Author's response to reviews

Title: GLUT1 gene is a potential hypoxic and prognostic marker in colorectal cancer patients

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Version: 5 Date: 6 February 2009

Author's response to reviews: see over
The Biomed Central Editorial Team

Object: MS: 2086800294216368 - GLUT1 gene is a potential hypoxic and prognostic marker in colorectal cancer patients.

Thank you for consideration of our manuscript for publication in your journal.

We have reviewed the above manuscript according to your reviewers’ comments.

Reviewer # 1 (Dr. Cornelis Sier)

We deeply appreciate your effort and patience in reviewing our manuscript, and we thank for giving us so many valuable comments. In response to your comments, detailed explanations and replies are as follows:

Major Compulsory Revisions

Question 1: All data together indicate that GLUT1 is enhanced in colorectal cancer, but there is not enough data to conclude anything about GLUT1 being a prognostic marker, as suggested in the title and discussion.

Answer: Thank you for mentioning this. In this manuscript, we found that GLUT1 mRNA expression could be stimulated by hypoxia and the expression is more predominant in the advanced CRC cell lines (UICC III) \textit{in vitro}. In addition, we found GLUT1 mRNA expression in the peripheral blood of CRC patients. The advanced cancer stage was significantly related to GLUT1 over-expression ($P = 0.004$), the risk of which was higher in stage II and III than in stage I (RR: 12.482; $P= 0.001$). GLUT1 over-expression in the blood also correlated with advanced tumor stages. According to the previous results, we concluded that GLUT1 may be a potential prognosis marker that could be determined by a non-invasive
method.

**Question 2:** The data that were indicated in the legend of table 5 were not present.

**Answer:** In the Results section, we have already presented the results of table 5 (line 3, p.18). We have added more description for table 5 in the Results section (line 6, p.18).

**Question 3:** The most interesting peripheral blood data were not discussed at all.

**Answer:** Thanks for pointing this out. We have added more discussion of interesting peripheral blood data in the Discussion section (line 10, p.22).

**Question 4:** The description of the peripheral blood preparation is missing. This is essential because you only want to isolate mRNA from circulating tumor cells, not white blood cells.

**Answer:** The description of the peripheral blood preparation has been added in the Method section (4th paragraph, line 20, p.10).

mRNA was extracted from whole blood, including CTC and WBC. Even though CTC and WBC were included, both the SAGE clone and EST clone analysis of GLUT1 Virtual Northern can prove that the WBC expression in GLUT1 was almost undetectable in normal subjects and in cancer patients. Altenberg *et al.* as well as Marayou *et al.*, published separately in 2004 and 2007 in *Genomics* 84:1014~1020 and the *European Journal of Clinical Investigation* 37: 282~290 respectively, found that the over-expression of GLUT1 in white blood cells would not be observed in both a normal subject and cancer patients; further, it would not show increased expression even in elevated serum insulin stimulation during an infection. On the other hand, the GLUT1 expression was almost undetectable in
our analysis of the blood from the normal population; therefore, the expression of GLUT1 in CTC would not be interfered with even if whole blood mRNA was used as the testing material. Furthermore, the membrane array technique for the detection of GLUT1 mRNA was a patented platform previously developed by us for the detection of minute serum CTC levels. The detection limitation was 5CTCs/ml blood (Chen et al. 2007. Oncology 70:438-446; Sheu et al. 2006 Int. J. Cancer 119[6]: 1419-1426). Therefore, the detection of serum GLUT1 should not encounter much trouble.

Question 5: The calculation of the percentage of over-expression is unclear.

Answer: Over-expression (%) = [(the number patients with over-expression) / (total number of patients)] × 100. Gene expression ratio of colorectal cancer tissues (normalized with corresponding β-actin signal) were compared with gene expression ratio of paired normal colorectal tumor tissues (normalized with corresponding β-actin signal). In the footnote of table 4, we have added a description that defines over-expression and our method for calculating the percentage of over-expression (Table 4) (line 9, p.40).

Question 6: There are obvious mistakes in the calculations. My remark about the medians reflected to the following standard error: Either means±SE, as also stated in the M&M, or medians plus range.

Answer: Thank you for your careful attention to the mistakes in the calculations. We have corrected the error in the Methods section (line 7 and 19, p.10).

Question 7: Because the authors did not discuss their peripheral blood data, we could not raise any questions about how the authors interpret that cells that are a
certain time in the circulation and therefore probably adapted to the oxygen-state in the peripheral blood could still reflect the oxygen-state in the peripheral blood could still reflect the oxygen-state in the (section of) the tumor where they from.

Answer: Thank you for pointing this out too. We have added more discussion of interesting peripheral blood data in the Discussion section (line 10, p.22). In our previous paper, we investigated gene expressions involved in the glycolytic pathways in colorectal cancer. We concluded that the glycolytic pathways and glycolysis-related genes (including GLUT1) may play an important role in the tumorigenesis of colorectal cancer (Oncology Reports 2008 Jan; 19(1):81-91). In addition, it was reported by Weidner (1993) that active angiogenesis may occur in cancer tissues growing to 2 mm in diameter. Evidence is accumulating that primary cancers begin shedding neoplastic cells into the circulation at an early stage (Smirnov et al., 2005); ~10^6 cells are shed daily per gram of tumor (Chang et al., 2000). Thus, circulating tumor cells is a potential source for the noninvasive and early diagnosis for cancer patients. O’Sullivan et al. (1997) indicated that preoperative detection of micrometastases may reflect either transient shedding of tumor cells, metastatic potential, or residual disease. Based on the concept that circulating tumor cells may be present in the bloodstream in very low numbers, a significant increase of GLUT1 mRNA in the peripheral blood of stage II and III CRC patients over that of stage I patients not only presents an advanced state of the disease but also the potential role of tumor progression.
Reviewer # 2 (Dr. Gemma Dominguez)

