**Author's response to reviews**

**Title:** Usefulness of 18F-fluorothymidine for early and accurate monitoring of antiproliferative effect of gefitinib in human tumor xenograft: Comparison with Ki-67 and phospho-EGFR expression

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**Author's response to reviews:** see over
Re: BMC Cancer – MS: 5717212398910526

Title: *Usefulness of $^{18}$F-fluorothymidine for early and accurate monitoring of antiproliferative effects of gefitinib in human tumor xenograft: Comparison with Ki-67 and phospho-EGFR expression*

Authors: Songji Zhao et al.

Dear Dr. Solera:

We have revised our manuscript (MS: 5717212398910526) entitled “*Usefulness of $^{18}$F-fluorothymidine for early and accurate monitoring of antiproliferative effects of gefitinib in human tumor xenograft: Comparison with Ki-67 and phospho-EGFR expression*” in accordance with the comments and suggestions of the reviewers. On a separate sheet, we have provided point-by-point responses to the comments and suggestions made by the reviewers. RED TEXT was used to indicate all the changes within the manuscript itself, and deletions have been noted in the point-by-point reply.

We look forward to the publication of our manuscript in BMC Cancer.

Sincerely yours,

Yuji Kuge, Ph.D.
On behalf of all the authors

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We are grateful to the reviewers for their insightful comments and useful suggestions that helped us improve our manuscript. As indicated in the responses that follow, we have taken all of the comments and suggestions into account in preparing the revised version of our manuscript.

Reviewers’ comments and our point-by-point responses are written below each comment.

Reviewer #1:

In the current manuscript, the authors assessed the use of ³H-fluorothymidine as a biomarker for response assessment to gefitinib. The uptake of ³H-fluorothymidine was compared with the uptake of 18F-FDG, Ki67 and EGFR. The manuscript is overall well written, the experiments were performed carefully and the obtained data are interesting. I believe this paper can be accepted for publication, although I do have some comments that need to be addressed.

Minor Essential Revisions

General:
1) Throughout the whole manuscript the authors seem to mix up ³H-FLT with 18F-FLT. If I understood the paper correctly, it should be ³H-FLT since it is injected together with the 18F-FDG. This should be corrected everywhere.

Response:
We thank the reviewer for the useful comment. As pointed out by the reviewer, in this study, ³H-FLT was injected instead of 18F-FLT together with 18F-FDG. To avoid confusing ³H-FLT with 18F-FLT in this study, we have changed 18F-FLT to ³H-FLT in several places in the revised manuscript as follows:

Corrections:
(1) Page 1, line 1 in the “Title”:
From: “Usefulness of ¹⁸F-fluorothymidine for …”
To: “3H-fluorothymidine can be used for …”

(2) Page 3, line 3 in the “Abstract” section:
From: “3'-deoxy-3'-18F-fluorothymidine (18F-FLT) can be used for …”
To: “3'-deoxy-3'-3H-fluorothymidine (3H-FLT) can be used for …”

(3) Page 4, lines 14 to 15 in the “Abstract” section:
From: “Thus, the usefulness of 18F-FLT for …”
To: “Thus, it was indicated that 3H-FLT as a surrogate biomarker can be used for …”

(4) Page 9, lines 3 to 4 in the “Background” section:
From: “Thus, in the present study, to evaluate whether 18F-FLT can used for …”
To: “Thus, in the present study, to evaluate whether 3H-FLT as a surrogate biomarker can be used for …”

(5) Page 18, lines 7 to 8 in the “Discussion” section:
From: “Thus, measurement of tumor proliferative activity using 18F-FLT may enable early …”
To: “Thus, measurement of tumor proliferative activity using 3H-FLT may enable early …”

(6) Page 19, lines 8 to 9 in the “Discussion” section:
From: Thus, our findings suggest that 18F-FLT can reflect EGFR activation and can be a predictor of the tumor response to gefitinib.
To: Thus, our findings suggest that 3H-FLT can reflect EGFR activation and can be a predictor of the tumor response to gefitinib.

(7) Page 22, lines 16 to 18 in the “Conclusions” section:
From: “Thus, the usefulness of 18F-FLT for monitoring the antiproliferative effect of gefitinib was indicated in a mouse model of human epidermoid cancer.”
To: “Thus, it was indicated that 3H-FLT as a surrogate biomarker can be used for monitoring the antiproliferative effect of gefitinib in a mouse model of human epidermoid cancer.”

