



Original Article

Implication of Pre- and Post-radiotherapy ctDNA Dynamics in Patients with Residual Triple-Negative Breast Cancer at Surgery after Neoadjuvant Chemotherapy: Findings from a Prospective Observational Study

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Purpose This study aims to determine the association between pre- and postoperative radiotherapy (PORT) circulating tumor DNA (ctDNA) dynamics and oncological outcomes in patients with residual triple-negative breast cancer who underwent surgery after neoadjuvant chemotherapy (NAC).

Materials and Methods Between March 2019 and July 2020, 11 nonmetastatic patients with residual disease who underwent surgery after NAC were prospectively enrolled. In each patient, tumor specimens obtained during surgery and blood samples collected at three time points during PORT (TO: pre-PORT, T1: 3 weeks after PORT, T2: 1 month after PORT) were sequenced, targeting 38 cancer-related genes. Disease-free survival (DFS) was evaluated and the association between DFS and ctDNA dynamics was analyzed.

Results At TO, ctDNA was detected in three patients (27.2%). The ctDNA dynamics were as follows: two showed a decreasing ctDNA variant allele frequency (VAF) and reached zero VAF at T2, while one patient exhibited an increasing VAF during PORT and maintained an elevated VAF at T2. After a median follow-up of 48 months, two patients experienced distant metastasis without any locoregional failures. All failures occurred in patients with ctDNA positivity at TO and a decreased VAF after PORT. The 4-year DFS rates according to the TO ctDNA status were 67% (positive ctDNA) and 100% (negative ctDNA) ($p=0.032$).

Conclusion More than a quarter of the patients with residual disease after post-NAC surgery exhibited pre-PORT ctDNA positivity, and ctDNA positivity was associated with poor DFS. For patients with pre-PORT ctDNA positivity, the administration of a more effective systemic treatment should be considered.

Key words Circulating tumor DNA, Neoadjuvant therapy, Triple-negative breast neoplasms, Radiotherapy

Introduction

Breast cancer is one of the most commonly diagnosed malignancies in women, and its incidence is increasing [1]. The treatment of many types of cancer largely depends on the histology and disease stage. In breast cancer, subtypes based on immunohistochemistry are also associated with prognosis and are important for determining treatment strategies [2]. Efforts have been made to further classify the disease and personalize the treatment for patients with breast cancer. Recent advancements in liquid biopsy techniques have enabled the development of novel approaches for cancer diagnosis and treatment. Liquid biopsy uses peripheral blood instead of directly obtaining tumor tissue, making it less invasive and allowing easy sample collection throughout the disease course [3]. Circulating tumor DNA (ctDNA), which is cell-free DNA originating from tumor cells, can be analyzed using liquid biopsy. ctDNAs can be used to detect

minimal residual disease (MRD), which is associated with poor prognosis in some cancer types. Tracking MRD may enable the tailoring of treatment based on recurrence risk, thereby potentially avoiding adverse events in patients with favorable prognosis and improving treatment outcomes in patients with unfavorable prognosis [4].

In patients with locally advanced breast cancer, neoadjuvant chemotherapy (NAC) is administered prior to surgery [5]. Achieving complete response after NAC is associated with improved prognosis, particularly in the triple-negative subtype [6]. Adjuvant capecitabine is indicated for therapeutic intensification in patients with residual tumors after NAC, as this indicates poor survival [7]. Further risk stratification may be possible using ctDNAs. Assessing ctDNA levels after definitive treatment may provide additional information on MRD, allowing for the prediction of poor prognosis and adjustment of treatment decisions. We conducted a prospective observational study to analyze ctDNA dynamics

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before and after postoperative radiation therapy (PORT) [8]. In this report, we present additional follow-up data for the patient cohort because intriguing results were observed during the follow-up period.

