J Med Sci, Volume 50, No. 4, 2018 Oktober: 411-423

Expression of circulating miR-200c and vascular endothelial growth factor-A (VEGF-A) mRNA as potential biomarker in human hepatocellular carcinoma

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DOI: http://dx.doi.org/10.19106/JMedScie/005004201805

ABSTRACT

Hepatocellular carcinoma (HCC) is a common liver disease that causes significant public health problems throughout the world, including in Indonesia. The HCC is the six most common cancers and second cancer-related deaths among men in the world. Recently it was reported that the microRNA is an important player in hepatocarcinogenesis. The expression of MiRNA-200c is often regulated in primary HCC and HCC cell lines. Vascular endothelial growth factor-A (VEGF-A) is a regulator of angiogenesis that has been reported as miR-200c target gene. This study was conducted to measure expression levels in miR-200c and mRNAVEGF-A and their potential role as biomarkers at HCC. A total of 36 HCC patients and 36 healthy subjects were included in this study. The relative expression of miRNA-200c and mRNA VEGF-A was quantified using reverse transcription real time quantitative PCR (qRT PCR). Relative expression was calculated using . Unpaired t-test was used to compare the expression levels of circulating miRNA-200c and mRNA VEGF-A in HCC patients and healthy subjects. Pearson test was used to determine correlation between circulating miR-200c expression and mRNA VEGF-A expression levels. The expression levels of circulating miR-200c in HCC patients were lower compared to healthy subjects although it was not significant ($p = 0.258$). Conversely, the expression levels of circulating mRNA VEGF-A in HCC patients were significantly higher compared to healthy subjects ($p = 0.001$). The relative expression levels of circulating miR-200c were negatively correlated with mRNA VEGF-A in HCC patients. In conclusion, the expression levels of mRNA VEGF-A in HCC patients are significantly deregulated in compared to that in healthy subjects. Negative correlation between circulating miRN-200c and mRNA VEGF-A expression levels are reported in HCC patients.

ABSTRAK

Hepatocellular carcinoma (HCC) adalah penyakit hati yang umum dan menyebabkan masalah kesehatan masyarakat yang signifikan di seluruh dunia, termasuk di Indonesia. Karsinoma hepatoseluler adalah penyebab kematian keenam akibat kanker umumnya dan kedua pada laki-laki di seluruh dunia. Saat ini microRNA dilaporkan berperan penting dalam hepatokarsinogenesis. Ekspresi MiRNA-200c mengalami deregulasi pada jaringan HCC primer dan sel HVCC. *Vascular endothelial growth factor-A* (VEGF-A) adalah regulator angiogenesis yang dilaporkan sebagai gen target miR-200c. Penelitian ini dilakukan untuk mengkaji ekspresi miR-200c dan mRNA VEGF-A serta peran

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potensialnya sebagai biomarker HCC. Sebanyak 36 pasien HCC dan 36 subjek sehat diambil dalam penelitian ini. Ekspresi relatif mRNA miRNA-200c dan VEGF-A diukur menggunakan *reverse transcription real time quantitative PCR* (qRT PCR). Ekspresi relatif dihitung menggunakan . Uji t tidak berpasangan digunakan untuk membandingkan tingkat sirkulasi miRNA-200c dan mRNA VEGF-A pasien HCC dan subjek sehat. Uji Pearson digunakan untuk menentukan korelasi antara sirkulasi ekspresi miR-200c dan tingkat ekspresi mRNA VEGF-A. Tingkat ekspresi miR-200c yang bersirkulasi pada pasien HCC lebih rendah dibandingkan dengan subjek sehat meskipun tidak bermakna (p = 0.258). Ekspresi level sirkulasi mRNA VEGF-A lebih tinggi secara bermakna dibandingkan subjek sehat (p = 0.001). Tingkat ekspresi relatif miR-200c dalam sirkulasi memiliki hubungan negatif dengan VEGF-A mRNA pada pasien HCC. Dapat disimpulkan, tingkat ekspresi mRNA VEGF-A mengalami deregulasi secara bermakna pada pasien HCC dibandingkan pada subjek sehat. Ada hubungan negatif antara tingkat ekspresi miRN-200c dalam sirkulasi dan mRNA VEGF-A pada pasien HCC.