Background:
1) paragraph 4, page 6: “However, these anatomical imaging techniques have limited value due to the relatively long periods required to obtain tumor size shrinkage the case of successful drug therapies.” This sentence should be rewritten.

Response:
We thank the reviewer for the useful comment. In accordance with the reviewer’s comment, we have rewritten the sentence in the “Background” section of the revised manuscript (page 7, lines 3 to 5).

**Corrections:**

**From:** “However, these anatomical imaging techniques have limited value due to the relatively long periods required to obtain tumor size shrinkage the case of successful drug therapies.”

**To:** “However, these anatomical imaging techniques have limited value because a relatively long time is required to obtain sufficient tumor size shrinkage with successful drug therapies.”

2) **paragraph 4, page7:** the authors refer to a reference of 1998 by ‘recently’. I don’t think a paper of 1998 can be called recently, so this should be referred to differently.

**Response:**

We thank the reviewer for the useful comment. We have deleted “recently” in the original manuscript and revised the sentence in the revised manuscript as follows (page 7, lines 12 to 14).

**Corrections:**

**From:** “Recently, the thymidine analog 3’-deoxy-3’-18F-fluorothymidine (18F-FLT) has been developed as a PET tracer for imaging tumor proliferation in vivo [16].”

**To:** “On the other hand, the thymidine analog 3’-deoxy-3’-18F-fluorothymidine (18F-FLT) was also developed as a PET tracer for imaging tumor proliferation in vivo [18].”

3) **In the background section (last paragraph):** Thus, in the present study, to evaluate whether 18F-FLT can BE used for the early and accurate detection...

**Response:**

We thank the reviewer for this correction. Accordingly, we have modified the sentence in the “Background” section of the revised manuscript (page 9, lines 3 to 4).

**Corrections:**
From: “Thus, in the present study, to evaluate whether \(^{18}\text{F-FLT}\) can be used for the early and accurate detection…”

To: “Thus, in the present study, to evaluate whether \(^{3}\text{H-FLT}\) as a surrogate biomarker can be used for the early and accurate detection…”

Materials and methods:
1) I would mention somewhere the origin of the A431 cells (lung?).
Response:
We completely agree with the reviewer. In accordance with reviewer’s comment, we have provided the origin of the A431 cells in the “Materials and methods” section of the revised manuscript as follows.

Added statements:
Page 10, line 16 to page 11, line 1:
(1) A431 is a human cell line established from an epidermoid carcinoma of the vulva of an 85-year-old female patient, which has gene amplification and an unusually high number of EGF receptors [27].
Page 11, lines 2 to 3:
(2) A431 xenograft is a recognized model for the testing of the biological effects on EGFR signaling [29].

Added references:
We have added related references and rearranged the number of references in the revised manuscript as follows (page 30, lines 11 to 14 and page 31, lines 1 to 3).


Discussion
1) 2nd paragraph: the last sentence should be rewritten
Response:
We thank the reviewer for the useful comment. In accordance with the reviewer’s comment, we have rewritten the sentence in the “Discussion” section of the revised manuscript as follows (page 18, lines 3 to 6).

**Corrections:**

**From:** These early changes in tumor proliferation activity were confirmed by our pathological studies; namely, immunohistochemical staining of the Ki-67 (Fig. 3) and phospho-EGFR assay (Fig. 4).

**To:** These early changes in tumor proliferation activity were confirmed by our pathological studies **that including** immunohistochemical staining of the Ki-67 (**Fig. 2**) and phospho-EGFR assay (**Fig. 3**).

**Discretionary Revisions**

1) **Just a suggestion for future in vivo studies.** It seems better to me to measure the mice tumours in 3 dimensions (\#/6 * (length*width*depth)) rather than using the smallest diameter for both the width and the depth. To my opinion there is a big difference between a tumour that has a width of 3cm and a depth of 3cm with a tumour that has a width of 3 cm but only 1 cm depth. This is not taken into account with the current formula.

**Response:**

We thank the reviewer for the useful suggestion concerning future in vivo studies. In accordance with the reviewer’s suggestion, we have added a statement on this point to the “Discussion” section of the revised manuscript as a limitation of this study (page 22, lines 4 to 7).

**Added statements:**

It is better to measure the mice tumors in three dimensions (3.14/6 x (length x width x depth)) rather than using the smallest diameter for both the width and depth (3.14/6 x longest diameter x (smallest diameter^3)). Thus, to measure accurate tumor volumes, in vivo studies are necessary.

