Author's response to reviews

Title: Expression of phosphoenolpyruvate carboxykinase linked to chemoradiation susceptibility of human colon cancer cells

Authors:

Ji-Won Park (sowisdom@gmail.com)
Seung Cheol Kim (onco@ewha.ac.kr)
Won Ki Kim (kimwk@ncc.re.kr)
Jun Pyu Hong (babu2080@gmail.com)
Kyung-Hee Kim (kyunghee@ncc.re.kr)
Hyun Yang Yeo (yeowoo0410@ncc.re.kr)
Jae Yong Lee (jylmd@hanmail.net)
M. Sun Kim (msunkim@ncc.re.kr)
Jong Heon Kim (jhkim@ncc.re.kr)
Se Young Yang (bibiysy@gmail.com)
Dae Yong Kim (radiopiakim@hanmail.net)
Jae Hwan Oh (jayoh@ncc.re.kr)
Jae Youl Cho (jaecho67@gmail.com)
Byong Chul Yoo (yoo_akh@ncc.re.kr)

Version: 3
Date: 17 February 2014

Author's response to reviews: see over
Dear Dr. Dafne Solera,

First of all, we would like to express our heartfelt thanks to the editorial board and the referees for very helpful and critical comments to strengthen our manuscript. Point-by-point responses to the reviewers’ comments are attached. All criticisms raised have been carefully addressed and incorporated into the revised manuscript using red font. Furthermore, ethical approval has been clearly addressed in the revised manuscript.

We hope our manuscript is properly revised and formatted for the publication in the journal.

Sincerely yours,

Byong Chul Yoo, Ph.D.
Colorectal Cancer Branch
Research Institute
National Cancer Center, Korea
Point-by-Point Responses

Reviewer’s report

Original title: Expression of phosphoenolpyruvate carboxykinase linked to chemoradiation susceptibility of human colon cancer cells

Version: 2

Date: 6 December 2013

Reviewer: Colleen M Croniger

Reviewer’s report:

Ji-Won Park et al present evidence for the possible role of PEPCK in the resistance to 5-Fluorouracil (5-FU) in colorectal cancer patients. Using proteomics, the investigators found that the mitochondrial form of PEPCK was reduced in patients with chemoradiation resistance. The study is interesting and has significance but several questions need to be addressed:

The authors sincerely thank the reviewer for the helpful comments. All questions raised have been answered and incorporated into the revised manuscript.

Major Compulsory Revisions:

1. Methods- protein homogenation and isolation of cytosolic and mitochondrial fractions need to be described in more detail.

Based on the reviewer’s comment, the fractionation methods have been revised for clarity and greater detail as follows:

(Before)
Mitochondria subcellular fractionation
For isolation of an enriched, functional mitochondrial fraction from cells, Mitochondria Isolation Kit (Sigma, Saint Louis, MO) was used as recommended by the manufacturer.

(After)
Whole-protein extraction and subcellular fractionation
Cells or tissues were homogenized in four volumes of cell lysis buffer (Pro-Prep; iNtRON Biotechnology, Gyeonggi, Republic of Korea) using a Sample Grinding Kit (GE Healthcare, Piscataway, NJ). The total homogenate was incubated on ice for 20 min and centrifuged at $600 \times g$ for 5 min. The supernatant was used as a whole
protein extract. The cytosolic fraction was obtained by centrifugation of the whole protein extract at 11,000 × g for 10 min.

For isolation of an enriched, functional mitochondrial fraction from cells, the Mitochondria Isolation Kit (Catalog No. MITISO1; Sigma, Saint Louis, MO) was used as recommended by the manufacturer. Briefly, cells were suspended with 10 volumes of the extraction buffer (20 mM MOPS, pH 7.5, containing 110 mM KCl, 1 mM EGTA, and 0.25 mg/ml trypsin) and incubated on ice for 3 min. The cells were then centrifuged for a few seconds. The supernatant was removed by aspiration, and eight volumes of the extraction buffer were added. After incubation on ice for 20 min, the albumin solution was added to a final concentration of 10 mg/ml to quench the proteolytic reaction. The solution was then centrifuged for a few seconds. The supernatant was removed by aspiration, and the pellet was washed with 8 volumes of the extraction buffer. This step was repeated. The pellet was then homogenized and centrifuged at 600 × g for 5 min. The supernatant liquid was transferred to a new tube and centrifuged at 11,000 × g for 10 min. The pellet was suspended in the storage buffer (10 mM HEPES, pH 7.4, containing 250 mM sucrose, 1 mM ATP, 0.08 mM ADP, 5 mM sodium succinate, 2 mM K₂HPO₄, and 1 mM DTT [~40 ml per 100 mg of tissue]) and used as a mitochondrial fraction.

