Peer Review File

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Reviewer A

In this article entitled, "ZR-75-1 breast cancer models to study the utility of 18F-FES by PET imaging" the authors evaluate the breast tumor imaging potential of the xenograft ZR-75-1 because this is an ER+PR+ cell line. In contrast, most studies evaluating 18F-FES utilize the MCF7 cell line, which is ER+PR-. Thus, ZR-75-1 is more reflective of the clinical scenario and, hence, imaging this tumor could be more reflective of the capabilities for 18F-FES to diagnose breast cancer tumors.

The studies are all straight forward and the data of good quality.

Weakness. The Discussion requires major reworking. There is no discussion of how the results compare with the literature that evaluated MCF7 tumors. You must have a comparative discussion if you are to use the word "model" in the title. In addition, there should be discussion with how the results compare with other investigation 18F-FES derivatives/tracers, since there are reports now in existence.

Reply reviewer A: Thank you very much for your advice. This is indeed our weakness. We have made the positive control using MCF-7 modes in our experiments. According to your suggestion, we add this part of the experiment into the article and further modify the discussion part. Thanks again.
Changes in the text: see Page 8,lines 146-148.Page 16,lines 206-210.Pages 17-18,lines 294-302.

Reviewer B

This manuscript aims to evaluate the evaluate the potential of 18F-FES as an imaging agent for the in vivo monitoring of the estrogen receptor (ER) expression and its potential predictive value in a model of ZR-75-1 human ductal carcinoma. The authors have used Fulvestrant, a well-known steroidal estrogen antagonist binding to the estrogen receptor thereby resulting in decreased estrogen biding, to prove that 18F-FES radiotracer can be used to reliably predict the efficacy of endocrine therapy. The reviewer believes the topic has been thoroughly introduced by the authors and enough background has been provided in order to put into context the conducted experimental work. Some of the limitations of the presented study have been identified by the authors and acknowledged in the manuscript.

Below are comments to the manuscript and points the reviewer believes need to be addressed:

1. In this study 40 mice where subcutaneously injected with ZR-75-1 cancer cells, but only 6 were used for the in vivo and ex vivo evaluation of the 18F-FES radiotracer and a total. of 10 mice were used for the competitive inhibition study. How have been used the remaining 24 animals in this study?

Reply 1：

Thank you very much for pointing out this mistake. After checking the experimental records, we found that 15 mice should not be included in the experiment. The mice used in the experiment were continuously marked, 40 mice were directly written according to the final number. We initially purchased 15 mice for experiment.However, some mice in the animal center were suspected to be infected with norovirus, so the animal breeder suggested that the mice should be executed. After the animal room was completely disinfected, we completed the experiment finally.

After perfecting the experimental scheme, 22 mice were actually used in the experiment, which has been corrected in this paper.Among them,12 mice were randomly divided into two groups and inoculated with ZR-75-1 cell line and MCF-7 cell line respectively. ZR-75-1 breast cancer-bearing mice were continued to carry out ex vivo experiments.Another 10 mice were randomly divided into two groups for competitive inhibition test. In order to ensure the progress of the experiment, we prepared three more mice than planned.

Changes in the text: see Page 6, line 110.

2. Add settings of CT in the sentence "..and the scanning energy window was…": tube voltage, exposure time and projections. The missing information is the time between radiotracer administration and PET-CT.

Reply 2：The missing information has been supplemented.

Changes in the text: Page 8, lines 136-138.

3. The reviewer thinks it would be beneficial to display maximum intensity projections of the imaged mice in order to better interpret the tracer distribution over time.

Reply 3: The figures have been replaced by maximum intensity projections.

Changes in the text: see Page 15, Fig.3.

4. The uptake in the tumor is very low, even at later time points (similar to areas where the there should not be any radiotracer uptake, i.e. joints, shinbone, muscle, spleen). How can you explain this?

