Peer Review File

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Reviewer A

Common 1: Congratulations on the very good article and I am happy to say no further questions this stage. Good article and in position to be published in my view.

Reply 1: Thank you very much for your kind words and positive feedback on the article. Your encouragement is greatly appreciated, and I look forward to its potential publication. Thank you again for your time and consideration.

Reviewer B

In this paper, ctDNA at baseline was shown to be a prognostic marker for patients with stage I-III breast cancer. Although the number of patients was small, the results suggest the need for further research.

Common: In discussion, the authors emphasized that ctDNA at baseline was useful to predict prognosis early breast cancer. However, in subgroup analysis, the result did not show usefulness in stage I and II breast cancer (Fig 6D). So I recommend the authors to modify the construction of articles.

Reply: Thank you for your advice. In our study, patients with high-level ctDNA at baseline had significantly lower RFS compared to those with low-level ctDNA. Although there was no statistically significant difference in RFS between the ctDNA negative group and ctDNA positive group, patients with ctDNA positive had lower RFS. Among patients with stage I/II, the RFS of patients with ctDNA positive was also lower than patients with ctDNA negative, however, this difference is not statistical significance (Figure 6D). We analyzed possible reasons for this outcome. As described in discussion, previous studies primarily enrolled patients at T2/T3 stage, which indicates a relatively larger tumor burden at the time of diagnosis. In our study, all patients were at T1/T2 stage. The earlier stage of these patients may result in a relatively lower number of RFS events in our study. This may explain why the differences in the prognosis between patients with ctDNA positive and ctDNA negative were not statistically significant. We reviewed literature again and found that previous studies have indicated a correlation between baseline ctDNA levels and prognosis in colorectal and ovarian cancers. It may be suggested that we should focus on not only the detection of ctDNA but also the level of ctDNA in patients with early breast cancer. We have also supplemented in the discussion. Although our preliminary research findings suggest that ctDNA levels at baseline could potentially serve as a biomarker to predict the prognosis for patients with early breast cancer, further studies with larger sample sizes are needed for validation. Following your advice, we have revised the statement in the first paragraph of the discussion section (see Page 10, line 311-313) and modified the construction accordingly (see Page 10-12, line 331-392). We are deeply grateful for your valuable advice.

Changes in the text:

Page 10, line 311-313: "The present study suggested that ctDNA at diagnosis, before any

treatment, could potentially serve as a biomarker to predict the prognosis for patients with early breast cancer. High-level baseline ctDNA was associated with worse outcomes."

Page 10-12, line 331-392: "Although the prognostic value of ctDNA has been investigated in previous studies, it remains uncertain whether detection of ctDNA at diagnosis has predictive potential for the prognosis of patients with early breast cancer. Magbanua et al. (16) examined the utility of ctDNA for predicting the risk of metastatic recurrence in 84 early breast cancer patients treated in the neoadjuvant I-SPY 2 TRIAL. Their results showed that detection of ctDNA at the time of diagnosis was not associated with an increased risk of metastatic recurrence. Garcia-Murillas et al. (19) used digital PCR (dPCR) to detect ctDNA in a prospective cohort of patients with early breast cancer undergoing NAT. Plasma samples were collected at the time of diagnosis from 42 patients. The study indicated that the detection of ctDNA at diagnosis was not predictive for disease-free survival (DFS) in patients with early breast cancer. However, Garcia-Murillas et al. (20) subsequently conducted another study with larger cohort of 101 patients with early breast cancer receiving NAT, which found that the detection of ctDNA at diagnosis, before any treatment, was associated with RFS. Similarly, Li et al. (14) showed that patients with ctDNA positive at diagnosis had significantly worse outcomes than those with ctDNA negative. In our study, the result showed that patients with ctDNA positive at baseline had a worse prognosis compared to those with ctDNA negative, although the difference was not statistically significant.

These previous studies mentioned above primarily enrolled patients who had relatively higher tumor burden at the time of diagnosis. For instance, in the study conducted by Garcia-Murillas et al. (20), the tumor stage of 101 patients was mainly T2 (n=51, 50.5%) and T3 (n=15, 14.9%), with 51 patients having positive lymph nodes. Similarly, in Li's study (14), 44 patients mainly presented with tumor stages of T2 (n=23, 52.3%) and T3 (n=14, 31.8%), with the majority having positive lymph nodes (n=35, 80%). In contrast, our study focused on patients at an earlier stage, with all the breast cancer patients at T1 (57.1%) and T2 (42.9%), and only 39.3% of patients having positive lymph nodes. The relatively low number of RFS and iDFS events observed in our study may be attributed to the earlier stage of the patients. This may also explain why the differences we observed in the prognosis between patients with ctDNA positive and ctDNA negative were not statistically significant.

