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Association between serum midkine levels and tumor size in Indonesian hepatocellular carcinoma patients: a cross-sectional study

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Background: The incidence of liver cancer is increased worldwide with 75%–85% diagnosed as hepatocellular carcinoma (HCC). Current practice has low sensitivity limitations to diagnose the early stages of HCC, thus urging the need for a biomarker with higher sensitivity to detect HCC, specifically in the early stage. This study aimed to determine the association between midkine levels and progressiveness of hepatocellular carcinoma (HCC), according to tumor size, Barcelona Clinic Liver Cancer (BCLC), and presence of portal venous thrombosis.

Methods: This cross-sectional study involved 100 patients in Adam Malik General Hospital diagnosed with HCC, collected with a consecutive sampling method, whose diagnoses were confirmed by findings of hypervascular on arterial phase imaging and portal vein or delayed phase washout triple-phase CT Scan. Samples are later categorized according to Barcelona Clinic Liver Cancer (BCLC) stages, tumor size, and presence of portal venous thrombosis. Blood samples were drawn to measure serum midkine using ELISA. Kruskal-Wallis and Mann-Whitney U tests were conducted to determine the difference of midkine levels based on tumor size, BCLC staging, and presence of portal venous thrombosis.

Results: Serum midkine level shows a significant difference over tumor size (p=0.014), no significant difference found compared to BCLC stages and presence of portal venous thrombosis.

Conclusion: Serum midkine levels are associated with the tumor size of HCC, thus helping physicians determine treatment plans.

Key words: midkine, liver neoplasms, hepatocellular carcinoma, biomarker, cytokine.

What is new? What is important?

Serum midkine level shows a significant difference over tumor size, no significant difference found compared to BCLC stages and presence of portal venous thrombosis.

INTRODUCTION

A worldwide trend of increased incidence of liver cancer is reported. Half of the cases in 2018 occurred in East Asia, of which 75%–85% of all cases are diagnosed as hepatocellular carcinoma (HCC) [1]. Failure to conduct HCC surveillance and screening is related to increasing HCC mortality, regardless of access to diagnostic facilities and treatment modalities [2]. Serum alpha-fetoprotein (AFP) is most widely used in clinical practice as a diagnostic biomarker [3], with limitations of low sensitivity to diagnose in early-stage HCC [4], and the level may alter in patients with exacerbation of underlying inflammation processes, including liver disease or cirrhosis [5].

These limitations urge the need for a biomarker with higher sensitivity to detect HCC, especially in the early stages. One study observed the importance of very early-stage HCC detection and treatment plans, resulting in 5-year survival rates of up to 80% after patients receive curative treatments [6]. Few studies were performed to assess novel biomarkers with higher sensitivity, especially in AFP-negative results [7–9]. One novel biomarker that yields better outcomes is midkine.

Midkine, encoded by the MDK gene in chromosome 11 [10], is a heparin-binding cytokine of low molecular weight. midkine is involved mainly in carcinogeneses, such as proliferation, anti-apoptosis, mitogenesis, and angiogenesis [11]. Subsequently, it is overexpressed in many types of malignancy with solid tumors, including HCC [12].

The Barcelona Clinic Liver Cancer (BCLC) staging system is widely applied worldwide in clinical practices. Its algorithm enables physicians to plan treatment, thus predicting the prognosis of patients [13]. Some limitations of the BCLC staging system

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must be taken into consideration. The algorithm was less refined, especially those categorized under the intermediate stage, as it does not comprise all promising treatment options available at the practice. However, there was no current evidence proving that patients treated outside BCLC guidelines survived better than those treated accordingly [14].

This is the first study comparing midkine levels among HCC patients based on tumor size in Indonesia, upbringing novel knowledge regarding the utilization of midkine level as a diagnostic tool for HCC. This study aims to determine the association between midkine levels in patients with HCC and the progressiveness of cancer, according to the presence of portal venous thrombosis, tumor size, and Barcelona Clinic Liver Cancer (BCLC) staging system.

