Author's response to reviews

Title: Large-scale proteomic identification of S100 proteins in breast cancer tissues

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Author's response to reviews:

We wish to thank the Referees for constructive comments. After careful reading of their specific observations we send attached our reply.

Comments of Reviewer 1

Essential revisions

1. Quantification of S100A proteins expression in tumors vs non-tumors/normal tissue would be of importance to show (Fig 4, page 8; as an option – N%V values). Such quantification would help in evaluation of the correlation of S100A proteins expression and tumorigenesis and metastasis (Figures 5 to 7).

Reply: A figure with S100A spot quantification has been introduced. The data in the graphs are expressed as median ±SD. This new figure is now numbered as Fig. 5, and the previous ones are shifted consequently.

2. The similar comment about quantification – for data shown in Figures 5 and 6 (pages 8 and 9). It is difficult to trace each case from Figure 5, where there is no quantitative information, to the image in Figure 6. This comment is also linked to the analysis of relationship in expression between various S100A proteins (Figure 7; page 9). There description and analysis is virtually absent, i.e. only 2 short sentences (page 9, “Relationship...” section). This manuscript may benefit from more thorough analysis of quantitative information in relation to clinico-pathological data. It may be that the authors have already done that (page 10; was correlation analysis based on quantitative data?), then it would be good
to show this quantitative analysis.

Reply: Concerning this point, we would like to mention that it is not within the aim of this study (which is not a clinical report) to trace quantitative information for individual patients.

However, in reply also to Reviewer 3, we added a table with % of patients expressing the different S100 protein forms (Figure 6B).

In relation to the comment on Figure 7 (now Fig. 8), we would like to point out that our analysis was performed to ascertain if the expression levels of different S100 proteins, identified in the present study, were correlated to each others. Indeed, our results suggest a collective deregulation of this gene family in breast cancer. We added in the text a comment on this point, as well as in the figure legend the information on quantitative data (N%V) and the p value. We have also deleted from Figure 7 (now Fig. 8) the term NO, which means no correlation, leaving the p value > 0.05.

3. Reported correlations with metastasis (pages 9-10, Fig 8) are highly interesting. Why the authors referred to the expression level 1.07 (page 10, top of the page) if it is not significant (p>0.05)?

Reply: The text and the diagram in Fig. 9 (previous fig 8) were aimed to show the general tendency of higher S100 protein expression in metastatic patients, compared to the disease-free group.

Statistical significance, assessed by unpaired F-test, was obtained for S100A2, S100A11 c, S100A8, S100A7 and S100A4. We believe that the information in this form is sounder than giving only the significant differences.

Mentioned correlations with Ki67 and nodal status would be beneficial to show (page 10, the last sentences of the top paragraph)

Reply: The information about correlations with Ki67 and nodal status is now presented as a separated paragraph with a new table (Table 2).

4. Reference to documentation related to ethical permits is required (page 5; clinical specimens). To mention that the recommendations were followed is not enough. These recommendations should be referred (number, decision-taking body, legislation followed, etc).

Reply: We added the request information in clinical specimens page 5.

Discretionary Revisions

5. Conditions of MALDI TOF MS have to be described, e.g. notes about digestion of proteins, conditions for generation and collection of spectra, parameters of searches, etc (page 6).

Reply: Conditions of MALDI TOF MS were described in detail in the “Material and Methods” session, page 7.

6. Please provide annotation of Figure 7. What mean “No” and “Yes”? If “No”
means no correlation, than what is the reason to show it?

Reply: This point has been modified as requested.

6.a Table 1 requires more annotations, as example, it is not obvious to all readers what is the Mowse score, and how you obtained numbers of peptides and coverage.

Reply: We integrated annotation of table 1 as requested. Entry names, protein names, accession numbers and theoretical MW and pl are from the Swiss-Prot database. Mowse score represent \(-10^{*}\log (P)\), where P is the probability that the observed match is a random event. Protein score greater than 67 are significant \((p < 0.05)\). Number of mass value matched and the \% of sequence coverage are given by Mascot database in the protein view session.

6b. Figure 3, mass spectra – Spot 10 is annotated twice. Please correct \((S100A7a\ seems\ to\ be\ spot\ 8)\).

Reply: Done

7. The authors are advised to check the text to avoid misuse or misspellings. As examples: p.2, methods section – “Tissue extraction was ...”, probably “Tissue extract was..”; p. 3, background, second paragraph – “S100 proteins are small, acidic proteins of Ca2+ binding proteins...”; p. 6, Protein identification – “Mass spectrometric sequencing...”, while in the text peptide mass fingerprinting was described; p. 8, top of the page – “...focalize.” or “localize”?; what is “...mass-screaning..”?

