Author's response to reviews

Title: Wnt/beta-Catenin Pathway Regulates ABCB1 Transcription in Chronic Myeloid Leukemia.

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Author's response to reviews: see over
To Dr. Jigisha Patel, Series Editor of the “BMC Cancer Journal”

Dear Dr. Patel,

First we would like to thank you for the opportunity of deadline extension that made possible to perform all experiments requested by the reviewers. Second we would like to thank the reviewers for the relevant comments and suggestions which improved our manuscript.

We are re-submitting the manuscript titled, “Wnt/β-Catenin Pathway Regulates ABCB1 Transcription in Chronic Myeloid Leukemia,” with all the changes and experiments inclusion requested by reviewers.

The changes in the revised manuscript are described below.

Authors’ answers to reviewer 1, Michelle Perugini:

Major Compulsory Revisions

1. The authors should justify the use of the K562 and Lucena cells for the studies in Figure 6. In the raw (un-normalised) data, is ABCB1 mRNA expression in Lucena cells higher than that of the K562 controls? It would be necessary for this to be the case in order for this cell line to be an appropriate model for response and resistance phenotypes in CML. This data should be included in Figure 6 along with assessment of increased b-catenin activity in the Lucena cell line compared with K562 cells.

Lucena is a multidrug resistant cell line derived from K562 cells by increased doses of Vincristine (VCR) treatment. Answering to your question, Lucena cells over-express ABCB1 mRNA levels (800.0-fold increase) and Pgp protein (45.0-fold increase) compared to its parental cell line. These data have been published by Corrêa and co-workers (Corrêa et al., 2012 Proteome Sci). In addition, we demonstrated in the same paper that Lucena cells are cross-resistant to imatinibe mesylate (IM) through Pgp efflux, making this cell line an appropriate model for resistance phenotype in CML. As our goal (in previous Figure 6, actual Figure 7) was
to evaluate ABCB1 mRNA levels under WNT1 knockdown, we used these cell lines as models for responsive (K562) and resistant (Lucena) phenotypes. The information about the level of ABCB1 expression was inserted in Introduction section, lines 90-93.

Moreover we added the assessment of increased b-catenin expression and activity in Lucena cells compared to K562 cells by immunofluorescence, flow cytometry, real time quantitative PCR and western blot assays in Figures 3 and 4, in Results section, β-catenin binds to the ABCB1 promoter at the TCF-binding site subsection, lines 267-271.

2. The authors should describe the differences in the patient cohorts between IM-responsive patients and IM-resistant patients. The IM-responsive cohort has no BC patients and a high proportion of CP patients, whereas the resistant group comprises nearly half BC-patients and only one CP patient and therefore is not representative of primary resistant CP CML. The authors need to justify these differences.

This result was deleted from the manuscript as it was included in another paper (Correa et al., 2012). The underlying information was referred in Introduction section lines 90-93.

3. Methods, Bone marrow samples – more information should be included for disease endpoints ie. What is considered major molecular response and complete hematologic and cytogenetic responses, and how are they measured?

This methodology was excluded from the manuscript.

4. Methods, Statistical analysis – the authors have used a paired t-test for comparison of mRNA levels and cell viability between K562 and Lucena cells from different assays. An unpaired t-test would be more appropriate given the analysis is between two different cell lines, and the authors should use this test for their analysis. The authors should also describe the statistical tests used for comparing expression levels in the patient samples.
As requested, the author used an unpaired t-test in order to analyze differences between the two cell lines from all experiments performed in this manuscript. This methodology is described in Methods section, Statistical Analysis subsection, lines 238-242.

5. Results, b-catenin binds to the ABCB1 promoter at the TCF-binding site – Need to justify the inclusion and meaning of SMAD8 in Figure 2.

In EMSA, SMAD8 was used as control (unrelated transcription factor), since the TCF-oligonucleotides did not contain SMAD8 binding site. In ChIP assay, SMAD8 was used as positive control, given that the amplified region (by real time quantitative PCR) contained a SMAD8 binding site. The authors cited these in Methods section, Electrophoretic Mobility Shift Assays (EMSAs) subsection, lines 135-136.

6. Authors should discuss the significance of the various TCF binding sites and their location in the promoter. Sites shown to be important in other cancers. Do any of the binding sites exhibit stronger binding to b-catenin than others, and why have the authors chosen 4 and 5 for the complete analysis, there is little discussion about the additional data in Supplementary Figure 1.

