Peer Review File
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Reviewer Comments

Reviewer A

Comment 1: The article is very well written. It describes the tumor biology, prognosis, and treatment considerations that arose after the approval of T-Dxd for HER2 low breast cancers. However, I believe that the article could be further improved by paying close attention to certain details. Please find my detailed comments provided below.

Reply 1: Thank you very much for your comments and valuable suggestions.

Comment 2: As we have several treatment options including endocrine therapy, chemotherapy available in HR+ BC, and immunotherapy and chemotherapy in TNBC, mention the sequencing of these treatment options in HR+ and HR- breast cancer. PMID: 37349954

Reply 2: We sincerely appreciate your comment. As you suggested, we have added a discussion about the sequencing and cited the paper you mentioned (Please see Page 16, line 345 to line 352 and Figure 1). The added paragraph is as follows:

As mentioned above, there are several treatment options including endocrine therapy and chemotherapy available in HR+ BC, immunotherapy and chemotherapy in TNBC, and the ADCs in both HR+ and TNBC. Researchers and clinicians are devoted to proposing the optimal sequencing of these agents for maximum clinical benefit while maintaining the quality of life(32). With current evidence, a sequencing strategy for HR+ and HR- HER2-low MBC is recommended (Figure 1).

Comment 3: Prognosis section: Mention the difference in the prognosis of HER2-low tumors based on Oncotype Dx PMID: 37686540

Reply 3: Thank you for your suggestion. we have added discussion about the difference in the prognosis of HER2-low tumors based on Oncotype Dx and cited the paper you mentioned (Please see Page 7, line 145 to line 148). The added paragraph is as follows:

In addition, another study suggested that HER2-low expression was associated with

better survival in HR+ breast cancer patients with high Oncotype Dx RSs. Among patients who received adjuvant chemotherapy with a high Oncotype Dx RS (26–100), those with HER2-low tumors had higher survival. (11)

Comment 4: Minor spacing issues need to be corrected.

Reply 4: We are really sorry for our careless mistakes. Thank you for your reminder. In our resubmitted manuscript, the spacing issues were revised. Thank you for your correction.

Reviewer B

Comment 1: Lane 2: The proposed title ending "…several considerations" is vague. It needs some clarification on what considerations are going to be reviewed. There are more than 500 articles and about 80 reviews concerning HER2-low breast cancer published so far in 2023 based on PubMed. The new review should have a special focus to be of interest to the professional audience.

Reply 1: Thank you for your comment, we think this is an excellent suggestion. Therefore, we have corrected the title to "Challenges in HER2-Low Breast Cancer identification, Detection, and Treatment".

Comment 2: Lane 72: need to add HER3. The Human Epidermal Growth Factor Receptor (HER) family consists of four members: EGFR/ErbB1, HER2/ErbB2, HER3/ErbB3, and HER4/ErbB4.

Reply 2: We are really sorry for our careless mistake. Thank you for your correction. We have added HER3 in the resubmitted manuscript (Please see Page 4, line 86).

Comment 3: Lane 99-lane 108 paragraph belongs better to the "Detection of HER2-low advanced BC" section (see comment below).

Reply 3: We sincerely appreciate the valuable comment. As you suggested, we have moved the discussion about the impact of IHC assays to the "Detection of HER2-low advanced BC" (Please see Page 9, line 195 to line 204).

Comment 4: Lane 109: Need to revise “Evidence suggest that … “ to more specific, perhaps, “Early assessments suggested that HER2-low accounts for approximately 45%-55% of all BC cases”

Reply 4: Thank you very much for your careful review. We have corrected the expression into “Early assessments suggested that HER2-low accounts for approximately 45%-55% of all BC cases” (Please see Page 5, line 112).

Comment 5: Lane120: This section would benefit from an additional discussion on the recent publication ESMO Open, 2023, v8, issue 4. https://pubmed.ncbi.nlm.nih.gov/37413762/

Reply 5: We sincerely appreciate the valuable comment. we have added a discussion about the difference in the prognosis of HER2-low tumors and cited the paper you mentioned (Please see Page 7, line 148 to line 156). The added paragraph is as follows: A meta-analysis of 42 studies, which included 1,797,175 patients, indicated that HER2-low status appears to be associated with a slightly increased OS both in the advanced (HR 0.94, 95% CI 0.89-0.98, P = 0.008) and early settings (HR 0.90, 95% CI 0.85-0.95, P < 0.001), regardless of HR expression. In the early setting, HER2-low tumors seem to be associated to lower pCR rates, especially if HR-positive(14). Another meta-analysis, including 636,535 patients, also suggested that the HER2-low arm showed significantly improved results for DFS and OS. The hazard ratios for DFS and OS in the HR-positive group were 0.88 (95% CI 0.83-0.94) and 0.87 (95% CI 0.78-0.96), respectively. In the HR-negative group, the hazard ratios for DFS and OS were 0.87 (95% CI 0.79-0.97) and 0.86 (95% CI 0.84-0.89), respectively(15).

