Dear Dr. Edmunds:

The original research manuscript MS: 1592166994173597, by Yang, R. et al, entitled “Quantitative Correlation between Promoter Methylation and Messenger RNA Levels of the Reduced Folate Carrier” is re-submitted for consideration for publication in BMC-Cancer. Revisions have been made in the light of reviewer’s comments as will be detailed subsequently. In addition, the manuscript has been reformatted according to the BMC journal guidelines as following:

1. Title page: Each author’s affiliation is indicated using superscript numbers, and is given in full description accordingly. The corresponding author (RG) is
indicated with a symbol, and e-mail address is provided. Each author’s email address is also listed.

2. The abstract: The abstract is reformatted according to medical journal in the BMC-series style. The abstract is re-structured into Background, Methods, Results, and Conclusions. The total word count is 244.

3. The Manuscript text: The text is reformatted into Background, Methods, Results, Discussion, and Conclusions.

4. Competing interests: A statement of competing interests is made following the manuscript text on page 14, line 17-18.

5. Authors’ contributions: Each author’s contribution is specified on page 14, line 21-23, and page 15, line 1-3.

6. Acknowledgements: An acknowledgement is made on page 15, line 6-10.

7. The source of funding for this study is indicated on page 15, line 12-13.

All the authors have reviewed the manuscript and agreed to its content.

Thank you for your consideration, and I look forward to hearing from you.

Sincerely,

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To Referee 1 (Dr. Matherly):

1). “page 4, 4th line from bottom, …”

This statement has been corrected on page 4, line 18-19, into “Transcription of the RFC starts from at least four distinct promoters, (designated A, B, C, and D),…”. A reference [13] is added, which gives a most recent review on the RFC transcription regulation and clinical implications.
2). “page 5. Cell culture. I assume all the cell lines…”

All the cell lines were grown in standard media with 2.3 µM folic acid. In M805 cells, the down-regulation of RFC was stable after transferring into media containing physiological level of leucovorin (20nM). Although we did not test the methylation level after grown cells in media with low folate, we assume it is not changed since the mRNA level of RFC was not apparently changed. This effect is stated on page 9 line 16-19.

3). “page 7. Methylation specific PCR. In this section and elsewhere it would be most helpful if the authors …”,

4). “page 8. Quantitative Real-time Methylation-specific PCR. Again it would be helpful if the positions …”

We have tabulated all primers and probes we designed in this study into Table 1, with their correspondent position indicated numerically relative to published sequence NCBI accession number U92868, in response to referee’s comments. For primers used in MSP to amplify RFC promoter B, a reference [23] is cited in the table.

5). “The assumption is that the cell lines used in this study use almost exclusive promoters A and B…”

Although attempted, we have not be able characterize the 5’UTR sequence of RFC transcript to determine which promoter was utilized by these cell lines using technique, such as 5’RACE as cited in reference [13]. The assumption of promoter A and B utilization was based on literature [15-20], which also suggested that promoter B was dominantly used in cancer cell lines [16, 19]. However, by examining both promoter B and promoter A using methylation specific PCR in all 8 cell lines, we found that there was no selective methylation in relationship to promoter utilization. We acknowledge that future study should be performed to clarify the role of methylation in other promoters cited in the literature most recently [14] (promoter A, D) on the section of Discussion on page 13 line 6-8.

6). “page 10. lines 9 and 10. PCR products in length of 132 bp for promoter A, and 110 BP for promoter B…”

10). “Figure 2. Why are results with HT1080 not included here? These should be shown in the modified figure (see above)…”

A composite image that shows both sets for promoter A (132,134bp) and B (110bp) PCR products is presented in the figure 2 in response to reviewer’s
7) “page 10. 3rd line from bottom. After the treatment of 5-aza-2’-deoxycytidine, What happened to RFC levels in the M805 cells upon treatment with this demethylating agent? ”

There was a 2.9-fold increase of RFC mRNA expression after 5-aza-2’-dCt treatment. This was done in the same context with with MDA-MB-231 cells. This effect is described on page10 line12-14.