**Reviewer #2**

**Major Compulsory Revisions**
1) The title does not describe the content of this study. For example, F-18 labeled FLT was not used in this study. In addition, a study with a single animal model may not likely to provide information on ‘usefulness’.

Response:
We completely agree with the reviewer. In accordance with the reviewer’s comment, we have revised the pertinent words concerning these points in the “Title”, “Abstract”, “Background” and “Discussion” sections of the revised manuscript as follows.

Corrections:
(1) Page 1, line 1 in the “Title”:
From: “Usefulness of 18F-fluorothymidine for …”
To: “3H-fluorothymidine can be used for …”
(2) Page 3, line 3 in the “Abstract” section:
From: “3'-deoxy-3'18F-fluorothymidine (18F-FLT) can be used for …”
To: “3'-deoxy-3'3H-fluorothymidine (3H-FLT) can be used for …”
(3) Page 4, lines 14 to 15 in the “Abstract” section:
From: “Thus, the usefulness of 18F-FLT for …”
To: “Thus, it was indicated that 3H-FLT as a surrogate biomarker can be used for …”
(4) Page 9, lines 3 to 4 in the “Background” section:
From: “Thus, in the present study, to evaluate whether 18F-FLT can be used for …”
To: “Thus, in the present study, to evaluate whether 3H-FLT as surrogate biomarker can be used for …”
(5) Page 18, lines 7 to 8 in the “Background” section:
From: “Thus, measurement of tumor proliferative activity using 18F-FLT may enable early …”
To: “Thus, measurement of tumor proliferative activity using 3H-FLT may enable early …”
(6) Page 19, lines 8 to 9 in the “Discussion” section:
From: “Thus, our findings suggest that 18F-FLT can reflect EGFR activation and can be a predictor of the tumor response to gefitinib.”
To: “Thus, our findings suggest that 3H-FLT can reflect EGFR activation and can be a predictor of the tumor response to gefitinib.”
(7) Page 22, lines 16 to 18 in the “Conclusions” section:
From: “Thus, the usefulness of 18F-FLT for monitoring the antiproliferative effect
of gefitinib was indicated in a mouse model of human epidermoid cancer.”

To: “Thus, it was indicated that $^3$H-FLT as a surrogate biomarker can be used for monitoring the antiproliferative effect of gefitinib in a mouse model of human epidermoid cancer.”

(8) As the reviewer suggested, in this study, we used $^3$H-FLT instead of $^{18}$F-FLT. We also used a single animal model (A431); thus, it may not provide information on ‘usefulness’. $^{18}$F-FLT and other tumor models should be used to provide information on ‘usefulness’ of FLT for early and accurate monitoring of the antiproliferative effect of gefitinib in human tumor xenografts. We have added a statement on this point to the “Discussion” section of the revised manuscript of our study as a limitation (page 22, lines 7 to 11).

Added sentences:
Other limitations of our study were that $^3$H-FLT was used instead of $^{18}$F-FLT and only one tumor model (A431) was used to compare the uptake of $^3$H-fluorothymidine with the uptake of $^{18}$F-FDG, Ki67 and phospho-EGFR after the treatment with two different doses of gefitinib. $^{18}$F-FLT and other tumor models should be used to confirm our present results.

2) In the abstract, FDG data are missing.

Response:
We thank the reviewer for the useful comment. In accordance with reviewer’s comment, we have added $^{18}$F-FDG data in the “Abstract” section of the revised manuscript.

Added statements:
(1) page 3, line 13 in the methods of “Abstract” section:
   “Biodistribution of $^3$H-FLT and $^{18}$F-FDG (%ID/g/kg) was …”
(2) page 4, line 3 in the results of “the Abstract” section:
   “… respectively; p<0.01 vs. control), but those of $^{18}$F-FDG were not.”

3) In the conclusion section of the abstract and the text, it was stated that FLT uptake levels dose-dependently decreased before a significant change in tumor size was observed. However, this statement is not supported by data. Both dosages showed the similar results on FLT uptake, FDG uptake, Ki67, pEGFR,
and tumor size within three days. I do not see any significant differences between two doses of gefitinib.

Response:
We greatly thank the reviewer for the useful comment. In accordance with the reviewer’s comment, we have corrected the statement in the conclusion sections of the “Abstract” and the text of the revised manuscript.

