Circulating tumor DNA as a predictive marker of recurrence for patients with stage II-III breast cancer treated with neoadjuvant therapy

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Abstract

Background:

Patients with stage II to III breast cancer have a high recurrence rate. The early detection of recurrent breast cancer remains a major unmet need. Circulating tumor DNA (ctDNA) has been shown to be a marker to detect disease progression in metastatic breast cancer. We aimed to evaluate the prognostic value of ctDNA in the setting of neoadjuvant therapy (NAT).

Methods:

Plasma was sampled at the initial diagnosis (defined as before NAT) and after NAT and breast surgery (defined as after NAT). We extracted ctDNA from the plasma and performed deep sequencing of a target gene panel. The detection of alterations, such as mutations and copy number variations, were considered to indicate ctDNA positivity.

Results:

A total of 95 patients were enrolled in this study; 60 patients exhibited ctDNA positivity before NAT, and 31 patients had ctDNA positivity after NAT. A pathologic complete response (pCR) was observed in 13 patients, including one ER(+)Her2(-) patient, six Her2(+) patients and six triple-negative breast cancer (TNBC) patients. Among the entire cohort, multivariate analysis showed that an N3 classification and ctDNA positivity after NAT were independent risk factors that predicted recurrence (N3, hazard ratio (HR) 3.34, 95% confidence interval (CI) 1.26 – 8.87, p = 0.016; ctDNA, HR 4.29, 95% CI 2.06 – 8.92, p < 0.0001). The presence of ctDNA before NAT did not affect the rate of recurrence-free survival. For patients with Her2(+) or TNBC, non-pCR breast cancer patients were associated with a trend of higher recurrence (p = 0.105). Advanced nodal status and ctDNA positivity after NAT were significant risk factors for recurrence (N2 – 3, HR 3.753, 95% CI 1.146 – 12.297, p = 0.029; ctDNA, HR 3.123, 95% CI 1.139 – 8.564, p = 0.027). Two patients who achieved a pCR had ctDNA positivity after NAT; one TNBC patient had hepatic metastases six months after surgery, and one Her2(+) breast cancer patient had brain metastasis 13 months after surgery.

Conclusions:

This study suggested that the presence of ctDNA after NAT is a robust marker for predicting relapse in stage II to III breast cancer patients.

Introduction

Although breast cancer prognosis has improved greatly during the past two decades, breast cancer-related death remains a major cause of cancer-related mortality in women[1, 2]. The main reason is that a significant proportion of breast cancer patients develop recurrence and distant metastases[3, 4]. Once clinical overt and symptomatic metastases occur, the disease is usually characterized by multiorgan

involvement and drug resistance to chemotherapy and/or endocrine treatment, which results in an even worse outcome than before metastases[5].

Early detection of recurrence and a robust marker predicting recurrence are the unmet needs for patients with breast cancer[6]. In the neoadjuvant setting, pathological complete response (pCR) is a favorable prognostic marker in patients with Her2 (+) and triple-negative breast cancer (TNBC)[7]. However, a proportion of patients with pCR still had a recurrence or metastasis, and not all of the non-pCR patients had a recurrence[7, 8]. Recently, circulating tumor DNA (ctDNA), which are circulating DNA fragments that carry tumor-specific sequence alterations found in the cell-free fraction of blood, are a promising and sensitive tool for targeted monitoring [9–13]. Dynamic quantitative changes in ctDNA occur earlier than radiologic changes in tumor size; therefore, the detection of resistance mutations using ctDNA can also occur significantly earlier than radiographic progression[14]. In previous reports on metastatic cancer patients, serial quantification of ctDNA allowed for noninvasive assessment of therapeutic response and understanding of the resistance mechanism [9, 12, 15, 16]. Two additional studies reported that tumor ctDNA in plasma can be used to detect minimal residual disease (MRD) in early-stage breast and colon cancer[17, 18]. However, for breast cancer patients receiving neoadjuvant therapy (NAT), the prognostic value of ctDNA is uncertain and the comparison in between ctDNA and pCR is unclear. In this study, we collected patients' plasma before and after NAT. We used next-generation sequencing (NGS)-based deep sequencing to detect ctDNA and evaluated the impact of ctDNA on disease recurrence.