The previous studies have explored the correlation between baseline ctDNA levels and patient prognosis in different types of cancers. Dobilas et al. (40) explored the association of baseline ctDNA levels with prognosis of ovarian cancer patients. They found that the overall survival (OS) of patients with high-level ctDNA was significantly worse than patients with low-level ctDNA. Phallen et al. (41) examined whether baseline ctDNA may be related to disease recurrence and survival of colorectal cancer patients. This study found that patients with increased ctDNA had a shorter progression-free survival (PFS) and overall survival (OS) compared to patients with lower ctDNA level. In our study, patients were categorized into two groups based on baseline ctDNA levels. The result indicated that patients with high-level ctDNA have significantly lower RFS compared to those with low-level ctDNA. It is noteworthy that all RFS events occurred in patients with high-level ctDNA. Taking into account the findings from previous studies, we believe that exploring the prognostic value of ctDNA at

diagnosis in patients with early breast cancer should not only focus on the detection of ctDNA but also on the ctDNA levels."

Reviewer C

Overall very informative and interesting article. Methodology and terminology of RFS,iDFS explained well. Results are very interesting implying this biomarker could be used for patients stratification after surgery. I have certain queries in article

Common 1: Quality check for article

Reply 1: Thank you for your advice. We have checked the quality for article and revised the statement in the first paragraph of the discussion section (see Page 10, line 311-313).

Changes in the text: The present study suggested that ctDNA at diagnosis, before any treatment, could potentially serve as a biomarker to predict the prognosis for patients with early breast cancer. High-level baseline ctDNA was associated with worse outcomes.

Common 2: cfDNA concentrations showed significant results but not mentioned in discussion section

Reply 2: Thank you for your advice. We have expanded the discussion of cfDNA concentrations in manuscript (see Page 10, line 314-321).

Changes in the text: In our study, we observed a significantly higher concentration of cfDNA in patients with invasive breast cancer compared to healthy women. This finding aligned with results of previous studies. Hashad et al. (35) found that the cfDNA concentration in patients with breast cancer was significantly higher than that in patients with benign breast diseases and healthy individuals. Madhavan et al. (36) compared the cfDNA concentrations between 82 patients with early breast cancer and 100 healthy women. The study found that patients with breast cancer had a significantly higher plasma cfDNA concentration than the healthy women.

Common 3: ctDNA positive/negative and ctDNA high/low levels could be discussed in under different for better readability and understanding.

Reply 3: Thank you for your advice. In our study, patients with high-level ctDNA had significantly lower RFS compared to those with low-level ctDNA. Although there was no statistically significant difference in RFS between the ctDNA negative group and ctDNA positive group, patients with ctDNA positive had lower RFS. We analyzed possible reasons for this outcome. As described in discussion, previous studies primarily enrolled patients at T2/T3 stage, which indicates a relatively larger tumor burden at the time of diagnosis. In our study, all patients were at T1/T2 stage. The earlier stage of these patients may result in a relatively lower number of RFS events in our study. This may explain why the differences in the prognosis between patients with ctDNA positive and ctDNA negative were not statistically significant. We reviewed literature again and found that previous studies have indicated a correlation between baseline ctDNA levels and prognosis in colorectal and ovarian cancers. It may be suggested that we should focus on not only the detection of ctDNA but also the level of ctDNA

in patients with early breast cancer. We have also supplemented in the discussion. Although our preliminary research findings suggest that ctDNA levels at baseline could potentially serve as a biomarker to predict the prognosis for patients with early breast cancer, further studies with larger sample sizes are needed for validation. Following your advice, we have modified the discussion (see Page 10-12, line 331-392). We are deeply grateful for your valuable advice.

Changes in the text:

Although the prognostic value of ctDNA has been investigated in previous studies, it remains uncertain whether detection of ctDNA at diagnosis has predictive potential for the prognosis of patients with early breast cancer. Magbanua et al. (16) examined the utility of ctDNA for predicting the risk of metastatic recurrence in 84 early breast cancer patients treated in the neoadjuvant I-SPY 2 TRIAL. Their results showed that detection of ctDNA at the time of diagnosis was not associated with an increased risk of metastatic recurrence. Garcia-Murillas et al. (19) used digital PCR (dPCR) to detect ctDNA in a prospective cohort of patients with early breast cancer undergoing NAT. Plasma samples were collected at the time of diagnosis from 42 patients. The study indicated that the detection of ctDNA at diagnosis was not predictive for disease-free survival (DFS) in patients with early breast cancer. However, Garcia-Murillas et al. (20) subsequently conducted another study with larger cohort of 101 patients with early breast cancer receiving NAT, which found that the detection of ctDNA at diagnosis, before any treatment, was associated with RFS. Similarly, Li et al. (14) showed that patients with ctDNA positive at diagnosis had significantly worse outcomes than those with ctDNA negative. In our study, the result showed that patients with ctDNA positive at baseline had a worse prognosis compared to those with ctDNA negative, although the difference was not statistically significant.