Corrections:
(1) Page 4, lines 11 to 13 in the conclusion of the “Abstract” section:
From: “In our animal model, $^3$H-FLT uptake levels dose-dependently decreased before a significant change in tumor size was observed.”
To: In our animal model, $^3$H-FLT uptake levels significantly decreased after the treatment with two different doses of gefitinib before a significant change in tumor size was observed.”
(2) Page 18, lines 2 to 3 in the “Discussion” section:
From: “After the treatment with gefitinib, the $^3$H-FLT uptake levels in the tumor were significantly decreased in a dose-dependent manner (Fig. 1).”
To: “After the treatment with two different doses of gefitinib, the $^3$H-FLT uptake levels in the tumor were significantly decreased at an early time point (Table 1A).
(3) Page 22, lines 13 to 15 in the “Conclusions” section:
From: “In our animal model, the $^3$H-FLT uptake level dose-dependently decreased before significant change in tumor size occurred.”
To: “In our animal model, the $^3$H-FLT uptake level significantly decreased after the treatment with two different doses of gefitinib before a significant change in tumor size was observed.”

4) The authors referred the study with erlotinib and cetuximab (ref 20-22). The effect on Ki67 and pEGFR (the aim of this study) by these agents does not seem to be different from those by gefitinib. Sohn’s study already indicated the usefulness of FLT for gefitinib. The authors should address more specifically why this study is required, i.e. mode of action, drug kinetics, imaging schedule, cell model, etc.

Response:
We greatly thank the reviewer for the useful comment. We completely agree with the
reviewer. In accordance with the reviewer’s comment, we have stated more specifically why this study is required, and added a statement on this point to the “Background” section in the revised manuscript.

As pointed out by the reviewer, although several studies have indicated the usefulness of FLT for monitoring the effect of gefitinib [Shon et al. (25), Su et al. (26)], whether FLT PET as a surrogate biomarker can be used for accurately monitoring the effect of gefitinib by comparing the level of FLT uptake with those of other proliferation or predictive markers, such as Ki-67 or phosphorylated EGFR, in an early phase of treatment has not been fully validated under a pathological condition. Thus, in this study, to clarify whether \([^3]H\text{-}FLT\) as a surrogate biomarker can be used for the early and accurate detection of the antiproliferative effect of gefitinib, we determined the changes in FLT uptake level after the start of treatment using different doses of gefitinib in comparison with those in \(^{18}\)F-FDG uptake, Ki-67 expression, and phospho-EGFR levels in a human tumor xenograft (A431), which is a recognized model for the testing of the biological effects on EGFR signaling.

Corrections:
Page 8, line 10 to page 9, line 2:
From: “However, there have been no studies of the usefulness of \(^{18}\)F-FLT PET for monitoring the antiproliferative effect of gefitinib, except for one report by Sohn et al. They demonstrated that \(^{18}\)F-FLT PET can predict early responses to gefitinib treatment in patients with advanced pulmonary adenocarcinoma [23].
To: “However, there have been no studies on the usefulness of \(^{18}\)F-FLT PET for monitoring the antiproliferative effect of gefitinib, except for two reports [25,26]. Sohn et al. demonstrated that \(^{18}\)F-FLT PET can be used to predict early responses to gefitinib treatment in patients with advanced pulmonary adenocarcinoma [25]. The effect of gefitinib on \(^3\)H-FLT uptake in vitro was studied previously by Su et al. [26]. Although, several studies have indicated the usefulness of \(^{18}\)F-FLT or \(^3\)H-FLT for monitoring the effect of gefitinib [25,26], whether \(^3\)H-FLT as a surrogate biomarker can be used for accurately monitoring the effect of gefitinib by comparing the level of \(^3\)H-FLT uptake with those of other proliferation or predictive markers, such as Ki-67 or phosphorylated EGFR in an early phase of treatment has not been fully validated under a pathological condition.”

Added references:
We have added related reference and rearranged the number of references the revised manuscript (page 30, lines 7 to 10).


5) The introduction can be shortened significantly.

Response:
We completely agree with the reviewer. In accordance with the reviewer’s comment, we have shortened the introduction.

List of deletions:
The following sentences of the “Background” section have been deleted from the original manuscript.

Page 5, lines 2 to 6:
(1) Drugs that target molecular abnormalities; namely, molecular-targeted agents, are now expected to hold the greatest promise for cancer therapy. Through recent advances in molecular targeted therapy of cancer, agents targeting the epidermal growth factor receptor (EGFR) are currently the most promising and well advanced in the clinical setting.

Page 7, lines 4 to 6:
(2) $^{18}$F-FDG is the most widely used tracer for tumor imaging by PET and can image viable tumor cells on the basis of glucose metabolism.

