Dear Editor,

We thank the editors and reviewers of the BMC cancer for taking their time to review our re-submitted article entitled: “Protein induced by vitamin K absence or antagonist-II production is a strong predictive marker for extrahepatic metastases in early hepatocellular carcinoma: a prospective evaluation”. We have made some corrections and clarifications in the manuscript after going over the reviewer's comments.

The changes are specified in enclosed summary of changes. We have responded to the reviewer’s comments and have incorporated all the modifications suggested by the reviewer into the revised version of the manuscript. The changes within the revised manuscript were highlighted in blue.

We hope the revised manuscript will better meet the requirements of the BMC cancer for publication. We thank again for the constructive comments by the reviewers.

Sincerely yours,

Thank you very much for your attention.

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SUMMARY OF CHANGES

Protein induced by vitamin K absence or antagonist-II production is a strong predictive marker for extrahepatic metastases in early hepatocellular carcinoma: a prospective evaluation

Refree 1: Takumi Uamabuki

1-1.Reviewer's comment: In this study, four different types of treatment modalities (TACE, RFA, PEI and Surgery) were performed in HCC patients. How about a subgroup analysis for extrahepatic metastases according to treatment modality? The author should discuss this point

Authors' response: We analyzed the subgroup analysis by four different types of treatment modalities (TACE, RFA, PEI and Surgery) according to the reviewer's suggestion. We specified predictive factors for extrahepatic metastasis according to treatment modality in Results section of revised manuscript from page 11, lines 3 to page 12, line 7 as following:

“Subgroup analysis according to treatment modality

The number of patients for extrahepatic metastasis was 59 (28.9%) for the TACE group, 7 (9.4%) for the PEIT group, 2 (4.7%) for the RFA group and 8 (23.5%) for the surgery
group. We analyzed the predictive factor for extrahepatic metastasis according to treatment modality. Serum PIVKA-II level (P < 0.001), AFP level (P = 0.027), portal vein thrombosis (P < 0.001), platelet count (P < 0.001) and PT (P = 0.018) were significantly associated with the presence of extrahepatic metastases in univariate analyses in the TACE group. It was consistently shown that PIVKA-II and platelet count were independent risk factors for extrahepatic metastases based on a multivariate analysis. Patients with PIVKA-II ≥300 mAU/mL had a 6.4-fold (95% confidence interval [CI], 3.255-12.711, P < 0.001) increased risk and those with platelet count > 130K a 1.8-fold (95% confidence interval [CI], 1.084-3.154, P = 0.024) increased risk in the TACE group. AFP level and portal vein thrombosis did not appear statistically significant for extrahepatic metastasis in a multivariate analysis.

The serum PIVKA-II level (P = 0.007) and AFP level (P = 0.032) were significantly associated with the presence of extrahepatic metastases based on univariate analyses in patients receiving surgery. However, only PIVKA-II level (P = 0.070) had an independent risk factor for extrahepatic metastases in multivariate analysis. A serum PIVKA-II level ≥ 300 mAU/mL had an 8.7-fold (95% CI, 4.793-15.699, P < 0.001) increased risk for extrahepatic metastasis. The serum PIVKA-II level (P = 0.012) and AFP level (P = 0.002) were significantly associated with the presence of extrahepatic metastases in patients receiving PEIT. A serum PIVKA-II level ≥ 300 mAU/mL had a 23.1-fold (95% CI, 2.175-245.979, P =0.009) increased risk for extrahepatic metastasis while a serum AFP level ≥ 400 mAU/mL had a 5.2-fold (95% CI, 1.000-27.742, P =0.050) increased risk. The serum PIVKA-II level (P = 0.019) was significantly associated with the presence of extrahepatic metastases in patients receiving RFA. However, no
statistical significance manifested in patients with serum PIVKA-II level ≥ 300 mAU/mL, which is due to the small number of patients.

1-2.Reviewer's comment: I’m a little confused by the characteristics that were adjusted in Table 3 and Table 4. The portal vein thrombosis was used for multivariate analysis in overall patients (Table 3), but the PT was used for univariate and multivariate analysis in subgroup analysis according to stage instead of the portal vein thrombosis (Table 4). I would like to know the explanation for this.

Authors' response: The reason we excluded portal vein thrombosis in subgroup analysis according to the stages in Table 4 is because AJCC stage I, II and BCLC A, B itself can not include patients with portal vein thrombosis by definition. If patients had portal vein thrombosis, their stage is AJCC stage III or BCLC stage C. Therefore we do not include portal vein thrombosis when we analyzed subgroup analysis according to stages.

