Author's response to reviews

Title: The human complement inhibitor Sushi Domain-Containing Protein 4 (SUSD4) expression in tumor cells and CD8+ T cells is associated with better prognosis of breast cancer patients.

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Author's response to reviews: see over
Dear Editor in Chief,

Regarding manuscript: 1938403625174604 entitled: The human complement inhibitor Sushi Domain-Containing Protein 4 (SUSD4) expression in tumor cells and infiltrating T cells is associated with better prognosis of breast cancer patients.

We are very grateful for the opportunity to resubmit our manuscript. In this extensively revised form, we have addressed all concerns of the reviewers by including new data and modifications in the text to make the manuscript more clear.

Please find a detailed response to the reviewers’ queries below.

We hope that the manuscript in its current form is suitable for publication in the BMC Cancer Journal.

Sincerely,

Anna Blom, professor
Answers to reviewers’ comments:

First, we would like to thank the reviewers for their insightful comments that have allowed us to significantly improve the quality of the manuscript.

Reviewer 1:

General comment: This paper shows that human SUSD4 expression in tumor cells and CD8+ T cells is associated with better prognosis of breast cancer patients. These findings provide novel clues into the function of SUSD4 in breast cancer. However, there are also several deficiencies as noted below, which the authors should address before further consideration.

Comment 1: For Figure 2, qPCR and FACS analyses indicates that the expression level of SUSD4 in the MDA-MB-231 cells is higher than that in BT20 cells, which is not consistent with the result of western blot. The author should provide control protein such as beta-actin to normalize it in western blot.

Answer: We apologize for the confusion in this figure, which is due to the fact that the experiments were done at different time points after transfection and that the MDA-MB-231 cell line has a tendency to decrease expression of SUSD4 over time. The FACS histogram in the initial manuscript showed MDA-MB-231 SUSD4a expression levels directly after transfection, while the western blot showed expression levels at the end of the experiments. Generally, the expression of SUSD4 has been stronger in BT20 cells as compared to MDA-MB-231 throughout all experiments. To adequately illustrate this point, the FACS histograms have been updated and now show SUSD4 expression levels at a similar time point as the western blots. For western blotting, we have used BCA assay to ascertain the same loading of the total protein for mock and SUSD4 transfected cells for both cell lines.

Comment 2: For Figure 3, the results of the original picture of cell migration and invasion should be provided.

Answer: The original pictures have been included in the figure.

Comment 3: The quality of Figure 4 needs to be improved.

Answer: The quality has been improved.

Comment 4: For Figure 5b, some cells are SUSD4 positive but CD8 negative; For Figure 5c, the mRNA expression level of SUSD4 in CD4+T cells is higher than that in CD8+T cells. Therefore, CD4 should be analyzed in Figure 5b and SUSD4 should also be analyzed at protein level in Figure 5c.

Answer: Thank you for this comment, which made us perform new experiments that yielded interesting and unexpected finding! Protein analysis by western blotting revealed that SUSD4 is upregulated upon T cell stimulation. This is completely opposite to what we see at mRNA level – presumably due to strong posttranscriptional regulation by for example micro RNAs. To make sure that this observation is true, we used T cells isolated from the same donor (at the same time point) for simultaneous mRNA and protein analysis. This analysis was performed for several different donors yielding consistent results for every one of them. Further, for the qPCR, we used three different primers specific for SUSD4a and all three
showed the same result. Thus, the final conclusion is that SUSD4a mRNA is definitely downregulated, while the protein expression is upregulated, when the T cell is activated.

Reviewer 2:

Comment 1: Figure 1A. The immunohistochemical staining for SUSD4 is staining the nucleus of the cells. SUSD4a has a transmembrane domain and is on the surface of cells. Why is SUSD4 staining nuclear and not membrane?

Answer: Importantly, we have established exact staining conditions using CHO cells expressing recombinant SUSD4 or lacking it and treated the same way as TMAs (see figure below, ref: Holmquist E, Okroj M, Nodin B, Jirstrom K, Blom AM. Sushi domain-containing protein 4 (SUSD4) inhibits complement by disrupting the formation of the classical C3 convertase. FASEB J. 2013;27(6):2355-66). We are therefore confident in the specificity of the staining. The staining for SUSD4 in TMAs is mainly cytoplasmic and perinuclear, but there is also a tendency of nuclear staining. Considering its domain structure, SUSD4a should be localized to the cell membrane as well as on the intracellular ER and Golgi membranes (effectively giving cytoplasmic staining). However, this protein is poorly studied and there is a possibility that the other splice form, SUSD4b, is also expressed. There is no information on potential localization of SUSD4b other than it lacks a transmembrane domain and therefore it is possible that it has intracellular and even nuclear localization. The antibody is generated against domains present in both SUSD4a and SUSD4b and does not distinguish between these two forms.

Comment 2: Figure 1B. Why does SUSD4 not stain in the epithelial cancer cells? Are the low and high examples shown here a Score 0 for SUSD4? How can you discern stained epithelial cells from stromal cells when SUSD4 has strong staining in Score 2 in Figure 1A?

Answer: Indeed, fig 1B shows score 0 for SUSD4 in epithelial cells. It was otherwise easy to discern stained stromal cells from epithelial cells because they stained much more intensely and were smaller in size.