2. Methods-state the catalogue number of the Santa Cruz antibody for PEPCK.

The catalog number of the Santa Cruz antibody for PEPCK has been added as follows:

(Before)

Western Blot analysis
Western Blot analysis was performed as previously described [10]. Supernatants of cell homogenates containing equivalent amounts of protein were subjected to SDS-PAGE, and transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, Bedford, MA). Membranes were incubated for 2 hr at room temperature with primary antibody against PEPCK (this antibody attaches to both cytosolic and mitochondrial PEPCK; Santa Cruz Biotechnology, Inc., Dallas, TX),.....

(After)

Western Blot analysis
Western Blot analysis was performed as described previously [10]. Supernatants of cell homogenates containing equivalent amounts of protein were subjected to SDS-
PAGE and transferred to polyvinylidene fluoride membranes (Millipore, Bedford, MA). The membranes were incubated for 2 h at room temperature with primary anti-PEPCK antibody that attaches to both cytosolic and mitochondrial PEPCK (Catalog No. sc-32879; Santa Cruz Biotechnology, Inc., Dallas, TX)

3. When using the PEPCK antibody label the kD for cytosolic (67kD) and mitochondrial (62kD) forms on the Western Blot. Is the PEPCK band that you are detecting actually both mito and cyto PEPCK forms together—what percentage PAGE did you use?

Many thanks for the helpful comments. The cytosolic and mitochondrial isoforms might be separated on a gradient gel, but we could not detect these two separate isoforms on the 4–12% gradient premade gels. As described in Figures 1 and 2 (Western Blot analysis of the fractionated samples), we did not require separation of isoforms in the Western Blot analysis. Despite the contamination of other fractions, the results represent the dominant isoform in each fractionation. As shown in Figure 3, transfection of mPEPCK siRNA reduced the expression of PEPCK. Because the two isoforms were not detected separately, we described the reduced immunoreactive bands as “PEPCK,” not “mPEPCK.” However, we are sure that the siRNA used in this experiment was constructed for specific suppression of mPEPCK, and the Western Blot results after siRNA transfection indicated decreased expression of mPEPCK and the unchanged cytosolic isoform of PEPCK. Figure 4 describes GFP-mPEPCK, which is of a different size. Figure 6 describes total PEPCK. Unfortunately, the amount of tissue was inadequate to obtain the mitochondrial fraction; additionally, the two isoforms were not detected separately by the PEPCK antibody. This limitation is clearly described and discussed in the revised manuscript.

4. Introduction- the statement that PEPCK is rate limiting step for gluconeogenesis is an old concept. Recent data from Burgess et al have shown that the gluconeogenic enzymes are regulated in a similar manner and together they modulate gluconeogenesis. The overview of PEPCK regulation is for the cytosolic form and should be stated as such. In addition a brief discussion of what is known about mitoPEPCK and its regulation should be included.

Based on the reviewer’s comments, parts of the Background and Discussion sections have been modified, as follows:
**Second paragraph in the Background**

*(Before)*

Phosphoenolpyruvate carboxykinase (PEPCK) is known to exist in both cytosol and mitochondria in mouse, human, and chicken and catalyzes the reversible decarboxylation of oxaloacetic acid with the concomitant transfer of the gamma-phosphate of GTP to form phosphoenolpyruvate (PEP) and GDP in the first committed step of gluconeogenesis and glyceroneogenesis [6, 7]. The expression of PEPCK is promoted by the mitogen-activated dual specificity protein kinase phosphatase 3 (MKP-3), increasing glucose production [8]. However, insulin-induced modification of transcription regulatory proteins is reported to prevent activation of PEPCK transcription, leading to rapid but reversible inhibition [9].