The reason we excluded PT in subgroup analysis in Table 4 was because they did not show statistically significance in univariate analysis in each subgroup, although overall patients showed statistically significance in univariate analysis and therefore included in multivariate analysis in overall patients (Table 3).

-Minor Essential Revisions

1-3.Reviewer's comment: The PT was not included in multivariate analysis of Table 3 (RESULTS, All patients, line 12).
**Authors’ response:** Table 4 is right. PT is not included in multivariate analysis.

According to reviewer’s comment, We removed PT and have changed the sentence in Results, all patients of revised manuscript on page 8, lines 12 as below.

We performed multivariate analysis with stage, portal vein thrombosis, PT, platelet count, and tumor markers according to cut-off values of tumor markers for AFP (≥400 ng/mL) and PIVKA-II (≥300 mAU/mL) ➔ removal of PT

We performed multivariate analysis with stage, portal vein thrombosis, platelet count, and tumor markers according to cut-off values of tumor markers for AFP (≥400 ng/mL) and PIVKA-II (≥300 mAU/mL).

**1-4. Reviewer’s comment:** The description “portal vein thrombosis (in AJCC)” should be changed to “portal vein thrombosis (in BCLC)” (RESULTS, All patients, line 14)

**Authors’ response:** According to reviewer’s comment, we have changed portal vein thrombosis (in AJCC) into (in BCLC) in revised manuscript on page 8, lines 14 as below.

It was consistently shown that PIVKA-II≥300 mAU/mL, portal vein thrombosis (in AJCC) and platelet count was independent risk factor for extrahepatic metastases based on multivariate analysis (Table 3).

It was consistently shown that PIVKA-II≥300 mAU/mL, portal vein thrombosis (in BCLC) and platelet count was independent risk factor for extrahepatic metastases based on
multivariate analysis (Table 3).

1-5. **Reviewer's comment**: The description “stage I, stage II and stage III” should be changed to “stage A, stage B and stage C”, respectively (RESULTS, subgroup analysis for extrahepatic metastases according to BCLC tumor stage, line 17,18).

**Authors’ response**: According to reviewer’s comment, We have changed stage I, II, III to stage A,B,C, respectively in revised manuscript on page 10, lines 24 as below.

The median time to extrahepatic metastases in those with a PIVKA-II ≥300 mAU/mL was 31.5 in **stage II** and 8.4 months in **stage III**, respectively, on Kaplan curves whereas **stage I** patients with a PIVKA-II ≥300 mAU/mL did not reach the median time to extrahepatic metastases during follow up periods. ➔

The median time to extrahepatic metastases in those with a PIVKA-II ≥300 mAU/mL was 31.5 in **stage B** and 8.4 months in **stage C**, respectively, on Kaplan curves whereas **stage A** patients with a PIVKA-II ≥300 mAU/mL did not reach the median time to extrahepatic metastases during follow up periods.

1-6. **Reviewer's comment**: The description “the groups II and III, group III” should be changed to “the groups B and C, group C”, respectively (RESULTS, Alteration of the PIVKA-II level ring follow-up, line 16,17).

**Authors’ response**: According to reviewer’s comment, We have changed stage I, II, III to stage A,B,C, respectively in revised manuscript on page 13, lines 22–23 as below.
The median time to extrahepatic metastases in groups II and III was 10.9 and 5.1 months, respectively (P < 0.001; Figure 4); however, group III did not decrease to the median time to extrahepatic metastases during the follow-up period.

The median time to extrahepatic metastases in groups B and C was 10.9 and 5.1 months, respectively (P < 0.001; Figure 4); however, group C did not decrease to the median time to extrahepatic metastases during the follow-up period.

1-7. Reviewer’s comment: The description “AFP>300” should be changed to “AFP>400” (FIGURE LEGENDS, Figure 1, line 6).

Authors’ response: According to reviewer’s comment, We have changed AFP >300 to AFP >400 in revised manuscript on Figure 1 legends

Refree 2: Hisanori Shinomi

Major Compulsory Revisions

2-1. Reviewer’s comment: The authors doesn’t show the state of the extra hepatic metastasis clearly, though they highlighted on it in their title. The population of the patients with extrahepatic metastasis in each groups, and correlation with PIVLA-II level, that is their point, can’t be reached from their data

Authors’ response: In our study, PIVKA-II levels were strongly associated with
extrahepatic metastases, especially in patients with early stage disease (BCLC stage A, AJCC I). As the tumor stage advances, the association between PIVKA-II and the risk for metastases may be weakened. We agree that the title of this study might mislead the conclusion, and it would be better to change our title to “Protein induced by vitamin K absence or antagonist-II production is a strong predictive marker for extrahepatic metastases in early hepatocellular carcinoma: a prospective evaluation.” We mentioned why the association between PIVKA-II and the risk for metastases may be weakened in advanced stage by the follow-up data in patients and discussion (page 15, line 1-20)

Revision: We changed the title to “Protein induced by vitamin K absence or antagonist-II production is a strong predictive marker for extrahepatic metastases in early hepatocellular carcinoma: a prospective evaluation”

2-2.Reviewer's comment: In “all patients” analysis, the authors didn’t show the state of the tumor size, number of the tumor, extra hepatic metastasis, micro vessel invasion, and many other factors are lacking.

