Author's response to reviews

Title: Combined mRNA expression levels of members of the urokinase plasminogen activator (uPA) system correlate with disease-associated survival of soft-tissue sarcoma patients

Authors:

Matthias Kotzsch (matthias.kotzsch@uniklinikum-dresden.de)
Viktor Magdolen (viktor.magdolen@lrz.tu-muenchen.de)
Thomas Greither (thomas.greither@medizin.uni-halle.de)
Matthias Kappler (matthias.kappler@medizin.uni-halle.de)
Matthias Bache (matthias.bache@medizin.uni-halle.de)
Christine Lautenschläger (christine.lautenschlaeger@medizin.uni-halle.de)
Susanne Füssel (Susanne.Fuessel@uniklinikum-dresden.de)
Alexander W Eckert (aw.eckert.wissenschaft@web.de)
Thomas Luther (t.luther@labor-ostsachsen.de)
Gustavo Baretton (Gustavo.Baretton@uniklinikum-dresden.de)
Peter Würl (familie.wuwr@googlemail.com)
Helge Taubert (Helge.Taubert@uk-erlangen.de)

Version: 2 Date: 28 May 2011

Author's response to reviews: see over
Dear Editor

Thank you very much for your letter dated from of April 14, 2011. We were pleased to learn that you consider our manuscript entitled “Combined mRNA expression levels of members of the urokinase plasminogen activator (uPA) system correlate with disease-associated survival of soft-tissue sarcoma patients” by Kotzsch et al., MS: 6385826635170727, acceptable for publication in *BMC Cancer* pending revision.

Please, find enclosed our modified manuscript and a point-to-point list of all changes made (as well as our reply/rebuttal) in response to the reviewers’ suggestions (changes we made in the manuscript are marked in “italic” in the revision letter, and by blue coloured text in the manuscript).

All co-authors have read and approved the revised manuscript. We hope that you will consider our revised manuscript suitable for publication in *BMC Cancer*. For editorial correspondence, please contact:

Dr. Kotzsch Matthias
Institut für Pathologie
Technische Universität Dresden
Fetscherstr. 74
D-01307 Dresden, Germany
Phone: +49 351 4583017; Fax: +49 351 4584358
e-mail: matthias.kotzsch@uniklinikum-dresden.de

We are looking forward to your reply.

Yours sincerely,

Matthias Kotzsch
Point-to-point list in response to the reviewers’ comments

Editorial comments

(1) Ethics

The Ethical committee of the Medical Faculty of the Martin-Luther-University has approved our study. We have added this to the Methods section, page 4:

The study adhered to national regulations on ethical issues and was approved by the Ethics Committee from the Medical Faculty of the Martin-Luther-University Halle-Wittenberg, Germany.

(2) Copyedit

We have asked American Journal Experts (AJE) to edit our revised manuscript. All their suggestions are now integrated in the revised manuscript.

Please, find attached the respective certificate of AJE.

Reviewer 1 (M.S. Benassi)

Minor essential revisions

(1) “Add and specify the 25-75th percentile for each median value reported (results page 6).”

We have specified the data on 25th and the 75th percentiles and added it to the Results section, page 7:

The normalized mRNA expression values of uPA, PAI-1, uPAR-wt, and uPAR-del4/5 ranged from 0-16.2 (median 0.23; 25th and 75th percentile 0.055 and 1.03, respectively), from 0-181.9 (median 0.73; 25th and 75th percentile 0.10 and 3.08, respectively), from 0-24.1 (median 0.18; 25th and 75th percentile 0.044 and 1.15, respectively), and from 0-0.29 (median 0.004; 25th and 75th percentile 0.0001 and 0.012, respectively), respectively.

(2) “The Authors should explain why they used the mRNA median value (50th percentile) as cut-off for survival analysis in all population and 33rd percentile in the R0 subgroup.”

The 50th percentile and the tercentiles (33% and 66% percentiles) are often used as cut-off points for survival analyses and are well accepted.

To present this fact more clearly, we have added the following explanation to the Results section on page 9:

… assessed whether some of the biological markers might add prognostic information for patients’ survival in the R0 subgroup of STS patients. In R0 patients, survival time is
inversely significantly correlated with expression of PAI-1 mRNA ($r_s = -0.337$, $P=0.014$) and of uPAR-del4/5 mRNA ($r_s = -0.346$, $P=0.013$) by bivariate correlation analysis using Spearman’s Rho test (Supplemental Data 3A). Furthermore, using linear regression analysis about a third of patients with lower expression rates for both markers showed the best survival (Supplemental Data 3B). Therefore it was implicated to apply the 33% percentiles as cut-off points for R0 patients according to their mRNA expression levels, i.e. for PAI-1 (33% percentile: 0.228) and for uPAR-del4/5 mRNA expression (33% percentile: 0.001). Accordingly, R0 patients (n=52) with mRNA values in the range of the 0% to the 33% percentile were allocated to…

Furthermore, Supplemental Data 3 were added to the Supplement section of the revised manuscript.