*(After)*

Phosphoenolpyruvate carboxykinase (PEPCK) is known to exist in both the cytosol and mitochondria in the mouse, human, and chicken. This enzyme catalyzes the reversible decarboxylation of oxaloacetic acid with the concomitant transfer of the gamma-phosphate of GTP to form phosphoenolpyruvate (PEP) and GDP [6, 7]. Cytosolic PEPCK has been investigated extensively and is considered to be a key enzyme in gluconeogenesis and glyceroneogenesis [8, 9]. In contrast, the mitochondrial isoform of PEPCK (mPEPCK) has a metabolic role that is complementary to but distinct from that of cytosolic PEPCK in the regulation of gluconeogenesis [9].

**New third paragraph in the Discussion**

Although the metabolic characteristics of mPECK remain largely unknown, recent reports suggest that mPEPCK plays various roles in gluconeogenesis and anaplerotic reactions. For example, mPEPCK plays a role in mitochondrial GTP synthesis with insulin release through anaplerotic PEP cycling [19] and cooperates with cytosolic PEPCK to adjust gluconeogenic/TCA flux in response to changes in substrate or energy availability [8]. At present, we cannot explain the molecular mechanism linking mPEPCK expression and susceptibility of CRC cells to 5-FU or radiation. However, expression of both cytosolic PEPCK and mPEPCK may indicate changes in gluconeogenic flux because PEPCK expression can be exquisitely coordinated in parallel with glucose requirements [20]. Our previous reports have shown that the slow energy metabolic process caused by downregulation of key enzymes, such as mitochondrial ATP synthase and pyruvate kinase M2, lead to
differential responses of cancer cells to anticancer drugs [17, 18]. Thus, mPEPCK downregulation could also lead to slow energy metabolism in CRC cells, and it may subsequently reduce the susceptibility of CRC cells to 5-FU or radiation.

Revision of References 8 and 9

(Before)


(After)


New References 19 and 20


5. Overexpression of mPEPCK in cytosolic fraction is not convincing data that the mitoPEPCK form is actually reaching or functioning in the mitochondria.

We agree with the reviewer’s comment. We are presently unable to explain why overexpressed mPEPCK is not targeted to the mitochondria. It is possible that linking of GFP to mPEPCK may affect the subcellular localization. mPEPCK located in the cytoplasm may not perform its role in chemoresistance effectively. Therefore, this weak point has been discussed as follows:

End of second paragraph in the Discussion

…… Although upregulated expression of mPEPCK showed the potential to slightly increase the 5-FU susceptibility in both the SNU-C4 and LoVo cell lines, the effects were not statistically significant (Figure 4). Because overexpressed mPEPCK was not found in the mitochondrial fraction (data not shown), further investigations are necessary to validate the effect of subcellular localization of this enzyme on CRT susceptibility.

6. What is mPEPCK doing? How does chemoradiation resistance correlate with reduced mPEPCK. There are primarily 3 functions of PEPCK listed below. Do you hypothesize one of these is the mechanism for your resistance? Please discuss this in your discussion a. Gluconeogenesis b. Glyceroneogenesis c. Anaplerosis

We sincerely thank the reviewer for the comment on the possible role of PEPCK in chemoradiation resistance. Further discussion has been added after the second paragraph in the Discussion section, as follows:

New third paragraph in the Discussion
Although the metabolic characteristics of mPECK remain largely unknown, recent reports suggest that mPEPCK plays various roles in gluconeogenesis and anaplerotic reactions. For example, mPEPCK plays a role in mitochondrial GTP synthesis with insulin release through anaplerotic PEP cycling [19] and cooperates with cytosolic PEPCK to adjust gluconeogenic/TCA flux in response to changes in substrate or energy availability [8]. At present, we cannot explain the molecular mechanism linking mPEPCK expression and susceptibility of CRC cells to 5-FU or radiation. However, expression of both cytosolic PEPCK and mPEPCK may indicate changes in gluconeogenic flux because PEPCK expression can be exquisitely coordinated in parallel with glucose requirements [20]. Our previous reports have shown that the slow energy metabolic process caused by downregulation of key enzymes, such as mitochondrial ATP synthase and pyruvate kinase M2, lead to differential responses of cancer cells to anticancer drugs [17, 18]. Thus, mPEPCK downregulation could also lead to slow energy metabolism in CRC cells, and it may subsequently reduce the susceptibility of CRC cells to 5-FU or radiation.

