Author's response to reviews

**Title:** Long-term cultivation of colorectal carcinoma cells with anti-cancer drugs induces drug resistance and telomere elongation

**Authors:**

Noritsugu Kuranaga (grd1409@gr.ndmc.ac.jp)
Nariyoshi Shinomiya (shinomi@ndmc.ac.jp)
Hidetaka Mochizuki (mochizuki@me.ndmc.ac.jp)

**Version:** 2  **Date:** 11 Jul 2001

PDF covering letter
June 12, 2001

Ruth King
Editorial Assistant
BioMedCentral
Middlesex House
34-42 Cleveland Street
London W1T 4LB

Re: Revision of the manuscript (BMC Cancer)
"Long-term cultivation of colorectal carcinoma cells with anti-cancer drugs induces drug resistance and telomere elongation" by N. Kuranaga, N. Shinomiya and H. Mochizuki

Dear Ruth:

We have studied the reviewers’ comments carefully and have made extensive corrections, which we hope to meet with your approval. I have also revised the revised manuscript and figures. Our answers to the reviewers' comments are described on the following pages.

All authors have approved the resubmission of this manuscript. We hope that these corrections are appropriate, and hope that our manuscript can again be considered for publication in BMC Cancer.

I apologize for the delay, but this is due to the extensive rewriting and rephrasing according to the reviewer's suggestion. Thank you very much for help and assistance.

Sincerely yours,

Nariyoshi Shinomiya

Nariyoshi Shinomiya
Answer to the report from Dr. Yasuhiko Kiyozuka:

According to the reviewer’s suggestion, the manuscript has been improved and re-written in blue type.

Major Criticisms:

1) According to the reviewer’s comments, we have inserted a few sentences in the Background, which clearly explain the reason why these anti-cancer agents were selected and only their co-treatment is presented in this study. We did not perform any experiments of the single use of each agent. Therefore, we can not answer the question “how are the conclusions with the single use of each agent?” We mentioned this point in the Discussion. In addition, we also mentioned the theoretical interpretation for determining the drug concentrations used in this study in the Materials and methods section.

2) As suggested, the definition of PDL and the method to determine PDLs were not clearly mentioned. Therefore, these points have been added to the Materials and methods as a new section “definition of the PDL”.

3) As suggested, the comparison of the sensitivities to anti-cancer drugs between parent cell lines and resistant cell lines was not clearly described in this paper. Unfortunately, we did not perform any precise comparisons regarding this point. But we chose drug concentration in terms of complete suppression of the growth of non-resistant parent cell lines. This point has been described in the section on anti-cancer drugs in the Materials and methods. Although the degree of resistance (resistant ratios) in the resistant cell lines is not clearly displayed, I think that it can be partly interpreted from the results of Fig. 2.

4) It has been reported by Papi S et al. (Cytometry 1996) that flow cytometric measurement of DNA content correlates well with the cytogenetic analysis. Therefore, this reference has been quoted.

5) According to the reviewer’s comments, the last sentence in the Conclusion of the Abstract does not match with the last sentence of Discussion. Although we do not think these are conflicting sentences, the expressions are not exactly appropriate. Therefore, the sentence in the Conclusion of the Abstract has been revised.

Minor Criticisms:

1) As suggested, this expression has been improved.
2) As suggested, “Genomic DNA from” has been deleted.
3) As suggested, this expression has been improved.
4) As suggested, no significance is presented in Fig. 4. Therefore, “Fig. 4” has been deleted from the sentence.
5) As suggested this expression was unclear. Therefore, this sentence has been improved.
Answer to the report from Dr. Monika Engelhardt:

According to the reviewer’s suggestion, the manuscript has been improved and re-written in red type.

Major Criticisms:

