Format for ANSWERING REVIEWERS

Title: Bortezomib Effect on E2F and cyclin family members in Human Hepatocellular Carcinoma Cell Lines

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The manuscript has been improved according to the suggestions of reviewers:

Please note that all modifications are reported in orange in the revised manuscript

Rewiever (1)

1. Title Page: Title could be more specific as E2Fs control a wide spectrum of genes. In addition, HCC cell lines could be specified as "human".
The title has been modified indicating the gene products evaluated and the word "human" has been introduced.

2. Introduction: This needs to be expanded by adding data in the following points:
a) current epidemiology of HCC (i.e. mEASL–EORTC HCC management guidelines, J Hepatol 2012)
Details about HCC epidemiology have been introduced (page 4 lines 2-13 from top) together with the novel reference no. 4 suggested by the referee.

b) sorafenib: mention type of inhibitor, how it improves patient survival
Details about sorafemib mechanisms of action and life span prolongation have been introduced on page 4 line 18-22 from top; additionally the novel reference no. 7 has been added.

c) BZB: how does it inhibit proteasome and where does it bind
This novel piece of information has been added on page 4 lines 6-7 from bottom.

d) specify type of hematological malignancies and of other malignancies that BZB is indicated for.
The specific hematological cancer pathologies for which BZB is indicated have been introduced on page 5 lines 1-2 from top.

e) current knowledge on the role of all E2F family members in cell cycle and related data in human HCC.
The cell cycle roles of E2Fs not sufficiently described in the original version of the manuscript have been added on page 5 lines 16-18 and on lines 24-27 from top. The available information about E2Fs involvement in HCC have been reported on page 5 lines 30-31 from top.

3. Material and Methods: mention the characteristics of HCC cell lines and the criteria according which their degree of differentiation was initially defined. Add related reference.

In the revised manuscript the sources of the used cell lines have been reported on page 7 line 3-7 from top. From the ATCC, catalogue No.: HB-8065 and Japanese Collection of Research Bioresources (JCRB), catalogue No.: JCRB1030, for HepG2 and JHH6, respectively, it is possible to gain the information requested by the reviewer. The papers which firstly described HepG2 and JHH6 have been also introduced (novel references no. 30 and 31, respectively) in the revised manuscript.

4. Data on positive and negative controls in a separate paragraph.
We are sorry but it is unclear to us the suggestion of the reviewer.

5. Reasoning of not extending experiments longer should be strengthened.
It is unclear whether the reviewer refers to the time point chosen for microarray/RT-PCR analysis (figures 1-2) or to the siRNA mediated depletion of E2F8 (figure 3). In the first case (figures 1-2) the two days time-point was chosen on the basis of our previous results (ref 20) which indicated this time point as optimal for the evaluation of BZB effects; in the revised manuscript this aspect has been further specified on page 8 lines 8-10 from top. In the second case (figure 3) the 48h time point was chosen for the reasons indicated on page 13 lines 15-19 from top. We also would like to remark the point that at longer time points the siRNA anti E2F8 in HepG2 and JHH6 did not significantly decrease the target mRNA level thus making the evaluation of its effect not appropriate.

6. Introduction: Add “liver” resection, “radiofrequency” ablation.
These correction were made in the revised manuscript
The selection criteria for HCC cell lines should be mentioned in “Material and Methods” section.
In the revised manuscript the sentence related to the selection criteria has been moved to the M&M section (page 7 lines 3-7 from top).
Replace “cyclin D dependent kinase” with “cyclin dependent kinases in complex with their cyclin partners”
In the revised manuscript the correction was introduced (page 5 lines 13-14 from bottom).

7. Material and Methods: Reference to origin of cell lines (Caucasian, Asian, other) and if they are affected by HBV or other hepatotropic viruses.
See response to reviewer point no.3

Brief description of statistical tests used is missing.
In the revised manuscript the names of the statistical tests used have been introduced on page 9 lines 2-3 from bottom.

