Author's response to reviews

Title: Reevaluation of the 22-1-1 antibody and its putative antigen, EBAG9/RCAS1, as a tumor marker

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

please find enclosed the resubmission of our manuscript MS: 126846151802571 in revised form, entitled "Reevaluation of the 22-1-1 antibody and its putative antigen, EBAG9/RCAS1, as a tumor marker", by Tatiana A. Reimer, Ioannis Anagnostopoulos, Bettina Erdmann, Insa Lehmann, Harald Stein, Peter Daniel, Bernd Dörken, and Armin Rehm.

The concerns and criticisms raised by reviewer 1, Satoshi Inoue, have been largely addressed in our response included in this resubmission letter. In contrast to this rev., we note that rev. 2, A. Jungbluth, raises essentially no concerns.

1.) Rev. 1 critizes the confusing employment of the terms RCAS1 and EBAG9.

Response: We take issue with the rev. opinion that both terms, RCAS1, as defined by the monoclonal antibody 22-1-1, and EBAG9 as a cDNA product, stand for the same structure and the same function, and we should therefore employ exclusively the term RCAS1. Although the rev. refers to numerous published reports on EBAG9 and RCAS1, in fact up to date more than 50 publications are...
listed in PubMed, we have unequivocal evidence that RCAS1, as defined by the monoclonal antibody 22-1-1, and the EBAG9 encoded gene product, are not the same. All the publications that rely on the synonymous employment of both terms rely on the wrong assumption published in Nakashima et al. (Ref. 3).

To review the rationale of our major contention briefly: the RCAS1 antigen was initially defined by the employment of a monoclonal antibody, 22-1-1. It was clear that the 22-1-1 defined antigen recognized is located predominantly at the plasma membrane. Then, Nakashima et al. (Ref. 3) employed expression cloning and found out that the 22-1-1 antigen correlated with a cDNA/cosmid clone in transfected cells that encoded a gene which was also termed RCAS1. Using a different cloning strategy, it was later found out that this cDNA encoded a gene which was named-alternatively- EBAG9. Therefore, the cDNA sequences for RCAS1 and EBAG9 are identical.

From these reports, a misleading picture emerged, and it was one major purpose of our current manuscript to revise this wrong view.

Our data strongly suggest:

a) the cDNA sequences for EBAG9 and RCAS1 are the same
b) the EBAG9/RCAS1 encoded gene product is a protein of 32 kD
c) the 22-1-1 antibody does not recognize the EBAG9/RCAS1 encoded gene product
d) the EBAG9/RCAS1 encoded gene product is located intracellularly, predominantly in the juxtanuclear Golgi region of cells
e) the 22-1-1 defined antigen is located predominantly at the plasma membrane, but also in the cytoplasm of mucus rich tissues and in secreted matter of glandular and epithelial tissue
f) the 22-1-1 defined antigen is identical with the glycan antigen Tn
g) overexpression of the Golgi-localized EBAG9/RCAS1 cDNA product leads -indirectly- to the expression of the Tn antigen, which is then recognized by the 22-1-1 antibody.

Based on our previous cell biological examination, as published in Engelsberg et al. and Rüder et al. (Ref. 10, 11), we maintain our view that the EBAG9/RCAS1 cDNA product has a modulatory function in vesicle transport that results indirectly in the appearance of the tumor-associated glycan antigen Tn. In the current manuscript, we provide additional morphological evidence for the subcellular localization of EBAG9/RCAS1. It follows that applying this strictly cell biological approach a reevaluation of all previously published reports on RCAS1/EBAG9 is mandatory. This reevaluation has to take into account that the 22-1-1 antibody recognizes a firmly established tumor-associated glycan antigen, Tn, which is by no means identical to the EBAG9/RCAS1 encoded gene product.
2.) The rev. requests to use the term RCAS1 only, but not the term EBAG9.

Response: The term RCAS1 stands for: "receptor binding cancer antigen expressed on SiSo cells". However, when this term was created it was believed that the monoclonal antigen 22-1-1 defines the RCAS1 encoded antigen. This is not the case, as shown in the current manuscript and in our previous report (Engelsberg et al., Ref. 10). Instead, monoclonal antibody 22-1-1 defines a very common tumor-associated O-linked glycan, Tn, which is by no means a novel tumor-associated antigen.