Reply: Done

8. Page 3. Conclusion should be about specific results reported in this manuscript. Only a general note about importance of this work is not sufficient. Please re-write this section to highlight specific findings.

Reply: Done

Comments of Reviewer 2

Minor mistakes

(Pag. 3) Sulfate is most common than sulphate.

Reply: Done

(Pag. 4) homeostasis1 ?

Reply: correct

Comments of Reviewer 3

Major Revisions
1. I am concerned with the quantification algorithm used. If I have understood the algorithm correctly (page 7), the spot volume (integrated dose intensity over the spot area) for one specific spot (e.g. S100A7) is divided by the sum of integrated dose intensities for all spots on the gel to obtain the value designated "%Vol". Thereafter, this value is divided by the %Vol value for actin to generate the value N%V. If this is correct, the spot volume is first normalized against the sum of spot volumes on the gel to correct for differences in gel staining, but then divided by a value (Actin %Vol) that is also normalized against the sum of spot volumes on the gel. Hence, the value N%V is equal to the spot volume of one specific spot divided by the spot volume for actin, and thus does not correct for differences in gel staining.

Reply: In the course of analyses we utilized two levels of normalization: the first (%Vol) is automatically generated by the algorithms of the system and corrects for differences in gel staining. The second normalization corrects for differences in the cellularity among the surgical samples, that is each spot divided for the actin content in the gel. On the other hand, normalization by %Vol actin is automatically corrected for the difference between gel staining. For us the N%V indicates a kind of ratiometric normalization.

2. This study is focused on the distribution of S100 protein expression in 100 breast cancer patients and association with patient outcome. Therefore, baseline patient characteristics (conventional clinical and histopathological parameters) must be presented, including the time period of inclusion for this study. Additionally, follow-up of patients must be described (e.g. how was metastatic disease defined, which radiological investigations were performed to detect metastatic disease, were the patients followed by the surgeons or their general practitioner, etc).

Reply: A new table with clinical-pathological characteristic of patients was introduced (Table 2).

All others requirements were added in the “Clinical Specimens” paragraph in page 5.

3. Validation of at least one of the differentially expressed S100 proteins in a set of patient samples should be performed, and this could easily be accomplished by immunoblotting using specific, commercially available antibodies (such as for actin in Fig 1).

Reply: A panel of WB with commercial antibody against S100A proteins was added (Fig. 7) and described in the paragraph Western blot validation of S100 proteins.

Minor Revisions

1. It is not clear whether the present study also include patient samples/results from the previous studies from the same group (such as ref 24). This should be clarified.
Reply: We believe that this information is not relevant for the reader. Indeed this study comprises new proteomic maps of about 20 old cases.

2. In the section "Clinical specimens" (page 5) the authors refer to bioethical recommendations. The name of the bioethical committee and the approval number should be stated.

Reply: See reply at comment 4 of reviewer 1.

3. In page 7, last line, the authors state that an area covering a pI/kDa range of 4-8/15-9kDa was analyzed, and the figure text states 4-6.5/15-9kDa, but the figure shows a pI of 4.5-7 and a Mw of 14-9 kDa. What is correct?

Reply: We correct figure and text with pI/kDa range of 4.7-7/15-9.

4. In Fig 3, no mass spectrum of spot 8 is shown, and two spectra are shown for spot 10.

Reply: See reply at comment 6b of reviewer 1

5. Fig. 5 is difficult to read and should be presented in a different way, such as a table with number of patients and percentages in the different categories.

Reply: We added the Fig. 5B showing a table with % of patients which expressed or not the different S100 proteins.

6. Statistical methods that incorporate the time period from surgery to development of metastatic disease are more suitable for analysis of patient outcome, such as Kaplan-Meier plots and log-rank test, and such analyses should be performed. The data and statistical method presented in Fig 8 are valid, but much information is lost when the time factor is not included.

Reply: Kaplan-Meier plots and log-rank test are better suited for longer time of follow up.

7. Correction for multiple testing should be performed in Fig 7.

Reply: In exploratory studies in which data are collected without a specific hypothesis, adjustment for multiple testing is not as essential. Moreover the % of S100 association remained unchanged with Bonferroni's Multiple Comparison Test or Tukey's Multiple Comparison Test.