Author's response to reviews

**Title:** Leptin as a critical regulator of hepatocellular carcinoma development through modulation of human telomerase reverse transcriptase

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**Author's response to reviews:** see over
To Dr. Rachel Neilan  
Assistant Scientific Editor  
BMC-series Journals

Feb 23, 2010

Dear Dr. Neilan,

We would like to submit our manuscript entitled: “Leptin as a critical regulator of hepatocellular carcinoma development through modulation of human telomerase reverse transcriptase” for further consideration in BMC journal.

Below please find our answers to all comments and suggestions raised by the reviewers, which helped us revise our manuscript.

Thank you very much for your co-operation and hoping that you will find our revised manuscript suitable for publication in your journal.

Sincerely,

Aspasia Tsezou  
Assoc.Prof.Med.Genetics
To Reviewer 1 (Jing Shen)

Thank you for the useful comments raised by you, which helped us revise our manuscript. Below please find our answers to all of your comments and suggestions. We hope you find our answers satisfactory.

**The rationale for establishing the relationship between obesity and HCC seems weak.**

If leptin was not expressed in healthy liver tissue, the higher expression of leptin found in HCC tissue may indicate that leptin is caused by cancer cells themselves, not due to obesity (leptin) to lead to HCC, i.e., leptin overexpression may be the result of HCC, not the cause. How to exclude this possibility needs more discussions.

**How to explain the observation that “blocked leptin’s expression in HepG2 cells……did not significant decrease hTERT mRNA levels and TA (Figure 2a, 2b)”?**

Concerning the relationship between obesity and HCC, we have included additional information in the Background, 3rd paragraph, first 5 lines.

Leptin’s expression in HCC liver tissues does not necessarily come from the adipocytes or other cells (stellate cells) surrounding the cancerous cells. It is possible that leptin is produced by the malignant cells (The HepG2 cells used in our study were found to express leptin) as a result of their malignant transformation. As we did not observe any correlation between leptin/leptin receptors expression in HCC liver tissues and BMI, we could assume that the production of leptin in HCC cells is not directly regulated by the adipose tissue deposit, but it also reflects the intricate interactions taking place into the tumorigenic microenvironment. This comment was added in the Discussion, page 11, 1st paragraph, last 4 lines.

In addition, as we observed that leptin’s knockdown by siRNA did not decrease hTERT mRNA expression, nor telomerase activity in HepG2 cells, it could be suggested that modification and upregulation of genes like hTERT and therefore carcinogenesis, are initially triggered by serum leptin (relationship with BMI) or by locally synthesized leptin (fatty liver) and then are strengthened by the endogenous leptin production in HCC cells. This comment has been added in the Discussion, page 11, 2nd paragraph, lines 7 until end of paragraph.

**DNA methylation occurred at gene promoter region is also an important regulating mechanism on gene expression. Its role need discussed in the text.**

DNA methylation is an important epigenetic mechanism and we have included in the text a relative comment in the Discussion, page 12, 2nd paragraph, last 4 lines.
Additionally, it would be very interesting to study more specifically its implication in hTERT regulation by leptin in future studies.

**Minor essential revisions:**

*Statistical analysis should be described briefly, not omitted.*

The reviewer is right as we had not discussed our statistical analysis in the manuscript. Brief statistical analysis is now provided in the Methods-Statistical analysis, page 7.

*Figure 1: it is unclear how to conduct statistical analysis for comparison leptin expression (unexpressed in healthy liver tissue compared with 5.9 in HCC liver tissues).*

Student’s t-test was used for the comparison of leptin’s expression between healthy and HCC liver tissues. Taking into consideration the statisticians’ opinion, this test is the appropriate one for these types of comparisons. This comment has been added in the statistical analysis section (page 7).

*The role of references [28, 29] cited in the text of “we sought to determine whether there is an association between leptin and hTERT mRNA expression” are unclear.*

We excluded references [28, 29] cited in the text of “..we sought to determine whether there is an association between leptin and hTERT mRNA expression”, as they were there by mistake.
To Reviewer 2 (Dawei Xu)

Thank you for the useful comments raised by you, which helped us revise our manuscript. Below please find our answers to all of your comments and suggestions. We hope you find our answers satisfactory.

1. **Based on the authors’ data, leptin is responsible for transcriptional regulation of both hTERT and MMPs genes. Moreover, the authors didn’t analyze any functional effects of leptin-treated HCC cells on cell migration and invasion. Therefore the title “Leptin as a critical regulator of hepatocellular carcinoma development and progression through modulation of human telomerase reverse transcriptase” is somehow misleading and needs to be rephrased.**

Taking into account the reviewer’s comments, we rephrased the title to “Leptin as a critical regulator of hepatocellular carcinoma development through modulation of human telomerase reverse transcriptase”.

