Author's response to reviews

Title: Increased expression of urokinase plasminogen activator and its cognate receptor in human seminomas

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Author's response to reviews:

Re: Ms # 2025934211324547 (Revised)

Title: "Increased expression of urokinase plasminogen activator and its cognate receptor in human seminomas"

Dear Editor,

Thank you for handling the manuscript and for your letter. I’m grateful to the Reviewers for their work and valuable comments. I have carefully revised the manuscript according to the comments and criticisms stated and I feel that the revised version is much improved. For clarity, all changes are reported in bold. Please, note that the revised manuscript has two additional authors which were by mistake omitted in the first submission and also contributed to the revision.

I hope that the revisions made in the manuscript are to the satisfaction of the Reviewers and that it will now be acceptable for publication.

Please, do not hesitate to contact me for any other information you may require.

Yours sincerely,

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Reply to Reviewer 1

We thank Reviewer 1 for the comments on the manuscript. Answers to the specific points are below reported. Please, note that all changes to the manuscript are reported in bold.

Major compulsory revisions:

1. There are no particular reasons. The number of samples analyzed by the different techniques simply reflect the number of samples available. To this regards it has been specified in the Methods section of the revised manuscript (page 6, lines 1-2) that the 10 samples tissues analyzed by immunohistochemistry are different from those reported in Table 1.

2. The Pearson’s correlation test is a useful method to analyze the correlation between two variables assuming that the data are normally distributed (Gaussian distribution). In our study the number of patients available is too small to verify if the data obtained for the different variable have a normal distribution or not. For this reason, and in agreement also with the advices of the Reviewer 3, we have repeated the correlation analysis using the non parametric Spearman correlation test, as reported in the Statistical analysis section of the Methods (page 9 of the revised manuscript). In this analysis we also analyzed the correlations of PAI-1 and PAI-2 with tumor size and patient’s age as suggested by the Reviewer 3. The results obtained showed that only PAI-1 significantly correlates with tumor size (p<0.05), while none of the uPAS components correlate with patient’s age, as shown in the revised figure 4. As a consequence we have modified the last paragraph of Results section (page 11) and the last paragraph of Discussion section (page 13).

Minor essential revisions

1. We agree with the Reviewer and replace TGCT with seminomas in the paragraph “Normal and tumoral human testicular tissues” of the Methods section (page 5). The abbreviation TGCT has been changed with seminomas also in the last sentence of page 4 and at page 12, lines 20 and 23.

Reply to Reviewer 2

We thank Reviewer 2 for the comments on the manuscript. Answers to the specific points are below reported. Please, note that all changes to the manuscript are reported in bold.

Major compulsory revisions:

1. As suggested by the Reviewer we have revised figure 3 reporting uPA and uPAR immunostaining also in the autologous normal testis surrounding the tumor. As described in the revised manuscript at pages 10,11 and 13,
spermatogonia appear to be negative for both uPA and uPAR

2. We cannot analyze the correlation with OCT4 and NANOG because we have run out most of the mRNA samples used to analyze uPAS members expression. However, we do not feel that the manuscript will suffer too much without this additional information. In addition, the main point raised by the Reviewer should be satisfied by the reply n.1.

Minor essential revisions

1. As mentioned in the Materials and Methods section (page 8/9) immunostained slides were analyzed and scored independently by two investigators (MM, ADB).

2. In figure 2A, uPA immuno-reactivity was not detected in control samples, but clearly present in tumor tissues. Thus the densitometric analysis in these conditions is not useful and do not add anything to the information already present. The same is true for figure 2C, in which only in one normal sample uPA activity could be detected. This is different from what observed in figure 2C where uPAR immuno-reactivity could be appreciated in all normal and tumor samples making densitometric analysis possible. For the latter, densitometric analysis of three independent experiments has been already reported in the Results section at page 10.

Discretionary revisions

1. I feel that the information reported in the Introduction section are appropriate. However, as also suggested by the Reviewer 3 we cited in the revised manuscript additional Reviews (see references 16, 17 and 26) dealing with the biochemical and biological functions of the uPAS.

2. As already specified in the Materials of the Methods section (see page 5), the monoclonal antibodies against urokinase plasminogen activator B-chain (uPA) and its receptor (uPAR) were purchased from the American Diagnostica Inc. (Stamford, CT).