MATERIALS AND METHODS

Study design and subjects

This cross-sectional study included 100 patients initially diagnosed with HCC at Adam Malik General Hospital, Medan, Indonesia, who signed an informed consent form from January to December 2018. Institutional Review Board approved this study of Universitas Sumatera Utara, register number 417/TGL/KEPK FK USU-RSUP HAM/2017.

The study includes all patients aged 18 or older with HCC diagnosed on radiologic findings. Patients diagnosed with cholangiocarcinoma, hemangioma, liver tumors other than HCC, and liver metastasis or had previously undergone treatment for HCC were excluded from the study. The diagnosis of HCC was confirmed by findings of hypervascular on arterial phase imaging and portal vein or delayed phase washout on triple-phase CT scan [15]. These findings were classified into BCLC stages (A, B, C, or D), tumor size, and presence of portal venous thrombosis. BCLC stages classified HCC into early-stage HCC (stage A), intermediate stage HCC (stage B), advanced stage (stage C), and terminal stage (stage D) based on tumor extension, liver functional reserve, physical status, and cancer-related symptoms [16]. The diagnosis of HCC was not obtained from histopathology examination. Besides the invasiveness of liver biopsy, it possesses the risk of tumor track seeding, with a study reported seeding rate of 2–3% [17]. Logical argument considering the abovementioned aspects would establish the diagnosis of HCC in subjects of this study without conducting the histopathological examination. Therefore, BCLC staging was used as it was based on imaging findings accompanied with clinical manifestations yet served clinicians therapeutic options along with patients prognoses.

Based on the largest tumor size found on imaging modalities, the patients are classified into tumor size (less than 3 cm, sized 3–5 cm, or larger than 5 cm.) The presence of portal venous thrombosis was based on findings in CT scans.

Laboratory examinations

Blood samples of as much as three ccs were drawn from the antecubital vein of patients after a proper aseptic procedure. Blood was placed in a tube containing EDTA and stored at 2–8°C. All samples were centrifuged for 15 minutes to obtain serum and then held at -20°C. Using the ELISA technique, samples were subjected to measure midkine level using Human midkine (MDK) ELISA kit (Glory Science Co, Texas, USA). After samples were added to the wells and combined with the specific antibody, a biotinylated detection antibody specific for midkine and Avidin-Horseradish Peroxidase (HRP) conjugate was added to each well and incubated. Free components were washed away, then substrate solution was added. Wells containing midkine, biotinylated detection antibody, and Avidin-HRP conjugate appeared blue. The enzyme-substrate reaction was halted by adding the stop solution when the color of the solution in wells turned yellow. The optical density (OD) value was measured at the wavelength of 450 nm. All of the procedures were conducted once for each sample [18].

Statistical analysis

Data were statistically analyzed using SPSS 22.0 software (IBM Corporation, New York, USA). The Kruskal-Wallis test and serial Mann-Whitney *U* test were used if the data were found not normally distributed. A p-value smaller than 0.05 is appraised as statistically significant.

RESULTS

This study was conducted on 100 patients with a mean age of 58.7 (SD=6.2) years. Most patients were male, with Hepatitis B as major etiology found from these cases. The median midkine level of this study was 0.72 ng/ml. (minimum-maximum value= 0.48-0.98). Most of the samples were categorized under stage C of BCLC (31%), followed by stage B (28%), stage D (21%), and stage A (20%). Portal venous thrombosis was found in 51% of the cases (Table 1).

Kolmogorov-Smirnov test revealed that the data was not normally distributed ($p < 0.001$); therefore, the Kruskal-Wallis test was conducted to perform nonparametric statistical analysis. There was a significant difference between serum midkine levels and tumor size in patients diagnosed with HCC ($p = 0.014$). midkine levels were significantly higher in tumor size > 5 cm compared to < 3 cm. There was no significant difference found between midkine levels and BCLC stages ($p = 0.074$) and the presence of portal venous thrombosis ($p = 0.148$) (Table 2).