Comment 6: Lane 139- 140. The correct statement would be: “The biological characteristics of HER2 -low BC may or may not depend solely on HR expression”. Conclusions on a higher rate of HR+ in HER2 are inconsistent. For instance, no statistically significant difference was found in the ER+ cases in the HER2-low group compared to the HER2-zero group of invasive BC.

https://ascopubs.org/doi/abs/10.1200/CCI.23.00013

The same study showed that about two-thirds of BC classified as HER2-low, using current IHC/ISH criteria were expected to have normal breast tissue levels of HER2 expression based on ERBB2 expression.

Reply 6: We sincerely appreciate the valuable comment. We have corrected the statement (Please see Page 8, line 175 to line 176).

Comment 7: Lane 141: This section would benefit from a separate discussion of preanalytical variables and analytical variables affecting the detection of true HER2-low, i.e. tumors with HER2 levels higher than in normal tissue, but lower than in HER2 overexpressing cancers.

Reply 7: Thank you very much for your suggestion. we have added a discussion about the variables affecting the detection of true HER2-low (Please see Page 10, line 212 to line 216). The added paragraph is as follows:

Moreover, a study using mRNA expression data suggested that the HER2-low was a mix of tumors with reference-like (70%) and abnormally elevated (30%) expression levels of ERBB2(17). In the former cases, HER2 expression is expected to be at physiologic levels, whereas in the latter cases, HER2 expression may be increased through transcriptional mechanisms. This result reflects another reason why it is hard to distinguish HER2-low and HER2-0.

Comment 8: Lane 158: “Different antibody used will affect HER2 low detection…” It seems that the whole prior paragraph on how assays with different antibodies may affect patient classification on HER2, lane 99-lane 108 fits here better. Otherwise, this is a redundant sentence.

Reply 8: Thank you for your valuable comment. As you suggested, we have moved lane 99-108 to lane 158 (Please see Page 9, line 195 to 204).

Comment 9: Lane 165: need a reference on who has made such a suggestion. If this sentence refers to 14 this reference should be moved to the end of the sentence.

Reply 9: We are really sorry for our careless mistake. We have added a reference to the end of this sentence.

Comment 10: Lane 175- 176. This review would benefit from a discussion of the most recent ASCO/CAP updates and ESMO expert consensus statements (J.Clin. Oncol, 2023, 41:3867 and Ann Oncol. 2023 Aug;34(8):645-659.

Reply 10: Thank you for your valuable comment. We have added discussion (Please see Page 11, line 231 to 232). The added paragraph is as follows:

The 2023 ASCO/CAP update and ESMO expert consensus statements highlight recommendations to distinguish IHC 0 from 1+(5,18).

Comment 11: Lane 184: "… the efforts to develop new quantitative HER2 assays." This could be a separate subsection. Towards this end, there are several studies describing novel methods that have the potential to complement the traditional pathological assessment and improve the accuracy of HER2 testing. These include but are not limited to:

1. Histopathology, 2022, 81, 770–785. https://doi.org/10.1111/his.14780 - This study refined the definition of HER2-low BC based on correlation with ErbB2 mRNA and distinguished between HER2 IHC score 1+ and score 0 tumors.

2. ASCO 2023, Poster Session Abstract 569 - The Suwen Biotech researchers used the MammaTyper qRT-PCR technology and reported a study at the ASCO 2023 meeting that IHC could not certainly reflect the amount of HER2 in some samples and that the prognosis of HER2-low cohort might be more related to ErbB2 mRNA expression.

3. JCO Clinical Cancer Informatics no. 7 (2023) e2300013. https://ascopubs.org/doi/abs/10.1200/CCI.23.00013 - This study proposed the diagnostic software approach to automatically compute precise cutoffs that could facilitate the detection of patients suitable for targeted therapies.

4. Lab Invest, 2022, 102(10): 1101-1108. - This study proposed a combination of quantitative immunofluorescence and mass spectrometry to measure Her2 protein in tissue sections.

5. Histopathology, 2023, 82(6): 912-924; Mod Pathol . 2023, 36(3): 100054. -
These studies are about AI-based digital pathology in the evaluation of HER2 IHC 0 and IHC 1+.

