Author’s response to reviews

Title: A proteomic analysis of serum-derived exosomes in rheumatoid arthritis

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Version: 1 Date: 18 Sep 2018

Author’s response to reviews:

Prof. Caroline Ospelt,
BMC Rheumatology,
Editor-in-Chief,

Dear Prof Caroline Ospelt,

Thank you for inviting us to submit a revised version of our manuscript. According to the reviewers’ comments, we carefully revised our original manuscript. In the revised manuscript, we added several experimental data.

Our responses to the comments of the reviewers are described in the following separated pages.
We believe that our revised manuscript is acceptable for the publication in BMC Rheumatology. We are looking forward to hearing good news so soon.

Sincerely yours,

Hirotaka Tsuno, MD, PhD.

Answers to the comments of Prof. Mojca Frank-Bertoncelj (reviewer 1)

We thank Prof. Mojca Frank-Bertoncelj (reviewer 1) for her useful suggestions. We have carefully our manuscript according to the comments. Owing to the suggestions we were able to improve our manuscript very much.

Major comments

[Comments] Purity and characterization of exosomes. ExoQuick can precipitate in addition to exosomes also other non-exosome protein components in serum, which could contaminate 2D-DIGE results. To determine to which extent DIGE results represent pure exosome proteins, I recommend additional characterization of exosome isolates and their purity e.g. by following the "Minimal experimental requirements for definition of extracellular vesicles and their functions: a position statement from the International Society for Extracellular Vesicles (Table 1, Lötvall J. et al. J Extracell Vesicles. 2014; 3: 10.3402/jev.v3.26913.) If possible, I recommend using a complementary method for exosome isolation, e.g. one of the gradient centrifugations, to confirm the major results.

[Answers]

We thank reviewer 1 for her comments.

We here used a relatively new method of Exoquick to isolate exosomes instead of traditional methods like gradient centrifugation, since Exoquick® was more powerful to isolate exosomes from large number of clinical samples. Exoquick® has been recognized as an alternative useful method to isolate exosomes and used in many recent studies. Of course, contamination of serum proteins cannot excluded as reviewer 1 commented. We would like to intensively investigate characters and functions of the individual identified proteins as exosomal protins in the future. We described this point in the last of discussion section of our revised manuscript (Line 4 of the page 21).

[Comments] Expected and predicted molecular weights and isoelectric points of identified proteins do not match, the authors should comment on this observation (Table 3). I would recommend confirming major results with Western blot if possible. How the observed results fit
We thank the reviewer 1 for her comments. We added description about differences between observed and theoretical MWs of the identified proteins in the discussion section of our revised manuscript (Line 6 of the page 20).

As she recommended, we tried to detect TLR3 fragment (24kDa) with western blot (WB). We first tried to detect TLR3 fragment (24kDa) in the active RA group by 2DE-WB. As a result, the TLR3 fragment (24kDa) was not detected, instead, several spots of TLR3 fragments (17-18kDa) were detected as shown in Figure 6A. This indicates that serum-derived exosomes in patients with active RA contain larger amounts of TLR 3 fragments than that in patients with inactive RA and OA and in healthy donors. We compared expression levels of the TLR3 fragments (17-18 kDa) by 1DE-WB among the 4 groups. As a result, high expression levels of the TLR3 fragments (17-18 kDa) were detected more frequently in aRA and iRA groups than in OA and HL groups(Figure 6B(ii) and (iii)). we added this new finding in the methods (Line 12 of the page 10 and line 9 of the page 11), results (Line 3 of the page 16), and discussion (Line 6 of the page 17) section of our revised manuscript.

According to the suggestion by reviewer 1, we confirmed the 6 proteins identified in this study have been registered in Vesiclepedia (Kalra H. et al. Vesiclepedia: a compendium for extracellular vesicles with continuous community annotation. PLoS Biol. 2012;10(12): e1001450. doi: 10.1371/journal.pbio.1001450) and added the description in the results section (Line 13 of the page 15).