Keywords: blood plasma - hepatocellular carcinoma - miR-200c - mRNA VEGF-A - biomarker

INTRODUCTION

Cancer is a non-infectious disease with significant impacts on overall morbidity and mortality in the world.[1] It was estimated in 2018 there are 18 million new cancer cases, 9 million cancer associated mortality worldwide. Hepatocellular carcinoma (HCC) is the most common cause of cancer deaths in the world (9.1% of total cancer related deaths or 746,000 in the world).[1] The HCC is also one of the problems in Indonesia, especially in males, with the second average mortality rate of 12,654 people/5 years. It is predicted to increase due to delays in the diagnosis, limitations of diagnostic methods and treatment.[1]

The development of early diagnostic methods for HCC has been widely applied. However, these current methods have some limitations in the practice.[2] Indonesia generally adopts the HCC diagnostic method based on the Asian-Pacific Association for the Study of the Liver (APASL). It is performed with observation of the typical vascular pattern of arterial enhancement with portal-venous washout obtained by dynamic computed tomography, dynamic magnetic resonance imaging or contrast enhanced ultrasonography.[3] The achievement of 5-year survival rate is still low only about 20% of all cases. This generally associated with late diagnosis of the HCC when the cancer has entered the advanced stage.[4]

MicroRNA is a class of small non-coding RNA molecule (22-24 nucleotides-long) that functions as a post-transcriptional regulator of protein coding gene expression. MicroRNA can bind to one or more mRNAs and cause the degradation and/or inhibition of protein translation.[4] About 30% of protein coding genes are known to be widely regulated by microRNAs.[5,6]

Dysregulation of microRNA expression has been identified in a variety of human cancers. This dysregulation is associated with clinical and biological functions specific to the process of carcinogenesis included HCC.[6,7] Many studies have shown that microRNA can potentially be a clinical diagnostic biomarker because it has a high degree of stability and can describe a biological change especially in cancer.[8,9] MicroRNA dysregulation can be detected in several ways using tissue, serum, plasma, or urine samples. High levels of stability in extracellular microRNAs can be encountered in body fluids especially plasma. MicroRNA can be used as non-invasive markers for HCC detection, prognostic and predict therapeutic response.[8]

Several microRNAs dysregulation

contribute to the pathogenesis of HCC. One of the microRNAs dysregulation reported in HCC is the miR-200 family. MiR-200c is downregulated in HCC tissue and it is associated with low survival rate of HCC patients in the early stages.[10] Decrease of miR-200c expression affects the expression of several oncogenes, such as Vascular Endothelial Growth Factor A (VEGF-A). An *in vitro* study reported VEGF-A, protein expression and cell proliferation significantly increased in HCC cells.[11] VEGF is a class of protein growth factor that plays a role in the angiogenesis of cancer cells, not only for the development of new blood vessels but also for the development of some types of cancer tissue.[12] Cancer cells produce angiogenic proteins such as VEGF that stimulates endothelial cells proliferation and migration.[13] Angiogenesis widely contributes in many cancer cells growth included HCC. The vascular endothelial cells develop at this stage when the tumor lesion has exceeded 0.5 mm. VEGF plays an important role in promoting of the endothelial cells proliferation, thus supporting neovascularization in and around cancer tissues.[14] The study aimed to measure expression levels in miR-200c and mRNA VEGF-A of HCC. The results of this study will be useful in the development of these genes as biomarker candidates of HCC.

MATERIALS AND METHODS

Design and sample collection

This was a cross sectional study using plasma samples from 36 new HCC patients in Dr. Sardjito General Hospital, Yogyakarta and Prof. DR. Margono Soekarjo General Hospital, Purwokerto. As control 36 plasma samples of healthy subjects obtained from Blood Bank Center of Dr. Sardjito General Hospital, Yogyakarta were used.

The inclusion criteria for HCC patients were new diagnosed with HCC, no history of other cancer, never received chemotherapy and radiotherapy, willing to participate in study, and the patient's clinical condition allowed to be taken his blood. The inclusion criteria for the healthy subjects were no history of HCC and other cancers, no family history of HCC, age 25-70 years, HBsAg negative, and no HBV and HCV infection history. The exclusion criteria were resigned as the respondent, and the patient's condition did not allow for blood sampling.