**New References 19 and 20**


7. You are using an antibody that detects both isoforms, therefore you should state that PEPCK protein (cytosolic and mitochondrial) expression is altered not just PEPCK expression. Overall the data is not convincing enough to state that it is the mitochondrial form of PEPCK that alters chemoradiation resistance. Your data suggests that either PEPCK cyto and/or mito may be involved.

We appreciate the helpful comments. Our response is identical to that to the third comment, as shown below:

Despite the contamination of other fractions, the results represent the dominant
isoform in each fractionation. As shown in Figure 3, transfection of mPEPCK siRNA reduced the expression of PEPCK. Because the two isoforms were not detected separately, we described the reduced immunoreactive bands as “PEPCK,” not “mPEPCK.” However, we are sure that the siRNA used in this experiment was constructed for specific suppression of mPEPCK, and the Western Blot results after siRNA transfection indicated decreased expression of mPEPCK and the unchanged cytosolic isoform of PEPCK. Figure 4 describes GFP-mPEPCK, which is of a different size. Figure 6 describes total PEPCK. Unfortunately, the amount of tissue was inadequate to obtain the mitochondrial fraction; additionally, the two isoforms were not detected separately by the PEPCK antibody. This limitation is clearly described and discussed in the revised manuscript.

8. All figures need statistics.

Based upon the reviewer’s comment, P values obtained from Student’s t-test were added to Figure 3b–d, Figure 4b and d, and Figure 6b.

In addition, the description of the results and the legend to Figure 3 have been modified as follows:

(Before)

Decreased cellular level of phosphoenolpyruvate (PEP) and reduced susceptibility to 5-FU/radiation induced by mPEPCK suppression in SNU-C4

mPEPCK siRNA transfection was performed using SNU-C4 to study the role of mPEPCK in 5-FU resistance. At 96hr after the siRNA transfection, significantly less PEPCK was detected in the mPEPCK-suppressed cells compared to the non-silencing (NS) control (Figure 3a). mPEPCK suppression itself reduced proliferation rate of SNU-C4 without any 5-FU and radiation treatment (Figure 3b). Furthermore, cellular level of PEP (an end product of PEPCK and substrate of pyruvate kinase) was also significantly decreased by the mPEPCK suppression in SNU-C4 (Figure 3c). Cell survival rates of the mPEPCK-suppressed SNU-4 cells after 1 µg/ml 5-FU treatment, 8 Gy radiation or combined treatment of 5-FU and radiation were higher than the control (Figure 3d).

Effects of mPEPCK overexpression using pEGFPc1-mPEPCK vector were also investigated. Overexpressed mPEPCK was localized in cytoplasm rather than mitochondria (Data not shown). Overexpression of mPEPCK in SNU-C4 and LoVo showed slightly increased susceptibility to 5-FU, but not to radiation or combined
treatment (Figure 4). The response of LoVo to 5-FU/radiation after mPEPCK overexpression showed similar pattern to that of SNU-C4 (Figure 4c and d).

**Figure 3 Effect of mPEPCK down-regulation on 5-FU response in SNU-C4 cells.**
(a) Suppressed mPEPCK expression in SNU-C4 transfected with mPEPCK siRNA. The PEPCK expression levels decreased over time in the mPEPCK siRNA-transfected cells, while the non-silencing (NS) control showed no apparent changes in the PEPCK expression levels. (b) Decreased cell proliferation rate after mPEPCK suppression. Without any treatment of 5-FU and radiation, artificial suppression of mPEPCK decreased the rate of cell proliferation. (c) Reduced cellular PEP level after mPEPCK suppression. (d) Increased 5-FU and radiation resistance after mPEPCK suppression in SNU-C4. MTT assay showed that the mPEPCK siRNA-transfected SNU-C4 cells exhibited higher survival rates than the control after either 1 μg/ml 5-FU treatment, 8 Gy irradiation, or combination treatment of 5-FU and radiation.