Methods

Patients and sample collection

Stage II or III breast cancer patients who received NAT were enrolled in this study. The clinical and pathologic characteristics were reviewed retrospectively from medical records. The presence of estrogen receptor (ER), progesterone receptor (PR), and Her2 was determined by immunohistochemical staining. The ER or PR status was considered negative when less than 1% of the tumor cells showed positive staining. For Her2 staining, a score of 0 or 1+ was considered negative; specimens with a score of 2+ were confirmed by fluorescence in situ hybridization analysis. The tumor histological grade was defined using the Nottingham combined histological grading system. This study was approved by the institutional review board (IRB number: 201704009RINC).

A 10-mL sample of blood was stored in an EDTA-containing tube at the initial diagnosis (defined as before NAT). All patients were treated with NAT and received breast surgery. Then, another 10 mL of blood was sampled after NAT and breast surgery (defined as after NAT). The plasma was collected after centrifugation at 1000´ G for 10 minutes within 3 hours of blood sampling and then stored at -80°C[19]. Cell-free DNA was extracted using a QIAamp Circulating Nucleic Acid Kit (Qiagen, Germantown, MD, USA) according to the manufacturer's protocol.

Library preparation and next-generation sequencing

The library was constructed using a QIAseq Targeted DNA Panel with a customized gene list. The customized panel was designed to amplify the coding regions of the following genes: *TP53*, *PIK3CA*, *Her2*, *GATA3*, *CDH1*, *PTEN*, *AKT1*, *ESR1*, *S100A7-9*, *ZNF703*, *B2M*, *CCND1*, *GATA3* and *c-MYC*. According to the manufacturer's protocol, 10 ng of DNA was digested briefly into small fragments by a fragmentation enzyme at 32°C and 72°C. The DNA fragments were added to QIAseq IL-N7 adapters, followed by target enrichment polymerase chain reaction (PCR) using the QIAGEN IL-Forward primer and targeted DNA Panel primers. Finally, the library was amplified with universal PCR. The DNA library was then checked by using an Agilent Chip High Sensitivity DNA kit. KAPA library quantification kits were used to quantify the final concentration. The final DNA library was sequenced with the following Illumina platforms: Illumina MiSeq Reagent Kit v2, 2 x 150 bp reads or Illumina NextSeq 550 system Mid-Output Kit, 2 x 150 bp reads.

Postsequencing analysis

Previously, we have constructed the analytic pipeline of post-NGS bioinformatics[20]. First, BWA software (version 0.5.9) was used to align the raw sequencing data to the reference human genome (Feb. 2009, GRCh37/hg19; SAMtools (version 0.1.18)). Picard (version 1.54) was used to perform the necessary data conversion, sorting, and indexing. GATK was used for variant calling with the Mutect2 and VariantFiltration parameters. Finally, ANNOVAR was used to annotate the genetic variants. Pathogenic and likely pathogenic variants were defined according to the American College of Medical Genomics and Genetics (ACMG) guidelines[21]. The presence of ctDNA was determined by the presence of pathogenic and likely pathogenic variants, which are also considered tumor mutations. For variants of uncertain significance, if the prevalence of the variants in the normal population was less than 0.01 in a genomic database (1000 Genomics, ESP6500 and ExAC) and predicted to be deleterious by computer software (SIFT, PolyPhen2, and CADD), then they were classified as "highly suspected deleterious". The above filtering analyses can remove the germline variants as much as possible[22]; these variants were highly suspected to originate from tumors, and the detection of these variants could be considered indicative of ctDNA.

