Author's response to reviews

Title: Prognostic relevance of mRNA levels of OPN splice variants in soft tissue sarcoma patients

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Author's response to reviews: see over
Dear Dr. Christna Chap,

we have now completed the revision of our manuscript „Prognostic relevance of mRNA levels of OPN splice variants in soft tissue sarcoma patients“. We have addressed the reviewers’ comments in a revised manuscript point-by-point to respond to the concerns, suggestions and helpful advice. Additionally, the manuscript was edited for proper English language, grammar and overall style by the highly qualified native English speaking editors at American Journal Experts. We would like to thank the editors and both reviewers for their helpful suggestions and comments. We feel that the manuscript has improved substantially and hope that we could adequately address all points of criticism.

Sincerely,

Antje Hahnel

Editorial requests - Ethics
Experimental research that is reported in the manuscript must have been performed with the approval of an appropriate ethics committee. A statement to this effect must appear in the Methods section of the manuscript, including the name of the body which gave approval, with a reference number where appropriate. Research carried out on humans must be in compliance with the Helsinki Declaration.

Our study was approved by the Ethics Committee of the Medical Faculty of the Martin-Luther-University Halle-Wittenberg. However it is not usual to get an approval number. We have added to the “Patients and Methods” chapter: The study was approved by the Ethics Committee of the Medical Faculty of the Martin-Luther-University Halle-Wittenberg and is in compliance with the Helsinki Declaration (p.5).

Referee 1 (Franz Rödel):

Major compulsory revisions:

1.) In their analysis, the authors compared mRNA-expression levels of the OPN splice variants in tumor of individual patients and in surrounding tissue. However, they did not address the ratio of splice variants to wild type (wt) OPN. Is there a more prominent expression of wt OPN as compared to splice variants? This correlation may be easily done with preceding data (Bache et al. BMC Cancer 2010) or should at least be performed in the corresponding tumor/mucosa pairs reported within this study.

In the study of Bache et al. (2010) we analyzed OPN protein expression in tumor and serum of STS patients. In addition, we used qRT-PCR to detect the total OPN mRNA level of 68 STS patients. We found in this limited analysis that total OPN mRNA level did not significantly correlate with prognosis. The rationale of this study is to analyze the impact of the mRNA level of the single splice OPN variants (a, b, c) in their prognostic and predictive impact in a larger patient analysis. Additionally, the mRNA expression level of total OPN and OPN splice variants show a significant correlation (n = 65, OPN-a: r = 0.32, P < 0.01; OPN-b: r = 0.30, P = 0.02; OPN-c: r = 0.26, P = 0.04). This sentence was added to the “Results and discussion” section (p.6)

Considering the remarks to the reviewer in “Discussion” section we added the sentence: “However, our preceding data of 68 STS patients show that mRNA expression level of whole OPN did not significantly correlate with prognosis [8].” (p.8)

Minor essential revisions:

2.) Figure1: to improve readability of the Figure, corresponding p-values should be included in the graphs and y-axis should be labeled with “Disease-specific survival”.
Figure 1 is now figure 2 in the manuscript. We added the corresponding “relative risks” and “p-values” in the graphs. Additionally, we changed the label of the y-axis from “survival” to “disease-specific survival” (see manuscript: section “Figures”, p.20).

Referee 2 (Maria Serena Benassi):

Major compulsory revisions

The Authors shows median values of splice variants in 124 tumours vs 15 paired normal tissues: what unpaired test was used to compare such different groups? No indications on the statistical differences are reported. Moreover, it would also be interesting to use a paired test (T test) to evaluate the statistical significance between the 15 tumours vs their 15 paired normal tissues.

Considering the remarks of the reviewer we analyzed statistical significance of the differences. We used a Wilcoxon signed-rank test to analyze the statistical differences of the 15 paired tumor and normal tissues. The test shows no difference between the median values of OPN-a in tumor tissues and normal tissues \((p = 0.80)\). For the OPN splice variants OPN-b and OPN-c the Wilcoxon test shows a trend for a statistical difference of the median values of tumor and paired normal tissues \((p = 0.07\) and \(p = 0.06)\).

The p-values were added to the manuscript (p. 6+7). Additionally in the “Patients and Methods” section we mentioned that we used the Wilcoxon signed-rank test (p. 6).

Since STS are very malignant tumours that frequently develop metastases in different sites, the A should report how many metastatic patients are included in the study apart from those with lymph node metastasis, also comparing mRNA values in all metastatic versus non metastatic tumours in order to assess the prognostic significance in terms of disease-free survival.

In our study, 50 of all 124 STS patients developed distant metastases (except lymph node metastases), whereas in 45 of 124 STS patients no distant metastases could be detected. On 3 patients we could not make a point about development of metastases or not. However, there was no correlation between the development of distant metastasis and the expression levels of OPN splice variants. The data were added to table 1 (p.16).