Second, considering the high abundance of the EBAG9/RCAS1 encoded gene product in essentially all tissues, we simply refuse to use a "functional" term like "receptor binding cancer antigen expressed on SiSo cells". We have shown that the EBAG9/RCAS1 encoded gene product is located intracellularly and the effects observed for recombinant EBAG9/RCAS1 on immune cells are most likely artifacts due to residual detergent (Engelsberg et al., Ref. 10; and this report). We strongly believe that the descriptive gene name EBAG9, which stands for "estrogen receptor-binding fragment-associated gene 9", is far less biased towards a function and should be used instead.

Third, the effects observed for tissue culture supernatant from 22-1-1 antigen positive cells, could not be reduplicated in our hands and we do not share the generally hold view on an apoptosis-inducing function of the 22-1-1 defined antigen. In fact, the tumor-associated glycan Tn can be associated with multiple polypeptides or glycolipid carrier molecules. It remains to be determined if one of these carrier molecules was responsible for the published effects. However, such an reexamination can not be the purpose of the present study.

Fourth, in our hands the 22-1-1 antigen is not suitable for immunoprecipitation or immunoblotting, and we failed to reduplicate the experiments showing that the 22-1-1 antibody (IgM isotype) recognizes a 78 kD antigen, as cited by the rev. To our knowledge, this experiment, which was already published 6 years ago, has not been repeated by others and therefore needs independent confirmation.

3.) Rev. 1 questions our immunohistochemical analysis and the conclusions drawn from these studies.

Response: First, we have decided to use a representative selection of different epithelial tumors and their normal counterparts. Our analysis included 10 cases of each of squamous cell carcinoma from the oral cavity, adenocarcinoma of the lung, gastric, colorectal, prostate carcinomas and breast carcinomas (data not shown for breast tissue). The selection included various degrees of differentiation and were always surrounded by various non-neoplastic tissues. We agree that this is not a fully systematic pathological study with an additional statistical evaluation.
However, we present a black-and-white picture on several different aspects of EBAG9/RCAS1 biology.

a) in a side-by-side comparison, we show that the cognate 22-1-1 defined antigen and the EBAG9/RCAS1 encoded antigen are distinguishable in situ in all tissues examined. Whereas the 22-1-1 antibody recognizes a plasma membrane localized antigen, but also in secretory active tissues cytoplasmic mucus and secreted matter from glandular tissues, Ab-1 was always negative for mucus. Instead, staining in benign and malignant tissues was seen in the cytoplasm, and in some cases a Golgi-like distribution could be visualized.

b) EBAG9/RCAS1, as defined by the monoclonal antibody Ab-1, which was raised against the recombinant protein EBAG9/RCAS1 and which is commercially available, is detectable in essentially all malignant and non-malignant tissues. This is in full agreement with published reports where normal murine tissues were examined (Ref. 24; and our own unpublished observation) and fits with the phylogeneic conservation of this molecule.

c) as could be expected for the antigen recognized by the 22-1-1 antibody (O-linked glycan Tn), we see tissue staining also in normal epithelia. Mucous secretions from prostate or colon often exhibit glycan epitopes that could easily include the Tn antigen. Intracellular staining of the intracellular mucin was most pronounced for signet ring cell carcinomas, where we found essentially no reactivity for the Ab-1 antibody. In addition, we would like to refer to Ref. 5 and 23 which also demonstrate 22-1-1 expression in non-malignant tissue. This has been properly cited in the "Discussion" section and might have escaped the attention of the rev.

Based on these findings, we take issue with the rev. opinion that this immunohistochemical study does not reflect representative examples that would support our conclusions. We do not feel that this evaluation requires further statistical support.

4.) Rev. 1 suggests to avoid evaluations of quantitative and tumor-specific roles of the RCAS1-defined antigen in tumors from the present data.