Materials and Methods

The details of the methodology are described in a previously published article [8], and a brief description is included in this report. Between March 2019 and July 2020, eleven triple-negative breast cancer patients who underwent NAC, surgery, and PORT were enrolled. All patients exhibited macroscopic residual disease in their surgical specimens. Anthracycline was used in 10 patients (90.9%) and taxane was used in all patients as the NAC regimen. The median time from NAC completion to surgery was 25 days (range, 20 to 32 days). PORT was initiated 4-5 weeks after surgery, and the PORT field encompassed the breast or chest wall and the regional lymph node area. All except one patient (90.9%) started the administration of adjuvant capecitabine 3-4 weeks after completion of PORT. Blood samples for ctDNA analysis were collected before PORT (T0), 3 weeks after PORT initiation (T1), and 1 month after PORT completion (T2). The median time from surgery to the first blood sampling was 32 days (range, 23 to 39 days). Ultra-deep sequencing (average depth 5,884.8 \times for cell-free DNA and 3,993.4 \times for genomic DNA) was performed on tumor tissues, three plasma samples, and leukocytes from each patient. Customized baits targeting 38 cancer-related genes were used (LiquidSCAN

v2-PanCancer panel, GENINUS, Seoul, Korea). The variant allele frequency (VAF) of ctDNA was measured and changes at different time points were evaluated. A schematic diagram of the study process is shown in Fig. 1.

Additional follow-up data were retrieved from the patients' medical records. Actuarial rates of disease-free survival (DFS), measured from the initiation of NAC, were calculated using the Kaplan-Meier method. The event for DFS was defined as any disease progression. Log-rank p-values were calculated for patients with and without ctDNA. The characteristics, ctDNA dynamics, and clinical course of patients who experienced recurrence are described. Statistical significance was set at $p < 0.05$. All statistical analyses were conducted using the R software ver. 4.2.1 (R Foundation for Statistical Computing, Vienna, Austria).

Results

The median follow-up period was 48 months (range, 36 to 54 months). At T0, ctDNA was detected in three patients (27.2%). Detailed ctDNA detection results have been described in previous publication [8]. Two patients (P1 and P8) with detectable ctDNA levels at T0 experienced recurrence. The actuarial DFS rates according to ctDNA detection are illustrated in Fig. 2. A statistically significant difference in DFS was observed between patients with and without ctDNA ($p=0.032$). The 4-year DFS rate was 67% for patients with detectable ctDNA at T0 and 100% for those without detectable ctDNA. Patient characteristics and treatment specifics are

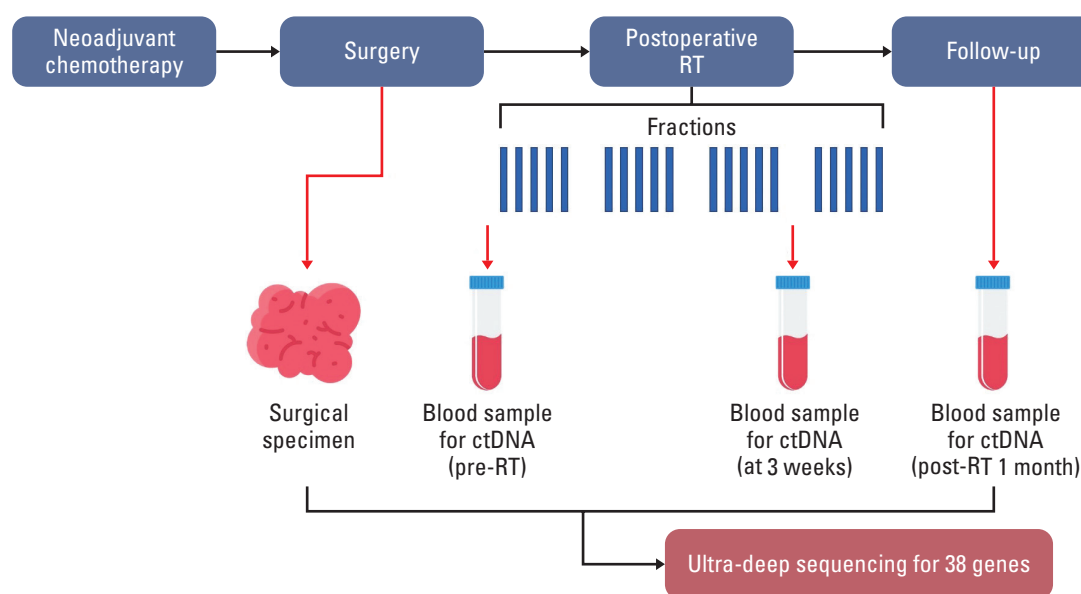


Fig. 1. Schematic diagram for process of this study. ctDNA, circulating tumor DNA; RT, radiation therapy.