Reply 4：The uptake values of various organs at 1 hour after ^{18}F-FES injection in our study were consistent with previous study(P=0.183).In which,^{18}F-FES uptake of tumors,bone and spleen were 3.12±0.31,0.83±0.08,1.05±0.12 respectively[1].^{18}F-FES was metabolized rapidly in the blood and reached the peak in 20-30 min after

injection[2]. Therefore, the uptake value of rich blood supply organs ,such as spleen and muscle, were similar to that of ER + tumor in the early stage.Then it decreased gradually and was significantly lower than the tumor uptake at 60min.Besides,the target-to-muscle ratio at 1 hour was 3.92 ±1.93, second only to the study by Dr.Amy M reported(T/M=4.5)[3].All these can prove that ^{18}F-FES is high-targeted.According to Somponpun's research [4] , the abundant ER in bone is related to the growth and development of mice, so joints and shinbone could take up ^{18}F-FES.

References：

[1].Bénard F, Ahmed N, Beauregard JM, et al. [18F]Fluorinated estradiol derivatives for oestrogen receptor imaging: impact of substituents, formulation and specific activity on the biodistribution in breast tumour-bearing mice[J]. Eur J Nucl Med Mol Imaging. 2008 Aug;35(8):1473-9.

[2].Kiesewetter D O, Kilbourn M R, Landvatter S W, et al. Preparation of four fluorine-18-labeled estrogens and their selective uptakes in target tissues of immature rats[J]. J Nucl Med, 1984,25(11):1212-1221.

[3].Fowler AM, Chan SR, Sharp TL, et al. Small-animal PET of steroid hormone receptors predicts tumor response to endocrine therapy using a preclinical model of breast cancer[J]. J Nucl Med. 2012 Jul;53(7):1119-26.

[4]Somponpun S J, Sladek C D. Osmotic regulation of estrogen receptor-beta in rat vasopressin and oxytocin neurons[J]. J Neurosci, 2003,23(10):4261-4269.

5. On the same note, the authors have not provided any in vitro functional characterization of the cells (radioactive uptake study) to convince the reviewer that the chosen ZR-75-1 breast cancer line does indeed uptake 18F-FES radiotracer. Also, please show the expression levels of ER in your ZR-71-5 cells compared to control lines. Both of the above aspects need to be addressed as they provide a rational to conduct the in vivo study.

Reply 5: Thank you for pointing out our shortcomings.The company that bought the cell line has provided us with authentication report. The cell lines were completely consistent with cell lines of ATCC by DNA comparison(see attachment).In our study, the expression rate of ER in ZR-75-1 cancer cells detected by immunohistochemistry was about 76%. The targeting of FES was proved by inhibition experiment. Besides, gamma count also confirmed ER expression in tumor. Therefore, the expression of ER in ZR-75-1 cells was evaluated both at the level of tissue and cell.

6. The in vivo study is lacking the right controls. In fact, the authors do not show the in vivo distribution of the chosen radiotracer in control mice: (i) non-tumor-bearing and ER- breast cancer model as negative controls; (iii) ER+ tumor as a positive control.

Reply 6: Thank you for your question. This is a very professional advice. We have made the positive control using MCF-7 modes in our experiments. According to your suggestion, we add this part of the experiment into the article and further modify the discussion part.

The purpose of this paper is to make a systematic study on ^{18}F-FES before clinical application in our center. According to the relevant research conducted by Fudan University Shanghai Cancer Center in 2011, the metabolic distribution of normal rats, ER- and ER + tumor bearing mice was basically the same except for tumor tissues. Therefore, we used ER + tumor bearing mice to study the biodistribution of ^{18}F-FES referring to the experience of their. Comparing the results of this paper with the literature, the biodistribution of FES in ZR-75-1 mice were consistent with that of normal rats one hour after injection（P=0.128)[1].Besides, the biodistribution of FES in ZR-75-1 mice were also consistent with that of ER- tumor bearing mice(P=0.314)[2].

References：

[1]YANG Zhong-yi, WANG Ming-wei, ZHANG Yong-ping,et al.The biodistribution and imaging of 16α-[18F]fluoroestradiol (18F-FES) in rats and breast tumor-bearing nude mice[J],Shanghai Medical Imaging, 2011,20(3):234-237.