Response: We fully agree with the rev., since he cites three studies (Ref. 8, 17, 18) that have employed a polyclonal antibody directed against the EBAG9/RCAS1 gene product or RT-PCR analysis to suggest the involvement of the EBAG9/RCAS1 gene product and its deregulation in cancer progression. In our last paragraph of the "Discussion" section (page 17), we have already fulfilled this demand of the rev., where we say: "It follows that studies on the correlation of EBAG9 expression and clinical prognosis were correct as long as their screens were based on RT-PCR or immunoblotting with a polyclonal anti EBAG9 antibody". 
The only claim we wish to maintain follows in the next sentence: "In contrast, functional and clinical studies on the 22-1-1 defined antigen (Tn) need to be revisited, and should be compared to other studies obtained with Tn-antibodies."

Since there are currently only three studies published (Ref. 8, 17, 18) on the role of the EBAG9/RCAS1 cDNA product (not the 22-1-1 defined antigen!) in tumor pathogenesis, we feel it is reasonable to suggest that further studies are needed to confirm a potential tumor involvement. This seems particularly interesting since the EBAG9/RCAS1 encoded gene product is expressed ubiquitously, but not tumor specific. Second, in Ref. 10 we suggest a physiological function of EBAG9/RCAS1 which might point to a link between O-linked glycan expression and vesicle transport.

5.) The rev. requests to see a proof that the polyclonal antibody and the monoclonal antibody raised against the EBAG9/RCAS1 encoded antigen indeed recognize the same antigen, both in immunoblot and in immunohistochemistry.

Response: We would like to point out that both antibodies, the polyclonal rabbit antibody used in this study for Western blots and the commercially available antibody Ab-1 employed in immunohistochemistry, were raised against the same EBAG9/RCAS1 fusion protein. The reason for the separate employment in both techniques was simply that the monoclonal antibody works better in immunohistochemistry, whereas the polyclonal serum can be used for dual purposes, immunohistochemistry and Western blot. However, in our previous publication (Ref. 10) we have shown that both antibodies recognize the same Golgi-localized antigen in HEK293 and MCF7 cells, but not a plasma membrane antigen.

For reviewing purposes, we add an additional Fig. (Fig_editor.jpg) where we show that in normal T lymphoblasts the Ab-1 antibody and in insulinoma INS-1E cells the polyclonal rabbit serum recognize the same Golgi-associated structure. This antigen is clearly separate from the antigen recognized by the 22-1-1 antibody (Ref. 10). Therefore, we feel that our claim that both antibodies recognize the same antigen is sufficiently supported and a complete repetition of the immunostainings with both antibodies is simply not reasonable.

6.) Rev. 1 proposes to perform experiments with the N-terminal GFP-tagged EBAG9/RCAS1 construct.

Response: In our recently published paper (Ref. 11), we show that the N-terminus of EBAG9/RCAS1
is palmitoylated and plays a decisive role in membrane attachment and in protein-protein interactions. Based on this observation, we have decided to place the GFP tag at the cytoplasmic C-terminus which minimizes a potential disturbance of the protein structure and protein-protein interactions.

7.) Supplemental Information for reviewing purposes only:
Figure legend for:
Fig_editor.jpg
Immunofluorescence staining for endogenous EBAG9/RCAS1 using the monoclonal antibody Ab-1 and the polyclonal rabbit anti EBAG9/RCAS1 serum. T lymphoblasts from mice (upper row) were fixed and stained with Ab-1 antibody. Bottom row, INS-1E insulinoma cells were stained with the polyclonal anti EBAG9/RCAS1 serum. The endogenous EBAG9/RCAS1 encoded gene product is found in a juxtanuclear Golgi region recognized by both antibodies.

8.) Minor revisions

Response: According to the rev. advice, we carefully corrected page 12, line 18.
In Figure 4, bottom panel, we corrected the legend with in the Figure: The treatment was with Nocodazole only, as already stated in the "Results" and "Methods" sections.

In summary, we feel that we have adequately dealt with the criticisms and we would like you to consider our revised version for publication in BMC Cancer.

Yours sincerely,

Armin Rehm, M.D.