Table 1
Demographic and clinical characteristics of HCC patients

Characteristic	n = 100
Mean age, years (SD)	58.7 (6.2)
Sex, n (%)	
Male	78 (78)
Female	22 (22)
Etiology, n (%)	
Hepatitis B	68 (68)
Hepatitis C	9 (9)
Non-alcoholic Fatty Liver Disease	23 (23)
Tumor size, n (%)	
< 3 cm	19 (19)
3 – 5 cm	39 (39)
> 5 cm	42 (42)
BCLC* stages, n (%)	
Stage A	20 (20)
Stage B	28 (28)
Stage C	31 (31)
Stage D	21 (21)
Portal venous thrombosis, n (%)	
Present	51 (51)
Absent	49 (49)
Midkine levels (ng/mL), median (min-max)	0.72 (0.48 – 0.98)

*BCLC: Barcelona Clinic Liver Cancer

Table 2
Median difference of serum midkine levels according to BCLC stages, tumor size, and presence of portal venous thrombosis

	Midkine levels (ng/mL) (IQR)	**P**
BCLC		
A	0.54 (0.22)	
B	0.695 (0.25)	0.074 [a]
C	0.74 (0.35)	
D	0.8 (0.16)	
Tumor size		
< 3 cm	0.6 (0.17)	0.014*[a]
3 – 5 cm	0.75 (0.23)	
> 5 cm	0.8 (0.3) [#]	
Portal venous thrombosis		
Present	0.78 (0.21)	0.148 [b]
Absent	0.67 (0.430)	

*p<0.05, [a]Kruskal-Wallis test, [b]Mann-Whitney U test,
[#] significant compared to tumor size <3 cm

DISCUSSION

Liver cancer contributed as second most deaths related to cancers [19], whereas HCC ranks the most among other types of liver cancer [20]. Males have a higher incidence of HCC due to low adiponectin levels [21], whose inhibition was mediated by C-Jun N-Terminal Kinase (JNK) [22]. Adiponectin is known to protect against liver cancer through activation of AMP-activated protein kinase (AMPK) and p38α [23]. In this study, the majority of patients (77.5%) with HCC were male, which was by the result from studies of Manieri *et al*. [21], Tangkijvanich *et al*. [23], and El-Serag *et al*. [24].

Despite the overall poor prognosis, patients diagnosed with early-stage of HCC (BCLC Stage A) have a relatively good prognosis, with a 5-year survival rate of > 70% and a 5-year survival rate (surgical intervention) was > 93% [25,26]. Difference among prognoses of early and late-stage HCC patients urges the need for more sensitive screening in the early stage of HCC, which is unable to be covered by AFP [27]. Current practice guidelines rely on a combination of imaging modalities (ultrasound) and α-fetoprotein (AFP) levels on diagnosing early HCC, with low sensitivity and specificity (63% and 45%) [28]. Midkine, a protein-secreting cytokine that is highly expressed in inflammation and cell proliferation, can be a diagnostic marker [29]. Detection of overexpressed midkine in blood serum or plasma would adequately serve as early malignancy screening [30]. Previous studies observed that serum midkine measures have higher sensitivity in the early stages of HCC than often-used measures [31]. Several studies determined cut-off value for midkine, ranging from 0.387 ng/mL with reported 90% sensitivity by Shaheen *et al*. [32] to 0.654 ng/mL by Nault [33] reported with the sensitivity of 86% and specificity of 90%. A study by Zhang *et al*. reported that midkine has a sensitivity of 85% and specificity of 83% in diagnosing HCC. This result was reported at a cut-off value of >0.5 ng/mL [34]. Another systematic review found that midkine is more superior compared to AFP in detecting HCC. The results were also applied in virus-related HCC. Midkine was also more sensitive and specific in detecting early HCC compared to AFP [35]. As shown from the median midkine levels in this study, all samples revealed results above the cut-off values; thus, this study concurred with previous abovementioned studies to support its potential as a novel biomarker that helps refine the diagnosis of HCC.