[2]Aliaga A, Rousseau JA, Ouellette R, et al. Breast cancer models to study the expression of estrogen receptors with small animal PET imaging[J]. Nucl Med Biol. 2004 Aug;31(6):761-70.

Changes in the text: see Page 7,lines 133-136.Page 10,lines 191-199.Page 15,lines 275-281.

7. The competitive inhibition results in Fig.2 need to be linked to text. More importantly, this figure is poorly generated, with no labels/info embedded to help the reader understand what he is looking at. MIPs are better suited in order to properly visualize the differences between mice before and after Fulvestran treatment(s). No proper quantification and related statistical analysis is shown in support of the author's claim that the treatment with fulvestran in this model is responsible for a statistically significant reduction of 18F-FES tumor uptake. This needs to be provided.

Reply 7: The figure has been replaced.The results of statistical analysis have been added to the article.

Changes in the text: see Page13, Fig.2. Pages 12,line 222.

8. In Fig.3 please display both in vivo quantification and ex vivo quantification as two separate graphs and the ex vivo quantification needs to be expressed in standard uptake value (SUV).

Reply 8: According to your professional suggestion, the original fig.3 didn't reflect the data very well, so we delete it.

$$\text{SUV}=\frac{Radioactivity\ in\ the\ region\ of\ interest(MBq/ml)}{Injection\ dose\ \ (MBq)\ /Body\ weight(Kg)},$$

$$\%\text{ID/g} = \frac{\text{Activity concentration in the region of interest}(\mu\text{Ci/g})}{\text{Injection dose}(\mu\text{Ci})}\times 100\%$$

the weight in SUV calculation is measured in 'kg", but the weight of the mice used in the experiment is lighter, and the error expressed by Kg is larger, so the SUV value is not used.

9.Please comment on the uptake in both uterus and ovaries adding to the %ID/g values the biological explanation of why this is seen in vivo.
Reply 9: FES is mainly metabolized by liver and intestine, so the uptake value in abdominal and pelvic cavity of mice is higher. The uterus and ovary are so small to be delineated under the background of high uptake in pelvic, so their radioactivity uptake can not be measured.The uptake values of uterus and ovary were measured by γ counter ex vivo:3.96±1.39，2.56±1.39%ID/g,which were similar with the study of Masayuki S.(uterus:3.34±0.39%ID/g)[1].That's because the uterus is a tissue rich in estrogen receptors.
Reference:
[1]Sasaki M, Fukumura T, Kuwabara Y, et al. Biodistribution and breast tumor uptake of 16alpha-[18F]-fluoro-17beta-estradiol in rat[J]. Ann Nucl Med. 2000 Apr;14(2):127-30.

10. Authors often provide irrelevant details (in particular in the Materials and Methods section, which is very lengthily) to the reader or discuss too extensively about them (for details, please refer to the section named “Minor corrections”). I encourage the authors to scrutinize the manuscript and aim to communicate both information and concepts in a clear and concise manner in order to meet the expectations of a scientific paper. Also, the manuscript would benefit from being reviewed/corrected by an English native speaker.
Reply 10: We have modified our test as advised.

11. Where applicable, remove subsections. Example: subsections “gamma counter”, “immunohistochemistry” etc. are not needed and not found in any scientific papers. All of these are part of the ex vivo tissue analyses section. If authors wish, they can keep “Immunohistochemistry” etc. but not as subsections (therefore “Ex vivo assay” need to be removed). The same thing applies also to the results section, where a number of subsections has been generated. Please address this.
Reply 11: The part of gamma counting in this experiment was to confirm the bioditribution of FES ex vivo. We have put it in a more appropriate position to make it easier to understand. In addition to hollow organs, gamma counting in vitro is accurate in some solid organs such as liver and spleen.Masayuki S[1], Antonio A[2],et al all carried out ex vivo experiments.
Reference:
[1]Sasaki M, Fukumura T, Kuwabara Y, et al. Biodistribution and breast tumor uptake of 16alpha-[18F]-fluoro-17beta-estradiol in rat[J]. Ann Nucl Med. 2000 Apr;14(2):127-30.
[2]Aliaga A, Rousseau JA, Ouellette R, et al. Breast cancer models to study the expression of estrogen receptors with small animal PET imaging[J]. Nucl Med Biol. 2004 Aug;31(6):761-70.