2. **It was known that leptin-deficient mice spontaneously developed obesity (Science, 1995, 269, 540; Genomics, 1991, 11, 1054), while the authors found increased expression of leptin in HCC. Therefore, the authors should briefly clarify the role of leptin in obesity in both humans and mice and association with HCC.**

In the revised manuscript, we tried to elucidate on the role of leptin system in obesity and clarify the evidence that make leptin a possible link between obesity and HCC in both human and mice. Relative comment has been included in the Background, page 3, 1st paragraph, and page 3, 3rd paragraph. Moreover, we propose that leptin is a pivotal player not only as an adipocyte-derived hormone (produced centrally or produced by adipocytes in liver tissue) but as a hormone that could also be produced by cancerous cells after their mutagenesis. We further support this by our observation of leptin expression in HEPG2 cells. Relative comments are included in the Discussion. page 11, 1st paragraph last 4 lines and 2nd paragraph, lines 7 until end).

The References in Science (1995) and Geneomics (1991) have been included (New References 5 and 6)
3. Regarding MYC/MAX/MAD network protein switch on the hTERT promoter (page 11), important papers in the area (PNAS, 2001, 98, 3826, FASEB J, 2010, 24, 579) should be referred to.

We added the proposed papers in the Discussion page 12, 1st paragraph and in References (new References 49, 50).

4. H3 in figure 3B should be acetylated H3 and labeling with “acH3” is clearer.

We have changed the label “H3” to “acH3” in figure 3.
To Reviewer 3 (Young Nyun Park)

Thank you for the useful comments raised by you, which helped us revise our manuscript. Below please find our answers to all of your comments and suggestions. We hope you find our answers satisfactory.

1. Non-alcoholic fatty liver disease (NAFLD)/non-alcoholic steatohepatitis (NASH), which can be induced by obesity, is considered to be a soil for HCC. To study the role of leptin in HCC development, the pathological information about the background liver whether NAFLD/NASH is present or not is important. However, there is no data about the presence of NAFLD/NASH in both HCC group and control group of this study. In addition, the study for the leptin expression in non-neoplastic liver of HCC patients is required.

   Indeed, the reviewer is correct and clinical data regarding NAFLD/NASH and metabolic syndrome would be important. As HCC prevalence in Greece is extremely low, we could not obtain fresh HCC tissues and we used HCC liver tissues provided by a tissue bank. Therefore, the above clinical data are missing. For the same reason leptin’s expression in non-neoplastic liver of HCC patients cannot be provided, as we do not have adjacent healthy liver tissues from HCC patients.

2. More detailed data about the patients’ BMI and metabolic syndrome including diabetes mellitus are required.

   Data about the patients’ BMI were added in the Methods section page 4. Indeed data regarding metabolic syndrome and diabetes mellitus could be useful but unfortunately cannot be provided, as is explained in the previous paragraph.

3. The etiologies of HCCs in this study are hepatitis B virus and hepatitis C virus. Is there any difference of leptin expression in HCC according to the different etiologies?

   We did not observe any difference in leptin expression levels between the HBV and HCV subgroups of the HCC group. We added this information in the Results page 7, end of 1st paragraph, that “leptin, OB-Rl and OB-Rs mRNA levels were not associated with age, BMI, sex, stage of HCC, or type of viral hepatitis.”
4. In this study, that leptin suppressed MMP-1 expression and triggered MMP-9 and MMP-13 expression in HepG2 cells. Please explain how decreased MMP-1 could contribute to a more favorable environment for invasion and metastasis of HCC in the cirrhotic liver.

The reviewer’s comment is valid and the misconception was due to the following mistaken statement in the original text ‘…leptin is able to suppress MMP-1 expression and trigger MMP-9 and MMP-13 expression in HepG2 cells, and this could contribute to a more favourable environment for invasion and metastasis of HCC in the cirrhotic liver’. We corrected the above statement and replaced it in the revised manuscript with ‘…leptin is able to suppress MMP-1 expression and trigger MMP-9 and MMP-13 expression in HepG2 cells. The observed elevated levels of MMP-9 and MMP-13 could contribute to a more favourable environment for invasion and metastasis of HCC in the cirrhotic liver’. This comment is found in the Discussion, page 13, 1st paragraph, last 4 lines.

5. There are several typing errors

We did our best to correct any typing errors in the text.