Total RNA extraction

About 5-6 mL of venous blood sample were taken and put into 2 EDTA tube vacutainer and then centrifuged at 1500 rpm for 10 min to obtain plasma sample. The plasma was then stored in the refrigerator at -80 ^{o}C. Total RNA was isolated from 200 µL of plasma using miRCURY RNA Isolation Kit-Biofluid (Cat No.300112, Exiqon) according to the kit procedure. Total RNA was then stored at -80 ^{o}C until cDNA synthesis.

qRT-PCR

As much as 4 µL the RNA isolated from plasma was used for cDNA synthesis using Universal cDNA Synthesis kit II, 8-64 rxns (Cat No.203301, Exiqon) according to the kit procedure. qRT-PCR was performed using ExiLent SYBR Green PCR master mix, 2.5 mL (Cat No. 203402, Exiqon) and specific microRNA primer (Exiqon), and cDNA template. The qRT PCR was performed using the Bio-rad Thermal Cycler CFX96 Real Time System (Bio-rad, USA). The qRT-PCR reaction was performed for 40 cycles with procedure as follow : predenaturation at 95°C for 10 min, followed by denaturation of 95°C for 10 sec, annealing, extension, and data measurement at 58°C for 1 min. Melting curve and melt peak curve analysis were performed to determine the specificity of the PCR product. method was used to measure miR-200c expression on plasma of HCC compared to healthy subjects with miR-16 used as reference gene in relative quantification of miR-200c expression.

Quantification of VEGF-A mRNA expression was performed by the One-Step qRT-PCR method using Bioline One-Step qRT-PCR master mix kit, VEGF-A mRNA

primer set (forward and reverse), RNA sample according to the kit procedure. was used to calculate the relative quantification of VEGF-A mRNA expression between plasma of hepatocellular carcinoma patients and healthy subjects. Beta actin mRNA (ACTB) was used as a reference gene for quantification of VEGF-A mRNA expression. The primer sequences of VEGF-A mRNA and ACTB mRNA used in this study were shown in TABLE 1.

TABLE 1. Primersequence *for* mRNA VEGF-A and mRNA ACTB

Gen	Forward	Reverse	Product length
VEGF-A	5'TGCAGATTATGCGGATCAAACC3'	5'TGCAATCACATTTGTTGTGCTGTA3'	80 bp
ACTB	5'GGGAATTCAAAACTGGAACGGT-GAAGG3'	5'GGAAGCTTATCAAAGTCCTCGGCCA-CA3'	136 bp

Expression analyzers miR-200c and VEGF-A mRNAs used Biorad CFX Manager TM Software to determine the value of quantification cycle (Cq), amplification curve, melting curve, melt peak curve from qRT-PCR results. The expression changes of miR-200c and VEGF mRNA in plasma of HCC patients and healthy subjects were calculated using the Livak Method formula.

Statistical analysis

Data were presented as mean ± standard deviation (SD). Unpaired t-test was used to evaluate the differences of miR-200c and VEGF-A mRNA expressions between HCC patients and healthy subjects. One way Anova were used for more than 2 groups of sub-analyzes of respondents' clinical characteristics included age, history of cirrhosis, history of HBV infection, HCV infection and BCLC stage. The correlation between miR-200c expression with VEGF-A mRNA expression was analyzed using Pearson correlation analysis. p value <0.05 and 95% confidence interval (CI) was considered significant.

RESULTS

Relationship between expression of miR-200c with characteristics and clinicopathology of HCC patients

Relationship between expressions of miR-200c based on clinicopathological characteristic of HCC patient was presented in TABEL 2.