8. Results: HepG2 should be accompanied by “cell line”.
In the revised manuscript the words “cell line” have been introduced after “HepG2” throughout the text.
Definition of E2F-1 upregulation: protein or mRNA level?
If the reviewer refers to the sentence of page 11 line 14-16 from top (discussion), the word “up-regulation” refers to the increased levels of mRNA and protein.
Grade of differentiation of HuH7 cell line is missing.
In the revised manuscript the HuH7 differentiation grade has been added on page 13 lines 2-3 from top

Results: Exact p values are missing.
Exact p values are not reported to avoid too many numbers in the legends; additionally knowing that each p value is lower than 0.05 is, from a mathematical point of view, sufficient to indicate the significance
Comments on Results should be mentioned in Discussion.
In the revised manuscript, according to reviewer request, we have deleted from the results the sentences:
-"This observation suggested that, at least in the HepG2 cell line and under our experimental conditions, E2F8 did not seem to be a major promoter of cell proliferation; consequently, it is unlikely that BZB anti-proliferative effects in the HepG2 cell line were mediated by E2F8 decrease", originally present on page 8 lines 14-17 from top.
-" Despite the BZB effects were quantitatively more relevant in HepG2 compared to JHH6, our data suggest that BZB may mediate cell proliferation inhibition via the down-regulation of E2F pro-proliferative members (E2F1 and E2F2) and the up-regulation of an E2F anti-proliferative member (E2F6)." originally present on page 8 lines 25-29 from top.

9. Discussion: For homogeneity, is advised to use the same terms throughout the text i.e. "activators and repressors E2Fs" or "anti-proliferative and pro-proliferative E2Fs
According to reviewer comment in the revised manuscript we have substituted on page 5 line 15 from top the words "activators" and "repressor" with the words "pro-proliferative" and "anti-proliferative", respectively; in all the other parts of the manuscript we have used the terms "pro-proliferative" and "anti-proliferative".

10. Figure legends: Abbreviations need explanation
We assume the reviewer refers to the abbreviation "BZB"; in the revised manuscript the word "BZB" has been substituted in the legends by the word "Bortezomib".

Rewiever (2)
1. Authors found that bortezomib treatment significantly increased E2F8 expression in JHH6 cells but significantly decreased it in HepG2 cells. E2F8 has been regarded as an anti-proliferation transcription factor. So the finding of bortezomib-elevated E2F8 could be used for explaining the cell suppression effects of bortezomib in JHH6 cells, but such finding put E2F8 as a contradicting role in HepG2 cells. The authors subsequently knocked down E2F8 gene levels and did not detect the change in cell number (Figure 3), concluding that decreased E2F8 expression had nothing to do with cell proliferation. To strengthen their statements, I urge the author repeat this experiments in JHH6 cells and discuss the results to the revised Figure 3.
According to reviewer request, in the revised manuscript data about the silencing of E2F8 in JHH6 have been introduced on page 8 line 1-2 from bottom (M&M), on page 10 lines 28-32 from top (results), on page 13 lines 10-11 from top and lines 12-14 from bottom (Discussion) and in the revised Figure 3 (panel 3AI and 3BI) and its legend. The novel piece of information about E2F8 silencing in JHH6 indicates the non-significant decrease in cell number upon E2F8 depletion.

2. The findings that bortezomib treatment induced differential expression of E2F family members between JHH6 and HepG2 cells are interesting. Considering the different phenotypes of these two cell lines, I am wondering whether their differentially responsive E2Fs expressions are related to epithelial-mesenchymal transition (EMT) of hepatocellular carcinoma. Since the authors have

already had microarray data in hand, I suggest them to analyze the bortezomib induced changes in EMT-related genes.
According to reviewer request, in the revised manuscript data about BZB effects on EMT gene expression have been introduced on page 11 lines 4-7 from bottom (results), on page 13 line 2 from bottom and page 14 lines 6-9 from top (Discussion) and in the novel Figure 5 and its legend. Once again it is confirmed the reduced effect on gene expression of BZB in JHH6 compared to HepG2.

3. A minor comment to this paper: English editing by a native English-speaking colleague could much improve the language quality of this paper.
As reported in the acknowledgment section the revised manuscript has been edited by a native English-speaking colleague, whose name can be provided if necessary.

Rewiever (3)
1) We are suggesting a few minor revisions such as spelling check..
See answer to point 3 of Rewiever (2)

2) ..and removal from legends text of reference to materials and methods section.
In the revised manuscript the reference to materials and methods in the figure legends have been removed.

3) For future papers, in order to confirm the data obtained and to avoid differences between human tumors and standardized tumor cell lines, I would suggest that the authors consider an in vivo study on bortezomib antitumor actions, using possibly derived patients cell lines.
We perfectly agree with the suggestion of the reviewer.

We trust that our revised manuscript will meet your and the reviewer's approval.
Looking forward to hearing from you soon, please receive my best regards

Sincerely yours,

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