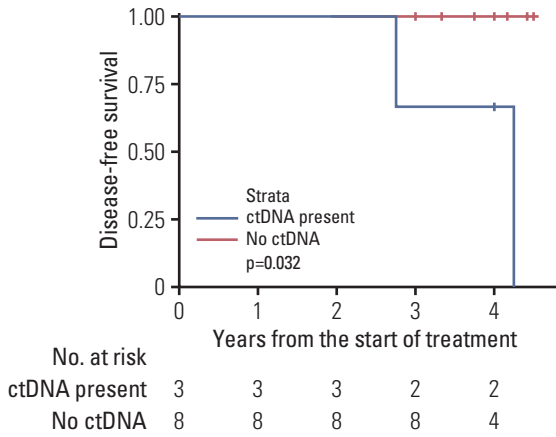


Fig. 2. Actuarial rates of disease-free survival according to detection of circulating tumor DNA (ctDNA).

summarized in Table 1. VAF in the plasma of these patients was the highest at T0 and later decreased, reaching zero at T2. Both patients with recurrence initially had advanced disease that spread to the internal mammary and/or supraclavicular lymph nodes. One patient (P9) had increased plasma VAF at T2, but no recurrence was reported at 48 months of follow-up.

One patient (P1) with recurrence had distant metastasis to the bone and pleura 33 months after NAC initiation. Palliative radiotherapy and chemotherapy were administered to the involved bone. However, brain metastasis progressed. The patient received intrathecal methotrexate, talazoparib, and whole-brain radiotherapy, although further disease progression was later reported. Another patient (P8) with a recurrence had distant metastases. Multiple liver, bone, and lymphangitic metastases were detected 51 months after NAC initiation. The patient received palliative chemotherapy.

Discussion

In a previous report, we hypothesized that the decrease in ctDNA VAF during and after PORT could be interpreted as the eradication of localized MRD by radiotherapy [8]. However, on further follow-up, both patients with decreasing ctDNA VAF levels experienced distant recurrence. In contrast, patients with increased ctDNA levels after PORT did not experience tumor recurrence. This result differs slightly from our initial expectations. The detection of ctDNA appears to be a strong predictive factor for recurrence. However, decreased ctDNA levels during and after PORT may not be a prognostic factor.

The detection of ctDNA and its prognostic significance in

Table 1. Patient characteristics and treatment

Case	Clinical stage	IMN involvement	SCN involvement	Pathologic Stage	Neoadjuvant CTx regimen	Adjuvant CTx	Variant in tumor	Variant in plasma	VAF change in study period	Recurrence
P1	cT3N3c	Yes	Yes	ypT2N3c	AC+D	Yes	MYC	EGFR	Decreased	Yes
P2	cT2N2a	No	No	ypT2N1	AC+D	Yes	(-)	(-)	(-)	No
P3	cT2N2a	No	No	ypT2N1	AC+D	Yes	TP53, RB1	(-)	(-)	No
P4	cT2N2a	No	No	ypT1N0	AC+D	Yes	(-)	(-)	(-)	No
P5	cT2N2a	No	No	ypT1N0	AC+D	Yes	TP53	(-)	(-)	No
P6	cT2N2a	No	No	ypT1N2a	CMF+PC	Yes	APC	(-)	(-)	No
P7	cT2N3b	Yes	No	ypT1N1	AC+PC	Yes	(-)	(-)	(-)	No
P8	cT3N3b	Yes	No	ypT2N3b	AC+PC	Yes	TP53, AKT1	CTNNB1	Decreased	Yes
P9	cT3N3b	Yes	No	ypT2N3b	AC+PC	Yes	TP53, PTEN, CSMD3	MAP2K4	Increased	No
P10	cT2N3b	Yes	No	ypT1N2b	AC+D	No	TP53	(-)	(-)	No
P11	cT2N3b	Yes	No	ypT1N3b	AC+D	Yes	TP53, PIK3CA	(-)	(-)	No

AC, doxorubicin and cyclophosphamide; CMF, cyclophosphamide, methotrexate, and 5-fluorouracil; CTx, chemotherapy; D, docetaxel; IMN, internal mammary lymph node; PC, paclitaxel and carboplatin; SCN, supraclavicular lymph node; VAF, variant allele frequency.

