Peer Review File

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Reviewer Comments

In this investigation, the authors aim at analysing the predictive value of FEN1 expression for tamoxifen resistant BC. FEN1 is an endonuclease involved in genome stability and therefore regarded as a tumour suppressor. In addition, FEN1 is supposed to interact with the oestrogen receptor. The authors have already published a set of papers on FEN1 and now add this investigation on FEN1importance for Tam-resistance.

Acquired Tam resistance is an important clinical problem and the identification of predictive biomarkers for this condition could greatly improve treatment. Therefore, this manuscript deals with a relevant topic for this journal. In addition, the authors investigated this topic from several sides, mRNA, protein and cell culture experiments.

Altogether I think this is paper can be published when a few points are clarified.

Several points are just the result of unclear description of the methods.

Comment 1: KM-plotter was used to perform a survival analysis. Second gene array analysis was done and GEO datasets analysed. All mRNA data point out that FEN1 mRNA abundance is important for survival, especially in ER-positive cases treated with tamoxifen. What was the cutoff for high and low expression - looks like the median was used, but this has never been stated. Cut off optimisation could lead to even data that are even more convincing. I had a short look at the GEO dataset. As it is not clearly stated on the NCBI web site, how was a Tam-relapse identified/defined?

Reply 1: Excellent questions. The cutoff for high and low expression of FEN1 mRNA was

the median values of mRNA expression levels (Please see Page 5, line 1-2). GSE9195 was the dataset of primary breast tumors patients treated by tamoxifen in adjuvant setting. We downloaded the relevant data from the GEO website, which is a complete data of 77 clinical information. We defined Tam-resistance (Figure 2A) as disease recurrence (N=13), and Tam-sensitive (Figure 2A) as disease-free recurrence (N=64).

Comment 2: A small cohort of 65 patients were analysed by immune-histochemistry. Again FEN1 high (separated at the median again, as it looks) was significantly correlated with survival in Kaplan-Meier, or uni- and multivariate cox regression. Here, I find it quite surprising that lymph node metastasis was not significant; in many if not most published cohorts this is the most significant factor. On the other hand, FEN1 positively correlated with lymph-node infiltration. Could you provide a Kaplan Meier analysis for FEN1 hi/lo with the stratum lymph node infiltration?

Reply 2: We appreciate your helpful comments and thank you for comments. The patients we enrolled are indeed small cohort and single-center data, but for the data with a median follow-up time of more than 152 months, we think it better reflects the prognosis of ER-positive early breast cancer patients receiving endocrine therapy. The results of previous large-scale clinical studies （such as SOFT and TEXT trial）on endocrine therapy for ER-positive early breast cancer tell us that for this part of patients, if adjuvant endocrine is effective, even if regional lymph node metastasis (especially N1), the risk of recurrence is still very low, but for patients with failure/resistance of adjuvant endocrine therapy, even with N0, still have a 15-20% risk of recurrence (1-3).

According to the updated meta-analyses of randomized trials of the efficacy of adjuvant tamoxifen, 5 years of tamoxifen for HR-positive disease (n=10,645) safely reduces 15-year risks of breast cancer recurrence [rate ratio (RR) 0.61, 95% confidence interval (CI): 0.57–0.65] and breast cancer death (RR 0.70, 95% CI: 0.64–0.75), regardless of quantitative ER and PR measurement, dose of tamoxifen, use of chemotherapy, entry age, nodal status, tumor differentiation, and diameter and site of first recurrence (4). The results of this study are also consistent with the results of previous clinical trials.

Furthermore, we agree with your excellent suggestions. However, due to the limited sample size of the study itself, it is impossible to conduct further stratified analysis. I hope to further explore in future research. I would like to thank the reviewer again for his guidance.

1. Swain SM, Jeong JH, Geyer CE Jr, et al. Longer therapy, iatrogenic amenorrhea, and survival in early breast cancer. N Engl J Med 2010;362:2053-65.

2. Francis PA, Pagani O, Fleming GF, et al. Tailoring Adjuvant Endocrine Therapy for Premenopausal Breast Cancer.; SOFT and TEXT Investigators and the International Breast Cancer Study Group. N Engl J Med 2018;379:122-37.

3. Absolute Improvements in Freedom From Distant Recurrence to Tailor Adjuvant Endocrine Therapies for Premenopausal Women: Results From TEXT and SOFT. J Clin Oncol 2020;38:1293-303.

4. Early Breast Cancer Trialists' Collaborative Group (EBCTCG). Aromatase inhibitors versus tamoxifen in early breast cancer: patient-level meta-analysis of the randomised trials. Lancet 2015;386:1341-52.

Comment 3: FEN1 KO was done by "siRNA" (method section) and further analysed by gene-array. However later, only lentiviral transfection is described. Please provide details on the vectors used. Otherwise, the experiments are hard to understand. See below my remark on GFP and band size.

Reply 3: Please forgive this mistake. The problems about the reagents and control that you mentioned are rigorous requirements. We have added content to address the existing deficiencies, and the revised content is marked in red in the manuscript (Please see Page 7, line 19; Page 8, line 3-10, 13-15). Thank you for reviewing our manuscript carefully.