A previous study from Vongsuvanh *et al*. has shown that adding midkine level as the measure has enabled higher sensitivity than AFP used alone [31], in which most of these cases were early stage (BCLC 0 or A) tumors [36]. The latter fact is crucial as these cases belong to a group prone to be disregarded. Group 0 or A has low sensitivity to current surveillance strategies, thus missing the chance of potentially curative treatment, which determines prognoses. This study shows the statistical difference of midkine level in association with tumor size upon early progressiveness of HCC. This finding is concordant with a previous study [37] that showed the correlation between midkine and tumor size. This study found that midkine level was not associated with the progressiveness of cancer, which was implied with advanced staging and the presence of thrombosis in portal venous. This finding is consistent with a study by Shimada *et al*. [38], which is associated with clinicopathologic features of HCC and serum midkine level. Therefore, serum midkine level could be used in clinical practices to diagnose HCC but not to measure the progressiveness of cancer in a patient. On its relevant function on malignant cell proliferation, midkine has the potential to be a therapeutic target in the era of personalized medicine.

However, there are several limitations to this study. The number of patients in each tumor sizes group was not equal, which invoked a potential of statistical bias. Furthermore, we did not include healthy subjects as the control population in this study. Therefore, we could not analyze the sensitivity and specificity of midkine level in diagnosing HCC. We also did not determine the level of AFP level in this study, therefore we were not able to determine the association between midkine and AFP. Further studies needed to be performed with a more significant number of cases and refined samples with recurrent HCC or newly diagnosed without underlying inflammation process, as well as to compare results of midkine as a promising biomarker with patients in different classification HCC.

CONCLUSION

Serum midkine level is associated with HCC tumor size. This finding helps physicians diagnose HCC, especially in patients with small size of tumor or when the invasive diagnostic method was unaccessible. Its ability to detect HCC in small tumor size helps physicians determine treatment plans and prognosis of patients even at early stages, thus yielding better patient-centered care.

Introducere: *Incidența cancerelor hepatice este crescută, iar majoritatea acestora sunt carcinoame hepatocelulare (HCC). Momentan stadiile timpurii ale HCC sunt limitate, fiind nevoie de biomarkeri sensibili. Scopul studiului a fost de a determina asocierea între nivelurile serice de midkine și progresia HCC conform mărimii tumorale, scalei Barcelona (BCLC) și prezenței trombozei venoase.*

Metode: *A fost realizat un studiu transversal pe 100 de pacienți din spitalul Adam Malik diagnosticați cu HCC. Diagnosticul a fost realizat prin examinare CT. Ulterior, pacienții au fost evaluați conform scalei BCLC, a prezenței trombozei venei porte, precum și a mărimii tumorale. Nivelurile de midkine au fost evaluate din ser prin metoda ELISA. Testele Mann-Whitney U și Kruskall-Wallis au fost utilizate pentru a evalua diferențele de midkine.*

Rezultate: *Nivelurile de midkine au fost diferite semnificativ în funcție de mărimea tumorală (p=0.014). Nu s-au observat diferențe între nivelurile de midkine și stadiile BCLC sau prezența trombozei de venă portă.*

Concluzii: *Nivelurile serice de midkinei se asociază cu mărimea tumorală a HCC, ajutând clinicienii să determine schemele de tratament.*