NAC for breast cancer has been the subject of several studies. Several studies have shown that ctDNA levels are significantly associated with the treatment outcomes. However, the exact time points for ctDNA detection suggested in these studies differed. Studies have demonstrated the prognostic significance of ctDNA detection at baseline [9], during or after NAC [10-13], and after surgery [14]. The prognostic significance of ctDNA detection was also observed in this study, as two out of three patients with detectable ctDNA in post-operative blood sampling experienced recurrence, whereas no recurrence was reported in patients without ctDNA. The results of this study contribute to the evidence support the prognostic significance of postoperative ctDNA detection in patients with breast cancer treated with NAC.

In contrast to the prognostic significance of ctDNA detection, the pre- and post-radiotherapy ctDNA dynamics observed in this study did not match our initial assumptions. Previous studies have shown that a decrease in ctDNA is associated with a better tumor response and treatment outcomes in various cancer types treated with chemotherapy, including breast cancer [12,15,16]. In radiotherapy, the dynamics of circulating viral DNA in cancers related to viral etiology have been observed in several studies. Better treatment outcomes were associated with the clearance of circulating viral DNA in patients who underwent definitive radiotherapy for human papillomavirus-related cervical cancer and Epstein-Barr virus-related nasopharyngeal cancer [17,18]. Post-radiotherapy ctDNA detection has also been associated with poor prognosis in patients who undergo definitive radiotherapy for esophageal cancer [19]. Based on these previous reports, we assumed that a decrease in ctDNA VAF may indicate MRD eradication and, therefore, correlate with better outcomes. However, in this study, patients with ctDNA detected after surgery and those with ctDNA clearance after PORT experienced recurrence. Differences in neoadjuvant, definitive, and adjuvant treatment settings may have caused this phenomenon. In neoadjuvant and definitive treatments, the clinically visible tumor burden remains, but in adjuvant settings, most of the tumor burden is removed. Therefore, the clearance of ctDNA after neoadjuvant and definitive treatments may have stronger prognostic implications than a decrease in ctDNA levels after PORT. Another possible explanation is that PORT may effectively eradicate locoregional MRD but have limited effects on distant metastasis. Both patients had distant metastases throughout their clinical course and no locoregional recurrence was reported. MRD occurring in the locoregional area may have been eradicated by PORT, leading to a decrease in ctDNA VAF. It should be noted that both patients had internal mammary and supraclavicular lymph node metastases, which made complete surgical removal challenging. However, this assumption

cannot be validated solely based on the observations of the present study.

In this study, one patient (P9) had an increased ctDNA VAF at the post-radiation therapy (RT) 1-month follow-up, but did not experience recurrence. Although this patient was receiving adjuvant capecitabine when blood was sampled, this change in ctDNA could not be explained as chemotherapy-related. Some studies have observed a transient peak in ctDNA during chemotherapy; however, this peak usually appeared a few days after the initiation of chemotherapy [20,21]. In this patient, the post-RT 1-month blood sampling was conducted three weeks after the initiation of adjuvant capecitabine. The absence of recurrence in this patient may have been due to chance.

While showing the prognostic significance of ctDNA detection, this study also demonstrated the discrepancies between tumor and ctDNA mutation profiles. In this study, the same 38 cancer-related genes were observed in tumor and blood samples, and the variants observed in ctDNA did not appear in tumors. The exact methodology for ctDNA detection varies among studies. Some studies utilized personalized or 'bespoke' ctDNA. This approach selects mutations from each tumor mutation profile, and ctDNA detection is performed for these selected mutations only. The detection of ctDNA using this methodology showed good correlation with treatment outcomes [10,11,22]. However, assuming discrepancies between tumor and ctDNA mutation profiles, such as those shown in this study, patient-specific ctDNA detection may have significant false negatives. One possible explanation for the discrepancies between tumor and ctDNA mutation profiles is tumor heterogeneity. Several studies reported tumor heterogeneity in breast cancer and its association of poor prognosis [23,24]. Due to spatial heterogeneity, samples obtained from specific sites of surgical tumor specimens may not contain mutations in metastatic clones. Researchers have observed differences in mutation profiles between tumors and ctDNA, and have suggested that analyzing ctDNA can help overcome tumor heterogeneity [25-27]. An optimal approach for ctDNA detection that considers intrapatient heterogeneity should be addressed in future research.