Changes in the text: see Page 8,lines 147-150.Page 13,lines 241-247.

12. In the discussion references need to be provided. For example, when commenting on liver and kidneys uptake, the authors say "…it was also observed in previous reports" but do not provide any referencing to that.
Reply 12：The relevant literature has been added.
Changes in the text: see Ref.25.

13. There is no list of funding sources/grants supporting the study.
Reply 13: Please refer to the Acknowledgments part for funding sources.

Minor corrections:
1. In the background section, please add more recent statistics and corresponding reference when discussing the number of breast cancer cases worldwide.
Reply 1: We added a new reference as suggested.
Changes in the text: see Ref.2.

2. Add an "s" to the word "cancer" in the sentence starting with "Estrogen receptor (ER),…".
Reply 2: We have added an "s" to the word "cancer".
Changes in the text: see Page 4, line 73.

3. Please delete the adjective "mature" to the sentence starting with "It plays a key role…" as it is non-scientific.
Reply 3:We deleted the adjective "mature"as advised.
Changes in the text: see Page 4, line 73.

4. Reformulate the sentence "It is sometimes not practical…". The limitations of the biopsy as a diagnostic and therapy assessment tool need to be stated in a more concise and clear manner
Reply 4: We have modified our test as advised.
Changes in the text: see Page 5, lines 79-80.

5. In the sentence "There is significant heterogeneity due to different measurement methods…", do provide original references.
Reply 5: We have modified our test as advised.
Changes in the text: see Ref.7.

6. Correct "Ecology" to "Oncology" in the sentence starting with "The American Society…".
Reply 6:We have corrected "Ecology" to "Oncology".
Changes in the text: see Page 5, line 84.

7. Provide original references of the studies involving 18F-FES as an imaging agent for ER+ tumors detection in the sentence starting with “In line with several studies…”. Referencing a review is not sufficient. Also, provide original reference from which the sensitivity and specificity of the above tracer have been taken from.
Reply 7: We have modified our test as advised.
Changes in the text: see Page 5, lines 93-94.

8. Remove “As far as we know” as this cannot be accepted in a scientific publication.
Reply 8: We have modified our test as advised.
Changes in the text: see Page 5, line 95.

9. Reformulate the sentence “ZR-75-1 cells have not been used as commonly as…” in a more concise manner, highlighting the clinical relevance of the ZR-75-1 model.
Reply 9: We have changed this expression as advised.
Changes in the text: see Pages 5-6, lines 95-96.

10. Do not use the formula “according to cancer statistics” and provide reference of the original work or source. The formula is also repeated several times throughout the manuscript and needs to be removed.
Reply 10: We have removed the sentence and provided reference of the original source.
Changes in the text: see Page 6, lines 96-97 and Ref.13.

11. In the Materials and Methods section, remove “Finally”.
Reply 11: We have removed “Finally”.

12. Add “were” to the sentence starting with “The remaining mice…”.
Reply 12:We have added “were” to the sentence starting with “The remaining mice…”.
Changes in the text: see Page 7, line120.

13. In the subsection “Micro-PET/CT imaging”, state the name of the center/facility in which the radiosynthesis has been carried out.
Reply 13: We have added the name of the center in our text.
Changes in the text: see Page 7, lines 123-124.

14. Remove “completed” and use “performed” or synonyms.
Reply 14: We have used “performed” as advised.
Changes in the text: see Page 7, line 130.

15. Correct “tail veil”. With “tail vein”.
Reply 15: We have corrected the wrong spelling.
Changes in the text: see Page 7, line 132.

