Author's response to reviews

Title: Role of the splicing factor SRSF4 in cisplatin-induced modifications of pre-mRNA splicing and apoptosis.

Authors:

Maude Gabriel (maude.gabriel@ulg.ac.be)
Yves Delforge (yves.delforge@ulg.ac.be)
Adeline Deward (adeward@ulg.ac.be)
Yvette Habraken (Yvette.habraken@ulg.ac.be)
Benoit Hennuy (benoit.hennuy@ulg.ac.be)
Jacques Piette (jpiette@ulg.ac.be)
Roscoe Klinck (roscoe.klinck@usherbrooke.ca)
Benoit Chabot (benoit.chabot@usherbrooke.ca)
Alain Colige (acolige@ulg.ac.be)
Charles Lambert (c.lambert@ulg.ac.be)

Version: 3
Date: 25 February 2015

Author's response to reviews: see over
Dear Editor,

We are pleased to submit a revised version of our manuscript “MS: 1653816502149879” entitled “Role of the splicing factor SRSF4 in cisplatin-induced modifications of pre-mRNA splicing and apoptosis”. We acknowledge the reviewers for their careful work and positive comments.

The revised manuscript is accompanied below by the detail of all changes and additions and by a point-by-point reply to the reviewers’ comments.

We thank you for your consideration and are looking forward to the editorial decision.

Sincerely yours,

M. Gabriel, MSc, corresponding author
A. Colige, Professor, promoter of the work
C. Lambert, PhD, promoter of the work
Changes and additions:

Abstract, line 45: “such as ATM, ATR…”

Result, line 190: “…of smaller splicing variants. The smallest variant had the expected size…”

Results, line 308/09: “the splicing changes induced by cisplatin in the events tested.”

Results, line 309-311: “The control siSCR and siSRSF6 alone had no effect. A similar reduction of the cisplatin-induced HNRNPDL exon 6 exclusion and exon 8 inclusion after knock-down of SRSF4 was observed in the breast cancer cell line BT549 (not illustrated).”

Discussion, line 354-355: “These discrepancies may be related to the different cell lines used, which may display different thresholds to elicit the DNA damage response.”

Discussion, line 372: “…indirect function, for example by…”

Discussion, line 394-400: “GO terms related to apoptosis were not highlighted by hierarchization analysis of the transcripts alternatively spliced upon cisplatin. We compared a list of transcripts related to apoptosis (GSEA [44,45]) with the list of transcripts with splicing affected by cisplatin treatment. Twenty-six actors involved in the regulation of apoptosis were common to both lists, as for example BAX, caspase-6, caspase-8 (pro-apoptotic) and MADD, API5 (anti-apoptotic). These examples illustrate that cisplatin-induced alterations of splicing may have both anti- and pro-apoptotic effects, and the net effect can’t be estimated on a theoretical basis.”

The reference number 44 and 45 has been added in the reference section.

The legend of figure 3 E-F, line 637: “(E: HNRNPDL-E6 p=0.13; F: AMZ2: p=0.49)” This is now indicated in the figure 3 E-F.

The legend of figure 5, line 655-657: “The histograms and errors bars represent mean and SD, respectively, illustrating the inter-experiment differences in the percentage of exon inclusion (n = 5 to 7). However, the statistics were made on the fold change measured in each independent experiment.”

The legend of figure 5 C-F, line 659-661, has been corrected to match with the panels described: “The splicing of MDM2 (C), HNRNPDL-E6 (**p=0.012; ***p=0.0013) (D), HNRPDLE-E8 (**p=0.0163) (E) and AMZ2 (**p=0.0002, **p=0.001) (F) was evaluated by end-point RT-PCR.”

The spacing between numbers and units has been checked.

