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Title: Prognostic Role of Plasma Level of Angiopoietin-1, Angiopoietin-2, and Vascular Endothelial Growth Factor in Hepatocellular Carcinoma

Dr. Subrata Ghosh & Dr. Andrzej S Tarnawski

Editors-in-Chief

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Dear Dr. Ghosh & Dr. Tarnawski;

We thank you for giving us the opportunity to revise our paper (62595) titled “Prognostic Role of Plasma Level of Angiopoietin-1, Angiopoietin-2, and Vascular Endothelial Growth Factor in Hepatocellular Carcinoma” and the helpful comments from the reviewers.

We have attached a revised version showing the changes in red font and separately listed our point-by-point responses. We feel that the comments have allowed us to improve the paper and hope you convey our gratitude to the reviewers for their time and effort in reviewing our manuscript.

Yours sincerely,

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Point-to-point responses to the specific comments of the editor and reviewers

Reviewer: 1

General comments:

The article analyzed the relation between the levels of Ang-1, Ang-2, and VEGF and the clinical characteristics, OS, and PFS. Only Ang-2 was a statistically significant with the clinical characteristics, OS, and PFS. Since Ang-2 was involved in tumor angiogenesis. MVD is a widely accepted marker of tumor angiogenesis. The comparison between Ang-2 and MVD was naturally indispensable for the convincing conclusion. I hope that author can supplement the data into the article.

Response 1: Thank you for your valuable comments. Conducting MVD using HCC tissue samples is a good idea; however, this study was not designed to test tumor tissue. Thus, IRB-approved tissue samples were not available. Therefore, we additionally addressed these points as one of the limitations and cited references in the Discussion section (see page 17, paragraph 1).

Changes in the text:

Page 17, Paragraph 2.

Fourth, this study did not evaluate other valuable angiogenesis markers such as microvessel density, PDGF/PDGFR, FGF/FGFR, and Endoglin (CD105). (Morse, M.A et al. *Clin Cancer Res* 2019;25:912 & Zhang, Q et al. *Front Med* 2020;7:584250) Further comprehensive studies including these angiogenesis markers are hence, needed.

Reviewer: 2

General comments:

This article is fresh in its perspective. It investigates the utility of Ang-2, AFP, Ang-1, and VEGF in terms of predicting the prognosis of HCC patients, but also in terms of OS and PFS. It is interesting to see the choices they've made and how they want to underline the importance of these biomarkers even in comparison with AFP, the most classic one when dealing with HCC. The authors raise many good points and are also good in recognizing the limitations of their study. I just wanted to underline this elements that could use a bit of work:

Comment 1. “However, the investigations on the prognostic value of blood angiogenesis biomarkers across all HCC stages are limited, especially in South Korea, where the hepatitis B virus is the dominant etiology of HCC(11)” – The temporal succession of this sentence is not clear. In this form, it seems like the authors suggest a connection between the presence of blood angiogenesis

biomarkers and HBV, without explaining it. Moreover, if the authors believe that a correlation is possible, that would affect the efficacy of the study, since a large majority of patients had HBV-related cirrhosis.

Response 1: Thank you for your valuable comments. To improve the clarity of the sentence in the Introduction, it has been corrected as follows :

Changes in the text:

Page 6, Paragraph 2.

However, investigations on the prognostic value of blood angiogenesis biomarkers across all HCC stages till date are limited, ~~especially in South Korea, where the hepatitis B virus is the dominant etiology of HCC(11).~~

The relationship between these angiogenesis markers and HBV etiology was additionally described in the results section (see page 10, paragraph 2) and S4 figure as follows. Moreover, we addressed these points as a limitation in the Discussion section (see page 16, paragraph 3).