Analysis of copy number changes

Since the *Her2*, *c-Myc*, *CCND1* and *S100A* genes can be amplified in some breast cancer tumors, we decided to use copy number alterations to indicate the presence of ctDNA[23-25]. Copy number variation (CNV) was analyzed by OncoCNV (https://github.com/BoevaLab/ONCOCNV) according to the authors' instructions. The baseline control consisted of 14 healthy people who were enrolled to generate their ctDNA BAM files. The ctDNA BAM files from breast cancer patients were compared to the BAM files from the control population by using OncoCNV's default *cghseg* segmentation algorithm[26]. The sequencing region of each targeted gene was divided into several segments. When the mean of all segments of each gene was significantly different from the baseline, such as when the copy number predicted was greater than three copies or fewer than one copy from the baseline, we considered that to indicate a CNV alteration, which indicated the presence of ctDNA.

Statistics

The chi-squared test and Fisher's exact test were used to calculate the significance of the variance between each group. Survival was estimated by Kaplan-Meier analysis. Cox proportional hazards regression analysis was used to estimate the hazards ratios of RFS with a corresponding 95% confidence interval (CI) for various factors. All *p* values are two-sided, and *p*-values less than 0.05 were considered statistically significant.

Results

Evaluation of assay performance

First, to confirm the accuracy of the NSG-based deep sequencing, we checked whether this method could distinguish the true existence of low-abundance mutants from background errors arising from polymerase chain reaction (PCR) or sequencing process. We constructed a *TP53* mutant (NM_000546.6: c.844C>A) as a reference sample; then we utilized this *TP53* mutant with serial concentrations of 100%, 10%, 1%, and 0.1% to test whether the experimental method could detect these mutants at these concentrations (Supplementary Methods). The results demonstrated that the signal from 0.1% mutant was significantly higher than background errors (Supplementary Figure S1A), suggesting NGS testing accurately detected mutants present at 0.1%. In addition, the mutation level could be measured with a linear fashion (R^2 = 0.9997, Supplementary Figure S1B).

Second, in deep cell-free analyses, another source of variants that make it hard to distinguish cancer mutations is clonal hematopoiesis of indeterminate potential (CHIP)[27-29]. Although CHIP mutations mostly occur in the *DNMT3A*, *TET2*, *ASXL1* and *TP53* genes, pathogenic variants of *TP53* are the main mutations in breast cancer tissue[30]. We examined the concordance of *TP53* genetic alterations between ctDNA and DNA from diagnostic tumor biopsies, and 100% of them were compatible (Supplementary Figure S2), suggesting that genetic alterations of ctDNA originated from tumors.

Patients

A total of 95 patients were enrolled in this study. The median age was 50.0 years old. Forty-one patients had ER(+) Her2(-) breast cancer, 29 patients had Her2(+) breast cancer, and 25 patients had triple-negative breast cancer (TNBC). Before NAT, tumors with T1, T2 and T3-4 size classifications were found in three, 54 and 38 patients of each population, respectively. Eighty-two patients had positive axillary lymph nodes. According to standard clinical practice, ER(+) Her2(-) breast cancer patients with large tumors were treated with NAT. Out of the 95 patients, 77 patients received anthracycline while 80 patients received taxane in their NAT regimens. All Her2(+) patients received only trastuzumab or trastuzumab in combination with pertuzumab. After NAT, 13 patients achieved a pCR of their primary breast tumors; 82 patients had a non-pCR status. Among the 13 pCR patients, there was one ER(+) Her2(-), six Her2(+) and six TNBC patients. The frequency of pCR was significantly higher in patients with Her2(+) breast cancer or TNBC than ER(+)Her2(-) patients (p = 0.002). CtDNA was detected in 60 patients before NAT and 31 patients after NAT. All of the clinical and pathologic characteristics are shown in Table 1.