Reply 11: Thank you very much for your suggestion. We have added discussion about novel method of the detection of HER2-low tumors (Please see Page 11, line 239 to line 247). The added paragraph is as follows:

Researchers have been contributing to explore the limitations of traditional diagnostic methods, propose advanced diagnostic approaches, and suggest novel techniques for precise measurement of HER2. qRT-PCR was employed to distinguish between HER2 IHC score 1+ and score 0 tumors(19,20). The result suggested that IHC may not accurately reflect HER2 levels in some samples, and ERBB2 mRNA expression might be more relevant to the prognosis of HER2-low cohort. In addition, Combination of quantitative immunofluorescence and mass spectrometry was found to be a more accurate measurement of HER2 protein in tissue sections(21). Furthermore, automated computation was also introduced to help determine precise cutoffs of HER2-low diagnosis(17).

Comment 12: Lane 198. This section is comprehensive. However, the side effects of anti-HER2-ADC treatments should be discussed at least in brief.

Reply 12: Thank you for your valuable comment. We have added a discussion about the side effects (Please see Page 17, line 368 to line 374). The added paragraph is as follows:

While these treatments can be effective, they may also be associated with side effects. The common ones are nausea and lowered blood counts, and these can be managed with medication. However, a small number of people who receive T-Dxd have interstitial lung disease (ILD). This risk has been noted from the very first studies of the drug. In the DESTINY-Breast04 trial, it occurred in about 12% of patients who received T-DXd(3). Based on available reports, a multidisciplinary guideline has been produced on proactive monitoring, diagnosis, and management of T-Dxd-related ILD. However, there are still many areas waiting for future investigation(42).

Comment 13: Lane 213. Abbreviate the first time" a median PFS (mPFS)…" and use this abbreviation consistently throughout the text.

Reply 13: We are really sorry for our careless mistake. The abbreviation has been checked throughout the manuscript.

Comment 14: Lane 306. The sentence needs minor correction:

"The data derived from various clinical trials indicates a ….statistically … significant survival advantage associated with T-Dxd."

Reply 14: Thank you very much for your careful review. We have corrected this sentence.

Reviewer C

Comment 1: This is a literature review whose main objective is to summarize the most recent findings of HER2-low clinicopathologic features, diagnostic concerns, and developments in treatment. The authors have done a very good job of summarizing the related clinical trials' main findings. However, it is my opinion that deeper research is needed particularly in the diagnostics section. Furthermore, many reviews covering this topic have been published recently, so I believe the authors need to clearly state in the rationale of the manuscript why their review is necessary and why it is different from the reviews that have been recently published. One good example is that they summarize the major clinicopathologic differences between HER2-0 and low, or the concept of HER2-ultralow. However, the literature investigation performed in this area could be expanded considerably.

Reply 1: Thank you very much for your valuable review.

Comment 2: 1. The authors need to clearly state the purpose of their review and highlight why it is necessary and why it is different from other reviews published so far.

Reply 2: Thank you very much for your comment. we have corrected the title to "Challenges in HER2-Low Breast Cancer Identification, Detection, and Treatment", to

clearly state the purpose of this review.

Comment 3: 2. A literature review of this caliber should include a search methodology clearly described in the Materials and Methods section. Perhaps the inclusion of a figure could also be necessary to explain step by step the literature search and the exclusion and inclusion criteria.

Reply 3: We appreciate your suggestion to include a detailed search methodology. However, after careful consideration, we have decided to maintain the current structure of the literature review. While we understand the value of a detailed presentation, the nature of this review does not necessitate an exhaustive breakdown in the Materials and Methods section.

Comment 4: Line 81. In this paragraph, the author defines the scores of HER2 IHC according to international guidelines. Being this a review, I would suggest making this paragraph more concise. For instance, describing the parameters of the criteria for positive ISH is unnecessary for the purpose of this manuscript.

Reply 4: Thank you for your suggestion. We have deleted the part of describing the parameters the criteria for positive ISH.

Comment 5: Line 109. This short paragraph about the proportion of HER2-low BC cases could fit better following this sentence from a previous paragraph: "Currently, in the vast majority of studies, including the phase 3 DB-04 trial, HER2-low BC is defined as BC with a HER2 IHC score of 1+ or 2+ and negative ISH result, and
this criterion has also been favored by most experts in the European Society for Medical Oncology".

Reply 5: Thank you for your valuable review. We have moved this short paragraph to the place you suggested (Please see Page 5, line 112-114)..

Comment 6: Line 99. The identification of HER2-low BC patients may be affected by more than the assays used. You mention this again further in the manuscript (paragraph

starting from line 142) such as interobserver variability, pre-analytical factors, tumor heterogeneity, etc. I suggest moving this paragraph to include it in that section. Furthermore, there are more recent references that could be included in this section. What other factors may affect interobserver variability between the HER2-negative cases? What are the difficulties that pathologists encounter when they see these cases in the microscope? What solutions have been proposed? Is AI or machine learning a feasible solution? Are other molecular tests an option?

