Author's response to reviews

Title: Threonyl-tRNA Synthetase Overexpression Correlates with Angiogenic Markers and Progression of Human Ovarian Cancer

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Author's response to reviews: see over
Response to Reviews:

The authors thank the reviewers for their careful review of our manuscript. We have considered the comments and have made changes to the manuscript that clarify the points raised by the reviewers and more accurately reflect the results presented (changes are indicated by line number). We agree that the data represent a new area that will benefit from further analysis to answer questions related to the role of TARS in inflammation and angiogenesis and its impact on clinical outcome. We also appreciate the limitation of this type of study and have now specified these limitations in the conclusions of the study. Below are descriptions of specific changes made to the manuscript and responses to reviewers’ comments.

Reviewer 1

1. What percentage of ovarian tumor tissues contain robust TARS staining in leukocytes and what is the outcome/survival of the patients with this staining? 32 of the 70 patient samples exhibited positive immune cell staining. There were very few tumors that had observed immune cell infiltration that did not have TARS staining. As with the tumor TARS, there was positive relationship between TARS and survival, but the confidence in this correlation was reduced in the multivariate analysis. We have clarified this result in the manuscript. We are confident that the staining in immune cells is not surface staining, and there is a published report indicating that TARS is among proteins found within exosomes secreted by immune cells (Buschow et al Immunol. Cell. Biol. 2010). We are very interested in characterizing the effects of TARS on the immune response to ovarian tumors. We are thus currently undertaking a full study of TARS and characterization of immune cell infiltration in a mouse model of ovarian cancer and in freshly isolated lymphocytes, but those results are beyond the scope of this manuscript. (lines 224-225; 335)

2. What effect do hypoxia and TNF-alpha have on mRNA stability in Fig. 4C? Others have shown that the primary effect of hypoxia and TNF-alpha on VEGF and IL-1beta respectively is through increased transcription through HIF-1 and NFkB rather than mRNA stability, however we did not directly measure mRNA stabilization. Regardless, the impact of VEGF and TNF-alpha on TARS secretion is not likely due to increased transcript level. We have clarified the text in the results to reflect the possible impact of mRNA stability on the observed mRNA levels. (lines 244-245)

3. Is there a correlation between CA-125 and TARS expression? There was a positive relationship between CA-125 and TARS (r=0.214), but it was not statistically significant (p-value > 0.1). We did not include these data because there were a limited number of samples that had CA-125 values (33), thus we decided to wait to report this correlation until we can obtain more study samples to raise the confidence level.

4. What is the function of TARS in normal ovaries? TARS is expressed in all cells and functions to charge threonine onto tRNA. It has only recently been described to have secondary functions as a signaling
molecule, results which inspired the current study. According to the Human Protein Atlas, TARS is moderately expressed in the ovary in general, but highly expressed in the glandular cells of the Fallopian tube and in ovarian, prostate and colon cancers. Our hypothesis is that TARS is overexpressed and secreted by tumor cells responding to nutrient or other stresses. We have adjusted the text in the introduction and discussion to clarify this concept. (lines 104-105; 195-197; 296-299)

Reviewer 2:

1. Please explain in more detail the method for detection of overexpressed TARS vs background levels. We appreciate the opportunity to clarify this important point. The concentration of antibody used is within the parameters suggested by the manufacturer and even when used at levels that can detect low levels of TARS in normal cells, there was a dramatic difference in the tumor staining. We tested a range of antibody concentrations on benign and advanced tumors and chose the antibody concentration that gave us the best range of TARS staining intensity to improve our ability to differentiate between low and high levels of staining. We used this same method in determining the concentration of VEGF antibody in this and previous analyses with good success. Thus, we are confident that we are not measuring noise. The Methods section has been modified to include additional information regarding the choice of antibody concentration and its use in establishing the background level of TARS staining. We have also found that TARS is heavily overexpressed in ovarian tumor cell lines and correlates with their aggressiveness, but these data will be included in another manuscript related to the mechanism of TARS function. (lines 150-152, 157)

2. On the IHC analysis, is the intensity a function of number of cells stained or intensity within a few cells? The score is based on overall intensity in the tumor within each sample. After two independent blinded scores were assessed, the discrepancies were discussed to come to a consensus or an average. There were very few samples where the scores differed by more than 1. The weakness inherent in this type of scoring has been better described in the text, and is part of the reasoning for showing the data in Fig 2C (see response 3,4). (lines 150-152; 352-354)

3, 4. In figure 2, what is the rational for inclusion of 2C; what is stage 3.75? Why group the data in 2B? In 2C, the intent was to be transparent about the TARS staining scores by showing the raw data in a graphical format. Ovarian cancer stages now include A, B, and C to give additional information about the tumor advancement. The stages were thus recorded as 3.25, 3.5 and 3.75. We grouped the stage data in Fig 2B because there were very few samples in stage 2. The Pearson’s correlations reported in the tables take these parameters into consideration and is a stronger analysis, but showing average scores gives the reader additional information. We have edited the text that describes this figure and its method to clarify these points. (lines 139, 565)

5. One of the hypotheses is the correlation of TARS with angiogenesis, however I do not believe there is convincing evidence of this presented in the paper. We based our conclusion on the correlation between TARS and VEGF staining and on the presence of PECAM, but we agree that the analysis does not include quantification of microvascular density or functional blood flow. We have therefore edited the title, abstract and conclusions to reflect the correlation with angiogenic markers rather than angiogenesis. (lines 1, 54, 59, 208, 347-348)

6. Please change the heading on the section “Serum levels of TARS correlate with tumor TARS and markers of angiogenesis.” We agree with the reviewer and have removed the reference to angiogenesis
in this subsection title. As requested, we have also clarified the limitations inherent in the experiments and conclusions in the discussion section. (lines 352-354)

Thank you for your consideration.

Sincerely,

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