The presence of various TCF binding sites and their location in ABCB1 promoter have been poorly studied in other cancers. The existing data (Yamada et al., 2000; Flahaut et al., 2009; Bourguignon et al., 2009) have taken advantage of knockdown experiments in order to investigate the involvement of canonical pathway in ABCB1 regulation. Luciferase vector assay with the most proximal TCF binding site has shown to be part of this activation. These suggest that this gene could be regulated by canonical pathway and this regulation is probably conducted by b-catenin’s binding in different or redundant TCF sites among the different types of cancer.

In order to screen in vitro binding of protein extract from K562 and Lucena cells, we performed EMSA assay with all 7 TCF-oligonucleotides sites (Supplementary figure 1). It can be observed that the protein complex is binding with different affinities to TCF-oligonucleotides
in vitro. Complete analysis, including super-shift assays with b-catenin, were only performed in TCF4 and TCF5 sites because they presented clearly and stronger patterns of protein extract binding. In order to understand the role of some TCF binding sites we included in the manuscript transfection experiments with different TCF-containing constructions. Our results indicate that several TCF binding sites are active in ABCB1 promoter and that this activation is mediated by b-catenin. This information was described in Methods section, Reporter vectors design and Transient transfection and Luciferase reporter assay subsections, lines 220-237 and inserted in Results section, ABCB1 promoter TCF binding sites role in transcriptional activity subsection, lines 301-316.

7. The authors need to show that the LiCl in their hands results in increased b-catenin stabilization and nuclear translocation, to ensure the concentration used is correct and that it is working as it should.

We have performed immunofluorescence for b-catenin and western blot (for activated GSK3b) in both cell lines under treatment in order to provide evidence that LiCl treatment (10mM, 24h) results in b-catenin’s translocation to nucleus, as required by reviewer 1. These results were labeled as Figure 4 and 5, and added in Results section, The Wnt/β-catenin signaling pathway regulates ABCB1 expression subsection, lines 274-278. Their methodologies are described in Methods section, Western Blot analysis and Immunofluorescence staining and confocal laser microscopy subsections, lines 200-219.

8. Results, The Wnt/b-catenin signaling pathway regulates ABCB1 expression (first para, last sentence) – The authors should provide an explanation as to why the increase in ABCB1 mRNA was more significant in K562 cells than the Lucena cells. Is this because ABCB1 expression is already higher in the Lucena cells than in the K562 cells (see comment 1)?

Yes, you are right. The reason we observed a more significant increase in ABCB1 mRNA levels in K562 cells compared to Lucena cells, is that Lucena cells already have high
levels of \textit{ABCB1} mRNA compared to K562 cells (800.0-fold increase), and K562 cells do not present Pgp protein in their plasma membrane (Rumjanek et al., 2001; Corrêa et al., 2012).

All Minor Essential Revisions were addressed in revised manuscript.

1. Abstract (first paragraph, last sentence) – should read “b-catenin is…” line 37

2. Background (first sentence) – needs some references relating to the translocation that occurs in BCR-ABL in CML. Line 58

3. Background (second para, second sentence) – should read “Nevertheless, some patients do…” Line 74

4. Background (second para) – author name misspelled – should read Flahaut not Flauhaut. Line 84

5. Methods, Bone marrow samples – change “We selected six…” to “We selected 6…” Subsection removed from the manuscript

6. Methods, Electrophoretic Mobility Shift Assays – change “double-strained” to “double-stranded” Line 107

7. Methods, Chromatin Immunoprecipitation (ChIP) assays on Native Chromatin (second para) – change “unspecific binding” to “non-specific binding”. Line 147

8. Methods, Real-time Quantitative PCR (RT-qPCR) (second sentence) – change “RNA from cell lines was treat with…” to “RNA from cell lines was treated with…” Line 151

9. Methods, Real-time Quantitative PCR (RT-qPCR) (second to last sentence) – Cobertt misspelled, should be Corbett. Line 161
10. Discussion (first para, second sentence) – in the sentence “each of which has responds…” delete has. Lines 319-320

11. Discussion (second para, third sentence) – statement requires referencing. Line 325

12. Discussion (third para, last sentence) – change “corroborates” to “corroborate”. Line 336

13. Discussion (fourth para, last sentence) – requires referencing. Line 342

14. Reference 5 is missing journal name and issue details. Lines 434-436

15. Reference 48 is missing journal name and issue details. Lines 590-591

Authors’ answers to reviewer 2, Frank Thevenod:

Major Compulsory Revisions:

1. **Figure 1: Protein expression is required, e.g. by FACS analysis with ABCB1 antibodies.**

   Previous Figure 1 was excluded from the manuscript as this result has been included in Correa et al., 2012. Pgp expression and activity was investigated in CML patients in Vasconcelos et al., 2011.