Genetic alterations in tumor ctDNA

Among the 95 patients, 19 patients were found to have ctDNA before and after NAT; 41 patients had ctDNA only before NAT, 12 patients had ctDNA only after NAT, and 23 patients had ctDNA neither before nor after NAT (Supplementary Table S1). The most common genetic variants were in the *TP53* (n = 28), followed by *PIK3CA* (n = 16), *CDH1* (n = 15), and *Her2* (n = 7) genes. Eighteen patients had altered CNVs in their ctDNA, including of *AKT1*, *CCND1*, *CDH1*, *c-MYC*, *Her2*, *PIK3CA*, *S100A*, and *ZNF703,* either before or after NAT (Supplementary Table S1 and Figure 1). Before NAT, Patient #73 (Figure 1A) and Patient #24 (Figure 1B) exhibited copy number gains of the *S100A* and *Her*2 genes in ctDNA, respectively; after NAT, the copy numbers of these genes in ctDNA returned to normal levels. Patient #3 (Figure 1C) had a new copy loss of the *PTEN* gene after NAT. We observed gains of *Her2* and *c-MYC* in patient #27 (Figure 1D) before NAT that were only partially resolved after NAT.

Association between ctDNA and clinical characteristics

Patients in whom ctDNA was detected before NAT tended to have a larger tumor size than those in whom ctDNA was not detected (mean 5.0 cm vs. 4.3 cm, p = 0.104, Supplementary Figure S3). However, the presence of ctDNA after NAT did not correlate with the tumor size or LN numbers after NAT. Although the difference was not significant, patients with pCR had a lower proportion of patients with detected ctDNA after NAT than patients with no pCR (patients with pCR vs. non-pCR: 15.4% vs. 35.4%, p = 0.132). (patients with pCR vs. non-pCR: 15.4% vs. 35.4%, p = 0.132). Additionally, the presence of ctDNA was not correlated with the molecular type of breast cancer.

Impact of clinical factors and ctDNA on RFS

The median follow-up time of the entire cohort was 5.1 years, and the 5-year recurrence-free survival (RFS) was 58% (95% CI 48.0 – 68.0%). For clinical factors, Kaplan-Meier analysis showed that the residual tumor size after NAT and N classification after NAT were prognostic factors for RFS; patients who achieved a pCR tended to have a better RFS than patients who did not achieve a pCR (Figure 2A – C and Table 2). On the other hand, patients with ctDNA after NAT had significantly inferior RFS (p < 0.001, Figure 2D). Other factors, such as age, ctDNA detection before NAT, molecular type, initial tumor size before NAT and N classification before NAT, did not influence RFS. RFS was similar between patients with and without *TP53*, *PIK3CA* and *CDH1* mutations (Table 2).

We then analyzed the clinical and pathologic characteristics of patients with and without ctDNA after NAT, and no difference was found between the two patient groups (Supplementary Table S2). After incorporating the residual tumor size, N classification after NAT, pCR and ctDNA after NAT, multivariate analysis showed that an N3 classification and ctDNA positivity after NAT were independent risk factors that predicted tumor recurrence (N3, hazard ratio (HR) 3.352, 95% CI 1.267 – 8.870, p = 0.015; ctDNA, HR 4.135, 95% CI 2.014 – 8.491, p < 0.0001). Other factors did not significantly impact RFS (Table 2).

Next, we analyzed the 72 patients who had detected ctDNA, either before or after NAT. Patients with ctDNA positivity after NAT had a significantly inferior RFS compared to those without detectable ctDNA (Supplementary Figure S4, p<0.001). After adjusting for tumor size (after NAT), N classification (after NAT) and pCR, multivariate analysis with the Cox model revealed that ctDNA positivity after NAT was the most significant risk factor that predicted tumor recurrence (HR 8.02, 95% CI 3.24 – 19.86, p < 0.0001) (Supplementary Table S3).

The impact of ctDNA on disease recurrence in different molecular types of breast cancer

The median RFS of all the patients with ctDNA positivity after NAT was 1.19 years. When stratified by the molecular type, ctDNA positivity after NAT was associated with a significantly inferior RFS for ER(+) breast cancer or TNBC patients and a trend of higher recurrence rates for patients with the Her2 subtype (Figure 3A – 3C). The median RFS of ER(+) breast cancer, Her2 (+) breast cancer and TNBC patients with ctDNA positivity after NAT were 0.90, 2.52 and 0.74 years, respectively.