Page 8, lines 7 to 11:
(3) However, in that study, they only evaluated the changes in $^{18}$F-FLT uptake level in tumors after gefitinib therapy. No report has pathologically validated the usefulness of $^{18}$F-FLT PET for monitoring the effect of gefitinib, by comparing the level of $^{18}$F-FLT uptake with those of other proliferation or predictive markers, such as Ki-67 or phosphorylated EGFR (phospho-EGFR).

Corrections:
Page 5, lines 2 to 3 in the “Background” section of the revised manuscript:
From: “EGFR is a receptor tyrosine kinase that plays a crucial role in the signal
transduction pathway, …”

To: “The epidermal growth factor receptor (EGFR) is a receptor tyrosine kinase that plays a crucial role in the signal transduction pathway, …”

6) Biomarkers are already available to predict the therapeutic benefit of gefitinib. The reason why imaging tests are needed despite available predictive biomarkers should be described in the introduction section.

Response:

We greatly thank the reviewer for the useful comment. As the reviewer suggested, recently, EGFR mutation, EGFR copy number, and EGFR protein expression are the three EGFR-related biomarkers that have been reported to be associated with the therapeutic benefit of gefitinib [12]. However, the therapeutic effect of gefitinib is not confined to patients whose tumors harbor EGFR mutation and other predictors of efficacy of this agent. In general, about 80% of NSCLCs with EGFR mutation respond to EGFR-TKIs, whereas 10% of tumors without EGFR mutations do so [13]. Although this observation provides highly valuable insights into the molecular mechanisms underlying sensitivity to EGFR-TKIs, none of the known clinical or molecular tumor characteristics allows the accurate prediction of tumor response at an early phase of treatment with gefitinib in an individual patient. Therefore, there is a clear need for new approaches to identify patients who will benefit from treatment with EGFR-TKIs. In this respect, imaging techniques that can be used to predict treatment outcome in an early phase of treatment are warranted. Molecular imaging may enable alternative evaluation procedures for the drug and enable the early change to alternative therapy if no functional response is indicated.

In accordance with the reviewer’s comment, we have added a statement on this point to the “Background” section of the revised manuscript.

Added statements:

We have added related reference and rearranged the number of references the revised manuscript (page 6, lines 6 to 18).

Recently, EGFR mutation, EGFR copy number, and EGFR protein expression are the three EGFR-related biomarkers that have been reported to be associated with the therapeutic benefit of gefitinib [12]. However, the therapeutic effect of gefitinib is not confined to patients whose tumors harbor EGFR mutation and other predictors of efficacy of this agent. In general, about 80% of NSCLCs with EGFR mutation respond
to EGFR-TKIs, whereas 10% of tumors without EGFR mutations do so [13]. Although this observation provides highly valuable insights into the molecular mechanisms underlying sensitivity to EGFR-TKIs, none of the known clinical or molecular tumor characteristics allows the accurate prediction of tumor response at an early phase of treatment with gefitinib in an individual patient. Therefore, there is a clear need for new approaches to identify patients who will benefit from treatment with EGFR-TKIs. In this respect, imaging techniques that can be used to predict treatment outcome in an early phase of treatment are warranted.

**Added references:**

We have added related references and rearranged the number of references the revised manuscript (page 27, lines 6 to 12 and lines 13 to 14).


7) The effect of gefitinib on FLT uptake was studied previously (Figure 2, Su H et al. *Clin Cancer Res* **2006;12:**5659-67). The introduction section should be revised. It is also needed to add an explanation of the results of this previous study in the discussion section.

**Response:**

We greatly thank the reviewer for the useful comment. We completely agree with the reviewer. In accordance with the reviewer’s comment, we have revised the introduction and have also added an explanation of the results of this previous study in the “Discussion” section of the revised manuscript as follows.

**Corrections:**

Page 8, line 10 to page 9, line 2 in the “Background” section:
From: “However, there have been no studies of the usefulness of $^{18}$F-FLT PET for monitoring the antiproliferative effect of gefitinib, except for one report by Sohn et al. They demonstrated that $^{18}$F-FLT PET can predict early responses to gefitinib treatment in patients with advanced pulmonary adenocarcinoma [23]. However, in that study, they only evaluated the changes in $^{18}$F-FLT uptake level in tumors after gefitinib therapy.”

