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Answering reviewers

Reviewer 01940125:

This is a well-organized and well-written review that provides comprehensive information to address the clinical significance as well as potential pitfalls of ESR amplification in cancer research. Based on the thinking flow of the authors, the main purpose of this review is to discuss whether ESR a real cancer driver that could be utilized as a therapeutic target. From the point of functional genomics, a true cancer driver should be defined with biological relevance from genomic/post-genomic levels, translational/clinical correlation to functional validation. Therefore, it is highly recommended that the authors also include some important conclusions based on cellular functional assays. Then, the readers will be able to find the solution of the 25-year debate. Some minor points are as following 1. Labels in Table 1 are confusing: a. There is "ER- %" in this table, so how about "%"? dose not mean ER+, ESR amplification or others? b. Id "%" indicates ER+ frequency, why the sum of ER+ and ER- dose not equal 100. Some were undetectable? c. The data of Li 2013 should be removed since the data of both "%" and "ER-%" are not available. 2. "et al." in the text should be Italic.

a. I thank the Reviewer for this important recommendation. The functional cellular assays concerning *ESR1* amplification published are now reviewed in the manuscript and regarding conclusions are discussed in chapter *Response or Resistance*:

"Other tumors might amplify a gene specifically driven by the tumors addiction to the respective pathway [19]. Indeed, this mechanism has been suggested in two independent studies that observed focal *ESR1* amplifications of low-level copy number change in long-term estrogen-deprived (LTED) MCF7 breast cancer cell lines, with use of DNA-specific GeneChips and qPCR for *ESR1* copy number determination. And another experimental study showed that breast-cancer-derived xenografts respond to estrogen treatment of tumor cells that harbor *ESR1* amplification, as determined by NGS [84, 97].

Furthermore, in one clinical phase II study for evaluating anti-estrogen treatment, a focal *ESR1* amplification appeared after therapy in one out of 49 tumors analyzed by NGS [98]. These functional studies provide strong evidence for the potential clinical

relevance of *ESR1* amplification as a mechanism of ERα pathway regulation. And one additional study used LTED MCF7 cells to show a change of *ESR1* gene status detectable by FISH; however, the FISH signals were RNase-sensitive and no *ESR1* copy number increase was detectable by *ESR1* qPCR, suggesting that the FISH results may have been due to probe hybridization to abundant mRNA [99]."

b. Table 1 and its legend were revised for clarification.

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Reviewer 00742507:

An excellent review of an important topics. The literature is adequate. The only problem is in the lack of any conclusions. Future directions are important, but the review should end in some kind of conclusion, so the reader will understand which output to take with him after reading the paper.

a. I thank the Reviewer for highlighting the need of conclusions. One concluding remark is therefore concretized in the manuscript:

"The debate on *ESR1* amplification in breast cancer is mainly based on methodological issues, including technical limitations, quality of application, and interpretation of results using the standard methods that are available today. The controversy on the frequency of low level *ESR1* amplifications in particular, highlights the need for methodically advanced and sensitive approaches that will allow consistent findings."

Another important conclusion was also clarified:

"As such, the nature of the *ESR1* gene status on the level of nucleic acids (DNA or RNA) might appear to be of secondary importance when considering a reproducible phenomenon that has an established standard diagnostic method and that is potentially applicable as a clinical marker. [3]. In contrast, studies on the potential clinical significance of detectable phenomena seem to be rather reasonable. In this context, the robustness and predictive power of a clinically applicable marker may be more important than its molecular properties."

Reviewer 02104609:

The article needs to be revised for English. Considerable sentences are too long to be followed. The readers may get lost. Here is an example: "A comparison of two different reference genes (ESR2 versus SOD2) with similar deletion frequency (~30%) according to TCGA, display similar copy number ratio pattern of tumors, with and without ESR1 amplification determined by FISH, over cases, but a huge difference in dynamic range of approximately a dimension within samples, suggesting rather technical issues of PCR approaches to be responsible for differences in study outcome than deletion frequency of the qPCR reference gene [35] (supplementary tables S1+S2 and supplementary graphs S1 - S4)."

a. **Too long sentences were shortened throughout the entire manuscript.**

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Reviewer 00227350:

Well written Review.

a. **I thank the Reviewer for his comment.**

Reviewer 00289387:

The authors submitted a review article discussing the 25-year debate of estrogen receptor alpha gene amplification (ESR1) in breast cancer. This is an interesting topic that is directly associated with breast cancer diagnosis and hormonal therapy. Overall, the paper is well-organized and written with several beautiful images illustrated in figures. A few minor issues need to be addressed. 1)Table 1. ERa negative (%) presented in both correlation and no correlation studies needs to be clarified, what does it mean for ER- as low % exists in these two-type studies? 2)Is it possible to add any evidence showing the correlation between ESR1 amplification and worse clinical outcomes without any hormonal treatment in Fig 5? 3) The authors should discuss more about ESR1 as a marker for both hormone sensitivity and resistance. For example, the nature of the gene low cope number and the tumor tissue heterogeneity may be the key factors that are accounted for initial sensitiveness to anti-ERa agents. Then some or most of the tissue lack of ESR1 amplification develop tumors that are resistant to the drugs, displaying tumor resistance. 4) A few places of typos should be corrected.

1. Table 1 and its legend where revised for clarification.

2. The data shown in figure 6 (former 5) base on a study including only patients that received anti-estrogen treatment. Thus data for patients without hormonal treatment are not available for this study. However, a study showing worse outcome for patients with tumors that harbored *ESR1* amplification but did not receive hormonal treatment is discussed:

"Additionally, a qPCR study found the worst outcome for patients whose tumors are ERα-negative and have *ESR1* amplification, while there was no association between survival in ERα-positive cancers that received Tamoxifen treatment [94]."

3. I thank the reviewer for raising this point. The aspect of tumor heterogeneity is now also discussed in regard to therapy response and resistance as follows:

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"And while the threshold for therapy response was determined at a doubled gene dose in the case of *ERBB2*, amplifications of other genes (e.g. *EGFR*, *ERBB3* (HER3), and *PIK3CA* in lung cancer) might be relevant at lower levels [38, 69, 110-119]. This and even a tumor's heterogeneity regarding the amplification status of a gene, should be taken into account when considering gene amplification as a maker for therapy response or resistance."

4. Typos were corrected.