(3) “They should state the follow-up end point used for Cox’ univariate analysis.”

The follow up end point was the disease-associated survival of the STS patients.

We have specified the disease-associated survival and added this fact to the Methods section, page 6:

… the disease-associated survival of STS patients was used as the follow-up end point. The disease-associated survival was defined as the time from the day of primary surgery to tumor-related death of the patients.

**Discretionary Revisions**

(1) “It would be interesting include a table with the mRNA median values of the single components in the different STS histotypes.”

To meet the reviewers’ suggestion, we added a supplemental table containing the median mRNA values of the uPAR system components in the different STS histotypes as Supplemental Data 2 to the Supplement section of the revised manuscript.

We specified this in the comments to Table 1 as follows:

d Abbreviations: LS – liposarcoma, …, Syn – synovial sarcoma; the median mRNA values of the uPAR system components in the different STS histotypes are presented as Supplemental Data 2
Reviewer 2 (A. Scorilas)

(1) “Background” section: Clinical and key biological data regarding soft tissue sarcomas should be described. On the contrary, the role of the components of the uPA system in soft tissue sarcoma patients is described in the “discussion” section as well and should be removed from the introduction or presented more briefly.“

To meet reviewers’ comments on the Background section, we added clinical and key biological data regarding soft tissue sarcomas to the Background section, page 3:

Soft-tissue sarcomas (STS) are malignant mesenchymal neoplasias with an incidence of about 1% among all human malignancies [1]. STS enlarge leading to the appearance of a pseudo capsule composed of an inner compression zone and an outer reactive zone at formation of fingers, which give rise to satellite lesions several centimeters away from the primary tumor [2]. The major clinical problems in the treatment of STS are the propensity of the tumor to recur locally, and the fact that many patients without obvious clinical metastases harbor occult micrometastases that become clinically evident. Lymph node metastases are rare in STS patients [3, 4]. Despite adequate local control of the primary tumor, about 50% of sarcoma patients will succumb to distant metastatic disease [5].

The new references were added to the References section.

(2) “More data should be presented about the characteristics and the putative role of uPAR-del4/5 variant compared to the “wild type” transcript.”

To meet the reviewers’ suggestions on this topic, we added some data on the putative tumor biological role of the uPAR-del4/5 splice variant to the Background section on pages 3, and 4:

… are also associated with poor prognosis in various cancer types, however, the prognostic impact of uPAR expression is not as pronounced as that of uPA and PAI-1 [12, 13]. In contrast, the expression of a mRNA splice variant of wild-type uPAR (uPAR-wt) lacking exons 4 and 5 (uPAR-del4/5) has been demonstrated to be a highly sensitive, independent prognostic marker in breast cancer patients [14-16].

Whereas wild-type uPAR consists of three structurally homologous domains, in the uPAR-del4/5 variant the complete domain II of uPAR is deleted, and the uPAR-del4/5 protein does not interact with either of its ligands uPA or vitronectin [17]. However, in breast cancer cells the overexpression of the uPAR-del4/5 protein profoundly affects the in vitro invasion capacity of cells through a Matrigel matrix, the adhesion to extracellular matrix proteins and also lung colonization in an in vivo metastasis model. These observations strongly suggest that uPAR-del4/5 displays biological activity modulating tumor biological relevant processes [17].
“Methods” section: Real time PCR methodology is described very briefly. More data should be shown regarding the type of chemistry used, the characteristics of primers/probes (sequence, Tm, site of hybridization etc), the efficiency of the reaction for each transcript, the specificity of the products, cycling conditions, concentration of cDNA, primers/probe etc.

The RT-PCR methodology for the components of the uPA system including the uPAR-del4/5 variant has been reported in detail in previous publications (Luther et al., 2003: uPAR, uPAR-del4/5; Biermann et al., 2007: uPA, PAI-1). However, we agree with the reviewer that the RT-PCR methodology has been described very briefly in the present manuscript.

Therefore, we added the following to the Methods section on pages 5, and 6:

For quantification of uPA, PAI-1, uPAR-wt and uPAR-del4/5 mRNA, gene-specific hybridization probes and highly sensitive RT-PCR assays (LC FastStart DNA Master Hybridization Probes, Roche Diagnostics, Mannheim, Germany) applying LightCycler technology (Light Cycler 1.0; Software Version 3.5, Roche) were used as previously described [14, 23]. Briefly, the PCR assays were performed using 2 µl of a 1:10 dilution of the respective cDNA products. Primer sequences, concentration of primers and probes as well as PCR conditions for amplification are shown as Supplemental Data 1. All HPLC-purified primers were purchased from TibMolBiol (Berlin, Germany). Five-log-range calibration curves were generated for each PCR run using eight glass capillaries coated with defined numbers of linearized plasmid molecules (10^1 – 10^6 molecules), carrying either the uPA, the PAI-1 or the uPAR (wild-type or -del4/5) cDNA (Roboscreen, Leipzig, Germany).

Furthermore, Supplemental Data 1 were added to the Supplement section of the revised manuscript.