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REFERENCES

1. CHEN LT., MARTINELLI E., CHENG AL., PENTHEROUDAKIS G., QIN S., BHATTACHARYYA GS., *et al. Pan-Asian adapted ESMO clinical practice guidelines for the management of patients with intermediate and advanced/relapsed hepatocellular carcinoma: a TOS-ESMO initiative endorsed by CSCO, ISMPO, JSMO, KSMO, MOS and SSO*. Ann Oncol. 2020;**31**(3):334–351.
2. HASAN I., SULAIMAN AS., GANI RA., LESMANA CR. *Risk factors for hepatocellular carcinoma and its mortality rate: a multicenter study in Indonesia.* Arch Cancer Res. 2019;**7**(1):2.
3. LOHO IM., SIREGAR L., WASPODO AS., HASAN I. *Current practice of hepatocellular carcinoma surveillance*. Acta Med Indones. 2018;**50**(4):353–360.
4. SHU H., LI W., SHANG S., QIN X., ZHANG S., LIU Y. *Diagnosis of AFP-negative early-stage hepatocellular carcinoma using Fuc-PON1*. Discov Med. 2017;**23**(126):163–168.
5. PAUL SB., GULATI MS., SREENIVAS V., MADAN K., GUPTA AK., MUKHOPADHYAY S., *et al. Evaluating patients with cirrhosis for hepatocellular carcinoma: value of clinical symptomatology, imaging and alpha- fetoprotein*. J Oncol. 2007; **72**(suppl 1):117–123.
6. YANG JD. *Detect or not to detect very early stage hepatocellular carcinoma? The western perspective. Clin Mol Hepatol*. 2019;**25**(4): 335–343.
7. DUAN J., WU Y., LIU J., ZHANG J., FU Z., FENG T., *et al. Genetic biomarkers for hepatocellular carcinoma in the era of precision medicine.* J Hepatocell Carcinoma. 2019;**6**:151–166.
8. OCKER M. *Biomarkers for hepatocellular carcinoma: what's new on the horizon?* World J Gastroenterol. 2018;**24**(35):3974–3979.
9. HODEIB H., ELSHORA O., SELIM A., SABRY NM., EL-ASHRY HM. *Serum midkine and osteopontin levels as diagnostic biomarkers of hepatocellular carcinoma*. Electron Physician. 2017;**9**(1):3492–3498.
10. WANG P., MAO YM., ZHAO CN., WANG JB., LI XM., YE DQ., *et al. Association of midkine and pleiotrophin gene polymorphisms with systemic lupus erythematosus susceptibility in Chinese Han population*. Front Immunol. 2020;**11**:110.
11. EI-EDEL RH., TAWFIK GA., NORELDIN RI., EL-JAKY MA., EISAA DA. *Evaluation of serum midkine as a novel marker in hepatocellular carcinomas*. Menoufia Med J. 2018;**31**(3):1094.
12. PARKIN DM., BRAY F., FERLAY J., PISANI P. *Global cancer statistics, 2002*. Ca-Cancer J Clin. 2005;**55**(2):74–108.
13. KINOSHITA A., ONODA H., FUSHIYA N., KOIKE K., NISHINO H., TAJIRI H. *Staging systems for hepatocellular carcinoma: current status and future perspectives*. World J Hepatol. 2015;**7**(3): 406–424.
14. RICHANI M., KOLLY P., KNOEPFLI M., HERRMANN E., ZWEIFEL M., TENGG-KOBLIGK HV., *et al. Treatment allocation in hepatocellular carcinoma: assessment of the BCLC algorithm*. Ann Hepatol. 2016;15(1):82–90.
15. MARRERO JA., KULIK LM., SIRLIN CB., ZHU AX., FINN RS., ABECASSIS MM., *et al. Diagnosis, staging, and management of hepatocellular carcinoma: 2018 practice guidance by the American Association for the Study of Liver Diseases*. Hepatology. 2018;**68**(2):723–750.
16. TELLAPURI S., SUTPHIN PD., BEG MS., SINGAL AG., KALVA SP. *Staging systems of hepatocellular carcinoma: a review*. Indian J Gastroenterol. 2018;**37**(6):481–491.
17. DOYLE A., SHERMAN M. *Liver biopsy for hepatocelullar carcinoma (HCC): should this be a routine?* Curr Hepatol Rep. 2017;**16**:46–50.
18. HNASKO R. ELISA: *Methods and protocols*. USA; New York: Humana Press, 2019.
19. WORLD HEALTH ORGANIZATION. *World cancer report 2014.* http://www.who.int// (accessed 23 May 2020)
20. BALOGH J., VICTOR III D., ASHAM EH., BURROUGHS SG., BOKTOUR M., SAHARIA A., *et al. Hepatocellular carcinoma: a review*. J Hepatocell Carcinoma. 2016;**3**: 41–53.
21. MANIERI E., HERRERA-MELLE L., MORA A., TOMÁS-LOBA A., LEIVA-VEGA L., FERNÁNDEZ DI., *et al. Adiponectin accounts for gender differences in hepatocellular carcinoma incidence*. J Exp Med. 2019;216(5):1108–1119.
22. SAXENA NK., FU PP., NAGALINGAM A., WANG J., HANDY J., COHEN C., *et al. Adiponectin modulates C-jun N-terminal kinase and mammalian target of rapamycin and inhibits hepatocellular carcinoma*. J Gastroenterol. 2010;**139**(5):1762–1773.