Authors’ response:

According to reviewer’s comment, we specified tumor size (<5cm versus ≥ 5cm), number of the tumor (1,2-3,>3) in Table 1.

The information of extra hepatic metastasis already was included in Results, Baseline characteristics of revised manuscript on page 7, line 17-20 as below.

“Of 354 patients, 76 (21%) had extrahepatic metastases during the observation period.
Most common places for metastases to occur are the lungs (57%), bones (20%), metastatic lymph nodes (36%), adrenal gland (7%), peritoneal seeding (5%), spleen (3%) and the brains (3%).

2-3. Reviewer's comment: The authors mentioned that “association of PIVKA-II with extra hepatic metastasis was maintained in the subgroup analyses”, but not in BCLC stage B (Table 4).

Authors’ response: Our results showed the association of PIVKA-II with extra hepatic metastasis was maintained in the subgroup analyses except BCLC stage B. This can be explained by the intrahepatic tumor burden, which itself increases the value of PIVKA-II regardless of the presence of metastases as we mentioned. As the tumor stage advances, the association between PIVKA-II and the risk for metastases may be weakened. However, in case of AJCC stage II and III, BCLC C patients, they included vascular invasion (portal and hepatic vein invasion) by definition, which probably can affect tumor metastasis. In case of BCLC stage B patients, they define large multinodular tumor without vascular invasion. Therefore, the high PIVKA-II value in this group probably reflect only intrahepatic tumor burdens and not affect extrahepatic tumor metastasis.

2-4. Reviewer's comment: In the 2nd paragraph of Discussion, the authors described that “In addition, our study demonstrated that PIVKA-II level ~ portal vein thrombosis and invasion”, but not in BCLC stage B (Table 4).
Authors’ response: We agree the reviewer’s comment and have changed the sentence in discussion section of revised manuscript on page 14, line 17-20 as below:

In addition, our study demonstrated that PIVKA-II levels may be strongly associated with extrahepatic metastases in patients with **AJCC stage I or II HCCs and BCLC stages A and B** who did not have portal vein thrombosis and invasion.

“In addition, our study demonstrated that PIVKA-II levels may be strongly associated with extrahepatic metastases in patients with **AJCC stage I HCCs** and **BCLC stages A** who did not have portal vein thrombosis and invasion”

2.5 Reviewer’s comment: The authors had better to mention about the cut off level of the AFP and PIVKA-II. Since their populations are relatively large, they can describe that they are certain value showing receiver operating characteristic curve.

Authors’ response: We agree the reviewer’s comment and specified this in Discussion section of revised manuscript on page 16, line 12-15 as below.

In our study, we set the cutoff value for PIVKA-II levels at 300 mAU/mL and AFP level at 400 which was determined by the lowest value of $((1 - \text{sensitivity})^2 + (1 - \text{specificity})^2)$ as 349 mAU/mL and 420 ng/mL, respectively by using receiver operating characteristic curve of extrahepatic metastasis at the 12 months after curative treatment.

- Minor Essential Revisions
2-6. Reviewer's comment: Do the patients who are taking warfarin contain in this group?

Authors’ response: There was no one taking warfarin in our study. According to reviewer’s comments, we added it to exclusion criteria and specified it in Patients and Methods, “Patients” of revised manuscript on page 4, lines 5–6

Among these patients, 64 in whom serum levels of PIVKA-II and AFP at the time of diagnosis were not available, 43 who had metastases at the time of diagnosis, and 15 who were untreated were excluded. We also excluded the patients with taking warfarin, which was no one. Accordingly, 354 patients were enrolled into a prospective cohort study. This study was approved by the Institutional Review Board of Seoul National University Hospital.

2-7. Reviewer's comment: The manuscript contains 10 figures, but figure legends are only from figure 1 to 4.

Authors’ response: The number of figure are ten including figure1, 2a,2b,2c,2d,2e,2f,3,4, although figure are from 1 to 4.