2. **Figure 2: Competition by Opt and beta-catenin is variable and not convincing for both TCF4 and TCF5 probes. Why was Smad8 tested?**

   Opt oligonucleotide is only the TCF consensus binding site sequence so the competition assay can be variable as the protein complex in each TCF binding site can use adjacent sites. To confirm the EMSA results we performed ChIP assays as shown in actual Figure 2.
Beta-catenin binding in super-shift assay is variable between K562 and Lucena’s protein extracts because as we show in actual Figure 3, Lucena cells present higher expression of beta-catenin.

In EMSA, SMAD8 was used as internal control (unrelated transcription factor), since the TCF-oligonucleotides did not contain SMAD8 binding site. In ChIP assay, SMAD8 was used as positive control, given that the amplified region (by real time quantitative PCR) contained SMAD8 binding site. These data were described in Methods section, Electrophoretic Mobility Shift Assays (EMSAs) subsection, lines 135-136.

3. Figure 3: The left panel probably the results of quantitative RT-PCR, the right panel the results of a qualitative RT-PCR. The right panel is not convincing (very weak signal in bound and unbound experiment. Why was a qualitative RT-PCR not shown for ABCB1?

The signal of bound and unbound fractions showed in the right panel of previous figure 3B is weak because they are fractions of input DNA. This result was incorporated in order to demonstrate the differences between qualitative and quantitative analysis. As requested by reviewer 2, we exchanged the qualitative analysis in the right panel for ABCB1 evaluation, which is the final interest of ChIP experiment (actual Figure 2) This result was included in Results section, β-catenin binds to the ABCB1 promoter at the TCF-binding site subsection, line 266.

4. Figure 4: The Figure is redundant and could be described in the text.

We removed figure 4 from the manuscript and described its information in the text as suggested by reviewer 2, in Results section, The Wnt/β-catenin signaling pathway regulates ABCB1 expression subsection, lines279-280.

5. Figure 5: These data are not clear. The “ctrl” is normalized to 1 in both parent and multidrug resistant cells. I assume that the ABCB1 levels are higher in control “Lucena”
compared to control “K562”. The interpretation (saturation of Wnt signaling) would be convincing only if all the data are normalized to the “K562” control only.

Indeed Lucena’s ABCB1 mRNA levels are higher compared to K562 (800.0-fold increase). Data normalization was changed to the “K562” control as required by reviewer 2. In order to make this data clear we re-draw the graphic showing ABCB1 mRNA levels in each condition (actual Figure 6). These results are described in Results section, The Wnt/β-catenin signaling pathway regulates ABCB1 expression subsection, lines .281-284

6. Figure 6: It is astonishing that siRNA against Wnt1 is so effective in multidrug resistant cells, but has no effect in parent cells considering that at least 19 Wnt ligands have been identified. Why did the authors exclusively focus on Wnt1? Are the other Wnt ligands irrelevant? A more credible strategy would be to knockdown a more down-stream target, e.g. beta-catenin or TCF4/5.

As requested by Reviewer 2 we knockdown b-catenin and we could confirm that ABCB1 mRNA levels are dependent of Wnt/b-catenin signaling. As supposed by reviewer in K562 cells other Wnt ligands are probably active, as Wnt1 knockdown was not capable to inhibit ABCB1 expression. We introduced this result as figure 7, in Results section, The Wnt/β-catenin signaling pathway regulates ABCB1 expression subsection, lines 285-295.

7. To demonstrate increased activity of Wnt signaling a TCF luciferase reporter gene assay is mandatory.

In order to demonstrate the increased activity of Wnt signaling we included in the manuscript transfection experiments with different TCF-containing constructions. Our results indicate that several TCF binding sites are active in ABCB1 promoter and that this activation is mediated by b-catenin as we observed a decrease in TCF activity in treated cells (LiCl 10 mM). This treatment increases b-catenin translocation to nucleus, diminishing the available citoplasmatic b-catenin. These data were described in Methods section, Reporter vectors design and Transient transfection and Luciferase reporter assay subsections, lines 220-237 and inserted
in Results section, ABCB1 promoter TCF binding sites role in transcriptional activity subsection, lines 301-316.

Please let me know if I successfully addressed all queries requested. If any more changes are needed, I will be pleased to try to correspond.

Hope to hear from you soon.

Best regards,

Stephany Corrêa.