TABLE 2. Relationship between expressions of miR-200c based on clinicopathological characteristic of HCC patient

Characteristic	HCC		*p-Value*
	n	Expression of miR-200c (Mean±SD)	
Age (n=36)			
• ≤ 50	7	7.90 ± 1.53	0.057
• >50	6	9.73 ± 1.55	
BCLC stage (n=29)			
• Early stage	3	9.54 ± 1.75	0.679
• Advance stage	8	9.11 ± 1.38	

Gender (n=36)			
• Male	8	8.80 ± 1.94	0.893
• Female	5	8.66 ± 1.63	
Smoking history (n=29)			
• Yes	3	9.99 ± 0.93	0.297
• No	8	8.94 ± 1.50	
Alcohol consumption (n=29)			
• Yes	1	7.95 ± 0	0.368
• No	10	9.36 ± 1.41	
Hepatitis (n=33)			
• Yes	7	8.86 ± 1.91	0.896
• No	5	9.00 ± 1.60	
Cirrhosis hepar (n=29)			
• Yes	3	8.61 ± 1.21	0.401
• No	8	9.46 ± 1.48	
AFP (ng/ml) (n=31)			
• < 200	6	9.29 ± 1.60	0.476
• >200	6	8.55 ± 1.89	
Nodul (n=28)			
• Single	5	8.42 ± 2.14	0.419
• Multiple	7	9.28 ± 1.40	
TACE (n=29)			
• Pre	4	9.62 ± 1.74	0.512
• Post	7	9.00 ± 1.28	
Metastasis (n=32)			
• Yes	4	7.52 ± 1.62	0.038*
• No	8	9.62 ± 1.34	

The miR-200c expression showed significantly deference between the metastasis groups compared to the non-metastatic group ($p < 0.05$). The relative expression of miR-200c in the metastasis group ($\Delta Cq = 7.52 \pm 1.62$) was significantly higher than the relative expression of miR-200c in the non-metastatic group ($\Delta Cq = 9.62 \pm 1.34$). However, the relative expression of miR-200c did not differ significantly on the basis of other sociodemographic and clinicopathologic characteristics.

Expression of microRNA-200c in blood plasma of HCC patient and healthy subject

To evaluate the factors that influence the loss of miR-200c expression on blood plasma of HCC patients, a logistic regression analysis was performed based on sociodemographic and clinicopathologic characteristics as shown in TABLE 3.

TABLE 3. Logistic regression analysis of miR-200c expression that detected and undetected in blood plasma of HCC patients (n=36)

							85% C.I Exp(B)	
Parameter	B	Stand. err	Wald	df	Sig	Exp(B)	*Lower*	*Upper*
HBV	-3.047	1.648	3.417	1	0.065	0.047	0.002	1.201
Multiple nodul	-0.761	0.654	1.35	1	0.245	0.467	0.130	1.684

The variables in the equation of independent variables, p value of wald test had significance > 0.25. Those value of HBV (p = 0.065) and nodule (p = 0.245) meant that each variable of HBV and nodule had significant partial influence to loss of miR-200c expression on HCC patients. The quantification results of the fold change expression miR-200c in the blood plasma of HCC patients compared to blood plasma of healthy subjects shown in TABLE 4.

TABLE 4. Relative expression of miR-200c in the blood plasma of hepatocellular carcinoma patient compared to healthy subject (n=26)

Sampel	ΔCq miR-200c (Mean±SD)	ΔΔCq miR-200c	*Fold Change*($2^{-\Delta\Delta Cq}$)
HCC(n=13)	8.75 ± 1.75	0.89	0.53
Healthy subject(n=13)	7.8 ± 2.11		

The value of $2^{-\Delta\Delta Cq}$ was less than 1 (0.53), then the calculation of fold change was continued by using the calculation with the result of 1.85. It could be concluded that the miR-200c expression in the blood plasma of HCC patients was down regulated 1.85-fold compared to that of healthy subjects.

The difference of relative expression (ΔCq) miR-200c plasma between HCC patients and healthy subjects was showed by TABLE 5. No significant difference in the relative expression of miR-200c in the blood plasma between HCC patients and healthy subjects was observed (p>0.05).

TABLE 5. Comparison test between relative expression of miR-200c in the blood plasma of HCC patients and healthy subjects (n=26)

Group	ΔCq miR-200c (Mean±SD)	p
HCC (n=13)	8.75 ± 1.75	0.258*
Healthy subject (n=13)	7.6 ± 2.11	

Expression of mRNA VEGF-A in blood plasma of HCC patients and healthy subjects

The expression of mRNA VEGF-A in blood plasma of HCC patients and healthy subjects was showed in TABLE 6. The expression of VEGF-A mRNA in the blood plasma of HCC patients increased 4.42 fold compared to healthy subjects.

