Author’s response to reviews

Title: Pitavastatin suppresses diethylnitrosamine-induced liver preneoplasms in male C57BL/KsJ-db/db obese mice

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Version: 2 Date: 27 April 2011

Author’s response to reviews: see over
Dr Andrzej K. Bednarek,

Section Editor, *BMC Cancer*,

Re: Revision of *BMC Cancer* manuscript MS # 5930295664816320: “Pitavastatin suppresses diethylnitrosamine-induced liver preneoplasms in male C57BL/KsJ-db/db obese mice”

Dear Dr. Bednarek,

Thank you for your email of January 28, 2010, indicating that the above cited paper can be accepted for publication in *BMC Cancer* after major revision. Enclosed please find our revised manuscript, including new data (new Figs. 1B, 2, and 3C), which we believe addresses all of the concerns raised by the two reviewers, and a point-by-point list of our responses to the criticisms. In the revised manuscript, we added a new co-author, Dr. Takahiro Kochi, who reperformed statistical analyses that are requested by Reviewer #2.

We thank you and the reviewers for these very helpful and constructive criticisms and we would like to request that our revised paper may again be considered for publication in *BMC Cancer*.

With best personal regards,

Sincerely,

Masahito Shimizu, M.D., Ph.D.
Responses to Reviewer #1 (Dr. Diego F. Calvisi)

We are pleased that in the overall comments this reviewer found our paper is novel and well written, and the methodologies used are appropriate. We responded to concerns raised by the reviewer #1 as follows.

Minor Essential Revisions:
1.) The Authors determined the levels of total and activated (phosphorylated) AMPK protein. Is this AMPK-α? Please clarify this point in the revised version of the manuscript.

We used primary antibodies for AMPK-α and phosphorylated AMPK-α in the present study. We clarified this point (Page 3, line 15, Page 7, line 16, Page 11, lines 14 and 25, Page 14, line 20, Page 15, line 4, Page 17, line 4, Page 23, line 25, Page 24, line 7, and Fig. 3B).

2.) Authors should briefly summarize either in the Introduction or in the Discussion section the findings of aberrant lipogenesis in human HCC with the appropriate references.

Following this suggestion, we revised the “Background” (Page 4, lines 12 to 14) and “Discussion” sections (Page 14, line 25 to Page 15, 3) with citing the good reference (see new Reference #8). We thank your valuable advice.

3.) In the Materials and Methods section, the Authors should briefly describe the criteria
to define foci. This would be highly helpful to the Readers who are not expert in the field.

Following this suggestion, we revised the “Materials and Methods” section (Page 7, lines 1 to 3).

4.) In Figure 1B only the graphs of FCA number in treated and untreated mice are shown. The Authors should add a low magnification picture from livers of both groups (treated and untreated) showing the morphologic differences.

This reviewer suggested that we add a low magnification picture from livers to show the morphologic differences, i.e. presence of FCA. However, the size of FCA is too small (300 – 400 µm diameter, Fig. 1A) and the appearance of this lesion becomes to be unclear in lower magnification field than Fig. 1A. Therefore, we didn’t include a low magnification picture in Fig. 1B.

5.) Authors should determine whether pitavastatin treatment results in decrease of proliferation and/or increase of apoptosis in FCA. These data will add mechanistic evidence to the present study.

Following this suggestion, we performed additional experiments (see new Fig. 2) and found that suppressive effect of pitavastatin on the development of FCA was associated with the induction of apoptosis in the liver and the inhibition of proliferation in this lesion (Page 3, line 14, Page 7, lines 5 to 11, Page 7, lines 18 to 19, Page 8, line 3, Page 10, line 24 to Page 11, line 12, Page 14, lines 13 to 15, Page 23, lines 8 to 24).
We appreciate your very important suggestion.

6.) It is known that antineoplastic activity of statins might be also partly independent on their activity toward cholesterol levels. The Authors should briefly comment this issue in the Discussion section.

Following this suggestion, we revised the “Discussion” section (Page 14, lines 2 to 9).
Responses to Reviewer #2 (Dr. Neeraj Saxena)

We wish to thank the Reviewer #2 because in the overall comments this reviewer found our study is interesting. In the overall comments, this reviewer advised that our manuscript needs more in-depth analysis of results. Following this advice, we performed additional experiments and obtained new findings that may fortify our previous conclusions (see new Fig. 2). We also conducted a statistical analysis again as suggested by this reviewer and obtained new results (see new Figs. 1B and 3C). We thank this reviewer for these constructive criticisms. We responded to concerns raised by the reviewer #2 as follows.

Major compulsory revisions:

Figure 1, FCA values shows significant decrease at 1ppm (p<0.05) and 10ppm (p<0.001). SD bars basically nullifies each other and shows no differences between the three groups. Same thing is true for Data in Figure 2 for FFA and total cholesterol levels.

This reviewer raised questions about the results of statistical analysis in Fig. 1B and old Fig. 2C. In order to clarify these points, we conducted a statistical analysis again using appropriate method (Bonferroni multiple comparisons criteria, Page 9, line 3) and obtained new results (see new Figs. 1B and 3C). Based on these new results, we revised the text (Page 3, line 12, Page 10, lines 16 to 22, Page 12, lines 2 to 5, Page 23, lines 6 to 7, and Page 24, lines 13 to 14). We deeply appreciate your very important suggestion.

Very importantly, control experimentation using wild type mice needs to be included in
this study to show the importance and relevance of obesity in DEN induced hepatocarcinogenesis.

This reviewer suggested that this study needs control experimentation using wild type mice. We fully understand the importance of this suggestion. Regarding this concern, in our previous study, we have already confirmed that neither C57B6 nor C57BL/KsJ-++/+ mice, genetic controls for db/db mice, showed the development of FCA and liver neoplasms and induction of liver dysfunction by DEN administration (see Reference #22). Therefore, control experimentation using these mice was not conducted in the present study. We clarified this point in the “Materials and Methods” section (Page 6, lines 18 to 20).

Minor Essential Revisions:

Improvement in adipocytokines imbalance (increased adiponectin and decreased leptin) is a cause or the effect of Fat resolution?

This reviewer asked how pitavastatin improves adipocytokine imbalance. We presume that improvement of adipocytokine imbalance by this agent is associated with the reduction of BMI and decrease of TNF-α (Page 15, lines 14 to 18 and new References #30 and 31).