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MEDICINE

Epigenetic activation of the prostaglandin receptor EP4 promotes resistance to endocrine therapy for breast cancer

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INTRODUCTION

In the manuscript titled 'Epigenetic activation of the prostaglandin receptor EP4 promotes resistance to endocrine therapy for breast cancer' Jeffrey F. Hiken et al investigated the changes in methylation in the breast cancer cell genome upon acquisition of endocrine therapy resistance, and successfully identified one potential target gene, PTGER4, encoding prostaglandin E2 receptor 4 (EP4) for treating aromatase inhibitor (AI) resistant breast cancer. The authors in this study have reported a global decrease in methylation in their model of AI resistant breast cancer, the long term estrogen deprived MCF7 cells (MCF7-LTED cells). Along with the global hypo-methylation in MCF7-LTED cells the authors noticed significant changes in expression levels of number of genes. They mapped and correlated these expression changes with variations in methylation, and PTGER4 was identified to be overexpressed in MCF7-LTED cells owing to significant hypo-methylation in its promoter region. EP4 overexpression in MCF7-LTED cells was validated and it was hypothesized that EP4 expression is necessary for the proliferation of LTED cells. The hypothesis was tested and it was found that estrogen independent proliferation in MCF7-LTED cells was indeed dependent on EP4, as knockdown of EP4 by specific si-RNAs and blocking of EP4 by specific inhibitors reduced the proliferative rate of MCF7-LTED cells, but failed to influence parental MCF7 cells. The authors extended their study to investigate the survival pathway influenced by overexpression of EP4 and discovered that EP4 drives the cellular proliferation through Adenylyl Cyclase-cAMP-Protein Kinase A signaling pathway.In addition, the authors showed that EP4 mediated PKA activation leads to EP4 activity-dependent association of CARM1, a coactivator of Estrogen Receptor alpha (ERα), with ER α and PKA, and that both ERα and CARM1 show increased binding to promoters of Estrogen responsive genes pS2 and PGR in the presence of an EP4 agonist in MCF7-LTED cells. Finally, the authors validated the clinical relevance of their study by comparing the EP4 methylation and expression status between the AI responders and non-responders (pre- and post-treatment) among 104 samples and also in two other sample sets. The expression of EP4 was increased in the non-responders compared to responders and was also found to be increased after AI regimen treatment. Thus, this study establishes the significance of EP4 overexpression in promoting resistance to endocrine therapy, and identifies a potential mechanism through association through CARM1. Though the experiments in the study were carefully designed and the manuscript at its present state does justice to the work performed, we while reviewing the

article came to the conclusion that inclusion of additional information and minor changes in arrangement will further improve the manuscript and will make it easier to follow for the readers. Below we list some suggestions that we have for the authors of the article.

MAJOR COMMENTS

· Would it be possible to check for EP4 status in other estrogen dependent cell lines after long term estrogen deprivation? An increased expression of EP4 as in MCF7-LTED in additional cancer lines would further consolidate the claims made in this study.

· In the section *'LTED Cells Up-regulate ERα response genes and Potential Resistance genes'* the authors report that expression of WISP2 is decreased in MCF7-LTED cells due to hyper-methylation in its promoter. WISP2 is an estrogen dependent gene, so, it is possible that estrogen-starvation would downregulate the Wisp2 expression regardless of methylation. Though the authors report an estrogen independent ER activity, showing the constitutive downregulation of WISP2 in MCF7-LTED cells in presence and absence of estrogen would be helpful.

· An ontology pathway analysis of upregulated genes or references to support the section title 'LTED Cells Up-regulate ERα response genes and Potential Resistance genes' would be helpful.

· In figure 3b, the authors stated that they *'applied the EP4 specific agonist L-902,688 to MCF7-LTED cells and observed a dramatic increase in cAMP which went away upon inhibition of EP4 with antagonists (Fig. 3b)*' which led us to interpret that antagonists were added after agonist mediated cAMP increase, but the opposite was outlined in the methods with respect to order of agonist/antagonist application. The methods section states *'For combined antagonist/agonist treatments, cells were pre-treated 10 min with ONO-AE3-208 before addition of L-902,688'*. This discrepancy might lead to confusion while trying to understand the particular experiment. As the main aim of the experiment was to prove that EP4 works through the Adenylyl Cyclase-cAMP-PKA axis, the authors might also consider using other inhibitors of the pathway to check if it achieves a similar effect on MCF7-LTED proliferation.

· In the text of the section 'Functional analysis of EP4 signaling in MCF7-LTED cells' the authors mention, *'cAMP activates CARM1 via phosphorylation by PKA and drives ligand-independent ER transcriptional activity to promote resistance to tamoxifen*' and '*phosphorylated CARM1 binds directly to ERα*'. A blot against p-CARM1 in MCF7-LTED cells in the presence and absence of EP4 agonists and antagonists would strengthen this claim.

· The authors showed that EP4 mRNA level was increased in 74 AI inhibitor resistant samples, and we were curious if the authors have attempted to stain for EP4 in the tissue sections. If possible, immunostaining data comparing the AI resistant samples and the responders may strengthen the manuscript.

MINOR COMMENTS

· Though the establishment of estrogen independent MCF7 lines has been reported in other studies, this study was entirely dependent on the MCF7-LTED cell lines, and so a mention of the time duration of estrogen deprivation to generate MCF7-LTED line will be helpful for the readers. It will be important information for those who would want to carry out similar experiments.

· In Figure 1d, the immunofluorescence image does not have a scale bar or a mention of the magnification. We recommend including a scale bar-both in this figure and in Figure 2d.

· We suggest that Figure S2b be included with Figure 1d, as it shows an increase of EP4 expression at transcriptional level.

· FigureS4 was referred to in the text before Figure S2. A rearrangement of the supplementary figure sequence will make it easier for the readers to follow. In addition, Figure S4d and Figure S4e is missing in the figure legend, and including them is necessary for a complete figure.

· In Figure 2, AlamarBlue assays were performed after 7 days after siRNA knockdown of EP4. For additional clarification of their methods, the authors might consider including the time point at which the knockdown of EP4 level in cells was checked.

· In the second line of second paragraph of results section *'Functional analysis of EP4 signaling in MCF7-LTED cells'*, reference support is needed for the statement 'AC activity can induce ERα phosphorylation'.

· Figure 3d,e has been mistakenly cited as Figure 3c,d, in the text. Also reconstruction of the sentence *'We further found that ERα showed a strong interaction between CARM1 and ERα in MCF7-LTED cells based ……with an ERα-specific antibody (Fig. 3c)'* is needed.

· The authors mentioned that Fulvestrant was effective on MCF7-LTED cells. The authors might want to discuss why targeting EP4 would be a preferred therapeutic intervention for AI resistant tumors (driven by estrogen independent ER) over Fulvestrant?

· There was no mention of Figure S5 in the text. The author might want to include in discussion what might be the reason that there was no significant difference in EP4 level between responders and non-responders before treatment in the dataset shown by this figure.