TABLE 6. Relative expression of mRNA VEGF-A in the blood plasma of HCC and healthy subjects (n=72)

Group	ΔCq mRNA VEGF (Mean±SD)	ΔΔCq mRNA VEGF	$2^{-\Delta\Delta Cq}$
HCC(n=36)	-0.95 ± 1.97	-2.14	4.42
Healthy subject (n=36)	1.19 ± 1.80		

The difference in the relative expression (ΔCq) of VEGF-A mRNA between HCC patients and healthy subject showed in TABLE 7. A significant difference in relative expression (ΔCq) of VEGF mRNA between blood plasma of HCC patients and healthy subjects was observed ($p<0.001$). The lower the ΔCq value, the higher the mRNA expression. It can be interpreted that the relative expression of mRNA VEGF-A was significantly upregulated in plasma of HCC patients compared to that in healthy subjects.

TABLE 7. Comparison test between relative expression of mRNA VEGF-A in the blood plasma of HCC and healthy subject (n=72)

Group	ΔCq mRNA VEGF (Mean±SD)	P
HCC(n=32)	-0.95 ± 1.97	<0.001*
Healthy subject(n=25)	1.19 ± 1.80	

Correlation between expression miR-200c and VEGF-A mRNA in blood plasma

TABLE 8 shows the correlation between miR-200c expression and VEGF-A mRNA expression in HCC patients and healthy subjects. The relative expression of miR-200c in blood plasma of HCC patients ($p = 0.576$) and also in healthy subjects ($p = 0.874$) were not correlated with the relative expression of VEGF-A mRNA. However, it appeared that the relative expression of miR-200c and VEGF-A mRNA had a negative correlation. It shown that the lower the relative expression of miR-200c, the higher the relative expression of VEGF-A mRNA was observed. Furthermore, the higher the relative expression miR-200c, the lower the relative expression of VEGF-A mRNA was observed.

TABLE 8. Correlation between miR-200c expression and VEGF-A mRNA expression

Variable	ΔCq mRNA VEGF-A (mean ± SD)		p	r
ΔCq miR-200c (mean ± SD)	HCC	HCC	0.576	-0.171
	8.75 ± 1.75	-0.95 ± 1.97		
	Healthy subject	Healthy subject	0.874	-0.049
	7.86 ± 2.11	1.19 ± 1.8		

DISCUSSION

Hepatocellular carcinoma development takes a long time with accumulation changes in the genetic and epigenetic levels that lead to oncogenes activation or inactivation of tumor suppressor genes. In terms of sex, the majority of HCC patients were males as many as 23 people and females as many as 13 people which mean having a 2:1 ratio with only one of the most dominant risk factors is the majority of HCC patients infected with hepatitis B virus. The HCC patients with various risk factors such as being in endemic areas of hepatitis, alcoholics, smokers, cirrhosis, aflatoxin contamination and age, male and female comparison rates to 4:1.[15] The dominance of male HCC patients is in line with the high mortality rate of HCC patients that majority is males in pacific Asia. The dominant age range of HCC patients in both men and women was ≥50 years age group (69.4%). In addition, the incidence of HCC in Asia-pacific increased both men and women aged ≥40 years.[16]

The HCC patients in this study were diagnosed at an early stage (BCLC B) of 9 people (32.7%) and advanced stage (BCLC C and BCLC D) were 20 (52.6%). In this study, no HCC patients were diagnosed with BCLC A stage. At very early stage of HCC, one-third of patients showed no specific symptoms when examined and only a quarter complained of discomfort and abdominal pain. Advanced stages are characterized by the presence of extra hepatic spread, vascular invasion, and the presence of cancer-related symptoms.[17]

In this study, the majority of HCC patients were also positively infected with the hepatitis B virus. The hepatitis B is the most dominant risk factor for HCC cases. Approximately 60-80% of people with hepatitis B infection developed into HCC in Asia.[18] In Indonesia as one of the developing countries in Asia, the incidence of HBV infection is at a high percentage of about 8% of the population.[19] In this study the incidence of cirrhosis only 9 people (31%) the rest of the results of the examination did not contain cirrhosis. In theory, about 80% to 90% of patients with cirrhosis can developed into HCC. Patients with chronic hepatitis B infection accompanied by cirrhosis may continue to develop into HCC, especially patients with hepatitis B virus that actively replicate.[20]

