

Peer Review File

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In this study entitled “Extracellular ubiquitin promotes hepatoma metastasis by mediating M2 macrophage polarization via the activation of CXCR4/ERK signaling pathway” the authors have shown that extracellular ubiquitin could promote hepatoma metastasis by promoting M2 macrophage polarization via the activation of CXCR4/ERK signaling pathway. The manuscript also suggest the importance of revising the personalized transfusion strategy needed for the treatment of HCC patients considering the neutralization of ubiquitin in stored red blood cell (RBC) units as it could be the responsible of the detrimental clinical outcomes induced by stored RBCs transfusion.

Despite the important significance of the results of the paper, I must say that it is very poorly written. I do not only mean innocuous typos or spelling mistakes. The manuscript is ultimately readable, but I have needed to re-read sentences or paragraphs several times to infer the meaning. I strongly suggest seeking a professional editor before publication. Furthermore, apart from a good proofreading by a native English specialist, I would like to provide some other minor considerations:

Comment 1: In the last paragraph of the “Introduction”, authors state that “Recently, we have demonstrated that eUb might be the link between allogeneic blood transfusion and poor prognosis of cancer patients, eUb promoted tumor metastasis in melanoma mouse model and this progress might be related with the immunomodulation mediated by eUb (16)”. However, as the study only used a mouse model authors should avoid referring to patients.

Reply 1: Thanks for the Reviewer’s advice and we have modified our text as advised.

Changes in the text: The sentences have been changed to “Recently, we have demonstrated that eUb might be the link between allogeneic blood transfusion and poor cancer prognosis; in the melanoma mouse model, eUb promoted tumor metastasis, which might be related to eUb-mediated immunomodulation (22).” (see Page 5, line 90-93).

Comment 2: In the “Methods”, in the explanation about the statistical analysis, are they assuming a normal distribution of the data? Could authors explain it?

Reply 2: Thanks for the Reviewer’s reminding and the data have been explained in the revised manuscript.

Changes in the text: The “Methods” has been changed to “GraphPad Prism 7 (GraphPad Software, La Jolla, CA) was used to perform statistical analysis. Non-parametric data (the expressions of CXCR4 and Ub in clinical tissues) were shown as median (25th-75th interquartile range), and comparisons between groups were performed with Wilcoxon matched-pairs signed rank test and Kruskal-Wallis test. Correlation of non-parametric data were

analyzed using Spearman Correlation test. Parametric data were expressed as mean \pm standard deviation (SD) and comparisons between groups were performed with Student's t-test and one-way ANOVA. A p-value of <0.05 was considered statistically significant." (see Page 13, line 236-244).

Comment 3: In the "Results", in the fourth paragraph about "Ub was up regulated in HCC tissues and positively related with CXCR4", authors state that "The results showed that the expressions of Ub and CXCR4 were dramatically stronger in HCC tissues than adjacent nontumor and normal liver tissues (Fig. 1A)". However, when I look into Fig. 1A the sample #4 does not really seem like that. Moreover, authors forgot to mention the GAPDH in the figure legend.

Reply 3: Among 40 cases of tissues, the expression of Ub in 14 cases of HCC tissues was lower than the corresponding adjacent nontumor liver tissues, and the expression of Ub in 13 cases of HCC tissues was lower than the corresponding normal liver tissues (Fig. 1B). After the statistical analysis of the expression of Ub in 40 cases of HCC tissues, adjacent nontumor and normal liver tissues, we concluded that "The results showed that the expressions of Ub and CXCR4 were dramatically stronger in HCC tissues than adjacent nontumor and normal liver tissues".

Changes in the text: The sentence has been changed to "The results showed that the expressions of Ub and CXCR4 were strikingly stronger in HCC tissues than in adjacent nontumor and normal liver tissues (Fig 1A), and there was no significant difference between their expressions in adjacent nontumor tissues and normal liver tissues (Fig 1B, 1C)." (see Page 13, line 252-255). And GAPDH has been mentioned in the "Figure legends".

Comment 4: In the "Results", in the fourth paragraph about "eUb promote d the metastasis of hepatoma cells in tumor bearing model", authors write "through H&E staining" but nothing about histology is explained in the "Methods" chapter. Furthermore, in the same paragraph authors write "Meanwhile, we harvested the lung tissues and detected the expression of metastasis related proteins, E cadherin was down regulated while Vimentin and N cadherin were increased in eUb group, as well as CXCR4." It is impossible for me to understand this sentence. Maybe, is it possible that the authors could really want to say this: "Meanwhile, we harvested the lung tissues and detected the expression of metastasis related proteins. E cadherin was down regulated while Vimentin and N cadherin were increased in eUb group, in relation to the control group, or regarding the control group."

Reply 4: We are very sorry that we harvested the lungs to perform H&E staining, but didn't evaluate histology at that time and we will pay attention to this point in the future study.

Changes in the text: The sentences have been changed to "Meanwhile, we harvested the lung tissues to detect the expressions of metastasis-related proteins and found that E-cadherin was downregulated; however, Vimentin, N-cadherin, and CXCR4 were upregulated in the eUb group compared to the control group." (see Page 16, line 300-302).

Comment 5: In the “Results”, in the fifth paragraph about “eUb up regulated the ratio of M2 macrophage in tumor bearing mice” authors forgot to refer Figures 5D and 5E.

Reply 5: Thanks for the Reviewer’s reminding, the results of Fig 5D and 5E have been added in the revised manuscript.

Changes in the text: The following sentences have been added: “The concentration of TGF- β in the serum of the eUb-treated mice was upregulated; however, the concentrations of TNF- α and IL-10 were not obviously changed compared with the control mice (Fig 5D). Furthermore, the results of Western blot showed that iNOS (M1 macrophage marker) and Arg-1 (M2 macrophage marker) were both enhanced upon eUb exposure; yet, Arg-1 was more significantly upregulated than iNOS (Fig 5E).” (see Page 16, line 317-321) . “Meanwhile, the expressions of CXCR4 and p-Erk1/2 were increased in the lung tissues of eUb-treated mice compared with the control mice (Fig 5E).” (see Page 18, line 345-347).

Comment 6: In the “Discussion”, for me the first paragraph is information that should be part of the “Introduction” instead of the “Discussion”.

Reply 6: Thanks for the Reviewer’s advice and the content of the first paragraph in the “Discussion” has been moved to the “Introduction”.

Changes in the text: see Page 4, line 72-73, 76-81, 86-88; Page 5, line 89-93.

Comment 7: Authors should clarify the meaning of the some initials the first time used. E. g. Red blood cell (RBC); Glyceraldehyde-3-phosphate dehydrogenase (GAPDH); Mitogen-Activated Protein Kinases (MAPK)

Reply 7: Thanks for the Reviewer’s reminding and the abbreviations have been clarified.

Changes in the text: The abbreviations have been clarified in the revised manuscript.

Comment 8: There are two tables at the end of the .pdf file (Table 1 and Table 2) but they are not referred in the manuscript.

Reply 8: The two tables have been referred in the “Methods”.

Changes in the text: see Page 5, line 107-108; Page 11, line 223-224.