“Results” section: Why did the authors use the 33th as a cut off? Was any statistical test used for this reason (for example X-tile analysis etc)?“

Please, see our comments in point (2) of Reviewer 1

“Results” section: The part of the analyses where the combination of PAI-1 and uPAR-del4/5 mRNA is used should be described more thoroughly, because it is quite confusing as it is. The authors should consider establishing 2 groups (high expression for both transcripts, low expression for both transcripts). Does any other combination of the molecules assessed in this study provide additional prognostic information?“

To present the data on the combination of PAI-1 and uPAR-del4/5 mRNA levels more clearly, we re-phrased the respective paragraph at the end of the Results section on page 9:

… whether a combination of PAI-1 and uPAR-del4/5 mRNA values might improve the prognostic impact for patients’ survival. Again, the mRNA expression levels of both PAI-1 and
uPAR-del4/5 were divided into groups with low (0-33% percentile) versus high (> 33% to 100% percentile) values, respectively.

and on page 10:

… when combined PAI-1/uPAR-del4/5 mRNA expression levels in tumor tissue were used. The risk of tumor-related death for R0 patients with a low/high mRNA expression (high PAI-1/low uPAR-del4/5 and low PAI-1/high uPAR-del4/5) was not significantly different from that for R0 patients with a low PAI-1/uPAR-del4/5 mRNA expression in their tumors. We did not observe any significant correlation for either group with the disease-associated survival of all STS patients or for the R1-STS patients. The combination of other factors did also not provide any additional prognostic information.

Accordingly, the figure legend to Figure 1 was re-phrased to present the data more clearly:

… For each, the PAI-1 and the uPAR-del4/5 mRNA level, the 33% percentile of the relative expression ratio was used as cut-off point.

Subgroup 1 (n=13), low expression of PAI-1/uPAR-del4/5 mRNA values; subgroup 2 (n=8), one of the mRNA values was either low or high; subgroup 3 (n=30), high expression of PAI-1/uPAR-del4/5 mRNA values. STS patients of subgroup 3 (high expression of PAI-1/uPAR-del4/5 mRNA) showed a 19-fold increased risk of tumor-related death (RR=19.1, 95%CI=1.1-335.3, P=0.044) compared to subgroup 1 with low expression of PAI-1/ uPAR-del4/5 mRNA.

(6) “Figure 1”: The figure should presents Kaplan Meier survival curves, rather than Cox’s regression analysis. Additionally, the p value calculated by the log rank analysis for survival should be presented.“

We have applied the multivariate Cox regression hazard analysis to adjust for other prognosis relevant factors as tumor stage, tumor type, and tumor localization. Kaplan-Meier analysis as an univariate analysis model does not consider the effect of other tumor biological or clinical parameters that are also associated with prognosis. In the mathematical model of the multivariate analysis the other clinically prognostic parameters included in the base model are considered as well and, thus, it describes better the complex biological interactions of tumor biological and clinical factors than an univariate Kaplan-Meier analysis model that considers only the tumor biological factors. Figure 1, in fact, depicts the results of the multivariate Cox regression analysis as a graph in a similar manner as described by Abeler et al., Histopathology 2009; 54:355-364. Similarly, we recently have published our data for the protein expressions of the uPA system components and their association with disease-associated survival in multivariate Cox regression analysis (Taubert et al., Br J Cancer 2010; 102:731-737). We think that this way the results for the protein expression of uPA system
components are better comparable to the data on the mRNA expression in tumor tissue of STS patients.

We specified this in the Results section, page 9, as follows:

... values, respectively. In multivariate Cox regression analysis, we found that patients with low mRNA values of both factors were characterized by a much better disease-associated survival than patients with tumors in which one or both mRNA values were high (Figure 1). R0 patients with high PAI-1/uPAR-del4/5 (n=30) revealed a significant 19-fold increased risk of tumor-related death (RR=19.1, 95%CI=1.1-335.3, P=0.044) compared to R0 patients who showed low PAI-1/uPAR-del4/5 mRNA expression levels (n=13).

(7) “Table 3”: The RR for the relevant clinical prognostic factors used in the statistical model should also be presented. Additionally, the authors do not explain the use of the 66th percentile. It is only mentioned that “Relative expression ratio mRNA/HPRT according to 33% and 66% percentiles. Subgroup 1 (low): up to 33% percentile, subgroup 2 (high): from 33% percentile up to 100%)”

(i) According to the reviewers’ suggestions, we have added the data for the relevant clinical factors used in the multivariate base model to Table 3.

(ii) The mRNA expression values of biological factors were divided into groups using the 33% percentile as described in the Results section, page 9: group Low: 0 - 33% percentile; group High: > 33% - 100% percentile.

To present this fact more clearly, we re-phrased the respective sentence in the comment on Table 3 as follows:

Relative mRNA expression ratio of the respective marker / HPRT divided into groups using the 33% percentile; Low: 0 - 33% percentile; High: >33% - 100% percentile.