The impact of ctDNA on disease recurrence in patients with and without a pCR

For the entire cohort, the presence of ctDNA after NAT was a significant risk factor associated with recurrence in both pCR and non-pCR patients (Figure 3D and 3E, all p < 0.001). Because pCR was previously reported as a surrogate marker for survival in patients with Her2(+) and TNBC[7], we analyzed these patient subgroups. Between the two patient populations, pCR was related to a trend of improved survival than non-pCR (HR 3.328, 95% CI 0.777 – 14.243, p = 0.105, Supplementary Table S4). Multivariate analysis showed that advanced nodal status and ctDNA after NAT were independently correlated with high risk (N2-3, HR 3.753, 95% CI 1.146–12.297, p = 0.029; ctDNA, HR 3.123, 95% CI. 1.139 – 8.564, p = 0.027), and pCR status did show a not significant correlation with recurrence (Table 3). A potential reason for this phenomenon is that pCR only represents the therapeutic efficacy of local breast tumor and the ctDNA may indicate that an occult lesion is present that is not effectively treated with NAT. In our study, 13 patients achieved a pCR after NAT, and among those patients, two exhibited ctDNA positivity after NAT. One patient (case #50) had TNBC and received neoadjuvant docetaxel/epirubicin (four cycles) and achieved a pCR for their primary breast and axillary tumors. However, she had hepatic metastases at 6 months after mastectomy (Supplementary Figure S5). The other patient (case #5) had Her2-positive breast cancer and received neoadjuvant docetaxel/trastuzumab (four cycles) and epirubicin/cyclophosphamide (four cycles). The pathology showed no residual tumors. Trastuzumab was continuously maintained for one year. At the end of trastuzumab treatment (13 months after mastectomy), a cerebellar metastasis was found. The other 11 patients who achieved a pCR did not have ctDNA after NAT nor did they experience recurrence or metastasis.

Discussion

Our data suggested that the presence of ctDNA after NAT is a prognostic factor that predicts breast cancer recurrence after mastectomy. Traditionally, the therapeutic response to NAT was considered a marker for predicting prognosis[7]. In our study, multivariate analysis showed a greater predictive value

for ctDNA than the response of the primary breast tumor to NAT treatment. ctDNA seems more representative of the therapeutic efficacy of primary and potential micrometastatic tumors treated with NAT.

During the median 5.1-year follow-up, the overall positive predictive value of ctDNA positivity after NAT for disease relapse was 70.9%, which was higher than the predictive value of 48.8% for relapse in non-pCR patients. After stratifying patients into pCR and non-pCR populations, ctDNA positivity after NAT remained a significant risk factor for RFS among the two patient groups (Fig. 3D and 3E). Although non-pCR patients usually had a significantly inferior RFS than pCR patients, ctDNA negativity after NAT in non-PCR patients was associated with a better RFS (Fig. 3E), compatible with previous findings that ctDNA clearance associated with the improved survival in non-pCR patients[31]. In contrast, pCR after NAT was a surrogate marker for predicting disease-free Her2(+) and TNBC patients. However, in our cohort, two patients (one Her2(+) and one TNBC) who achieved a pCR and exhibited ctDNA positivity after NAT developed distal metastasis at six months and one year, respectively. A possible reason is that the pCR was assessed using only primary breast tumor detection without evaluating systemic micrometastatic tumor cells. ctDNA is more suitable than pCR for representing the overall disease state and could be a robust marker for predicting the survival rate.