To: “However, there have been no studies on the usefulness of $^{18}$F-FLT PET for monitoring the antiproliferative effect of gefitinib, except for two reports [25,26]. Sohn et al. demonstrated that $^{18}$F-FLT PET can be used to predict early responses to gefitinib treatment in patients with advanced pulmonary adenocarcinoma [25]. The effect of gefitinib on $^3$H-FLT uptake in vitro was studied previously by Su et al. [26]. Although, several studies have indicated the usefulness of $^{18}$F-FLT or $^3$H-FLT for monitoring effect of gefitinib [25,26], whether $^3$H-FLT as a surrogate biomarker can be used for accurately monitoring the effect of gefitinib by comparing the level of $^3$H-FLT uptake with those of other proliferation or predictive markers, such as Ki-67 or phosphorylated EGFR, in an early phase of treatment has not been fully validated under a pathological condition.”

Added statements:

(1) Page 19, lines 4 to 8:
Su et al. [26] reported that the growth inhibitory effect of gefitinib was parall to the inhibition of EFGR phosphorylation in a gefitinib-sensitive cell line (NSCLC H3255). These data strongly support our results in confirming the proof of the mechanism of the EGFR inhibitor gefitinib.

(2) Page 20, lines 10 to 13:
Su et al. [26] also showed that a marked decrease (~ 90%) in $^3$H-FLT uptake in NSCLC H3255 cells was observed 2 days after exposure to two different doses of gefitinib. The in vitro data supported our results in confirming the proof of the mechanism of the EGFR inhibitor gefitinib.

Added reference:
We have added related references and rearranged the number of references the revised manuscript (page 30, lines 7 to 10).
8) **Provide a rationale for using 100 and 200 mg doses of gefitinib.**

**Response:**
We greatly thank the reviewer for the useful comment. In accordance with the reviewer’s comment, we have provided a rationale for using 100 and 200 mg/kg/day doses of gefitinib.

The antitumor activity of gefitinib was demonstrated in tests with tumor xenografts derived from a range of different human tissues. Gefitinib was particularly effective against A431 xenografts, a recognized model for the testing of the biological effects on EGFR signaling [29]. Many previous studies demonstrated that gefitinib monotherapy was well tolerated in mice, and a partial regression dose of gefitinib was 100 mg/kg/day and the maximum tolerated dose was 200 mg/kg/day. In this setting of greater initial tumor burden, a single-agent dose of gefitinib (100 mg/kg/day) resulted in only partial tumor growth inhibition without any complete tumor remission. A single-agent dose of gefitinib (200 mg/kg/day), which is the highest published dose used in xenograft models, was reported to induce complete, but transient, tumor regression [30]. The results have been used to rationalize the approval of gefitinib in Japan [30]. However, in an early phase of treatment with gefitinib, no significant difference in tumor size was observed after treatment with the two different doses of gefitinib. In athymic nude mice bearing A431 xenografts, p.o. treatment once a day with gefitinib inhibited tumor growth in a dose-dependent manner. Similarly, gefitinib inhibited the growth of different tumor xenografts in a dose-dependent manner. Accordingly, 100 mg/kg/day as a partial regression dose of gefitinib and 200 mg/kg/day as the maximum tolerated dose have been widely used to evaluate the effects of gefitinib on human tumor xenografts [29-31].

In accordance with the results shown in previous studies, we selected two different doses of 100 and 200 mg/kg/day in our study to evaluate whether $^3$H-FLT as a biomarker can be used for the early and accurate detection of the antiproliferative effect of gefitinib.

**Added statements:**
In accordance with the reviewer’s comment, we have added a statement on this point to the “Materials and methods” section in the revised manuscript as follows (page 11, lines 9 to 11).

The doses of gefitinib have been widely used to evaluate its effects on human tumor
xenografts [29-31].

**Added references:**

We have added related references and rearranged the number of references the revised manuscript (page 31, lines 1 to 3; lines 4 to 8 and lines 9 to 12).


9) **Provide a rationale for selecting cell line for this study.**

**Response:**

In accordance with the reviewer’s comment, we have provided a rationale for selecting the cell line (A431) used in this study. A431 is a human cell line established from an epidermoid carcinoma of the vulva of an 85-year-old female patient, which have a gene amplification and unusually high number of EGF receptors [27]. The antitumor activity of gefitinib was demonstrated in tests with tumor xenografts derived from a range of different human tissues. Gefitinib was particularly effective against A431 xenografts, a recognized model for the testing of the biological effects on EGFR signaling [29]. In the case of gefitinib, the chosen dose was 200 mg/kg/day, the highest published dose used in the A431 xenograft model, which had been reported to induce complete, but transient, tumor regression [30]. The results have been used to rationalize the approval of gefitinib in Japan. Thus, the EGFR-dependent human A431 tumor xenograft model was used in this study.

