Reviewer’s report

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

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Reviewer: Dr Monika Engelhardt

Level of interest: A paper of limited interest

Advice on publication: Other (see below)

The article Long-term cultivation of colorectal carcinoma cells with anti-cancer drugs induces drug resistance and telomere elongation by N. Kuranaga et al describes cell growth kinetics of 2 colorectal cell lines cultivated with two anti-cancer drugs with respect to population doublings, telomere length, telomerase activity, mRNA expression of telomerase components and acquisition of mdr expression.

Major criticisms:

1. The English phrasing and grammar needs much more attention and correct spelling, grammar and expressions/wording would greatly improve the manuscript. Some of these major flaws are mentioned in the section 'minor criticism', but could not be named in full detail by the reviewer. In general, the whole paper needs rewriting and rephrasing to ameliorate understanding its contents.
2. In the 'background section', the authors claim that the relationship between the role of telomerase activation in the progression of cancer cells has not been explained, this seems inadequately phrased, since there are numerous papers focusing on that aspect, please either rephrase or take out.
3. The cancer drugs used in the paper, such as 5-FU or CDDP should be fully mentioned and explained in its action in the background and methods section. It also does not help, if these are not given by their full name, but abbreviation in the whole text, even in the incomplete abbreviation list on page 14, these are not explained, please name and explain all words when used in abbreviated terms throughout the entire manuscript.
4. On page 5, the authors should name why the fluorescence at 560nm and 590 nm wavelength was measured, in order to determine cell numbers?
5. Since it is well known that best distribution of telomeres is performed in low concentration agarose gels, the high concentration of 1% is a little surprising, please explain why lower concentrations as previously published were not used. Moreover, running the gel at 50V for only 75 min seems very short indeed, time error here?
6. (page 7, statistical analysis): The two groups that were tested by the Student's t-test remain unclear, are CDDP plus 5-FU in both cell lines or each as compared to its control meant? Please clarify.
7. Why in both tumor cell lines - with addition of both anti-cancer drugs - the authors conclude that
growth kinetics and speed were almost as high as in the controls, remains unclear, since this is most certainly not the case. Please correct. It would also be of interest to mention what happened with both cell lines, both the control and with addition of both anti-cancer drugs beyond d 100 as well as with the addition of each single anti-cancer drug alone.

8. It remains unclear to the reviewer why different cut-offs for PDL were chosen for the measurement of the proliferative activity of both cells lines, namely 19 and 39 vs. 27 and 58 rather than using exactly the same ones to better compare both with each other.

9. The effect of clonality measured by DNA histogram analysis does not completely convince the reviewer that in each cell line chromosomal changes did not occur, this seems to be better excluded using FISH analyses rather than by DNA histogram pattern, please comment.

10. Figure 4 shows the expression of mdr-1 and MRP, but neither convincingly demonstrates their upregulation nor does it show potential changes in the control cell lines grown without anti-cancer treatment. Please improve or exchange the figure.

11. The elongation of telomeres in figure 5 is not completely convincing for DLD-1 cells, especially the claimed telomere elongation from 7.96 to 9.3kbp, the latter even appears shorter or at the utmost both seem similar in length. Please also give mean TRF values as compared to peak TRFs and comment on their differences.

12. In figure 6, telomerase activity does not seem to be extremely or as given 'strongly' upregulated, rather than increased at moderate values. Also, in DLD-1 telomerase at all time points, with 7, 27 and 56 PDL seem very comparable or similar, please change the text and interpretation accordingly or improve/exchange the figure.

13. In figure 7 the upregulation of mRNA expressions are not convincingly displayed either. Please exchange figure to better display these changes.

14. The plateau that is mentioned by the authors in the discussion (page 10) seems misleading, since both cell lines with CDDP and 5 FU addition did not reach a plateau at all, however, grew slower but gradually as compared to the control.

15. The authors claim that their cell lines after some time after the addition of both anti-cancer drugs acquired drug resistance. How can they safely prove that? What was the half life of both drugs? How often was each anti-cancer drug renewed in the culture to ensure their activity on both cell lines up to late PDs, or was it once added and never later again? Please comment in detail.

16. Please rephrase: These may be different conclusions from one in which there is active telomere elongation. This is not well understood and needs to be rephrased.

17. Although this may be the first report on drug resistance and telomere elongation as claimed by the authors, these results have substantial shortcomings, seem less well understood and are certainly not very well presented: first the continuous and/or frequent addition of both drugs to both cell lines does not seem to have taken place, secondly telomere elongation at least for DLD-1 seems less well documented and is not convincing and thirdly telomerase changes / activation during cell growth was very moderate. Bearing these substantial shortcomings of the manuscript in mind, the authors should be much more carefully when interpreting their results than performed so far.