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groups based on baseline ctDNA levels. The result indicated that patients with high-level ctDNA have significantly lower RFS compared to those with low-level ctDNA. It is noteworthy that all RFS events occurred in patients with high-level ctDNA. Taking into account the findings from previous studies, we believe that exploring the prognostic value of ctDNA at diagnosis in patients with early breast cancer should not only focus on the detection of ctDNA but also on the ctDNA levels.

Common 4: PIK3CA gene mentioned in discussion section but not present in result section?

Reply 4: Thank you for your reminder. In the results section, PIK3CA and its mutation frequency were presented in Figure 2 (see Page 19, line 554-556), but we did not describe in paragraph. Following your advice, we have made revisions to describe PIK3CA in the results section (see Page 8, line 262).

Changes in the text: Additionally, there were 16 genes such as PIK3CA et al. with mutation frequency of 3.51% and 61 genes with mutation frequency of 1.75% (Figure 2).

Common 5: Is there any difference in clinicopathological characteristics of pts with recurrence vs no recurrence

Reply 5: The correlation of RFS with clinicopathologic characteristics in patients with invasive breast cancer (n=84) is shown in Table 1:

Table 1. The correlation of RFS with clinicopathologic characteristics in patients with invasive breast cancer

Clinicopathologic characteristics	Patients without RFS events (n=80), n (%)	Patients with RFS events (n=4), n (%)	P value
Age			
≤50 years	33 (41.3)	0	0.151
>50 years	47 (58.8)	4 (100)	
Histological type			
Invasive ductal carcinoma	59 (73.8)	2 (50)	0.301
Specified carcinoma	21 (26.3)	2 (50)	
Histological grade			
I	6 (7.5)	0	0.716
II	45 (56.3)	3 (75)	
III	29 (36.3)	1 (25)	
Lymph-vascular space invasion (LVSI)			
Negative	61 (76.3)	2 (50)	0.237
Positive	19 (23.8)	2 (50)	
T Stage			
T1	46 (57.5)	2 (50)	0.767
T2	34 (42.5)	2 (50)	

Lymph node status			
Negative	49 (61.3)	2 (50)	0.653
Positive	31 (38.8)	2 (50)	
Stage			
I	30 (37.5)	1 (25)	0.033
II	43 (53.8)	1 (25)	
III	7 (8.8)	2 (50)	
ER status			
Negative	21 (26.3)	0	0.237
Positive	59 (73.8)	4 (100)	
PR status			
Negative	29 (36.3)	2 (50)	0.578
Positive	51 (63.8)	2 (50)	
HER2 status			
Negative	62 (77.5)	2 (50)	0.208
Positive	18 (22.5)	2 (50)	
Ki-67			
≤20%	48 (60)	0	0.018
>20%	32 (40)	4 (100)	
Molecular subtype			
$HR^+/HER2^-$	47 (58.8)	2 (50)	0.273
$HR^+/HER2^+$	12 (15)	2 (50)	
$HR^-/HER2^+$	6 (7.5)	0	
$HR^-/HER2^-$	15 (18.8)	0	
ctDNA detection			
Negative	27 (33.8)	0	0.158
Positive	53 (66.3)	4	
ctDNA level			
Low-level	56 (70)	0	0.004
High-level	24 (30)	4 (100)	

The results showed significant statistical differences in stage, ki-67, and ctDNA level between patients with and without recurrence. The ctDNA level may partially reflect the tumor burden and may be correlated with traditional clinicopathologic characteristics. Therefore, we believe that ctDNA cannot completely replace traditional prognostic indicators. But, it may potentially serve as a supplementary prognostic indicator in patients with invasive breast cancer.

Common 6: Is cox hazard model analysis included for RFS/iDFS?

Reply 6: Thank you very much for your advice. We attempted to conduct Cox hazard model analysis. However, the results presented in the Table 2 for RFS did not present a significant P value. Additionally, we noticed that the values of HR and 95% CI seemed unusual. Upon careful analysis, we think that the out` comes may be attributed to the absence of RFS events in patients with low-level ctDNA and those with Ki-67≤20% (refer to the table in Reply 5). For

iDFS, the Omnibus test result for Cox model coefficients using stage, Ki-67 and ctDNA levels as variables is not significant (P=0.078). The relatively low number of RFS/iDFS events may be attributed to the earlier stage of the patients. We sincerely anticipate that longer follow-up and additional RFS/iDFS events will be necessary to draw a definitive conclusion in the future. And we have acknowledged this limitation in the discussion.

Table 2. Cox hazard model analysis for RFS

	B	P value	HR	95% CI
TNM I		0.707		
TNM II	-1.155	0.415	0.32	0.02-5.07
TNM III	10.816	0.871	49833.38	0.00-3.11E+61
Ki-67	19.770	0.882	385385809.96	0.00-1.96E+122
ctDNA level	20.569	0.895	856697467.58	0.00-4.17E+141