFR-2 level in liver cirrhosis was correlated with increasing of Child Pugh Turcotte (CPT) score, Model of End-Stage Liver Disease (MELD) score and alanine aminotransferase (ALT) level, significantly.

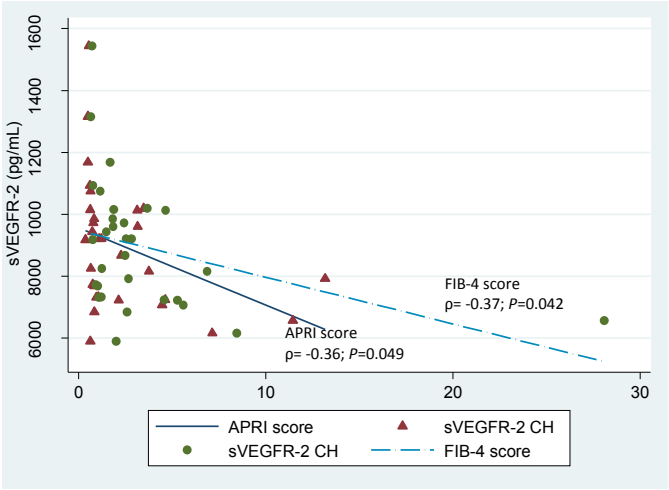

Figure 4.
Increasing of sVEGFR-2 level in chronic hepatitis (CH) was correlated with decreasing score of APRI and FIB-4, significantly.

DISCUSSION

The cancer related death is a major public health problem in Indonesia and accounts for the seventh largest cause of death. In present study, the average age was approximately fifth decade and the prevalence of male was more frequent than that of women in all groups. The distribution of age and sex was almost similar compared with previous studies. [17,23-25] The most frequent etiology in all subjects was hepatitis B virus (HCC 56.52%; LC 50%; CH 65.79%). The research results correspond to a report of the national basic health research that the prevalence of hepatitis in Indonesia has increased twofold from 0.6% in 2007 to 1.2% in 2012 (19.3% HAV, 21.8% HBV, 2.5% HCV, and 1.8% others respectively).[25]

VEGF and its receptor, VEGFR-2, are important angiogenic factors in several cancers and the measurement of these factors has been useful for the tumor growth and prognosis. sVEGFR-2 and VEGF serum are representative angiogenesis soluble factors (ASF) in which the increase in the serum level of VEGF will be followed by a decrease in the level of sVEGFR-2, or the serum level of VEGF correlates negatively with the sVEGFR-2 level.[19] Because the binding of VEGF and VEGFR-2 has a down-regulation process, the increase of VEGFR in the serum will affect to the decrease level of sVEGFR-2.[26-27]

The soluble form of VEGFR-2 (sVEGFR-2) contains the extracellular domains of the receptor but lacks the tyrosine kinase domain. Recently, it was reported that sVEGFR-2 is a potential predictive biomarker for VEGF signaling inhibitors among several cancers, such as colorectal cancer and breast cancer.[26-27] The sVEGFR-2 level in present study among HCC, LC, and CH subjects was lower than that in the healthy subjects. This result could

be suggested that the expression of VEGF and VEGFR-2 on the hepatic tissues among HCC, LC, and CH subjects was increased compared with healthy control, although we did not evaluate VEGF in the serum and the expression of VEGF and VEGFR-2 in the hepatic tissues.

The sVEGFR-2 levels in LC subjects were the lowest among other subjects, and it was suggested that the expression of VEGF and VEGFR-2 in LC tissues would be increased; otherwise the process of fibrogenesis was more dominant compared with the neo-angiogenesis process in the LC subjects. Previous report also supported that the expression of VEGFR-2 of the hepatic tissues increased in the hepatitis and cirrhosis subjects.[28] The increased expression of the angiogenesis and fibrogenesis factors in cirrhosis occurs in a condition when the hepatocyte undergoes regeneration, not in the fibroblast of the hepatic fibrosis.[16]

The increase in the expression of VEGF and VEGFR-2 in the cirrhotic tissues indicates the neo-angiogenesis process resulting in the generation of HCC. Previous research revealed that the expression of VEGFR-2 was increased in non-tumor cirrhotic tissues, but it was not connected with the existence of tumor invasion and relapse after transplantation, suggested of the poorly up-regulation process in the differentiation and progressivity of tumor.[29] In the cirrhosis with nodular lesion, the expression of VEGF was increased in comparison with lesion of non-tumor but it is lower than the lesion of HCC, which is caused by a decrease of vascularization in the cirrhotic tissues compared with the HCC tissues.[18,29]

There has been no previous paper on a comparison between the sVEGFR-2 levels in cirrhosis, chronic hepatitis, and healthy subjects. The two previous papers studied the sVEGFR-2 level as a seromarker of relapse in HCC.[21,22] The previous two papers concluded that sVEGFR-2 as a response predictor of therapy in HCC was significant compared with the other seromarkers of angiogenesis (sVEGFR-1, VEGFR, angiotensin-2 (Ang-2) and placental growth factor (P1GF).

According to the ROC-AUC analyses, the clinical cut-off points was 7215 pg/mL between LC and HCC, 7237 pg/mL between CH and HCC, and 7237 pg/mL between CH and LC. The OR point was statistically significant (>1) between LC and HCC (5.87), and between CH and LC (4.63) groups. This result showed that sVEGFR-2 could be made as a predictive factor for the generation of HCC and the progression of LC from chronic hepatitis especially HBV and HCV. The hepatocyte of hepatitis B with pre-S mutation (ground glass hepatocytes/GGH) contains pre-S deletion mutants in the endoplasmic reticulum and restricts the activity of biologic cells, and it proves to activate the VEGF-A of the hepatic tissues and has the potential to change to pre-neoplastic cell with the risk of becoming carcinoma cell.[30-32]

The phenotypic change of DNA repair genes in cirrhosis and hepatic fibrosis that undergoes active inflammation is related to progressivity towards HCC.[33] As in CHB, CHC also risks progressivity towards cirrhosis and/or HCC. *In vitro* study showed that HCV could stimulate the activity of the gene promoter of VEGF as the consequence of the stability of the activity of hypoxia-inducible factor-1 (HIF-1) and inducted the expression and secretion of VEGF-A ligand.[34] Although present study did not show the positive correlation, it was reported that HCV-related HCC had a significant increased level of VEGF in comparison with HBV-related HCC, and VEGF levels in HCV subjects were higher than those in healthy subjects.[35] It was found, however, that there was a significant correlation, using the MELD, CPT and ALT. Significant correlation was also shown in CH subject using APRI and FIB4 score.

There are some limitations in this study. This research employs a hospital-based with cross sectional method, which cannot prove causality (cause-effect relationship), and is unable to represent real situations in the general population. Biopsy is an invasive act that is often avoided by subjects; an operative act (hepatectomy) and transplantation are very seldom conducted so that a researcher cannot obtain a sample of hepatic tissue to prove the expression of VEGF, VEGFR, and MVD in the hepatic tissue. There are still many factors that affect the angiogenesis process that were not examined in this research, such as: other angiogenesis factors (VEGF, sVEGFR-1, P1GF and Ang-2), genetic factor (VEGF polymorphisms) and environment.

In conclusions, there were significant differences of the median of serum sVEGFR-2 between hepatocellular carcinoma, liver cirrhosis and chronic hepatitis compared with healthy subjects. There were significant correlation between sVEGFR-2 with severity disease in LC (MELD, CPT and AST) and CH (APRI and FIB4). The serum sVEGFR-2 could be used as predictive factor progressing CH to LC, but not HCC.

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