Regarding Kaplan Meier analysis the test used for the statistical difference between survival curves is not documented (Breslow’s or Log-Rank test?).

We used the Log-Rank test for the statistical difference between survival curves regarding Kaplan Meier analysis.

We added a comment to the manuscript in the “Patients and Methods” section (p.5).

The authors state that the expression of OPNa is higher in females than in males. They should report the mean/median mRNA values and the statistical difference between the 2 groups.

We said: “Male patients have significantly lower mRNA expression levels of OPN-a in their tumors compared to female patients \((P = 0.02)\).” Actually we wanted to say: “More male patients got a low OPN-a mRNA expression level than female patients \((P = 0.02, \text {table 1})\).” This sentence was added to the “Results and discussion” section (p. 7).

Additionally, we compared the median values of female and male patients (0.83 vs. 0.43 copies OPN-a mRNA/copies HPRT mRNA) and conclude that there is no significant difference between the mRNA expression level of OPN-a in male and female STS patients \((P = 0.10, \text{Mann–Whitney U test})\). We did not include this non-significant result into the manuscript.

The authors should indicate the cut-off value for survival analysis in females and RT patients. In fact, only the cut-off (median values) for survival analysis in all population is reported in result section.
Considering the suggestion of the reviewer we recreated table 3 and added the median values for survival analysis of all patients, females and RT patients to the table (p.17).

The discussion of the results must be improved in order to explain the impact of this research on STS clinical management.

These are the first results to study prognostic relevance of OPN splice variants in STS patients. More data are necessary to evaluate the OPN splice variants in the clinical management of STS patients. We add this sentence in the conclusion (p.10).

Minor essential revisions

In the RESULT SECTION, paragraph “mRNA expression level and Disease survival”, there is confusion between Table 3 (Cox’s analysis) and FIG 1 (Kaplan Meier) where the captions are wrong or missing. Caption to Fig. 1 is that of Table 3. Caption to Fig. 1 is missing in the legends and Fig. 1a is not discussed in the results.

Table 3 showed the association of the mRNA expression of different OPN splice variants in different groups of patients (all STS patients, females and RT patients) with overall survival in Kaplan Meier analysis and multivariate Cox’s regression analyses. The median values of OPN-a, OPN-b and OPN-c were added to table 3 and used as cut-off points to divide the STS patients into two groups: one with high and one with low (reference group) OPN mRNA levels in tumor tissues. Figure 2 (previously Fig. 1) displays Cox’s regression hazard models (this is now added to the caption of Fig.2). Table 3 (Cox’s analysis) and figure 2 (previously Fig. 1) contain partly overlapping information. For a better understanding we recreated table 3 and the legend of Figure 2 (previously Figure 1). In addition, we have labeled the discussed data in the section “mRNA expression level and disease survival” with the appropriate table and / or figure.

Fig 1 and 2 should be switched around (in the results Fig. 2 is described before Fig. 1)

In accordance with the suggestion of the reviewer we switched the figures around. Figure 1 is now figure 2 and figure 2 is now figure 1 (please, see manuscript).

Fig. 1 supp. is a reduced copy of Fig. 2 (Box Plot)

In accordance with the suggestion of the reviewer we removed Fig 1 supp.

The Authors should document which tumour staging system was used

The tumors were staged according to the Union for International Cancer Control (UICC) system. We added this sentence to the “Patients and Material” section (p. 5).

Discretionary Revisions

It would be interesting include a table with the mean/median mRNA values of the single variants in the different histotypes although the difference is not significant.

The table below contains the median mRNA expression level of the OPN splice variants in the different histotypes of soft tissue sarcoma. The table was added to the supplementary data files and is number as supplementary figure 1 (see manuscript).
<table>
<thead>
<tr>
<th>Type of Tumor</th>
<th>OPN-a (median, range)</th>
<th>OPN-b (median, range)</th>
<th>OPN-c (median, range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liposarcoma</td>
<td>0.60 (0.04-85.29)</td>
<td>0.38 (0.04-17.64)</td>
<td>0.082 (0.02-3.08)</td>
</tr>
<tr>
<td>MFH/Fibrosarcoma</td>
<td>0.95 (0.05-55.55)</td>
<td>0.71 (0.04-17.43)</td>
<td>0.149 (0.00-2.09)</td>
</tr>
<tr>
<td>Neurogenic sarcoma</td>
<td>0.46 (0.02-20.03)</td>
<td>0.40 (0.03-9.07)</td>
<td>0.078 (0.01-4.03)</td>
</tr>
<tr>
<td>Rhabdomyo-/Leimyosarcoma</td>
<td>0.66 (0.00-65.31)</td>
<td>0.57 (0.00-16.20)</td>
<td>0.072 (0.00-1.22)</td>
</tr>
<tr>
<td>Other STS</td>
<td>0.50 (0.01-6.34)</td>
<td>0.20 (0.01-1.82)</td>
<td>0.029 (0.00-0.58)</td>
</tr>
</tbody>
</table>