Reply 6: I appreciate your suggestion very much. We have moved the paragraph to the detection section. In addition, we have added a discussion about novel methods of the detection of HER2-low tumors (Please see Page 11, line 239 to line 247). The added paragraph is as follows:

Researchers have been contributing to exploring the limitations of traditional diagnostic methods, proposing advanced diagnostic approaches, and suggesting novel techniques for precise measurement of HER2. qRT-PCR was employed to distinguish between HER2 IHC score 1+ and score 0 tumors(19,20). The result suggested that IHC may not accurately reflect HER2 levels in some samples, and ERBB2 mRNA expression might be more relevant to the prognosis of HER2-low cohort. In addition, the Combination of quantitative immunofluorescence and mass spectrometry was found to be a more accurate measurement of HER2 protein in tissue sections(21). Furthermore, automated computation was also introduced to help determine precise cutoffs of HER2-low diagnosis(17).

Comment 7: In the paragraph: clinicopathological features, Many and more recent studies have evaluated features between HER2-0 and HER2-low and adjusted by ER status. I recommend adding these references and contrasting them.

Reply 7: Thank you for your valuable review. We have added a paragraph discussing features between HER2-0 and HER2-low and adjusted by ER status (Please see Page 6, line 135 to line 140). The added paragraph is as follows:

A real-world study, including 65, 035 patients with BC, suggested that HER2-low tumors were significantly associated with histologic subtype, a higher ER, and lower

progesterone receptor expression in the ER+ cohort, whereas within the ER-cohort, HER2-low tumors were associated with a lower tumor grade(11). Another single-center study also indicated that HER2-low/ER+ early-stage BC was associated with a lower grade and Oncotype DX recurrence score(12).

Comment 8: In the paragraph: Prognosis. I recommend adding more references, focusing also on overall survival.

Reply 8: I appreciate your suggestion very much. We have added more discussion and references about prognosis(Please see Page 7, line 145 to line 156). The added paragraph is as follows:

In addition, another study suggested that HER2-low expression was associated with better survival in HR+ breast cancer patients with high Oncotype Dx RSs. Among patients who received adjuvant chemotherapy with a high Oncotype Dx RS (26–100), those with HER2-low tumors had higher survival(13). A meta-analysis of 42 studies, which included 1,797,175 patients, indicated that HER2-low status appears to be associated with a slightly increased OS both in the advanced (HR 0.94, 95% CI 0.89-0.98, P = 0.008) and early settings (HR 0.90, 95% CI 0.85-0.95, P < 0.001), regardless of HR expression. In the early setting, HER2-low tumors seem to be associated to lower pCR rates, especially if HR-positive(14). Another meta-analysis, including 636,535 patients, also suggested that HER2-low arm showed significantly improved results for DFS and OS. The hazard ratios for DFS and OS in the HR-positive group were 0.88 (95% CI 0.83-0.94) and 0.87 (95% CI 0.78-0.96), respectively. In the HR-negative group, the hazard ratios for DFS and OS were 0.87 (95% CI 0.79-0.97) and 0.86 (95% CI 0.84-0.89), respectively(15).

Comment 9: In line 181. You mention HER2-ultralow. There have been a couple of studies that have assessed the detection of HER2-ultra low with IHC and molecular tests. It would be interesting to expand on this in your review since it is a very important topic right now, especially while we await the Destiny 06 results. Furthermore, it could be a good opportunity to discuss how low can go and realistically speaking how good

the IHC technique is to detect these subtle differences.

Reply 9: Thank you for your thoughtful suggestion regarding the inclusion of information on HER2-ultralow. While I recognize the significance of discussing the nuances of HER2-ultralow detection and the capabilities of IHC techniques, I have carefully considered the scope and focus of the current review. Given the constraints of the manuscript, I have opted to maintain a specific emphasis on the primary objectives outlined in the initial plan.

Comment 10: In your 'advances in treatment section' perhaps you could also discuss the improvement in survival outcomes that T-Dxd showed in the Daisy trial, where around 30% of patients with HER2-0 had a good response.

Reply 10: Thank you for your insightful suggestion regarding the inclusion of the improvement in survival outcomes demonstrated by T-Dxd in the Daisy trial. While I acknowledge the significance of this trial and its findings, I have carefully considered the focus and structure of the 'Advances in Treatment' section within the current manuscript. At this juncture, I have opted to maintain a specific emphasis on the primary objectives established in the initial plan.