Author's response to reviews

Title: RASSF1A protein expression and correlation with clinicopathological parameters in renal cell carcinoma

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Author's response to reviews: see over
Responses to the comments of A. Sgambato:

Major revisions:

1) **It is not clear from the Abstract as well as from the Results and Table 2 in which direction is the association between RASSF1A immunopositivity and other pathological parameters (i.e., high protein expression is associated with low or high pT stage?)**

This issue has now been improved in the revised version of the manuscript. The following phrase has been added: *This indicates that higher expression of RASSF1A was positively correlated with the higher pT stage, histological grading, and pTNM group stage in the whole study group irrespective to the labelling index.*

2) **In the MATERIAL AND METHODS section in the paragraph Patient characteristics and follow up the Authors state that: “Survival analysis was carried out for 187 selected patients with pathologically proved clear cell carcinoma of RCC…..”. It is not well clear how those cases were selected. Moreover, it is not clear whether all the 318 cases were clear cell-RCC or not.** We have now also mentioned in the materials and methods section that all 318 patients were clear cell carcinoma. The survival analysis has been performed for 187 patients with complete follow up data but without any other selection as indicated now in the manuscript. *Survival analysis was carried out for 187 patients with complete follow-up data and pathologically proved clear cell carcinoma of RCC.*

3) **In the same paragraph, the Authors also state that: “Seventeen patients demonstrated metastasis at the time of diagnosis……………..”** however, 35 patients (out of 187) are indicated as M1 in Table 1.
Seventeen patients demonstrated metastases at the time of diagnosis and eighteen patients without primary metastasis developed metastases in the course of follow up. Thus thirty five patients with M1 in the table 2 indicates the total number of patients with metastasis included in the present study.

4) In the RESULTS section in the paragraph Expression of RASSF1A protein it is not clearly indicated whether analysis was performed on all 318 cases or only refers to the 187 clear cell-RCC. In the former case, it is extremely important to know whether all tumors were clear cell-RCC or not.

We have now explicitly indicated in the results section that all 318 cases have been analyzed: The presence of RASSF1A protein in primary tumor specimens and corresponding histologically normal surrounding parenchyma was investigated using immunohistochemical analysis of TMA slides of 318 patients with CC-RCC.

5) In the same paragraph, the Authors state that: “In tumors a mean positivity of 19% (median 11%) was detected……” but they do not mention how much it was in normal cells.

We have now described that an almost complete positivity for RASSF1A in proximal and distal tubules of normal epithelial cells was observed. An almost complete staining for epithelial cells of the proximal and distal tubules with labelling index of nearly 100% was observed…

6) Survival analysis was carried out using the cut-off value of 25% positive cells. As the Authors mention, 85% of tumors exhibited a
RASSF1A labelling index of 25%. Thus, the two groups are numerically very different and this might have biased the statistical analysis.

The present study aimed at a first identification of a possible correlation of RASSF1A expression with clinicopathological parameters. Thus no expression data were available in advance. Our study revealed that a subgroup of tumors exhibited increased expression using an arbitrarily cut off value. Consequently, the survival analysis in this study could only be carried out by comparing differently sized subgroups in statistical analysis.

We agree that analysis of numerically similar case and control groups in future studies following an appropriate pre-selection could improve the statistical validity of survival analysis.

**Discretionary Revisions**

1) **Manuscript should be checked throughout for English.** The manuscript has been edited for spelling errors.

2) **The thoughts regarding VHL pathway at the end of the DISCUSSION are not well integrated with the findings of the study.**

   The respective part of the discussion has been revised.
Responses to the comments of R. Dammann

Major revisions:

1. The paper has several flaws. It is unclear if correlations (Table 2) were made for a subset group of tumors with a higher expression of RASSF1A (immunopositivity) or a lower expression (reduced expression) compared to normal tissue.

   We have now added a statement in the results section as well as the heading of table 2 that expression analysis was carried out for the entire group of tumors (n=318). See also point 1) Reviewer Sgambato

2. The results are kept to short and al lot of details are missing: How many tumors and normal tissue were actually analysed? In the material and methods we have described that 318 cores of Tissue Microarray (TMA) from tumor and corresponding peritumoral normal specimens have been considered for immunohistochemistry.

3. Which antibody was utilized? Is this antibody RASSF1A specific?

   Figures of staining are missing! Immunohistochemical analysis, antibodies, specificity of antibodies and related figures have already been described by our group in an open access publication and cited in the manuscript (Peters et al., 2007).

4. How was the ,25% labelling index’ established? As described in the manuscript a cut off value of 25% labelling index was arbitrarily chosen to allow separation of tumor groups obviously exhibiting different expression characteristics (Fig.1). Moreover, the usage of cut offs other than 25% results in impaired statistics.
5. **How many cell were counted?** Regarding the cell count we would refer to the material and methods section of the manuscript describing the details: Evaluation of TMA`s was carried out by counting immunopositive and negative cells within the area of each tissue microarray punch. The diameter of each spot on the slides was approx. 1 mm. In tumor tissues only tumor cells were considered while in peritumoral tissue specimens the number of immunopositive and negative proximal and distal tubular cells but not glomerular cells was determined. The RASSF1A labelling index (similar to the 5th comment of the first reviewer) was calculated as the percentage of cells of interest displaying immunopositivity in the analyzed tissue samples (TMA`s spots).

6. **Why 25%? - Moreover it would be helpful to include the ‘Labeling-Index’ of normal cells.** On the revised manuscript we add in the result section, that the labelling index of almost 100% was observed in the normal tissue.

7. **Figure 1 is not understandable.** Frequency plots are widely used in literature providing primary data about the distribution of signals observed (here expressed as labelling index) to the readers. Figure 1 represents a frequency plot to illustrate the distribution of RASSF1A labelling index in the tumor group. Staining characteristics and signal specificity have already been published (Peters et al., 2007).

8. **Table 2 is incomprehensible: Which correlation (T1..T4, G1…. pTNMl…) were made? What was the ‘labelling index’?** Spearmans nonparametric analysis considers each of the subgroups (e.g. T1-T4) as independent variables for correlation analysis with the dependent variable labelling index.
Nonparametric analysis was chosen as the dependent variable is not normally distributed. Labelling index has been applied as the dependent variable in all statistical analyses.

**General comment of the authors to the review of R. Dammann**

In our view large part of the concerns of R. Dammann are due to fact that data presented in our previously published open access article (Peters et al., 2007) has not been considered when reviewing this manuscript.

In addition we have now acknowledged the contribution of our statistician B. Vaske (Department of Biometry, University Medical School Hannover) who supervised statistical calculations.

When taking into account that RASSF1A alterations represents one of the most frequently detected molecular alterations in CC-RCC development and, moreover, that CC-RCC is one of the most common lethal malignancy in humans we clearly do not agree with the reviewers opinion of an “insufficient interest” for publication of the present manuscript.