**Added statements:**
In accordance with the reviewer’s comment, we have added the statements on this point to the “Materials and methods” section of the revised manuscript as follows:

(1) Page 10, line 16 to page 11, line 1:
A431 is a human cell line established from an epidermoid carcinoma of the vulva of an 85-year-old female patient, which have a gene amplification and unusually high number of EGF receptors [27].

(2) Page 11, lines 2 to 3:
A431 xenograft is a recognized model for the testing of the biological effects on EGFR signaling [29].

Added references:
We have added related references and rearranged the number of references the revised manuscript (page 30, lines 11 to 14; page 31, lines 1 to 3 and lines 4 to 8).


10) The 2nd and 3rd paragraph of the discussion section can be shortened significantly. Introduction of phosphor-EGFR, and TK1 is redundant.

Response:
We completely agree with the reviewer. In accordance with the reviewer’s suggestion, we have shortened the 2nd and 3rd paragraphs of the discussion section.

List of deletions:
The following sentences on the 2nd and 3rd paragraph of the “Discussion” section have been deleted from the original manuscript:

Page 16, lines 13 to14:
(1) EGFR promotes cancer cell growth, proliferation, invasion, and metastasis, and inhibits apoptosis [1, 2].

Page 16, lines 16 to 17:
(2) Drugs targeting these tyrosine kinases block EGFR activation and the intracellular events that follow.

Page 17, lines 15 to 18:
(3) Owing to the phosphorylation of $^{18}$F-FLT by TK1, negatively charged $^{18}$F-FLT monophosphate is formed, resulting in intracellular trapping and accumulation of radioactivity [36]. Thus, this tracer is retained in proliferating cells through the activity of thymidine kinase.

**Deleted reference:**
The following reference of the “References” section has been deleted from the original manuscript (page 29, lines 7-10).


11) Please comment on the study limitations in the discussion section.

**Response:**
In accordance with the reviewer’s comment, we have commented on the study limitation in the “Discussion” section of the revised manuscript as follows (page 22, lines 7 to 11).

**Added sentences:**
Other limitations of our study were that $^3$H-FLT was used instead of $^{18}$F-FLT and only one tumor model (A431) was used to compare the uptake of $^3$H-FLT with the uptake of $^{18}$F-FDG, Ki67 and phospho-EGFR after the treatment with two different doses of
gefitinib. $^{18}$F-FLT and other tumor models should be used to confirm our present results.

Minor Essential Revisions
1) The symbol “*” may not be used in the abstract.

Response:
We completely agree with the reviewer. In accordance with the reviewer’s suggestion, we have removed the symbol “*” in the abstract in the original manuscript.

2) Indicate the tracer used on the y axis of Figure 1.

Response:
In accordance with the reviewer’s suggestion, we have indicated the tracer used on the y-axis of Figure 1. However, in accordance with the reviewer’s suggestion, Figure 1 has been deleted from the original manuscript.

Discretionary Revisions
1) Provide information on weight of animals used.

Response:
In accordance the reviewer’s suggestion, we have provided information on the weights of animals used in the “Results” section of the revised manuscript (page 15, line 15 to page 16, line 2).

Added statements:
No significant differences were observed in mouse body weights among the three groups before and 3 days after the start of treatment. Mouse body weights were 19.6 ± 1.1 g for the control group and 18.8 ± 1.5 g and 19.0 ± 0.9 g for the 100 and 200 mg/kg gefitinib groups, and 18.6 ± 1.3 g for the control group and 17.3 ± 0.9 g and 17.7 ± 0.8 g for the 100 and 200 mg/kg gefitinib groups before and 3 days after the start of treatment, respectively.

Corrections:
Page 11, lines 14 to 16 in the “Materials and methods” section of the revised manuscript:
From: “Mice in the control and treatment groups were intravenously injected with a mixture of $^{18}$F-FDG (7.4 MBq) and $^3$H-FLT (0.185 MBq) 24 hours after the second treatment under light anesthesia.”

To: “After overnight fasting, mice in the control and treatment groups were intravenously injected with a mixture of $^{18}$F-FDG (7.4 MBq) and $^3$H-FLT (0.185 MBq) 24 hours after the second treatment under light anesthesia.”