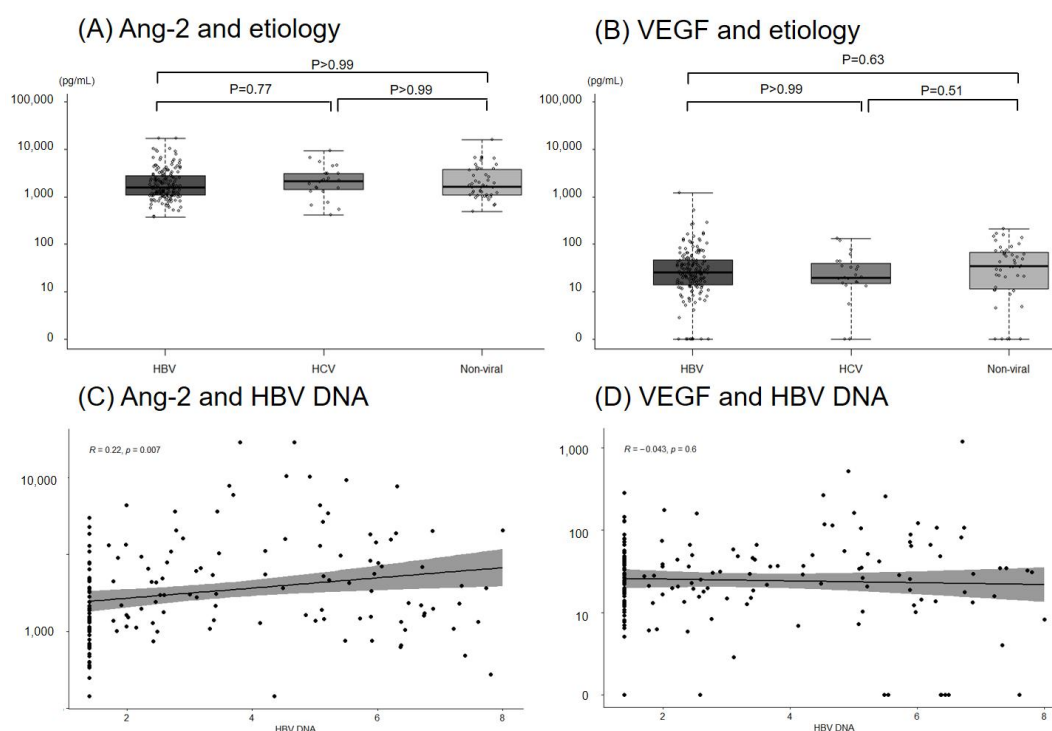
Changes in the text:

Page 10, Paragraph 2.

~~The median plasma levels of Ang-2 and VEGF did not differ according to the etiology of HCC (S4 Figure A and B). The correlation plot between each plasma level of the biomarkers and etiology is shown in S4 Figure C and D among CHB patients (n=156). HBV DNA levels were not significantly correlated with Ang-2 or VEGF levels.~~

Page 17, Paragraph 2.

~~This study has several limitations. First, this was a single-center study in an HBV-endemic area. Although there was no difference in Ang-2 and VEGF levels according to the etiology of HCC in this study, the results need external validation.~~



S4 Figure. Plasma levels of Angiopoietin-1 and Angiopoietin-2 according to etiology and HBV DNA level.

Comment 2. There are still a few grammar mistakes (very little things, overall, like “did not significant” or “the potential of each plasma level” Page 10, line 19: it should also be specified how the other patients evolved. Supplementary figure 4: Kaplan-Meier curve is misspelled.

Response 2: Thank you for your comment. We have corrected all the grammar mistakes in the related pages and figure. The manuscript has been edited by a professional native English editor repeatedly.

Changes in the text:

Page 10, Paragraph 2.

Meanwhile, the rho value between **a**Ang-1 or VEGF levels and liver function or tumor extent **was** not significant.

Page 17, Paragraph 1.

Recent studies suggested that predicting OS was made possible by periodically measuring serum angiogenic cytokines, especially **angiopoietin-2 Ang-2** or VEGF, in patients with HCC treated with sorafenib or lenvatinib.

Page 39, Supplementary figure S5. Kaplan-Meier curve for progression free and overall

survival

Reviewer: 3

General comments:

In this manuscript, the authors aimed to study the plasma levels of three angiogenesis markers in HCC patients, and further evaluate the association between their levels and patient's prognosis. The article is interesting. However, there are still some problems need to be resolved, as detailed below.

Comment 1. What are the plasma levels of these three factors in normal people and liver cirrhosis patients? It should be supplemented at the same time.