Changes in the text: We have supplemented the corresponding parts in method section: Small interfering RNAs (siRNAs) for FEN1 ordered from RiboBio Company (Guangzhou, China). The target sequence of FEN1 was 5'-GGGTCAAGAGGCTGAGTAA-3' (sense), 5'-UUACUCAGCCUCUUGACCCdTdT-3'(anti-sense), and negative control: 5'-UUCUCCGAACGUGUCACGUtt-3'(sense),5'-ACGUGACACGUUCGGAGAAtt-

3'(anti-sense). The siRNAs (100 nM) were transfected into cells using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Seventy-two hours after transfection, cells were harvested for the subsequent experiments. In brief, the lentiviral vectors LV-GFP-FEN1-RNAi, LV-GFP-FEN1-3FLAG and empty vector controls were synthesized (Genechem Company). The target sequence of FEN1 was the same as before. (Please see Page 7, line 19; Page 8, line 3-10, 13-15)

Comment 4: Fig. 3A, C: What does the double band for FEN1 mean; In OE it seems to be a GFP fusion? But what about non-transfected T47D – in 3C the smaller band is gone. Could you please provide the molecular mass of the bands on the blots?! Is this antibody specific? If not, there could be a problem with the immuno-histochemistry. How did you prove the specificity?

Reply 4: Thanks for your excellent comment. We have provided information to explain the double band for FEN1 mean, which were endogenous and exogenously expressed FEN1 in the manuscript. When OE-FEN1, the band above 43kDa (about 60 kDa) represents exogenous FEN1 expression. The double band for FEN1 in non-transfected T47D may be a cleavage band caused by protein degradation, rather than a double band in the true sense. The first antibody of FEN1 from GeneTex (San Antonio, TX) has been used in many studies (5, 6) and we believe that the antibody is specific. In addition, we have supplied molecular weight data in the Western blot shown in Figure 4 and Figure 6. The IHC antibody is an antibody that has been reported many times in the previous papers (7, 8). We believe that the antibody is specific.

5. Keqiang Zhang, Sawa Keymeulen, Rebecca Nelson, et al. Overexpression of Flap Endonuclease 1 Correlates with Enhanced Proliferation and Poor Prognosis of Non–Small-Cell Lung Cancer. Am J Pathol. 2018 Jan; 188(1): 242–251.

6. Lingfeng He, Libo Luo, Hong Zhu, et al. FEN1 promotes tumor progression and confers cisplatin resistance in non-small-cell lung cancer. Mol Oncol. 2017 Jun; 11(6): 640–654.

7. He L, Zhang Y, Sun H, et al. Targeting DNA Flap Endonuclease 1 to Impede Breast Cancer Progression, EBioMedicine. 2016 Dec; 14: 32–43.

8. Jianwei Wang, Lina Zhou, Zhi Li, et al. YY1 suppresses FEN1 over-expression and drug resistance in breast cancer. BMC Cancer. 2015; 15: 50.

Comment 5: Do not call the “relative MTT signal” a proliferation rate.

Reply 5: We appreciate your suggestions. We deleted the content about "The proliferation rate in each group was calculated" in the original text and added the context of cell viability.

Changes in the text: Deleted the content about "The proliferation rate in each group was calculated" (Page 9, line 6-8) in the original text. Added the context about "The percentage of cell viability was calculated" (Page 9, line6-8).

Comment 6: The 4OH-Tam concentrations are too high. It would be better to include concentrations down to 1nM.

Reply 6: Excellent questions. As you said, in vitro experiments such as MTT, the choice of tamoxifen concentration is very important. By referring to the previous papers on tamoxifen resistance (9-13), we selected such three more commonly used and classic tamoxifen concentrations, of which the 1uM concentration corresponds to the clinical pharmacological concentration.

9. Jian Liu Xiang Li Meng Wang, et al. A miR-26a/E2F7 feedback loop contributes to tamoxifen resistance in ER-positive breast cancer. Int J Oncol. 2018 Oct;53(4):1601-1612.

10. Yue Xue,Wenwen Lian,Jiaqi Zhi, et al. HDAC5-mediated deacetylation and nuclear localisation of SOX9 is critical for tamoxifen resistance in breast cancer. Br J Cancer. 2019 Dec;121(12):1039-1049.

11. Ang Gao, Tonghua Sun, Gui Ma, et al. LEM4 confers tamoxifen resistance to breast cancer cells by activating cyclin D-CDK4/6-Rb and ERα pathway. Nat Commun. 2018 Oct 9;9(1):4180.

12. Yanming Wu, Zhao Zhang, Mauro E Cenciarini, et al. Tamoxifen Resistance in Breast Cancer Is Regulated by the EZH2–ERα–GREB1 Transcriptional Axis. Cancer Res.

2018 Feb 1;78(3):671-684.

13. Yinghua Zhu, Yujie Liu, Chao Zhang, et al. Tamoxifen-resistant breast cancer cells are resistant to DNA-damaging chemotherapy because of upregulated BARD1 and BRCA1. Nat Commun. 2018 Apr 23;9(1):1595.

Comment 7: Fig. 3 B: I do not really understand this experiment. Please make it clear in this manuscript, why GFP is expressed after transfection.

Reply 7: Thanks for your excellent comment. The lentiviral vectors LV-GFP-FEN1-RNAi, LV-GFP-FEN1-3FLAG and empty vector controls were synthesized (Genechem Company).To estimate transfection efficiency, our experiments utilize LV-GFP-FEN1-RNAi, LV-GFP-FEN1-3FLAG and LV-GFP-NC in which GFP is expressed as a fusion. The percent GFP-positive cells is determined by florescent microscopy 120 h after transfection. Poor transfection can result in low translocation efficiency. Test several programs to optimize the transfection efficiency for each cell line. The details have been shown in the revised manuscript (In Method section, Page 8, line 15-21).