2D-DIGE: Could the authors provide more details about the characteristics of the spots (e.g. where there any changes in the horizontal/vertical positions of the spots, suggesting changes in the size/charge between the experimental group)s. Were the 204 spots identified in all 43 samples, can the authors provide the Suppl. Table with spot intensities in 43 samples? Were the overlapping spots (e.g. Fig 3Ai) changed in the same direction (e.g. increased). There seem to be three predominant sizes of exosome proteins, can the authors comment on this. Could the authors mark the differentially enriched spots on Fig 2. for all 4 experimental groups (or provide additional supplementary Figure)?

We thank the reviewer 1 for her comments. The 204 spots show positive intensity in all the 43 samples. We described additional information on the 204 spots (averages of each spots in the aRA, iRA, OA, and HL groups) as “supplementary Table 1” in the revised manuscript (Line 18 of the page 12).
To show the directions of spot intensity differences, we added information in Figure 3A(i) and 3B(i) in the revised manuscript. For example, when 12 and 8 spots showed higher and lower intensity respectively in the disease groups than in the HL group, we showed it as (↑12, ↓8). This would help understanding of the figures.

The 3 predominant sizes of exosome proteins are speculated to be albumin or IgG from their MWs, although they were not identified by mass spectrometry. Even though we tried to remove albumin and IgG by serum albumin and IgG removal kit (AurumTM Serum Protein Mini Kit, Bio-rad) as a preliminary experiment, they were only partially removed. Thereby the proteins were considered to be contained in exosomes or bound to exosomes.

As the reviewer 1 suggested, we added “supplementary Figure 1” to show location of the differently enriched spots on the Figure 2 in the revised manuscript (Line 10 of the page 14).

Minor comments

[Comments] Pre-analytical variables: The authors should provide details on pre-analytical factors during blood withdrawal (needle gauge, tubes used, temperature of the centrifugation, time between blood withdrawal and centrifugation, any haemolysis present, were all the samples treated same way).

[Answers]

We thank the reviewer 1 for her comments. We use needles of 21-23 gauges and Venegect II® tubes to take blood samples. The tubes were centrifuged at 1,500×g for 10 minutes at room temperature. The time between blood collection and centrifugation was largely within 15 minutes. We added short description on these points in the materials and methods section of the revised manuscript. (Line 7 and 9 of the page 8)

[Comment] The description of RA pathogenesis in the background is too simplified.

[Answer]

We thank the reviewer 1 for the suggestion. However, on the other hand, the other reviewer commented “the introduction was too long and diffuse”. Thereby, we tried to brush up the description of background without change of the whole volume of the background.

[Comments] Table 1, please notify significant differences in parameters between iRA and aRA, I would suggest commenting briefly on patient characteristics in Results

[Answers]
We thank the reviewer 1 for the suggestion. As reviewer 1 suggested, we compared the parameters of clinical feature between between iRA and aRA. The statistical significance of parameters in clinical features between the aRA and the iRA groups was calculated by Student’s t-test. We added the description on this point in the section of materials and methods and results and Table 1 in our revised manuscript. (Line 7 of the page 11 and Line 2 of the page 12)

[Comments] The authors suggest that the TLR3 fragment may be functional, which is speculative, it could be also a degradation product, can the authors comment on this. Is TLR3 fragment increased in exosomes from all patients with active RA?

[Answers]

We thank the reviewer 1 for the suggestion. We understood that our description of “the TLR3 fragment may be functional” was too speculative. Therefore, we revised the description in the discussion section of the revised manuscript (Line 1 of the page 19).

The expression level of the TLR3 fragment (24kDa) was increased in serum exosomes from 10 of 12 aRA patients. Additionally, high expression levels of the TLR3 fragments (17-18 kDa) were detected more frequently in aRA and iRA groups than in OA and HL groups (Figure 6B(ii) and (iii)).

[Comments] Recently, Poly(I:C), a TLR3 ligand, was shown to associate with extracellular vesicles with downstream effects on FLS (https://doi.org/10.3389/fimmu.2018.00028). How do the authors comment on this data with respect to their findings?

[Answers] We thank the reviewer 1 for proposing the most recent article describing the association between Poly(I:C) of a TLR3 ligand and extracellular vesicles in synovial fibroblasts. We cited this article (Line 9 of the page 18).