Response 1: We agree with your comment. In fact, we included 100 age-and sex-matched normal control samples obtained in 2006 with the HCC samples obtained in 2012-2016 and measured the three angiogenesis markers as below. Although the control samples were stored at -70°C, the absolute plasma levels of Ang-1, Ang-2, and VEGF in the 100 healthy subjects (1.4 ng/mL, 0.5, and 4.2 pg/mL, respectively) seemed to be lower than those previously reported in other studies (In Circulation paper [2004;43:423], the Ang-1, Ang-2 and VEGF levels in 40 healthy controls were 8.2 ng/mL, 3.3 ng/mL, and 27 pg/mL, respectively). Considering the relatively older samples in healthy controls compared to those of the HCC patients, we were not confident in comparing these two groups. Therefore, we decided to exclude the following analysis including the normal controls in this study, although the diagnostic potential of Ang-2 was also excellent, as shown below.

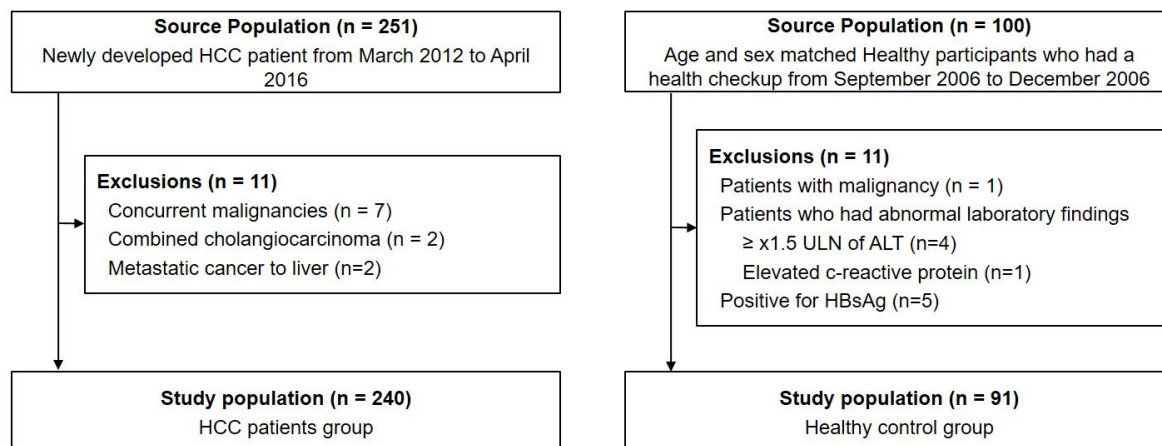


Figure. Patient flow gram

Table. Baseline characteristics of healthy control and hepatocellular carcinoma patients

Characteristic	Healthy control (n=91)	HCC patients (n=240)	P
Age, mean age \pm SD, year	58.9 \pm 9.4	60.9 \pm 11.2	0.13
Male sex, n (%)	72 (79.1)	193 (80.4)	0.79
BMI, mean \pm SD, kg/m ²	23.9 \pm 2.6	24.1 \pm 3.0	0.55
Laboratory results			
WBC, median (IQR), $\times 10^3/\mu\text{L}$	5.8 (5.1 – 6.7)	5.1 (4.0 – 6.6)	0.01
Hemoglobin, median (IQR), g/dL	15.1 (14.0 – 15.8)	13.6 (12.0 – 14.8)	<0.001
Platelet count, median (IQR), $\times 10^9/\mu\text{L}$	229 (197 – 263)	130 (88 – 184)	<0.001
Prothrombin time, median (IQR), INR	1.00 (0.98 – 1.03)	1.11 (1.04 – 1.18)	<0.001
AST, median (IQR), IU/mL	22 (19 – 27)	45 (32 – 73)	<0.001
ALT, median (IQR), IU/mL	22 (17 – 28)	38 (23 – 61)	<0.001
Albumin, ; median (IQR), g/dL	4.5 (4.3 – 4.6)	4.0 (3.5 – 4.2)	<0.001
Total bilirubin, median (IQR), mg/dL	1.0 (0.8 – 1.4)	0.8 (0.5 – 1.1)	0.68
Creatinine, median (IQR), mg/dL	1.1 (1.0 – 1.2)	0.8 (0.7 – 1.0)	<0.001
Plasma level of AFP and Angiogenesis markers			
AFP, median (IQR), ng/dL	2.4 (1.7 – 2.9)	17.9 (4.0 – 698)	<0.001
Ang-1, median (IQR), pg/mL	1433 (731 – 2477)	3216 (1565 – 6266)	<0.001
Ang-2, median (IQR), pg/mL	487 (265 – 723)	1684 (1107 – 3064)	<0.001
VEGF, median (IQR), pg/mL	4.2 (0 – 9.5)	26.5 (13.8 – 51.3)	<0.001