ctDNA testing may provide genotyping information that can aid the selection of appropriate targeted agents. The PlasmaMATCH trial allocated patients to different treatment arms with targeted agents based on ctDNA testing results, and the use of targeted therapies against *ERBB2* and *AKT1* mutations showed promising clinical feasibility [28]. In this study, ctDNA analysis revealed the presence of *EGFR*, *CTNNB1*, and *MAP2K4* mutations. However, clinical implications of these mutations remain unclear. While *EGFR* overexpression is often observed in triple-negative breast

cancer, *EGFR* mutations are rare [29]. Unlike for lung adenocarcinoma, there are currently no approved targeted agents for breast cancer with *EGFR* mutations. Additionally, there are no available agents that specifically target *CTNNB1* and *MAP2K4* mutations. Although ctDNA analysis has the potential to identify druggable targets in breast cancer, careful selection of target genes for sequencing is crucial for achieving this purpose.

Immunotherapy is actively used for the treatment of TNBC. The addition of pembrolizumab to cytotoxic chemotherapy has shown promising results for both operable and inoperable/metastatic TNBC [30,31]. ctDNA detection can also be integrated into immunotherapy. A recent trial, c-TRAK TN, aimed to assess the feasibility of ctDNA surveillance and the commencement of pembrolizumab immunotherapy. However, this trial failed to demonstrate the effectiveness of ctDNA-guided pembrolizumab, presumably because of the small number of patients who underwent treatment [32]. Other trials are also investigating the feasibility of integrating ctDNA detection and immunotherapy, such as the PERSEVERE trial (NCT04849364). Further data on this approach will be available in the future.

A major limitation of this study is the small number of patients enrolled. Although a significant difference in DFS was observed between patients with ctDNA detection and those without ctDNA detection, the interaction of other prognostic factors, such as initial disease extent, should be analyzed. Notably, both patients who had a recurrence in this study also had more advanced disease that metastasized to the internal mammary and/or supraclavicular lymph nodes at the initial clinical presentation. The small cohort size limited further statistical analyses of the independent association between ctDNA and treatment outcomes. The detection sensitivity of ctDNA in this study may have been limited by the analysis of only 38 genes. The observations from this study were hypothesis-generating rather than confirmative, and further studies are needed for clarification. Despite these limitations, ctDNA dynamics and their association with treatment outcomes in PORT settings for breast cancer have

rarely been studied. This report provides valuable information for these settings.

In conclusion, the results of this prospective study, after 4 years of follow-up, showed that ctDNA detection during PORT for breast cancer has prognostic significance. However, contrary to our initial assumptions, decreasing ctDNA levels during and after PORT did not necessarily indicate better treatment outcomes. Therefore, in the setting of PORT for breast cancer, ctDNA detection may have stronger prognostic implications than ctDNA dynamics. Nonetheless, as patients with ctDNA and recurrence did not experience locoregional recurrence, an explanation for localized MRD eradication with PORT is still plausible.



Ethical Statement

This study was approved by the Institutional Review Board of Samsung Medical Center prior to study initiation (approval number 2018-10-137). Written informed consent was obtained from each participant before enrollment.

Author Contributions

Conceived and designed the analysis: Kim H, Park WY.
Collected the data: Kim H, Park W, Cho WK, Kim N.
Contributed data or analysis tools: Kim YJ, Park WY.
Performed the analysis: Lee TH, Kim H, Kim YJ.
Wrote the paper: Lee TH, Kim H.

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Conflicts of Interest

Conflict of interest relevant to this article was not reported.

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