Taking these findings and our data into consideration, Poly(I:C) EV might contain the TLR3 fragments and have some function. Further studies are needed in this point.

[Comments] In addition, OA vs healthy comparisons are interesting, why do the authors not comment on this. How do the authors comment on their finding that iRA and OA exosomes differ least? Can the authors comment all 6 differentially enriched spots in discussion?

[Answers] We thank the reviewer 1 for the suggestion. As the reviewer 1 suggested, we add the description about cathepsin F, which was increased in OA group, in the discussion section of the revised manuscript (Line 1 of the page 20).

We would like to comment all 6 proteins identified in this study in the discussion section, however, the amount of description of discussion section has been already quite large. Therefore, we referred to only the 3 proteins (TLR3, Pro-neuregulin-3, membrane-bound isoform and Cathepsin F) of the 6 proteins.
[Comment] The electron microscopy image should be sharper.

[Answer] We thank the reviewer 1 for her comment. As reviewer 1 recommended, we prepared a sharper photo as Figure 1 in the revised manuscript (revised Figure 1).

[Comment] I would suggest using SD rather than SEM in Fig. 5, also dots would be more informative than bars.

[Answer] We thank the reviewer 1 for her comment. We changed SEM to SD and bars to dot plots to be more informative in Figure 5 (revised Figure 5).

We thank the reviewer 1, Prof. Mojca Frank-Bertoncelj, for giving us many useful suggestions. The suggestions from the reviewer 1 were very useful to improve our manuscript.

Answers to the comments of Prof. Gyorgy Nagy (reviewer 2)

We thank the reviewer 2, Prof. Gyorgy Nagy for giving us many useful suggestions. We have responded one by one to the major and minor comments from the reviewer, as described below. We revised our manuscript very carefully. The suggestions from the reviewer 2 improved our manuscript greatly. We appreciate the suggestions of the reviewer 2.

Major point:

[Comment] It would be essential to confirm the increased expression of TLR3 of RA exosomes by Western blot.

[Answer] As reviewer 2 recommended, we tried to detect TLR3 fragment (24kDa) with Western blot (WB). We first tried to detect TLR3 fragment (24kDa) in the active RA group by 2DE-WB. As a result, the TLR3 fragment (24kDa) was not detected, instead, several spots of TLR3 fragments (17-18kDa) were detected as shown in Figure 6A. This indicates that serum-derived exosomes in patients with active RA contain larger amounts of TLR 3 fragments than that in patients with inactive RA and OA and in healthy donors. We compared expression levels of the TLR3 fragments (17-18 kDa) by 1DE-WB among the 4 groups. As a result, high expression levels of the TLR3 fragments (17-18 kDa) were detected more frequently in aRA and iRA groups than in OA and HL groups (Figure 6B(ii) and (iii)). we added this new finding in the methods (Line 12 of the page 10 and line 9 of the page 11), results (Line 3 of the page 16), and discussion (Line 6 of the page 17) section of our revised manuscript.

Minor points:

[Comment] The introduction is too long and diffuse.
[Answer] We thank the reviewer 2 for the suggestion. On the other hand, the other reviewer commented “The description of RA pathogenesis in the background is too simplified.”. Thereby, we tried to brush up the description of background without change of the whole volume of the background.

[Comment] The references are not up-to-date, many important recent (from 2016-18) original papers were not cited.

[Answer] We thank the reviewer 2 for the suggestion. As the reviewer 2 suggested, we cited 5 recent original articles (from 2016-2018) in the revised manuscript as follow; Reference No.11 (Line 1 of the page 6), No.20, 21 (Line 4 of the page 7), No.31 (Line 9 of the page 18), and No.39 (Line 3 of the page 20).

[Comment] The manuscript needs English language editing by a native speaker.

[Answer] We thank the reviewer 2 for the suggestion. As reviewer 2 suggested, English language of our revised manuscript was edited by a native speaker.

We thank the reviewer 2, Prof. Gyorgy Nagy for giving us many useful suggestions. The suggestions from the reviewer 2 were very useful to improve our manuscript.