Author's response to reviews

Title: Effects of small interfering RNA targeting thymidylate synthase on survival of ACC3 cells from salivary adenoid cystic carcinoma

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Version: 4 Date: 23 July 2008

Author's response to reviews: see over
Letter to the Editor

Dear Prof. Melissa Norton
Editor-in-Chief
BMC Cancer

MS: 7699201882012193
Effects of small interfering RNA targeting thymidylate synthase on survival of ACC3 cells from salivary adenoid cystic carcinoma
Shin-ichiro Maruya, Takashi Shirasaki, Hiroki Mizukami, Seiji Kakehata, Hidekachi Kurotaki, Soroku Yagihashi and Hideichi Shinkawa

I am returning herewith the above manuscript revised according to your letter. We found the referees’ comments most helpful and have revised the manuscript accordingly. We added “competing interests” and “authors’ contributions” following editor’s suggestion (page 17). I also included a letter that responded to reviewers’ comments. We greatly appreciate if you take the publication of our manuscript into consideration.

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Letter to Reviewers

The comments of the three reviewers have been helpful in allowing us to revise our manuscript. We have attempted to address the questions raised by the referees as follows:
Referee #1:

1. The main purpose of present study is to evaluate the effect of TS silence in salivary adenoid cystic carcinoma cells. In this regard, we understand that it would be better to use several cell lines from ACC. However, it is very difficult to obtain several cell lines, because the number of available ACC lines is quite limited. However, ACC3 is a well investigated cell line and ACC3 has common molecular and morphological characteristics of adenoid cystic carcinoma. There are many reports of single usage of ACC3 cell as a representative cell line of salivary gland cancer (page 14, line 24 to page 15, line 2; Ref. 32). In addition, we presently tried to assess TS silence in ACC3 cells, because there was no experimental evidence that TS silence could be a therapeutic target in ACC (page 12, lines 3 to 6).

2. We succeed in Western blot of TS protein. Photograph is added in Figure 2C.

3. We actually agree that S-phase fraction rate of ACC3 is relatively high. In preliminary experiments, S-phase rate was higher in ACC3 than in other squamous cell carcinoma cell lines. We speculate that high S-phase population is a common characteristic of ACC. However, we currently don’t have idea of the reason why ACC3 cells have high population of S-phase. We added the data of other cell cycle phases such as G0/G1 and G2/M (Figure 3), following reviewer’s suggestion. No significant increase in sub-G1 was observed. Our data indicated that TS silence by siRNA induces specific S-phase accumulation. In preliminary experiment in other cell line, the pattern of cell cycle alteration was mostly similar.

4. Following reviewer’s suggestion, we added a Western blot analysis data showing up-regulation of wild-type p53 in ACC3 cell treated with TS siRNA(Figure 4). Furthermore, we added the discussion about the implication of p53 pathway in association with p21 activation, S-phase accumulation, and TS silence (page 13, line 24 to page 14, line 14). Unfortunately, we currently have no data about p53 mutation status of ACC3 cells. In this point, we quoted a reference describing the association between p53 mutation and resistance to TS inhibitor in other cells (Ref. 19, page 4, lines 20 to 22).

5. We agree that p21 activation is a main mechanism of cytostatic effects of TS silence. Our data seems to be consistent with previous investigations using 5-FU and antisense oligodeoxynucleotides (page 12, lines 18 to 21). However, there is a discrepancy between p21 up-regulation and S-phase accumulation, because p21 activation is thought to be associated with G1 arrest, not S-phase arrest. We speculated the involvement of other molecules or mechanisms and discussed possible participation of E2F, Cdc25A, and Chk1 in S-phase regulation (page 12,
6. We corrected figures by adding mean SD to real-time PCR data (Figure 2B and 4B). All analyses were confirmed several times.
Referee #2:

1. In the present study, we showed that our siRNA was effective in silencing TS mRNA and protein. However, as reviewer’s indication, we have to consider the limitation of gene silence when using siRNA, because siRNA transfection occasionally leads to non-specific gene silence. In this context, we further discussed the possibility of off-target effect, a non-specific gene silence. We also described our siRNA primarily suppressed TS mRNA expression in cDNA microarray analysis (page 12, lines 9 to 17). This result of cDNA microarray may guarantee the efficacy of the siRNA in specific silence of TS gene.

2. Our data suggested that TS silence led to S-phase accumulation without increase in sub-G1 population. We added data of other cell cycle fraction in Figure 3. According to previous reports, TS inhibition by 5-FU and antisense oligodeoxynucleotides results in S-phase accumulation (page 12, lines 18 to 21). These observations seem to be consistent with present data. However, its mechanism has not yet to be understood. We need to deeply investigate how TS silence leads to S-phase as a future project. We currently discussed the involvement of other cell cycle regulators including E2F, Cdc25A, and Chk1 in S-phase regulation (page 12, line 24 to page 13, page 13, line 24 to page 14, line 14).

3. As reviewer’s indication, we understand that there is a discrepancy between up-regulation of p21 and active caspase-3 and S-phase arrest. Although its mechanism has not been clarified, it appeared to be common responses in TS silence (page 12, lines 18 to 21). We added comments to explain this discrepancy and possible cell cycle mechanism (page 13, line 24 to page 14, line 14). To evaluate whether p21 up-regulation induced by TS silence is associated with p53 status, we added a data of Western blot analysis, showing up-regulation of wild-type p53 protein (Figure 4). This data suggests a possibility that p21 activation induced by TS inhibition may be controlled by its up-stream p53.
Referee #3:
1. We performed Western blot analysis for TS protein and showed reduced expression of TS protein in siRNA-transfected ACC3 cells in Figure 2C of revised version.
2. Main purpose of present study is to evaluate the effect of TS silence in salivary adenoid cystic carcinoma cells. In this regard, we understand that it would be better to use several cell line from ACC. However, it is very difficult to obtain several cell lines, because the number of available ACC lines is quite limited. ACC3 is a well investigated cell line typical model of ACC and ACC3 has common molecular and morphological characteristics of adenoid cystic carcinoma. There are many reports of single usage of ACC3 cell as a representative cell line (page 14, line 23 to page 15, line 1). We presently focused on TS silence in ACC3 cells, because there was no experimental evidence that TS silence could be a therapeutic target in ACC (page 12, lines 3 to 6).
3. When we evaluated 2 different siRNAs in a preliminary analysis, the effects on TS silence and cell viability were almost identical (data not shown). cDNA microarray analysis revealed that TS gene is primarily silenced gene in cells treated with our siRNA (page 12, lines 14 to 17). This result suggests that our TS siRNA effectively and specifically silenced TS gene expression.
4. The data of real-time RT-PCR was average of several experiments. Error bars were attached to a new version (Figure 2B and 4B).
5. Following reviewer’s suggestion, we corrected a sentence. We think that “expression” is more appropriate (page 10, lines 7-9). Moreover, we added a data of Western blot for p53 (Figure 4). This assay showed that TS inhibition by siRNA induced up-regulation of wild-type p53 and supported a possibility that p21 activation caused from TS inhibition is mainly controlled by p53 (page 13, line 24 to page 14, line 14).
Thank you for your consideration of the revised version.

Sincerely,

Shin-ichiro Maruya, M.D., Ph.D.