The majority of HCC patients experienced an increase in alpha fetoprotein (AFP) concentrations higher than the normal limit (≥200 ng / mL). The high AFP concentrations in HCC patients are associated with HCC patient status with hepatitis viral infection, especially hepatitis B virus. The upregulation and downregulation of AFP expression are closely related to BCLC status of HCC patients where in the early stages BCLC (O-A) concentration of AFP in normal limits.[21] The study also looked at demographic and clinicopathologic characteristics that affected the loss of miR-200c expression in 17 plasma samples of HCC patients using logistic regression. The HBV infection variable and the number of nodules have a significant partial effect on the loss of miR-200c expression in HCC. The loss of miR-200c expression in blood plasma of HCC patients was also reported by Okamoto *et al*.,[22] where HCV and HBV were known to regulate microRNA expression through epigenetic mechanisms by the occurrence of DNA hypermethylation in many gen promoters. Specific mechanisms of how the regulation of miR-200c expression was reported by Vrba *et al.*[23] In their study of DNA methylation of miR-200c and miR-141 in CpG islands causing loss of expression miR-200c which is a class of suppressor tumors microRNAin cancer.

Multiple nodules in HCC patients may be derived from multiple primary HCC nodules and metastatic intrahepatic nodules. Research conducted by Ng *et al.*[24] in HCC patients with multiple nodules found 36% of primary nodules and 64% of intrahepatic metastatic nodules. Previous studies have revealed that the miR-200 family has been shown to suppress the formation and growth of HCC cancer cells. *In vitro* study using melanoma cells revealed increased expression of miR-

200c and miR-205 capable of inhibiting cancer formation.[25] To support the growth of cancer cells requires angiogenesis activation for the formation of new blood vessels. The miR-200c is a negative regulator of VEGF expression at mRNA and protein levels.[26] In studies using ovarian cancer cells, miR-200c suppresses the expression of fas-associated phosphatase-1 (FAP-1), thus increasing the expression of CD95 which is an apoptotic receptor, thus increasing the sensitivity of cells to apoptosis.[27]

This study found that the relative expression of miR-200c in the blood plasma of HCC patients experienced downregulation of 1.85 times compared to that expression in healthy subjects. Previous studies have found that miR-200c expression in HCC tissues was downregulated compared to healthy tissue.[6,10] Research using circulating microRNA from serum HCC patients miR-200c is known to decrease expression than healthy individuals although not significantly different.[28] This suggests that decreased expression of miR-200c is one of the molecular mechanisms of the carcinogenesis process. The miR-200 family including miR-200c is a tumor suppressor microRNA, where its down regulation or loss of expression contributes to the process of carcinogenesis and cancer progression with increased expression of the oncogenic protein.[10] Research conducted by Li *et al*.[29] found that decreased miR-200c expression in HCC tissue and line-cell was associated with increased oncogen mitotic arrest deficiency protein 2 (MAD2L). Down regulation of miR-200c expression in cancer cells contributes to the epithelial mesenchymal transition (EMT) process, migration, metastasis by direct targeting of E-cadherin mRNA to inhibit transcription pathways from ZEB1 and ZEB2.[30]

MicroRNA deregulation, especially microRNA downregulation can be due to interference of microRNA biogenesis process in cancer cell. Melo *et al*.[31] revealed that mutations in the exportin-5 gene (XPO5) result in trapping of pre-miRNAs in the nucleus thus inhibiting the maturation of microRNAs in cancer cells. This study found the relationship between relative expression miR-200c with metastatic status of HCC patients. Metastasis is the spread of cancer cells originating from primary cancer tissue to the nearest organ or tissue of distant organs through blood vessels and lymphatic vessels.[32] The miR-200c is known to regulate many genes that support angiogenesis, invasive, and metastasis. The VEGF-A, DLC1, ZEB1/ZEB2, FLT-1, KDR, VIM, PLCG1, NTRK2, NTF3, FN1, MSN genes are regulated by miR-200c proven to play a role in cancer progression.[33] Panda *et al*.[34] reported how miR-200c regulates the expression of VEGF-A. using ishikawa-line cells (endometrial adenocarcinoma) increased miR-200cexpression followed by decreased expression of mRNA and VEGF-A protein. VEGF-A is a protein that plays an important role in the process of angiogenesis early in the development of cancer cells by supporting neovascularization of cancer tissue.