2) Figure 1 a duplicate of Table 1.

Response:
In accordance the reviewer’s suggestion, we have deleted “Figure 1” in the original manuscript and rearranged the number of figures in the revised manuscript.

List of deletions:
The following items related to Figure 1 have been deleted from the original manuscript.

(1) The uptake levels of $^3$H-FLT and $^{18}$F-FDG in the tumor are summarized in Figs. 1A and B (page 14, lines 8 to 9 of the “Results” section);
(2) Fig. 1A (page 14, line 11 of the “Results” section);
(3) Fig. 1B (page 14, line 14 of the “Results” section);
(4) Figure 1. $^3$H-FLT (A) and $^{18}$F-FDG (B) uptake levels in mice bearing tumor. Control, Gefitinib 100, and Gefitinib 200 indicate the control group, group treated with 100 mg/kg gefitinib, and group treated with 200 mg/kg gefitinib, respectively. Values given are mean ± SD. (page 31, lines 2 to 5 of the “Figure legends” section in the original manuscript)

Corrections:
(1) Page 15, lines 10 to 11 of the “Results” section:
From: The T/M ratios of $^3$H-FLT uptake were also significantly decreased to 72% and 60% of the control value, respectively (Fig. 1A).
To: The T/M ratios of $^3$H-FLT uptake were also significantly decreased to 72% and 60% of the control value, respectively (Table 1A).
(2) Page 15, lines 13 to 15 of the “Results” section:
From: The T/M ratios of $^{18}$F-FDG were not reduced significantly (102% and 97% of the control value for 100 and 200 mg/kg gefitinib, respectively) (Fig. 1B).
To: The T/M ratios of $^{18}$F-FDG were not reduced significantly (102% and 97% of the control value for 100 and 200 mg/kg gefitinib, respectively) (Table 1B).

(3) Page 18, lines 2 to 3 of the “Discussion” section:

From: After the treatment with, the $^3$H-FLT uptake levels were significantly decreased in a dose-dependent manner (Fig. 1).

To: After the treatment with two different doses of gefitinib, the $^3$H-FLT uptake levels in the tumor were significantly decreased at an early time point (Table 1A).

Journal Style Points

In accordance with the Journal Style Points for: MS:5717212398910526 [First Submission], we have revised our manuscript as follows.

In the title page of the revised manuscript:
1) We have removed the semicolons at the end of the authors’ affiliations and then listed author s’ affiliations in separate lines.
2) We have moved “*Corresponding author” to the end of the authors’ affiliations from the end of the authors’ e-mail addresses.
3) We have added “addresses” after “E-mail”.
4) We have removed the hyphens and added colons between the authors’ names and authors’ e-mail addresses.
5) We have removed the semicolons at the end of the authors’ e-mail addresses and then listed the author’s e-mail addresses in separate lines.
6) We have removed the details of the corresponding author and first author as follows.

List of deletions:
The following authors’ addresses have been deleted “Title” page from the original manuscript.

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In the “References” section of the revised manuscript:
We have removed “et al.” from the original manuscript and added all the authors’ names in the “References” section in the revised manuscript as follows.

1) Reference 6: page 25, line 17 to page 18, line 3

2) Reference 7: page 26, lines 4 to 8

3) Reference 9: page 26, lines 12 to 16
   The reference has been already published in the Cancer, so we have removed “in press”, and added published Yere, Volume and pages.

4) Reference 17: page 28, lines 8 to 12

5) Reference 20: page 29, lines 2 to 5

6) Reference 22: page 29, lines 8 to 12
   Ullrich RT, Zander T, Neumaier B, Koker M, Shimamura T, Waerzeggers Y,
Borgman CL, Tawadros S, Li H, Sos ML, Backes H, Shapiro GI, Wolf J, Jacobs AH, Thomas RK, Winkeler A:

7) Reference 40: page 33, lines 11 to 15
   Wagner M, Seitz U, Buck A, Neumaier B, Schultheiss S, Bangerter M, Bommer M, Leithäuser F, Wawra E, Munzert G, Reske SN:

8) Reference 42: page 34, lines 1 to 5
   Herrmann K, Wieder HA, Buck AK, Schöffel M, Krause BJ, Fend F, Schuster T, Meyer zum Büschenfelde C, Wester HJ, Duyster J, Peschel C, Schwaiger M, Dechow T:

We thank the reviewers for their insightful comments and useful suggestions that have helped us improve our manuscript.