18. The very low telomerase activity (page 11) during the first PDL are not shown and should probably be included in figure 5.

19. The final conclusion that telomere elongation was not due to abnormal clones rather than upregulation of telomerase seems very unlikely to be the only and final conclusion to the observed phenomenon and should be more carefully addressed. Clonal appearance was not carefully tested, nor telomerase upregulation very substantial. Other factors than those named might most likely have had a substantial input in the observed changes and discussion of these would greatly improve the manuscript.

Minor criticisms:
Abstract:
1. In the abstract, in the results section: please change: during the observation period, an abnormal.
2. Rephrase: Telomere length gradually increased with progressive PDL.
3. In the conclusions: and elongation of telomeres have some ..... Colorectal carcinoma cells with longer telomeres may be selected.

Background
4. We often encounter patients....
5. An acceleration of the....
6. (1-3) which have proved to play.....
7. MDR-1 and MRP genes have been reported.....and to frequently exist on.....
8. Telomere shortening has been reported to be involved in the.....
9. It has also been clarified that once tumor cells acquire telomerase activity telomere length is maintained.
10. Up to now, the relationship......and to various characteristics of tumor cells......
11. Instead of 'for a long period of time' use the exact time of observation, e.g. 100 days.
12. .....and obtained cancer cells that were highly resistant to anti-cancer drugs and grew rapidly.
13. .....including telomere length....

Material and methods
14. Take out......and used as study materials.
15. Rephrase (page 4): used for cell passage. The cell passage was performed when cells had reached confluence.
16. (page 4, last paragraph)...in triplicates.
17. (page 5): Ten ml of diluted Alamar blue were added to each well and incubated.....
18. (page 5): Telomerase activity was determined using a modified TRAP assay. .....with minor modifications.

Results
19. (page 8): growth was almost completely suppressed until (or: up to) day 14.
20. .....growth speed gradually increased.
21. Please note that in relative sentences, starting with who, which or that, there is no comma. Please change accordingly in the whole text.
22. (page 9) ... in the passages cells were observed.

Discussion
23. However, it is also possible that cells that possess high telomerase......
24. In their study, critically short telomeres in tumor-derived cell lines......
25. (page 11): This is the first report......and elongation of telomeres.

Specific questions:
1. Are the conclusions drawn adequately supported by the data shown: if not, what are the shortcomings and could they be overcome?
   As named in detail in the major comments sections from 1.-19., there are several shortcomings of the paper that need to be addressed.

2. Are sufficient details provided to allow replication of the work or comparison with related analyses: if not, what is missing?
Although there is sufficient data presented as well as enough details given in the methods section to allow replication of the data, numerous points as listed in 1.-19. need to be carefully addressed, such as the high concentration of the agarose gel and running the gel at 50V for only 75 min. Moreover, the statistical analyses are either unclear or not very extensively performed at all, these need to be performed with more regard and in more detail. It also remains unclear why in both tumor cell lines - with addition of both anti-cancer drugs - the authors conclude that growth kinetics and speed were almost as high as in the controls. As already explained above, it would also be of interest to mention what happened with both cell lines, beyond d 100 and with the addition of each single anti-cancer drug alone. Of concern is as well, why different cut-offs for PDL were chosen for the measurement of the proliferative activity of both cells lines, namely 19 and 39 vs. 27 and 58 rather than using exactly the same ones to better compare both with each other. As mentioned, especially figures 4, 6 and 7 do not adequately display what is meant to be described by the authors, these have to be improved or exchanged. Finally, the authors claim that their cell lines acquired drug resistance needs better clarification. How can they safely prove that? What was the half life of both drugs? How often was each anti-cancer drug renewed in the culture to ensure their activity on both cell lines up to late PDs, or was it once added band never later again? This needs to be addressed in detail.

3. Does the manuscript adhere to the relevant standards for reporting and data deposition: if not, in what ways?
Bearing in mind the extensive major and minor comments, these need to be addresses, if the authors can improve and/or clarify all of these comments raised this will help to greatly improve the manuscript and ensure that the relevant standards of reporting are met.

4. Is the writing acceptable?
The English phrasing and grammar needs much more attention. Correct spelling, expressions and grammar will greatly improve the manuscript. Some of these major flaws are mentioned in the section 'minor criticism', but could not be named in full detail by the reviewer. In general, the whole paper needs extensive rewriting and rephrasing to ameliorate understanding its contents.

Level of interest: limited

Advice on publication: accept after extensive revision

Quality of written English: poor, not acceptable for publication unless it is rewritten

Competing interests:

None declared.