Although patients with ctDNA positivity after NAT had inferior RFS, the length of RFS varied among patients with different molecular types. Among patients with ctDNA positivity after NAT, patients with Her2-type breast cancer had a significantly longer RFS than patients with TNBC and luminal breast cancers. The maintenance of anti-Her2 antibody therapy and the potential long-term preservation of antibody-dependent cellular cytotoxicity may explain the risk attenuation and delayed relapse of Her2-positive breast cancer patients[32]. In this study, all twelve Her2-positive breast cancer patients received postmastectomy adjuvant anti-Her2 therapy; one received trastuzumab emtansine, another received trastuzumab plus pertuzumab, and the remaining patients received trastuzumab for one year. For the eight TNBC patients, only one received adjuvant chemotherapy. Out of the eleven patients with ER(+) breast cancer, six received adjuvant chemotherapy, and all of them received hormone therapy. Notably, the median RFS of TNBC and ER(+) breast cancer patients was less than one year. This result might suggest that current standard chemotherapy and hormone therapy treatments were not effective for these patients. CtDNA has the potential to identify actionable genetic variants that provide sensitivity or resistance mechanisms for chemotherapy and/or targeted therapy; this information can be used to guide personalized therapy in the future[33]. Alternative adjuvant therapy options can be explored for these patients.

The concordance between pCR and the clearance of ctDNA was moderate. The ctDNA concentration usually decreases after NAT[18, 34]. In a previous report, the decrease in ctDNA levels in patients who achieved a pCR was greater than that in those who did not achieve a pCR[34]. Similarly, our data revealed that a lower proportion of patients who achieved a pCR exhibited ctDNA positivity after NAT than that in non-pCR patients (pCR vs. non-pCR: 15.4% vs. 35.4%, $p = 0.132$). Among the 72 patients with ctDNA positivity (before and after NAT), 81.0% of responders had a decrease in ctDNA (defined as a tumor size

reduction of more than 30% of the original size)[35], whereas 58.9% of nonresponders had a decrease in ctDNA concentrations (Pearson's chi-squared, p = 0.088, Fig. 4A and 4B).

A limitation of this study was that we only examined ctDNA before and after NAT and did not perform longitudinal monitoring; as a result, we were not able to detect late recurrence. In our cohort, 42 patients had disease recurrence. Out of those 42 patients, 22 exhibited ctDNA positivity after NAT. The 22 patients with ctDNA positivity had a significantly shorter time to recurrence than those with ctDNA negativity (with ctDNA vs. without ctDNA: 1.31 vs. 2.64 years, p = 0.004, Fig. 4C). A single-time point sample of ctDNA after NAT was a only significant predictor of only early recurrence. Longitudinally tracking ctDNA may improve the predictive value for both early and late recurrence[34].

Conclusions

We showed that ctDNA detection after NAT has great clinical utility potential as a prognostic marker in patients with breast cancer. CtDNA detection can identify and define a subset of high-risk patients. The next step is to determine the type of adjuvant therapy strategies that can effectively reduce recurrence. Since actionable genetic variants can be detected by ctDNA, further prospective trials should focus on incorporating ctDNA detection and exploring how to guide patient treatment, which could maximize the utility of ctDNA detection.

Abbreviations

ctDNA
Circulating tumor DNA
CHIP
Clonal hematopoiesis of indeterminate potential
ER
Estrogen receptor
Her2
Human epidermal growth factor receptor
NAT
Neoadjuvant therapy
pCR
Pathologic complete response
RFS
Recurrence-free survival
TNBC
Triple-negative breast cancer

Declarations

Ethics approval and consent to participate

Ethical approval was obtained from ethical committees of National Taiwan University Hospital (IRB number: 201704009RINC). All patients provided written informed consent. The study was conducted in accordance with the Declaration of Helsinki.

Consent for publication

Not applicable.

Availability of data and materials

The data on genetic variants in ctDNA are included in the supplementary information files. Other datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Competing interests

PH Lin reports receiving unrelated sponsored research grants, lecture fees and travel support from AstraZeneca and Pfizer. CS Huang reports receiving advisory board fees from Amgen, Eli Lilly, Pfizer and Roche and receiving travel support from Amgen, AstraZeneca, Pfizer and Roche. The other authors declare no competing interests.