(After)
Decreased cellular level of PEP and reduced susceptibility to 5-FU/radiation induced by mPEPCK suppression in SNU-C4
mPEPCK siRNA transfection was performed using SNU-C4 to investigate the role of mPEPCK in 5-FU resistance. At 96 h after siRNA transfection, significantly less PEPCK was detected in the mPEPCK-suppressed cells than in the nonsilenced (NS) control (Figure 3a). mPEPCK suppression itself reduced the proliferation rate of SNU-C4 without any 5-FU or radiation treatment (Figure 3b). Furthermore, the cellular level of PEP (an end product of PEPCK and substrate of pyruvate kinase) was also significantly decreased by the mPEPCK suppression in SNU-C4 (Figure 3c). The survival rates of the mPEPCK-suppressed SNU-4 cells after 1 μg/ml 5-FU treatment or 8-Gy radiation were higher than those of the control, but the difference did not reach statistical significance (P = 0.082 and 0.069, respectively) (Figure 3d). Suppression of mPEPCK significantly increased survival of SNU-4 cells after combined treatment comprising 5-FU and radiation (P = 0.0005) (Figure 3d).

The effects of mPEPCK overexpression using pEGFPc1-mPEPCK vector were also investigated. Overexpressed mPEPCK was localized in the cytoplasm rather than in the mitochondria (data not shown). Overexpression of mPEPCK in SNU-C4 and LoVo showed slightly increased susceptibility to 5-FU (P = 0.067), but
not to radiation or combined treatment ($P = 0.21$ and $0.39$, respectively) (Figure 4). The response of LoVo to 5-FU/radiation after mPEPCK overexpression showed a pattern similar to that of SNU-C4 (Figure 4c and d).

**Figure 3. Effect of mPEPCK downregulation on 5-FU response in SNU-C4 cells.**
(a) Suppressed mPEPCK expression in SNU-C4 cells transfected with mPEPCK siRNA. The PEPCK expression levels decreased over time in the mPEPCK siRNA-transfected cells, while the nonsilenced (NS) control showed no apparent changes in the PEPCK expression levels. (b) Decreased cell proliferation rate after mPEPCK suppression. With no 5-FU or radiation treatment, artificial suppression of mPEPCK decreased the rate of cell proliferation. (c) Reduced cellular PEP level after mPEPCK suppression. (d) Increased 5-FU and radiation resistance was observed after mPEPCK suppression in SNU-C4 cells. MTT assay showed that the mPEPCK siRNA-transfected SNU-C4 cells exhibited higher survival rates than the control after combination treatment comprising 5-FU and radiation.

9. Human patient data. Please include methods for RNA isolation and RT-PCR measurements for this data in the methods section. Please include primers used for this data. Were the primers for Pck1 and/or Pck2?

Ł Only the PEPCK protein level was evaluated in tissue from patients with colorectal cancer. We did not perform RT-PCR using RNA obtained from tissue samples.

**Minor Revisions:**
1. Please use Western Blot not western blot.

Ł This change was made throughout the manuscript.

**Level of interest:** An article of importance in its field

**Quality of written English:** Not suitable for publication unless extensively edited

Ł The English in this manuscript has been checked by two professional editors, both native speakers of English.
Statistical review: Yes, and I have assessed the statistics in my report.

Declaration of competing interests: I declare I have no competing interest

Reviewer's report

Original title: Expression of phosphoenolpyruvate carboxykinase linked to chemoradiation susceptibility of human colon cancer cells

Version: 2

Date: 17 December 2013

Reviewer: Li-Jen Su

Reviewer's report:

Major Compulsory Revisions - none.

Minor Essential Revisions

1. Manuscript needs some language corrections before being published.
   ✑ The English in this manuscript has been checked by at least two professional editors, both native speakers of English.

2. Some tables need arrangement.
   ✑ The manuscript contains only Table 1 and Supplementary Table 1. To assist the reader, the protein level of PEPCK normalized to actin was rounded off to the second decimal place.

(Before)
Supplemental table 1. PEPCK expression levels in 122 patients with rectal cancer.

*PEPCK expression (PEPCK/β-actin) was measured by Western blot, and optical density of blots were measured from a scanned image (Fig. 6c). PEPCK expression levels of SNU-C4 and HCT-116 were used as a control.

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(After)
Level of interest: An article whose findings are important to those with closely related research interests

Quality of written English: Needs some language corrections before being published.
- The English in this manuscript has been checked by two professional editors, both native speakers of English.

Statistical review: No, the manuscript does not need to be seen by a statistician.

Declaration of competing interests: I declare that I have no competing interests.