We deeply appreciated your effort and patience in reviewing our manuscript, and we thank you for giving us so many valuable comments. All of our authors are also grateful for your kind guidance. In response to your comments, detailed explanations are as follows:

**Major Compulsory Revisions**

**Question 1:** Authors have tried to answer the reviewers’ comments, and although I agree, in most cases, with the reply, sometimes the response is placed in a position in the text where it has no sense and without any connection with the rest of the content. For example, to clarify those authors used the tumor and the normal counterpart tissue of the same patient from 10 CRC, they place a sentence in line 20 page 19 of the Discussion. The sentence has no sense here, not only because it should be added in the Materials section, but also because it is added without any connection with the previous and the next text. Author should carefully revise the text in this way.

**Answer:** Thank you for this suggestion. We have completely reviewed the entire paper again and moved the above-mentioned sentences from the Discussion to the Materials section (line 17, p.12).

**Question 2:** I think that although the content of the manuscript is suitable for publication in this journal, the presentation in the current way is unacceptable.

**Answer:** The presentation has now been thoroughly edited.

**Question 3:** English style should also be deeply corrected.

**Answer:** In-depth English corrections have been made as well.
Reviewer #3 (Dr. Lothar Fecker)

We deeply appreciate your effort and patience in reviewing our manuscript, we thank you for giving us so many valuable comments. All of our authors are also grateful for your kind guidance. In response to your comments, detail explanations are as follows:

Minor Essential Revisions:

Question 1: Page 3; Abstract; lines 15-16: I think you should exchange SW480 (...) for (SW620 (…)

Answer: We have made this correction. (line 17, p.3)

Question 2: Page 7; lines 11: please write:….to investigate the correlation of hypoxia-related genes…

Answer: Thanks for your attention to these details. We have made this correction as well.

Question 3: Page 9; Chapter: Reverse transcription-polymerase chain reaction (RT-PCR). I suggest altering the order of the first sentences in the chapter in this way: RNA was extracted from colorectal cancer cell lines using........Total RNA (20µg) was reverse transcribed to cDNA and two microliters of each of these cDNA samples were used for each PCR reaction.

Answer: We will gladly make this adjustment and alter the order of the chapter’s first sentences in this way: “RNA was extracted from colorectal cancer cell lines using ISOGEN™ (Nippon Gene Co., Ltd., Toyama, Japan) and QIAmp® RNA Blood Mini Kit (Qiagen Inc., Valencia, CA). Total RNA (20µg) was reverse transcribed to cDNA, and two microliters of each of these cDNA samples were used for each PCR reaction” (lines 9 and 11, p.9).
**Question 4:** Page 12; lines 7-13: I think you can omit the sentence beginning with “Sixteen target genes (..)…” as this you have also written in the legend to figure 5. But if you do so, please mention in the following sentence that the housekeeping gene was β-actin and the bacterial gene was from Mycobacterium tuberculosis!

**Answer:** Thank you for this suggestion. We have omitted the sentence beginning with “Sixteen target genes (..)…” (lines 7-13, p.12). As suggested, we have mentioned that the housekeeping gene was β-actin and the bacterial gene was from Mycobacterium tuberculosis (line 14, p.12) in the following sentence.

**Question 5:** Page 13; line 2: Please describe the procedure of hybridization, incubation with antibodies and especially the blocking step (when? After incubation with antibodies??) more precisely.

**Answer:** We have made this correction by describing the procedure of hybridization, the incubation with antibodies, and especially the blocking step more precisely. The blocking step occurs ahead of incubation with antibodies (line 25, p.12).

**Question 6:** Page 19; lines 19-21: Please check the grammar of this newly introduced sentence. I don’t understand what you mean.

**Answer:** In accordance with the suggestions by Dr. Gemma Dominguez (Reviewer 2), we have removed the sentence in the Discussion and added it to the Materials section after checking the grammar and editing the sentence (line 17, p.12).

**Question 7:** Page 21; lines 21: You should make a correction here: ...from early stages through advanced stages of …
Answer: Thank you very much for mentioning this. We have made a correction here: ...from early stages through advanced stages of … (line 18, p.21).

**Question 8:** Legend to figure 2: a) Please describe shortly what was the control; b) Please describe how the relative expression ratio was calculated. It should be clear for the reader whether the measured values in a series were normalized to the \( \beta \)-actin signal of the respective control or whether each measured value was normalized to the corresponding (individual) \( \beta \)-actin signal.

**Answer:** Thank you for this insightful comment. a) In the legend to Figure 2, we have described the control condition; b) In addition, we have added the description of how the relative expression ratio was calculated in the legend of Figure 2 (line 20, p.32).

**Question 9:** Description of Table 4: Also, here you should describe more precisely how the ratio of gene expression was determined: Was it (??): Gene expression ratio of colorectal cancer tissues (normalized with corresponding \( \beta \)-actin signal) compared with gene expression ratio of paired normal colorectal tumor tissues (normalized with corresponding \( \beta \)-actin signal)

**Answer:** Thanks for mentioning this with such precision. In response, we have described more precisely how the ratio of gene expression is determined, in the footnote of Table 4 (Table 4, line 9, p.40).
Again, I would like to thank the reviewers for the time and effort that they invested in reviewing our manuscript. I am looking forward to hearing from you soon!

Sincerely yours,

Shiu-Ru Lin

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