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Author contributions:

CS Huang had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. Study concept and design: PH Lin and CS Huang. Patient collection: PH Lin, MY Wang, LW Tsai, C Lo, SS Kuo and CS Huang. Performing experiments and bioinformatics: TC Yen, TY Huang, CK Chen, K Yang, WC Huang and SC Fan. Acquisition, analysis, or interpretation of data: all authors. Drafting of the manuscript: PH Lin and CS Huang. Critical revision of the manuscript for important intellectual content: all authors.

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Tables

Table 1. Clinical and pathologic characteristics of enrolled patients stratified by molecular type

	All	ER(+)Her2(-)	ER(±)Her2(+)	TNBC
Number	95	41	29	25
Age (mean ± SD)	50.0 ± 8.8	49.2 ± 7.8	49.3 ± 8.7	52.0 ± 10.2
T classification (before NAT)				
T1	3	0	1	2
T2	54	19	16	19
T3-4	38	22	12	4
N classification (before NAT)				
N-negative	13	4	6	3
N-positive	82	37	23	22
T classification (after NAT)				
no tumor	13	1	6	6
T1	32	10	13	9
T2	29	16	6	7
T3-4	21	14	4	3
N classification (after NAT)				
N0	34	7	16	11
N1	29	9	10	10
N2	22	17	2	3
N3	10	8	1	1
Response				
pCR	13	1	6	6
non-pCR	82	40	23	19
NAT regimen				
Anthracycline	77	33	24	20
Taxane	80	29	29	22
Trastuzumab/pertuzumab	29	0	29	0
Presence of ctDNA				
before NAT	60	33	15	12

after NAT	31	11	10	10

Table 2. Univariate and multivariate analysis of recurrence-free survival of the entire cohort

variables	univariate				multivariate			
	HR	lower	upper	P value	HR	lower	upper	P value
Age (>50 vs. <50)	0.962	00.525	1.763	.899				
T classification (before NAT)								
T1-2	1							
T3-4	1.026	.553	1.903	0.936				
N classification (before NAT)								
N-negative	1							
N-positive	2.266	0.700	7.336	0.172				
T classification (after NAT)								
no tumor	1				1			
T1	2.536	0.568	11.333	0.223	1.963	0.333	11.575	0.456
T2	4.842	1.112	21.083	0.036	2.435	0.450	13.186	0.302
T3-4	4.158	0.929	18.604	0.062	2.338	0.488	11.202	0.288
N classification (after NAT)								
N0	1				1			
N1	0.953	0.401	2.263	0.914	1.378	.526	3.606	0.514
N2	1.750	0.798	3.838	0.163	1.418	.611	3.293	0.416
N3	3.055	1.246	7.487	0.015	3.352	1.267	8.870	0.015
Response								
pCR 1					1			
non-pCR	3.656	0.883	15.134	0.074	2.230	0.468	10.623	0.314
Molecular type								
ER/PR(+)Her2(-)	1							
ER/PR(+)Her2(+)	0.611	0.284	1.314	0.207				
TNBC	1.294	0.639	2.622	0.474				
ctDNA								
before NAT*	0.700	0.378	1.298	0.257				
after NAT*	3.894	2.113	7.177	<0.001	4.135	2.014	8.491	<0.001

Genes				
TP53#	1.156	0.609	2.197	0.657
CDH1#	0.669	0.263	1.704	0.399
PIK3CA#	1.313	0.607	2.837	0.489

*The presence of ctDNA vs. nonpresence of ctDNA; #gene mutation vs. nonmutation

Table 3. Multivariate analysis of recurrence-free survival in patients with Her2(+) breast cancer and TNBC

Variables	HR	lower	upper	P value
T classification (after NAT)				
no tumor	1			
T1	0.909	0.167	4.952	0.912
T2	2.461	0.435	13.917	0.308
T3-4	4.082	0.756	22.038	0.102
N classification (after NAT)				
N0	1			
N1	1.845	.633	5.378	0.262
N2-3	3.753	1.146	12.297	0.029
Response				
pCR	1			
non-pCR	4.082	0.756	22.038	0.102
ctDNA after NAT				
undetected	1			
detected	3.123	1.139	8.564	0.027