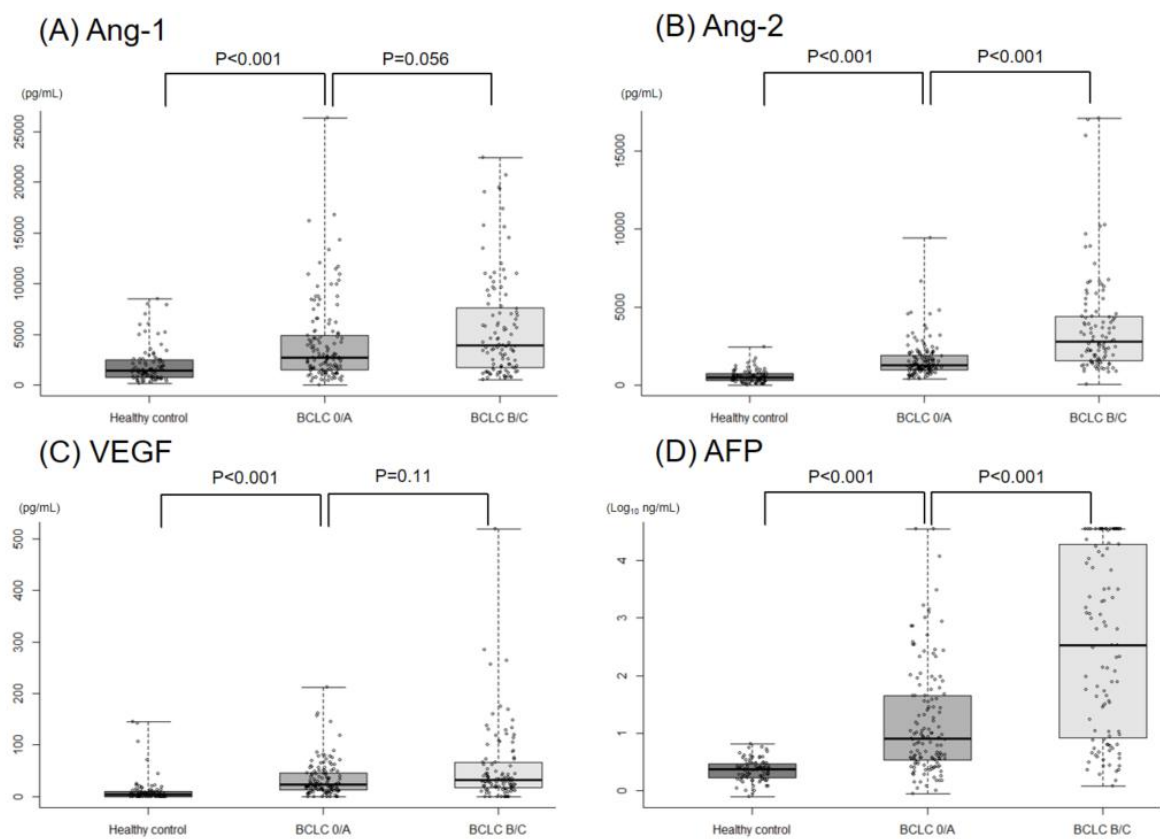
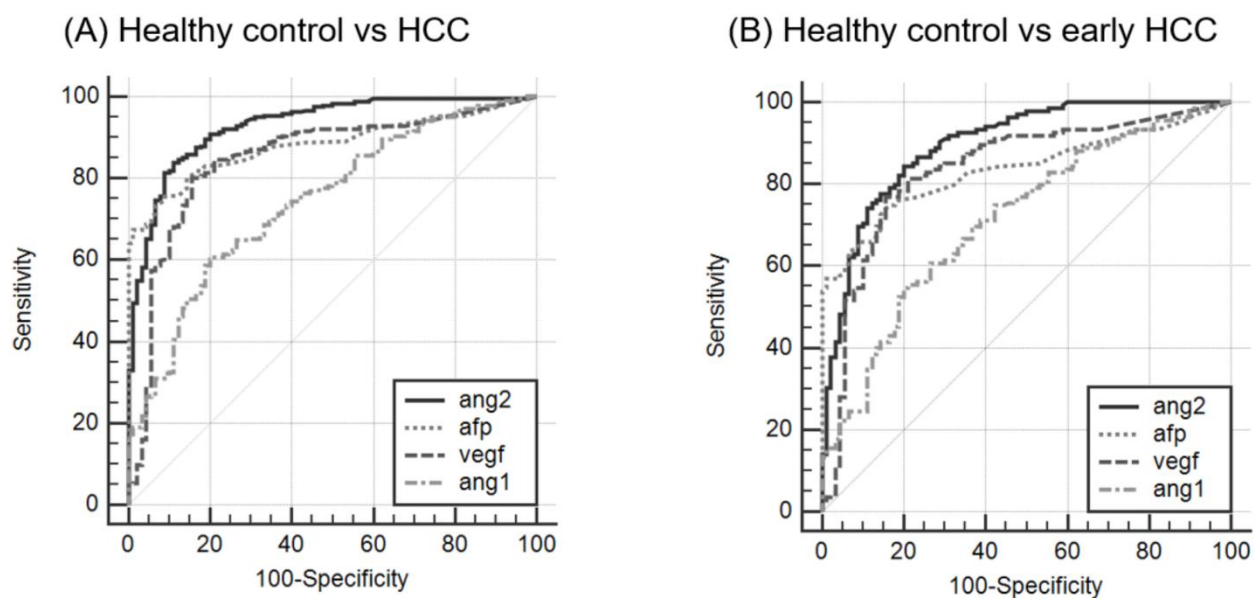