Comment 3: Figure 1D, F and H. How do you define survival versus recurrence? The survival data is strong and convincing. The recurrence data, by eye, looks the same as the survival data, but is not statistically significant. The authors could consider not showing the recurrence curves and only the survival curves. Taking the recurrence curves out of the manuscript will not change the conclusions of the paper.
**Answer:** Survival was defined as number of years the patient survived after diagnosis. Recurrence is defined as recurrence of the breast cancer after the first successful therapy, either in the initial site or as metastases, and thus not necessarily leading to death. In some cases these patients were treated successfully during recurrence.

The reason why survival is statistically significant and recurrence is not despite similar trend lies presumably in the relatively small number of the patients analysed. However, it still shows an important trend and therefore we would like to keep it in the manuscript. Recurrence is a parameter often analysed in this type of study and when excluded would generate questions in the reader.

**Comment 4:** Figure 2B. The x-axis for the flow cytometry curve is strange. How can the majority of the peak be below zero? The western immunoblot analysis in Fig. 2C is very impressive. Either change the scale on the x-axis for Fig. 2B or just remove the flow cytometry experiment from the paper.

**Answer:** Unfortunately the proprietary Partec flow cytometer software generates this type of result i.e. scale ranges from $10^{-1}$ to $10^3$ (from 0.1 to 1000, please note that none of the values are below zero). However, this does not affect the accuracy of the experiment. The fluorescent signal in the flow cytometer is always in arbitrary units and depends on the settings, such as the gain. Thus, the x-scale values per se are not informative, only the relative values between mock- and SUSD4-transfected cells, which clearly show a difference. We would like to keep the flow cytometry data since this method of protein detection is quite different from western blotting and shows among others that a portion of SUSD4 is on the cell surface, which cannot be ascertained by the blot. On the other hand, the blot shows that our antibodies detect protein of correct size, which is also valuable information. Thus the two methods are complementary.

**Comment 5:** Figure 3. Photos of the stained cells need to be included with the data for wound closure, migration and invasion. For wound closure, a time course showing the cells filling in the wound is necessary for a reader to properly evaluate the data. If a yellow pipet was used to scratch the monolayer, were the scratches equal in diameter for each cell line? Showing the cell counts in not sufficient. A photo of stained cells on the membrane for migration and invasion is also needed.

**Answer:** Requested representative photos have been included in each figure. Indeed the yellow tip generated scratches that differed a little in width but the results presented are always relative to the initial wound width and therefore this does not disturb the conclusions. For the wound closure we have included time course with two time points.

**Comment 6:** Figure 3. Please state for each experiment the number of times it was performed and whether it was performed in duplicate, triplicate, etc.

**Answer:** This information has now been added.

**Comment 7:** Figure 4. A scale bar needs to be put into all photos in this figure. The photos of the cells in Fig. 4B are not clear. One cannot distinguish a colony from a single cell. New photos should be taken with better definition.

**Answer:** Scale bars have been added and the quality of fig 4 was improved as requested. However, it is sometimes difficult to distinguish every separate cell in a colony, since the cells tend to grow on top of each other as the colony grows larger in size.
Comment 8: Figure 5. A photo showing staining of CD4+ cells and no SUSD4 staining should be shown. CD4+ cells do not stain for SUSD4 – correct? Page 17 lines 8 and 9 never state that SUSD4 does not stain in CD4+ cells: “Further stainings with anti-CD4 (data not shown) or anti-CD8 (Figure 5B), showed that cytotoxic CD8+ T cells expressed SUSD4.” This is an important statement to clarify since further experiments are performed to study SUSD4 in CD4 cells.

Answer: Thank you for this comment! After further optimization of the staining method, we can conclude that tumor infiltrating CD4+ T cells also are positive for SUSD4. The figure has been revised to include the CD4/SUSD4 staining.

Comment 9: In Figure 5B, red cells (SUSD4) with no green staining (CD8) are shown (two on the very bottom of photo) contrary to the text on page 17, line 10: “…cells positive for SUSD4 were also positive for CD3 and CD8.”

Answer: Indeed some of the SUSD4 positive cells were negative for CD8+. These cells could however be single tumors cells positive for SUSD4. This has been clarified in text now.

Comment 10: Figure 5A and B. A photo showing CD8 staining but no SUSD4 should also be included to represent the staining patterns of the cells.

Answer: The figure has been changed to include this.

Comment 11: Figure 5C and D. The results from these experiments are not clear. The expression profile of CD4+ (Fig. 5C) is identical to CD8+(Fig. 5D), but SUSD4 was shown to only be present in CD8+ cells in the above Fig A and B.

Answer: This discrepancy has now been resolved by improved immunochemical staining in which we detected SUSD4 expression by both CD4+ and CD8+ tumor infiltrating T cells.

Comment 12: Minor Essential Revisions

1. Typo page 5, line 4. cells cells
2. SUSD4 should be italicized when describing the gene or RNA.
3. Page 10, line 10: 10X magnification – does that include the ocular lens 100X total?
4. Page 15, line 15. Using two specific primers for qPCR not just one?
5. Page 19, lines 1 and 2 Nonetheless, since we could not expression?
6. Page 11, line 7 states 4X magnification. Is that total magnifications?
7. Page 11, line 23 states 20X magnification. Total? Ocular included?

Answer: The mistakes have been corrected.