Author's response to reviews

Title: High STAT1 mRNA levels but not its tyrosine phosphorylation are associated with macrophage infiltration and bad prognosis in breast cancer

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Author's response to reviews: see over
Ref.: Submission of the revised version of MS: 6944991661096899 - High STAT1 mRNA levels but not its tyrosine phosphorylation are associated with macrophage infiltration and bad prognosis in breast cancer

Dear Editors,

All authors concur with the submission. The material is original research, has not been previously published and is not under consideration for publication elsewhere. The authors declare no conflict of financial interest.

In response to the comments of the reviewers, we have now revised the text. Please find below our response to each question raised by the reviewers.

Yours sincerely,

Wolfgang Doppler, M.D.Ph.D.
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Response to the points of the reviewers

We thank both reviewers for their constructive criticisms and questions. We have now revised the text to better clarify the issues raised as outlined below.

Response to Reviewer Jean-Luc Davignon

"Interestingly, STAT1 Ser727 levels are associated with total STAT1 mRNA levels. On the other hand, STAT1 Ser727 appears not to be a marker for bad prognosis but is rather associated with a favourable outcome. Our data are discussed in the Discussion section, 1st paragraph. " However, the statement that Ser727 is associated with favourable outcome was not found in the Results. Indeed, the fact that STAT1 Ser727 is associated with total STAT1 mRNA levels BUT is associated with favourable outcome does not make sense to the reviewer. Please explain.

The association of STAT1 Ser727 with favorable outcome was shown in Figure 6A and described in the second last paragraph of the result section, end of page 11 of the previous version. We indicate in the text that association with favorable outcome was not significant possibly because of the low number of cases investigated and this is the reason why we do not want to discuss this result extensively. Part of a possible explanation why STAT1 protein levels in contrast to STAT1 mRNA levels are not significantly associated with bad prognosis is given in the Discussion when addressing the potential reason for the higher predictive power of STAT1 mRNA vs. STAT1 protein levels determined by IHC. We have now extended this discussion to better clarify the issue (p16).

Quality of written English: Not suitable for publication unless extensively edited

The paper was proofread by a native English speaker (Jonathan Vosper, Ph.D. University of Cambridge, England).

Response to Reviewer Hazem Ghebeh

The most important and major issue is that authors do not have enough evidence for an association between macrophage infiltration and STAT1 mRNA levels (Title). If both STAT1 mRNA and CD68 are associated with bad prognosis, this does not proof that they are associated with each other (Reply to reviewers, page 2 general comments). Therefore this should not be present in the title as their major finding. The data presented have merely showed an association with immune cell infiltration in general.

We do not claim that the association between STAT1 mRNA and macrophage infiltration is a causal relationship. There are two points that justify our focus on macrophages in the title: We show that expression of macrophage marker genes is correlated with STAT1 and STAT1 target genes. Furthermore, the predictive power of STAT1 is lost, when CD68 was taken as a confounder in the Cox Regression analysis (Fig. 6C). Such behavior of two correlated variables may indeed indicate an underlying mechanistic relationship as in case of the known STAT1 targets MX1 and CXCL10 in our study (Fig. 6C). We have modified the statement in the abstract regarding the loss of predictive power in multivariate Cox regression to better emphasize this result (2nd last sentence of Results section of Abstract).

We found that markers for other types of leukocytes, like FOXP3 or CD45, correlate with STAT1 expression as well. Yet we could not find any significant linkage to the survival or relapse prognosis for these transcripts (Figure 6AB).

1. The second subtitle of the results "No evidence for downregulation/mutation...(Page 9)

Having same level of IFN-gamma between tumor and adjacent tissues does not exclude the possibility that increased levels of mRNA STAT1 and IRF-1 are not due to immune cell
infiltiration. IFN-gamma is not always produced by immune cell infiltration. Some types of T-cell activation lead to the release of other cytokines (very common in cancer) like IL-10, TGF-beta without IFN-gamma. Interferon-gamma producing macrophages are not always present among tumor infiltrating immune cells.