1. According to the reviewer’s suggestion, the overall manuscript has been rewritten in terms of the English phrasing and grammar, and reviewed by a professional native speaker bio-medical editor.
2. As suggested, there are numerous papers focusing on the role of telomerase activation in the progression of cancer cells. Therefore, we have rephrased this sentence.
3. According to the reviewer’s suggestion, this expression has been improved. The action of CDDP and 5-FU was explained briefly in the section on anti-cancer drugs in the Materials and methods.
4. The alamar blue assay (cell proliferation assay) is similar to the “MTT assay”. It is well known that the fluorescence intensity at the emission wavelength well correlates with the number of viable cells in this method. Therefore, we used this assay kit in this study. The manipulations were performed strictly according to the manufacturer’s instructions. To provide a better understanding, the sentence has been rewritten.
5. It is true that many papers using lower concentration of agarose for the analysis of telomere length have been published. On the other hand, there have also been many papers using a relatively high concentration (0.8 - 1%) of agarose such as Oncogene (1995) 10: 937-944, Cancer Res. (1994) 54: 3557-3560, and so on. We simply performed the experiments according to the latter methods. Since we used the Mupid mini-gel system, running the gel at 50V for 75 min is correct. By using this system we could obtain a sufficiently good-quality image to analyze the telomere length.
6. The statistical analysis was applied only to the cell proliferation assay (Fig. 2). Therefore, this point has been clearly mentioned both in methods and in figure legends.
7. As to the growth kinetics and speed, we estimated these factors from the results of Fig. 1. Growth speed is considered to correlate with the slope of the Day-PDL-relationship curve. Namely, the slope represents the time required for the cells to reach the confluence. The description on the first paragraph of the Results is based on this idea. Regarding the results of both treatment of anti-cancer drugs beyond 100 days and the addition of each single anti-cancer drug alone, we unfortunately did not perform such experiments. Therefore, we could not clearly answer these questions. Since these questions are similar to the other reviewer’s comments, we added a few sentences on this point in the Discussion.
8. Since the growth speed of LoVo cells was much slower than that of DLD-1 cells, it was difficult to set the same cut-offs for PDL. Retrospectively, we think that this comment is legitimate, but it was very difficult to arrange the experiments according to this point in the actual experiments. But we believe that different cut-offs for PDL do not lessen the quality of the data.
9. We think the reviewer’s comment is a reasonable one, but we could not perform any additional experiments using FISH analyses because of the lack of cell storage of the corresponding PDL to this study. On the other hand, it has been reported by Papi S et al. (Cytometry 1996) that flow cytometric measurement of DNA content well correlates with the cytogenetic analysis. Therefore, this reference has been quoted in the Results section (ref. #20).
10. As to the Figure 4, we think the reviewer’s comment is a reasonable one, but we could not perform additional experiments because of the lack of the PDL-matched control cell lysates. However, we confirmed that a simple passage of these cells in the normal culture medium without any anti-cancer drugs has no effect on the expression of mdr-1 and MRP. Accordingly, this point has been added in the Results section.
11. As suggested, we calculated the mean TRF values and compare with peak TRF values as pointed out in this comment, the elongation of the telomeres in Figure 5 is not completely convincing for DLD-1 cells, especially the claimed telomere elongation from 7.96 to 9.3 kbp, because mean TRF values of them are 8.67 and 8.25 kbp, respectively. Therefore, we described this point in the Results section. We also added the mean TRF values in Figure 5.
12. As suggested, we have changed the interpretation and toned down the expression from “strongly upregulated” to simply “upregulated”. As telomerase activity of DLD-1 cells at 7, 27 and 58 PDL seems very similar, we also mentioned about this point.
13. We believe that our data have enough quality to display the upregulation of mRNA expressions described in the text. We think that deterioration of the picture quality may have occurred during the file exchanging process from JPEG to PDF form. Therefore, we reproduced a high-quality version.
14. Regarding the phrase “plateau”, we meant that increase in the growth speed reached the plateau level
(maximal level = the same speed as the control cells). But as pointed out in the reviewer’s comments, this expression is apparently inadequate and confusing. Therefore, the expression has been improved.

15. Although we could not tell the exact half-life of both drugs when it was added to the culture medium, we consider that drug concentration used in our experiment is adequate. Each anti-cancer drug was renewed at every passage, which means ca. every 3 days. The dose indicated in the text is sufficient to induce the drug resistance and to select the drug-resistant cells. In this dose normal control cells can not grow. These points have been added in the Methods section.

16. According to the reviewer’s suggestion, the sentence has been rephrased.

17. First, according to the method of treatment of the cells with anti-cancer drugs, we have described precisely in the Methods section. Secondly, as to the point that telomere elongation in DLD-1 cells seems less well documented, we added the analysis in terms of the mean TRF and have rewritten the text. As pointed out by the reviewer, the elongation of the telomere may be unclear, but it is clear that a remarkable elongation of the TRF was observed at least from 0 to 27 PDL. Thirdly, the reviewer mentioned that the telomerase change/activation during cell growth was very moderate. The sentences in the Results section related to this point have also been improved. We believe these changes will satisfy the reviewer.

18. Regarding the very low telomerase activity during the first several PDL, we confirmed this result by ELISA-based TRAP assay because we could not obtain enough number of cells for the analysis due to the very slow proliferation speed. Therefore, we can neither display the results of electrophoresis-based TRAP assay nor TRF assay (Southern blot). We can not include these results in Figure 5. This point has been added to the text.

19. According to the reviewer’s suggestion, the final conclusion in the Results has been rewritten.

Minor Criticisms:

Regarding the minor criticisms from No. 1 to No. 25, we have rewritten the manuscript according to the reviewer’s suggestions.

Specific questions:

Regarding the specific questions from No. 1 to No. 4, these points have already been pointed out in the major criticisms and minor criticisms, and the comments seem to be repetitive. We therefore believe we have already answered these items satisfactorily.