The line point of the error bars in all figures has been increased.
Reviewer’s response

Reviewer: Raya Huang

1. Figure 1, please explain why there are many non-specific bands in most of the cisplatin-treated samples?

The various bands observed in cisplatin-treated samples are not non-specific. Indeed, more than 40 different splice variants of MDM-2 transcript have been identified (Bartel F. et al, MDM2 and Its Splice Variant Messenger RNAs: Expression in Tumors and Down-Regulation Using Antisense Oligonucleotides, Mol Cancer Res, 2004). While the MDM2-alt1 splice variant is the most abundantly expressed in response to DNA damage inducers (UV and cisplatin), other variants co-exist (Chandler DS et al., Genotoxic stress induces coordinately regulated alternative splicing of the p53 modulators MDM2 and MDM4, Cancer res, 2006). The manuscript has been modified to clarify this point.

2. Figure 3 E and F, although the combine treatment did not reverse the splicing induced by cisplatin, the splicing events seemed increased in the combine treatment with cisplatin plus caffeine and DNA-PK inhibitor.

Indeed, we observe a tendency for increased exclusion of HNRNPDL exon 6 (FIG 3 E) and AMZ2 exon 3 (FIG 3 F) with the combined treatment as compared to cisplatin alone. However, the difference is not significant (p=0.13 for HNRNPDL exon 6 and p=0.49 AMZ2 exon 3). This is why it was not reported in the original version of the manuscript. This is now indicated in the figure 3.

3.a Figure 5 C-F legends do not match the panels described.

The legend of the figure has been corrected.

3.b It is also surprising that the error bars between –siSRSF4 and + siSRSF4 seem to overlap yet the significant difference value is **, p= 0.016 for HNRNPDL-E6, while the p values of one asterisk for the combine siSRSF4 and siSRSF6 was not indicated.

The p-value between control and combined siSRSF4/siSRSF6 samples is a “three stars” value. These stars have been omitted in the original figure which is corrected in the revised manuscript. The histograms and errors bars represent mean and SD, respectively, illustrating the inter-experiment differences (n = 5 to 7) in the percentage of exon inclusion. However, the statistics were made on the fold change measured in each independent experiment (siSRSF4 + cisplatin versus siSCR + cisplatin: mean fold change = 0.49±0.17, n=7, p = 0.012; siSRSF4/6 + cisplatin versus siSCR + cisplatin: mean fold change = 0.57±0.03, n=5, p = 0.0013). The lower p-value for siSRSF4/6 sample reflects the very low SD. The rationale for obtaining these values has been indicated in the legend of the figure 5.
4. It is surprising that the major splicing factor SRSF1 or SRSF2 are not involved in the cisplatin induced pre-mRNA splicing events since it has been shown that these factors are overexpressed or hyperphosphorylated in cisplatin resistant cell lines. Did they examine other SRSF factors in addition to SRSF4 and SRSF6?

We only investigated splicing factors SRSF4 and SRSF6 in the present study. A large siRNA-mediated screening of the involvement of 57 splicing factors in hnRNPDL exon 6 and 8 splicing in basal condition has been performed, and the participation of the identified splicing factors in the cisplatin-mediated splicing is underway (article in preparation).

5. In Discussion, third paragraph, the author claimed that PI3K pathway, but not DNA damage response and p53, is involved in the alteration of splicing by cisplatin. Although it is mentioned that other group (Shkreta et al. ref 30) has found that DNA damage response and p53 contribute to cisplatin-induced splicing of other genes, this demands more discussion.

In Shkreta’s work, the splicing of the reporter transcript (Bcl-X) in non-cancer 293 cells was shown to be dependent on ATM and p53 using pharmacological inhibitors and siRNA. Our data showing that ATM is not required were obtained using pharmacological inhibitors in the breast cancer cell line MCF7, and further ascertained using ATM deficient AT5BIVA cells. Similarly, the lack of requirement of p53 was shown using p53-deficient MG63 cells. Thus, the difference in the two studies may arise, as indicated in the text, from the different cell lines used, which may display different thresholds to elicit the DNA damage response. Consistent with this view is our observation that Bcl-X splicing is not altered in our cell line at the concentration of cisplatin used. The Discussion has been modified accordingly.