We agree with the argument of the reviewer that IFN-gamma cannot serve as an universal marker for infiltration with immune cells. We have changed the text on p11 accordingly.

2. The third subtitle of the results "Coordinate regulation of STAT1, STAT1 target genes.... (page 9 & 10)

The way genes are categorized looks confusing/not accurate:

a) What is the justification for grouping CXCL9, CXCL10, CXCL11 together? These chemokines can be produced by many cells in addition to epithelial cells and therefore cannot be claimed that they specifically linked with STAT1 expression by tumor epithelium.

Our justification is that these three CXCR3 ligands are chemokines known to be regulated by STAT1. We do not claim a specific expression of these genes in the tumor epithelium, this was only found for the subgroups of antiviral proteins and macrophage associated proteins (Fig. 4B and text in Result section).

b) What is the justification for grouping INF-gamma, CD45, and FOXP3 together? INF-gamma is not a specific lymphocyte marker as it is released by both lymphocytes and macrophages (see review by Schroder et al 2011). CD45 is not a lymphocytes marker, it is rather a pan-leukocyte marker (lymphocytes, neutrophils and monocytes all express CD45). FOXP3 is a marker of a subset of lymphocytes (T-reg) but it can be expressed by tumor cells (see Triulzi et al 2013).

We are aware of the fact that the three genes are reportedly expressed in other cell types than lymphocytes. For FOXP3 a new study documents that its expression in tumor cells of human breast cancer tissue is minimal in comparison to Tregs [Droeser RA, Obermann EC, Wolf AM, Wallner S, Wolf D, Tzankov A: Negligible nuclear FOXP3 expression in breast cancer epithelial cells compared with FOXP3-positive T cells. Clin Breast Cancer 2013, 13(4):264-270.] This reference is now included. However, we agree that other immune cell types than lymphocytes can substantially contribute to CD45 and IFN–gamma expression and have therefore changed the name of the subgroup to immune cell markers.

c) PD-L2 is expressed on activated T-cells and not macrophages and dendritic cells only as initially thought (reviewed by Rozali et al 2012).

We have included the reference and now changed the sentence in the Result section accordingly to mention the findings of PD-L2 expression also in other cell types (end of p12).

d) What is the significance of two clusters? What does this finding means? (I don't see what figure 5 is adding to the main findings of the paper...).

The hierarchical clustering analysis shown in Figure 5 provides further evidence for the link between expression of markers for macrophages, immunosuppression and STAT1 with a different method.

3. Discussion This part has to be improved as it is the most interesting finding in the paper

a) The significant correlation between STAT1 mRNA and STAT1 protein phosphorylated at Ser727 but not with Y701 should be discussed more as there might be difference in the impact on STAT1 activation by the site of phosphorylation. For example: recent findings by Barnholt et al. 2009 have shown that macrophage activation can be reduced by inhibition of Ser727 phosphorylation but not Y701 indicating a difference in the role of these two sites of phosphorylation.
We have extended the discussion on this topic (p16).

b) In addition, the mechanism and importance of the activation of STAT1 between different subset of cells in breast cancer should be discussed (see for example Koromilas et al 2013).

We have previously referenced these findings which are relevant for mouse models of breast cancer, where the impact of STAT1 deficiency was investigated, including a recent report by our group in the Introduction part. We have now added the Koromilas review reference (p5).

c) The fact that some genes are controlled posttranscriptionaly should be mentioned and caution should be made not to consider mRNA equal to protein expression always. Based on this, statements like "STAT1 mRNA levels as compared to STAT1 protein (IHC) provides an argument against a direct involvement..." (page 13) might not be justified as mRNA does not always mean protein. This is in addition that the type of cell producing this mRNA is not known.

Actually our statement was based on the discrepancy between mRNA and protein levels. We have now completely rewritten this statement to make it more clear (end of p 16).

d) Discussion in page 14 need to be improved, abridged (currently it has contradictory or not relevant sentences/paragraphs).

Discussion was substantially changed (now p17 and 18).