Deleted liver cancer-1 (DLC1) is a tumor suppressor gene and metastasis suppressor gene that govern the formation of actin and focal adhesion. The expression of DLC1 is also regulated by miR-200c.[35] miR-200c also suppresses the expression of fibronectin 1 (FN1), meosin (MSN), and leptin receptor (LEPR) so that cancer cell migration becomes inhibited.[36] miR-200c is found to regulate the epithelial to mesenchymal transition process that supports metastasis with direct targeting mechanisms ZEB1 and ZEB2 to suppress E-cadherin.[36] In this study, the relative expression of VEGF-A mRNA was significantly upregulated in blood plasma of HCC patients by 4.42 fold compared to that in healthy subjects. This is consistent with the results in the previous studies that found that VEGF-A mRNA expression has increased in HCC tissue compared to normal tissue and is strongly associated with vascularization of cancer and mortality incidence.[37] HCC patients with hepatitis B virus infection have increased VEGF-A mRNA expression and this correlates with recurrence after resection and mortality events in HCC patients.[38]

The correlation test results showed

that the relative expression of miR-200c was not significantly correlated with the relative expression of VEGF-A mRNA both in the blood plasma of HCC patients and of healthy subjects. In this study was found that miR-200c has the ability to regulate the expression of many protein coding genes, one of which is the VEGF-A gene. MicroRNA has the potential to regulate the expression of hundreds of genes, and one protein coding gene can be regulated by various microRNA. These contributions resulting many pathways to be cancer cells. There are currently over 1000 genes encoding microRNAs in the human genome, and the microRNAs play a role in regulating the expression of about 60% of the human protein coding gene.[39]

Not only the expression of miR-200c regulated by many factors but also the expression of VEGF-A mRNA. The miR200b expression in cancer cells is also known as the VEGF-A regulator by targeting VEGF-A directly and its receptor Flt1 and KDR. In experiment miR200b may act as an angiogenesis inhibitor in cancer cells.[40] This opinion is in agreement with Yeh *et al*.[10] found that 4 families of miR-200 (miR-200a, miR-200b, miR-200c, and miR-141) acting as suppressor tumor genes decreased expression in HCC. Cancer cells in hypoxic conditions have increased regulation of VEGF expression for the purpose of angiogenesis. In experiment miR-20b is known to decrease the regulation of VEGF expression in cancer cells. MiR-20b targeted directly at 3'UTR VEGF plays a role in regulating VEGF protein expression in cancer. MiR-125a directly regulates the expression of the VEGF-A gene at post transcription level by targeting the VEGF-A30 mRNA. In their experiment with restore the expression of miR-125a, it was able to inhibit the development of cancer malignancy significantly by decreasing the regulation of matrix metalloproteinase 11 (MMP11) and VEGF-A expression both *in vitro* and *in vivo*.[41] The presence of many other more dominant regulators in regulating VEGF-A gene expression besides miR-200c led to variations in the quantification results of relative expression of the two genes so no correlation was found between the relative expression of miR-200c and VEGF-A mRNA in blood plasma of HCC patients.

CONCLUSION

This study shows that the expression of miR-200c and VEGF-A mRNA can be detected and quantified in the blood plasma of HCC. There is a downregulation of miR-200c expression and upregulation of VEGF-A mRNA expression between plasma of HCC patients compared to that healthy subjects. However, there is no significant correlation between miR-200c expression and VEGF-A mRNA expression in the blood plasma of HCC patients.

ACKNOWLEDGMENTS

We would like to thanks for the patients who participate in this study. We also would like to thanks for Dr. Sardjito General Hospital Yogyakarta and Prof. DR. Margono Soekarjo General Hospital Purwokerto to facilitate this study for sample access.

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