Figure. Plasma levels of angiogenesis markers between healthy control and HCC patients



Marker	AUROC	95% CI	P
Ang-2	0.931	0.898 – 0.956	Reference
AFP	0.883	0.844 – 0.916	0.03
VEGF	0.846	0.803 – 0.883	0.01
Ang-1	0.745	0.695 – 0.791	<0.0001

Marker	AUROC	95% CI	P
Ang-2	0.897	0.849 – 0.933	Reference
AFP	0.836	0.781 – 0.882	0.06
VEGF	0.837	0.782 – 0.882	0.11
Ang-1	0.714	0.650 – 0.772	<0.0001

Figure. ROC curves for HCC diagnosis

If we intend to evaluate angiogenesis markers as diagnostic biomarkers, it is essential to compare the plasma levels of these markers among normal healthy subjects and patients with chronic hepatitis, cirrhosis, and HCC. However, since this study focused on and evaluated the role of angiogenesis markers as prognostic markers, we believe that the presented data are relatively sufficient to suggest the potential prognostic role of these angiogenesis markers. Therefore, we additionally addressed these points as a limitation in the Discussion section (see page 17, paragraph 1).

Changes in the text:

Page 17, Paragraph 2.

Third, this study did not evaluate the plasma levels of these three angiogenesis factors in normal control subjects or cirrhotic patients because we focused on the role of Ang-1, Ang-2, and VEGF as prognostic rather than diagnostic markers.

Comment 2. The author's results show that Angiopoietin-2 is significantly related to the prognosis of HCC patients. The changing trend of this factor after treatment (TACE or alternative drugs) should be classified.

Response 2: Thank you for your valuable comments. We agree that if a longitudinal evaluation of Ang-2 was performed, this would have been a better study. In this study, longitudinal evaluation was not possible because we stored only HCC samples at the time of diagnosis. Therefore, we have addressed these points as a limitation in the Discussion section (see page 16, paragraph 3).

“Second, the angiogenic biomarkers were measured only once at the time of HCC diagnosis, so the longitudinal profile according to tumor progression or treatment response cannot be analyzed.”

Comment 3. There are some format and grammar errors in the text (such as Ang-2, Angiopoietin-1). The manuscript needs to be revised thoroughly.

Response 3: Thank you for your comment. We have meticulously corrected the grammar mistakes.

Changes in the text:

Page 10, Paragraph 2.

Meanwhile, the rho value between aAng-1 or VEGF levels and liver function or tumor extent was not significant.

Page 17, Paragraph 1.

Recent studies suggested that predicting OS was made possible by periodically measuring serum angiogenic cytokines, especially ~~angiopoietin-2~~ Ang-2 or VEGF, in patients with HCC treated with sorafenib or lenvatinib.