Minor points:

1. Please increase the line points of the error bars in all figures. It is not easy to visualize at the current forms.

The manuscript has been modified accordingly.

2. Grammar and typos:

Abstract, line 45, such as ATM, ATR…

The manuscript has been modified accordingly.

Results, line 308/09, the splicing changes induced by cisplatin in the events tested.

The manuscript has been modified accordingly.
Discussion, line 372, …indirect function, for example by…

The manuscript has been modified accordingly.

3. Please check spacing between number and units, for example, 100 nM and 24_hours, in several figure legends.

The manuscript has been modified accordingly.
Reviewer’s response

Reviewer : Qi-En Wang

1) Figure 1G: The product of MDM2-FL after oxaliplatin treatment is increased as well as MDM2-ATL1. It seems like it does not have similar effect on MDM2 splicing as cisplatin. It is not clear why authors did not run these two samples in the same gen with the same amount of samples.

In the gel illustrated in the figure 1G, the overall expression of MDM-2 is indeed higher in oxaliplatin-treated sample than control sample. However, this was not confirmed in another experiment. This point is not addressed in the manuscript as we are mostly interested by the relative intensities of the signals for full-length and alt-1 splicing variants. In the figure 1G, the profile of RT-PCR products in oxaliplatin (50μM) treated cells differs from that of cisplatin (50μM) treated cells, but resemble more closely to cisplatin at 20μM (Compare Fig 1G and 1A). We hypothesize that MCF7 cells are less sensitive to oxaliplatin than to cisplatin, used at the same concentration. The two samples (CTL and oxaliplatin) were run on the same gel, but samples not illustrated here were in between, explaining why the two samples are separated by a vertical line.

2) The authors evaluated the role of SRSF4 in mediating the effect of cisplatin on AS by siRNA targeting SRSF4 in only MCF7 cell line, it is interesting to know the results in one or more breast cancer cell lines.

The effect of SRSF4 knock-down on cisplatin-induced HNRNPDL exon 6 exclusion and exon 8 inclusion was tested in the breast cancer cell line BT549. Knock-down of SRSF4 were by 75% and 89% in experiments 1 and 2, respectively.

The exclusion of exon 6 was reduced by 28% (exp. 1) and 45% (exp. 2).
The inclusion of exon 8 was reduced by 59% (exp.1) and 65% (exp.2).
Therefore, the data are similar to those obtained in MCF7 cells. The manuscript has been modified accordingly. The splicing of AMZ2 has not been investigated. Indeed, we observed that the signal of AMZ2 is lower in BT549 cells than in MCF7 cells, making the analysis of the data more difficult and dubious.

3) Figure 5: legends and figures do not match.

The legend of figure 5 has been corrected.
4) To address the potential involvement of SRSF4-dependent splicing events induced by cisplatin in apoptosis, the splicing of some apoptosis-related genes, e.g., caspase-2 and Bim, should be determined.

GO terms related to apoptosis were not highlighted by hierarchization analysis of the transcripts alternatively spliced upon cisplatin. We compared a list of transcripts related to apoptosis (GSEA, Gene Set Enrichment Analysis, Subramanian, Tamayo, et al. (2005, PNAS 102, 15545-15550) and Mootha, Lindgren, et al. (2003, Nat Genet 34, 267-273) with the list of transcripts with splicing affected by cisplatin treatment. Twenty-six actors involved in the regulation of apoptosis were common to both lists, as for example BAX, caspase-6, caspase-8 (pro-apoptotic) and MADD, API5 (anti-apoptotic). These examples illustrate that cisplatin-induced alterations of splicing may have both anti- and pro-apoptotic effects, and the net effect can’t be estimated on a theoretical basis.