8) “Figure 4. Although I am not a statistician, this analysis seems peculiar and hardly rigorous. The data essentially involves a correlation between …”

The statistical analysis was based on all 8 samples included in this study. If only 3 samples had been included, the correlation could have been closer to minor 1. We acknowledge that the statistical analysis is not robust due to limited samples. A statement is made on page 14 line 2-4, and this will be addressed in future studies.

9) “page 12. line 8. methotrexate is not a substrate for p-glycoprotein…”

“p-glycoprotein” is deleted in response to referee’s comments on page 12 line 4.

11) “Typo ;page 9, 2nd line from bottom in consistence should be consistent with. Likewise for page 10, line 4…”

All the typing errors have been corrected according to referee’s comments.

To referee 2 (Dr. Moscow)

“An alternative interpretation of the data is that RFC promoter methylation status is not related to RFC gene expression or MTX sensitivity in 6 of the 8 cell lines examined. Data that would help establish the context of the reported observation…”

The MTX resistant leukemia cell lines (CEMT, HL60R, and M316) and breast cancer cell line MDA-MB-231 had been characterized defined MTX resistance mechanism as cited in the reference [23, 24, 26, 27], other cell lines including CCRF-CEM, HT1080, and MCF-7 are MTX sensitive. A reference [24] describing the characterization of MD231 has been added on page 5 line 8, and page 12, line 10. M805 is relative a novel cell line with unknown MTX resistant mechanism. Moreover, among the 8 cell lines studied, only MDA-MB-231 and M805 showed a decreased RFC mRNA expression, and other cell lines had
relatively normal RFC mRNA. Therefore, DNA methylation may not played a role in RFC mRNA regulation and MTX resistance in these cell lines.

“…Also, it would be of interest whether the recently described low-pH folate transporter, which is also an important regulator of MTX uptake, is expressed in these cell lines, and whether it is also regulated by methylation”

There is certainly an increasing interest regarding to a low-pH folate transport route, named PCFT (Qiu A., et al, Cell. 2006, 127 (5):917-28). PCFT appears as an intestinal folate transporter with an optimal pH of 5.5, however, its function is not fully characterized, neither clinical implication in relationship to MTX resistance in the context of physiological pH environment. Our previous study also demonstrate that a 300-fold over-expression of folate receptor alpha would contribute only 10% increased MTX uptake, neither on MTX sensitivity [reference 30]. This further suggests that the low-affinity, high capacity RFC is perhaps the dominant route for MTX uptake and drug efficacy. One investigator in our lab is currently examining the status of PCFT in cell lines, in collaboration with the Goldman’s group. The preliminary data suggests that PCFT is expressed in these cell lines, however, its role in relation to MTX uptake and resistance is yet to be determined. The promoter region, as well as transcription regulatory mechanism is not clearly defined, it would lbe difficult to perform study on promoter methylation at current stage.

“Since most cell lines do not show RFC methylation, and since demethylation of M805 did not result in increased MTX sensitivity, the relevance of the incrementally new findings reported in the paper is not established. If clinical samples…”

Using 5-aza-2'-dCt as a demethylation agent increased the mRNA level of RFC in MDA-MB-231 and M805 cells, however, did not resulted in a increased MTX sensitivity, perhaps in association with increased MRPs expression after 5-aza-2'-dCt treatment as similarly reported in previous study [reference 23], this suggests that 5-aza-2'-dCt is not a agent with a specific target. A statement has been made reflecting referee’s comment on page 13 line8-11. Using the methodology developed in this study, we had measured the promoter methylation in a small cohort of clinical specimens. The manuscript of this study is submitted elsewhere. Furthermore, a recent study suggests that RFC promoter methylation is a potential mechanism in primary central nervous system lymphomas. [Reference 32] Altogether, it suggests that further study with larger volume of specimen is yet needed in the future study.