3. The explanation for the MDA-MB-231 is now given also at page 8 of the revised manuscript.

4. The single uPAR negative sample in the immunohistochemistry experiments showed a strong uPA immunoreactivity. This has been specified at page 11 of the revised manuscript.

Reply to Reviewer 3

We thank Reviewer 3 for the comments on the manuscript. Answers to the specific points are below reported. Please, note that all changes to the manuscript are reported in bold.
Major compulsory revisions:

1. We agree with the Reviewer on this point and, as already mentioned in the last sentence of the Discussion section the findings reported in the manuscript should be verified on a larger case study, especially for what concern possible correlations between variation in the expression of the uPAS components and clinical parameters. Nevertheless, we feel that the data reported may be of interest for basic research and clinicians dealing with testicular cancers.

2. The seminomas investigated by western blot were also investigated at the mRNA levels as for each patients two frozen samples were available and used for RNA or protein extraction. The samples used in immunohistochemistry, available at the Regina Elena Cancer Institute, were different from those used for the analysis of mRNA expression reported in table 1. This has now been specified in the Materials and Methods section of the revised manuscript (page 5).

3. References provided by the Reviewer have been cited in the revised manuscript as reference n. 16, 17 and 26.

4. As already specified in the Materials of the Methods section (see page 5), the monoclonal antibodies against urokinase plasminogen activator B-chain (uPA) and its receptor (uPAR) were purchased from the American Diagnostica Inc. (Stamford, CT).

5. There are no particular reasons. Having protein extracts only from 3 seminomas and 3 normal testis tissues it was easier and cheaper to analyze the expression by means of western blot. The available ELISA kits for uPAS components should be the method of choice for larger case study.

6. Cp means crossing point, and we intended it as the threshold Cp. However, in the revised manuscript to avoid confusion we change the ##Cp with ##Ct.

7. In each sample the threshold crossing points (Ct) of target genes were normalized against that of #-actin, used as reference gene, by the ##Ct method using the LightCycler relative quantification software. Then the values of normalized target genes in seminoma samples were divided by the average value or normalized target genes found in 6 normal testicular tissue samples, and reported as fold of variation. These has now been reported in the revised manuscript (see page 6 and 7).

8. The Pearson’s correlation test is a useful method to analyze the correlation between two variables assuming that the data are normally distributed (Gaussian distribution). In our study the number of patients available is too small to verify if the data obtained for the different variables have a normal distribution or not. For this reason, and in agreement also with the advices of the Reviewer 3, we have repeated the correlation analysis using the non parametric Spearman correlation test as reported in the Statistical analysis section of the Methods (page 9 of the revised manuscript). In this analysis we also analyzed the correlations of PAI-1
and PAI-2 with tumor size and patient’s age as suggested by the Reviewer 3. The results obtained showed that only PAI-1 significantly correlate with tumor size (p<0.05), while none of the uPAS components correlate with patient’s age as shown in the revised figure 4. As a consequence we have modified the last paragraph of Results section (page 11) and the last paragraph of Discussion section (page 13).

9. We thank the Reviewer for his observation. Statistical analysis have been now performed with the Fisher’s exact test, as reported in the Statistical analysis section at page 9 of the revised manuscript. The results confirm the statistically significant increase for uPAR, but not for uPA, as suspected by the Reviewer. This has been corrected in the revised manuscript (see the end of page 10 and the beginning of page 11). Accordingly, the Discussion has been modified (see page 13).

10. References provided by the Reviewer have been cited in the Introduction and Discussion sections of the revised manuscript as references n. 16, 17 and 26.

11. We should apologize about that. Indeed, in figure 1 the bars represent the median and not the mean value. This has now been specified in the legend of figure 1.

12. The molecular weight standards have been reported in figure 2C. We also attempted to run western blots for PAI-1 using a monoclonal antibody from the American Diagnostica Inc. (product n. 3780). However, the antibody gave in our western blot multiple no specific bands. For this reason and since it expression was not significantly modulated at the mRNA level it was decided to not go any further.

13. The 10 samples tissues analyzed by immunohistochemistry are different from those reported in Table 1 and this has been specified in the Materials and Methods section (page 6, lines 1-2).

14. See reply n. 7 and n. 8.

Minor comments

All the suggested corrections have been made in the revised manuscript.