16. Correct sentence “Each bed was scanned statically…” with “Mice were scanned…”.
Reply 16: We have modified our test as advised.
Changes in the text: see Page 8, line 136.

17. In the subsection 2.4 “Biodistribution of 18F-FES remove “manual” and reformulate the sentence. The information on waiting time between administration of radiotracer and PET-CT needs to be added in its relevant section (microPET-CT imaging).
Reply 17: We have modified our test as advised.
Changes in the text: see Page 9, lines 158-159.

18. Be consistent in writing the radiotracer name (keep 18 in superscript).
Reply 18: We have modified our test as advised.

19. The sentence “The gamma counter under evaluation…” does not have a scientific meaning and takes unnecessary space. It is sufficient to include the name of the gamma counter instrument in brackets in the sentence “The radioactivity of the samples was determined…”.
Reply 19: We have modified our test as advised.
Changes in the text: see Page 9, lines 162-163.

20. In the subsection “Immunohistochemistry” the authors use too many words to express simple info/concepts (example: “…Then these tissue blocks were embedded….”. Reformulate the sentences in a clear and condensed manner. Similar issues are throughout the whole manuscript and need to be addressed by the authors.
Reply 20: We have modified our test as advised.
Changes in the text: see Pages 9-10, lines 165-172.

21. Remove “the color was developed…” as this is not a scientific term.
Reply 21: We have removed “the color was developed…” as advised.

22. Again, the sentence “Then the samples were observed under a microscope” is not needed and information can be delivered in a more succinct and scientific way. Authors did not include the relevant information about the microscope/objective/software used.
Reply 22: We have removed the redundant sentences.
Changes in the text: see Page 10, lines 169-170.

23. In “Radiation dose estimation in the human body”, correct “Images obtained from biodistribution of…” as images have been generated from performing PET-CT imaging and not biodistribution analysis. The sentence as it stands is very confusing.
Reply 23: We have removed this inappropriate expression.

Changes in the text: see Page 10, line 178.

24. In the sentence “The remainder organ retention time…” 2.62 is hours/mins? Specify.
Reply 24: We have added the unit “MBq.h/MBq”.
Changes in the text: see Page 10, line 185.

25. Reformulate the sentence “Paired t test were used to compare the radiation dose in animal models and organs measured directly in human” as this does not make sense.
Reply 25: We have reformulated the sentence as advised.
Changes in the text: see Page 11, lines 199-200.

26. More concise regarding the software used to perform the statistical analysis. “SPSS version…was applied…” is not grammatically correct.
Reply 26: We have modified our test as advised.
Changes in the text: see Page 11, lines 200-201.

27. In “Results” were used to conduct the study. How have the remaining 24 mice been used? (see 2. in Major comments)
Reply 27: Please see reply 2 in Major comments.

28. Remove “especially soon after the injection” and be more specific giving the corresponding time point post tracer injection. Remove any generic comment present in the manuscript.
Reply 28: We have modified our test as advised.
Changes in the text: see Page 13, line 235.

29. What do you mean in Fig.1 with %ID/g-mean? Show %ID/g values.
Reply 29: Thank you for your advice. This error has been corrected.

30. The authors need to provide original references to their claims and not just redirecting to reviews.
Reply 30: We have modified our test as advised.

31. In the Abstract the authors write: “…Increasing the concentration of drugs or the number of transferred cells in tumor sites has been one of the primary goals. To address this issue,…”. The term “issue” is here being used inappropriately. Please replace with “these challenges”.
Reply 31: Sorry, we didn't find this sentence in the abstract. According to the suggestion, we have polished the abstract.
Changes in the text: see Pages 3-4, lines 39-64.

32. In “Radiation dose in human body” the authors say “The retention time…was relatively long…”. Please do not use it as it is inaccurate/generic comment. Please provide only specifics on how long.
Reply 32: We have removed this inaccurate comment.
Changes in the text: see Pagess 16-17, lines 279-280.