Author's response to reviews

Title: The clinicopathological and prognostic impact of 14-3-3 sigma expression on vulvar squamous cell carcinomas

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Author's response to reviews: see over
Please find enclosed the revised version of the manuscript “The clinicopathological and prognostic impact of 14-3-3 sigma expression on vulvar squamous cell carcinomas” by Zhihui Wang, Claes G Tropè, Zhenhe Suo, Gunhild Trøen, Guanrui Yang, Jahn M Nesland and Ruth Holm.

We have revised the manuscript according to reviewers recommendations. The comments of the reviewers have been dealt with in the following way:

**Reviewer 1 (Zannoni Gian Franco)**

**Reviewer's report:** While the issue is novel since no previous papers have investigated the role of this protein in vulvar cancer, many relevant criticisms needs to be raised. The authors declare that in vulvar carcinoma more 14-3-3 sigma protein moved from cytoplasm in the nucleus suggesting a potential role in tumorigenesis, but in Table 1 we can see a statistical significant correlation between increasing high nucleus and also cytoplasmatic levels of the protein and tumor diameter and depth of invasion. This finding suggests that no different tumor biological behaviour depend on cellular localization. In this context the conclusion seems to be not appropriated. Moreover no correlation was reported between survival and protein levels according on cellular localization, so a specific role of this peculiar pattern of expression in cancerogenesis of vulvar cancer seems to be still to demonstrate (page 13 line 10-14; pag 15 line 3-6).

We have included in the discussion “However, we found that high expression of 14-3-3σ in cytoplasm as well as in nucleus was significantly correlated to large tumor diameter and deep of invasion. Furthermore, neither 14-3-3σ expression in cytoplasm nor in nucleus was significant associated with clinical outcome. These results may indicate that the cellular localization of 14-3-3σ is not important for the progression of vulvar carcinomas. However, we cannot exclude the possibility that nuclear localization of 14-3-3σ protein may be an early event in carcinogenesis of vulvar carcinomas. Further studies are needed to clarify the importance of 14-3-3σ nuclear localization in development of vulvar carcinomas.” (page 13, paragraph 1, line 14-21).

We have changed the first line in the conclusion to: “Low levels of 14-3-3σ protein expression in 25% of the cases may indicate that 14-3-3σ protein expression may be associated with tumorigenesis in a subset of vulvar squamous cell carcinomas.” (page 15, conclusions, line 1-3).
In Methods page 5 line 19-21 the sentence is not clear and should be rewritten.

The sentences have been changed to: “All patients were followed until death or 31. December, 2006. One hundred and twenty patients (40%) died of vulvar cancer. The median follow-up time for all patients was 52 months (range, 0.4 to 346 months), whereas the median follow-up time for patients alive at last observation was 131 months (range, 11 to 346 months).” (page 5, line 18-22).

Reviewer 2 (CP Crum)

Reviewer's report: No revisions needed.

Reviewer 3 (Mong Hong Lee)

Reviewer's report: Wang et al. described the 14-3-3 sigma (sigma) expression in vulvar carcinomas. 14-3-3 sigma functions as a tumor suppressor, and it is important to characterize its role in cancer. The authors have tried to investigate the role of sigma in vulvar squamous carcinomas (VSCC). However, the manuscript is presenting negative data, and the studies are very superficial. Thus, the manuscript is not novel enough to warrant publication in BMC Cancer.

The following are other major concerns. The title is clinicopathological and prognostic impact of sigma on VSCC. All the results in this paper are negative. Although they found that sigma is highly expressed in VSCC, they found no correlation with sigma expression and survival of VSCC, reducing the interest of this study.

No previous papers have investigated the role of 14-3-3 sigma protein in vulvar cancers. Although we did not find any correlation with 14-3-3 sigma expression and survival these results are important to publish. If the journals are not publishing negative findings many other research groups will do similar studies due to lack of information. Furthermore, what happens if PhD students only obtain negative findings? They will not have their PhD without any publications. It is very important that researchers are honest with their results. This is easier to live up to if the researchers knew that also negative findings will be accepted by the journals. Most seriously, if journals focus on positive findings, excluding the negative ones, this may lead to wrong diagnosis/treatment of the patients.

Criticisms of Fig2, In this figure they show sigma expression by western from 2 VSCC cell lines but there is no loading control and nothing to compare levels of sigma with, example, no normal cell line sigma expression data. This observation did not provide any useful information.

We agree with prof. Mong Hong Lee that a normal cell line should be included to compare levels of 14-3-3 sigma. However, normal vulvar cell line is not possible to obtain. We have therefore excluded Fig. 2. The specificity of our antibody has previously been documented by the company. The sentence “The primary antibody is highly specific to 14-3-3σ and shows no cross-reaction with other isoforms of 14-3-3 (information from NeoMarkers).” has been included in Material and Methods (page 7, paragraph 1, line 2-3).

In Fig.3, the authors should address whether the low expression of sigma is due to the promoter methylation. Presenting the mRNA expression from random cases does not have any biological significance or mean anything.

When we started our study we planned to perform 14-3-3 sigma methylation analysis. However, our methylation study was not successful. Using two different primer sets we found methylation in all vulvar carcinomas examined and in normal blood, whereas the two vulvar squamous cell carcinoma cell lines SW-954
and CAL-39 were unmethylated for 14-3-3 sigma. 14-3-3 sigma methylation in all vulvar carcinomas and normal blood samples may be due to the normal lymphocytes in blood and the lymphocytes surrounding the epithelial tumors. Previously, Bhatia et al. Cancer Epidemiol Biomarkers Prev 12 (2003) 165-169 have demonstrated 14-3-3 sigma gene to be methylated in normal lymphocytes. To examine 14-3-3 sigma gene methylation it is necessary to use microdissection to obtain pure tumor cell population. However, we will need approximately 100,000 tumor cells for the methylation study. Since our vulvar carcinoma samples are very small in size we have not the possibility to perform microdissection. We have included in the discussion “We have identified 14-3-3σ methylation in 57/57 (100%) vulvar carcinomas and 5/5 (100%) normal blood samples, whereas the two vulvar squamous cell carcinoma cell lines SW-954 and CAL-39 were unmethylated for 14-3-3σ (unpublished data). 14-3-3σ methylation in all vulvar carcinomas and normal blood samples may be due to the normal lymphocytes in blood and the lymphocytes surrounding the epithelial tumors.” (page 12, paragraph 3, line 3-7)

Despite the lack of methylation data we found it important to include the association between 14-3-3 sigma mRNA and protein. Our findings indicate that 14-3-3 sigma expression is also regulated at post-transcriptional level (page 14, paragraph 4).

Yours sincerely
Ruth Holm, PhD