23. TANGKIJVANICH P., MAHACHAI V., SUWANGOOL P., POOVORAWAN Y. *Gender difference in clinicopathologic features and survival of patients with hepatocellular carcinoma*. World J Gastroenterol. 2004;**10**(11): 1547–1550.
24. EL-SERAG HB., RUDOLPH KL. *Hepatocellular carcinoma: epidemiology and molecular carcinogenesis*. J Gastroenterol. 2007;**132**:2557–2576.
25. MAZZAFERRO V., REGALIA E., DOCI R., ANDREOLA S., PULVIRENTI A., BOZZETTI F., *et al*. *Liver transplantation for the treatment of small hepatocellular carcinomas in patients with cirrhosis*. N Engl J Med. 1996;**334**(11):693–700.
26. TAKAYAMA T., MAKUUCHI M., KOJIRO M., LAUWERS GY., ADAMS RB., WILSON SR., *et al*. *Early hepatocellular carcinoma: pathology, imaging, and therapy*. Ann Surg Oncol. 2008;**15**:972–978.
27. KO YS., BAE JH., SINN DH., GWAK GY., KANG W., PAIK YH., *et al. The clinical significance of serum alpha-fetoprotein in diagnosing hepatocellular carcinoma in a health screening population*. Korean J Gastroenterol. 2017;**69**(4):232–238.
28. TZARTZEVA K., OBI J., RICH NE., PARIKH ND., MARRERO JA., YOPP A., *et al*. *Surveillance imaging and alpha fetoprotein for early detection of hepatocellular carcinoma in patients with cirrhosis: a meta-analysis*. J Gastroenterol. 2018;**154**(6):1706–1718.
29. JING X., CUI X., LIANG H., HAO C., HAN C. *Diagnostic accuracy of ELISA for detecting serum midkine in cancer patients*. PLoS One. 2017;**12**(7): e0180511.
30. ZHANG BH., LI B., KONG LX., YAN LN., YANG JY. *Diagnostic accuracy of midkine on hepatocellular carcinoma: A meta-analysis*. PLoS One. 2019; **14**(10): e0223514.
31. VONGSUVANH R., VAN DER POORTEN D., ISELI T., STRASSER SI., MCCAUGHAN GW., GEORGE J. *midkine increases diagnostic yield in AFP negative and NASH-related hepatocellular carcinoma*. PLoS One. 2016;11(5): e0155800.
32. SHAHEEN KY., ABDEL-MAGEED AI., SAFWAT E., ALBREEDY AM. *The value of serum midkine level in diagnosis of hepatocellular carcinoma*. Int J Hepatol. 2015; **2015**:146389.
33. NAULT J-C. Midkine as a new diagnostic tool in hepatocellular carcinoma. Transl Gastrointest Cancer. 2014;**3**(2):100–102.
34. ZHANG BH., LI B., KONG LX., YAN LN., YANG JY. *Diagnostic accuracy of midkine on hepatocellular carcinoma: A meta-analysis.* PLoS One. 2019; **14**(10):e0223514.
35. LU Q., CAO H., LV C., WANG X., CAO S. *Comparison of diagnostic accuracy of midkine and AFP for detecting hepatocellular carcinoma: A systematic review and meta-analysis.* Biosci Rep. 2020;**40**(3):BSR20192424.
36. ZHAO ZQ., YANG S., LU HS. *Expression of midkine and vascular endothelial growth factor in gastric cancer and the association of high levels with poor prognosis and survival*. Mol Med Rep. 2012;**5**(2):415–419.
37. ZHU WW., GUO JJ., GUO L., JIA HL., ZHU M., ZHANG JB., *et al*. *Evaluation of midkine as a diagnostic serum biomarker in hepatocellular carcinoma.* Clin Cancer Res. 2013;**19**(14):3944–3954.
38. SHIMADA H., NABEYA Y., TAGAWA M., OKAZUMI SI., MATSUBARA H., KADOMATSU K., *et al*. *Preoperative serum midkine concentration is a prognostic marker for esophageal squamous cell carcinoma.* Cancer Sci. 2003;**94**(7):628–632.

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