Figures

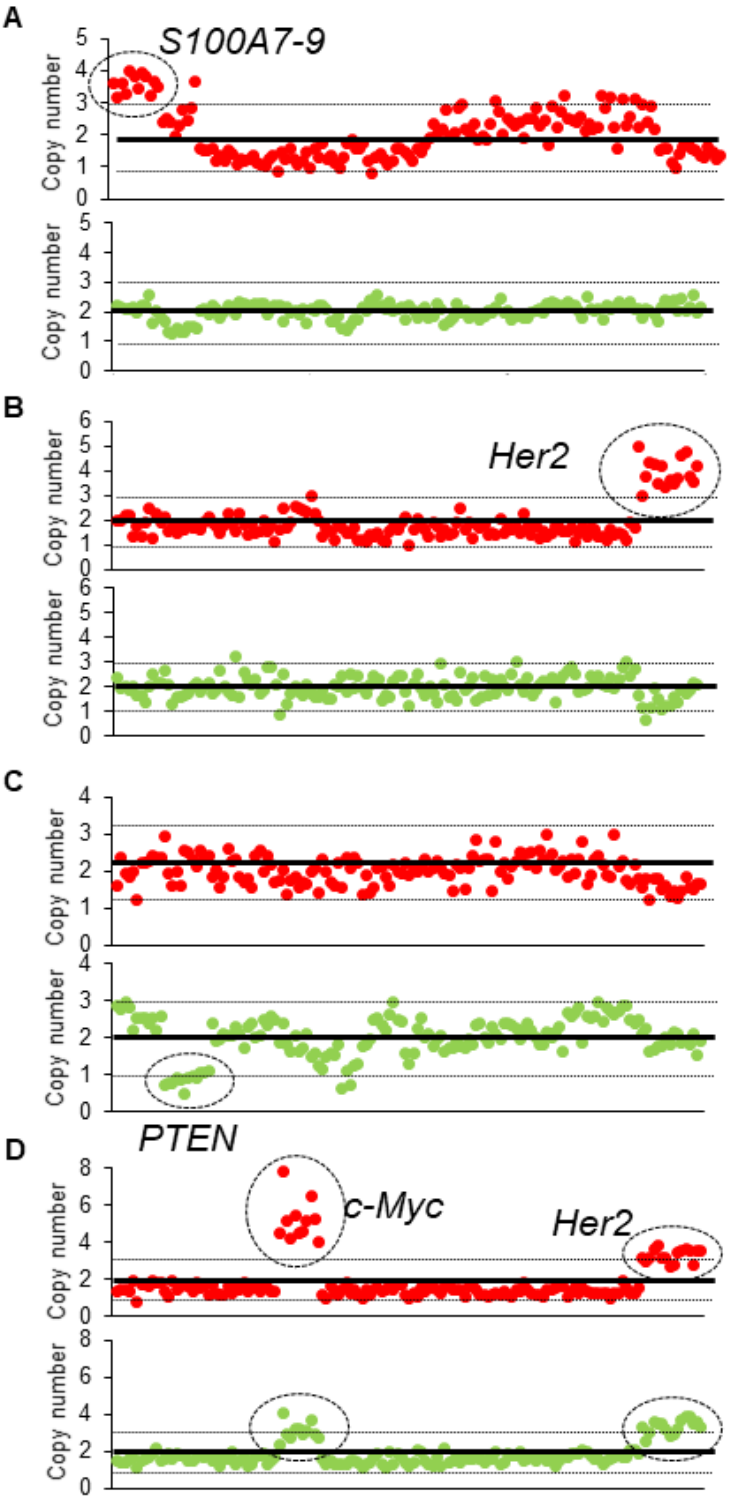

Figure 1

The CNV of four patients before and after NAT (A – D). The red dots represent the CNV before NAT, and green dots represent the CNV after NAT.

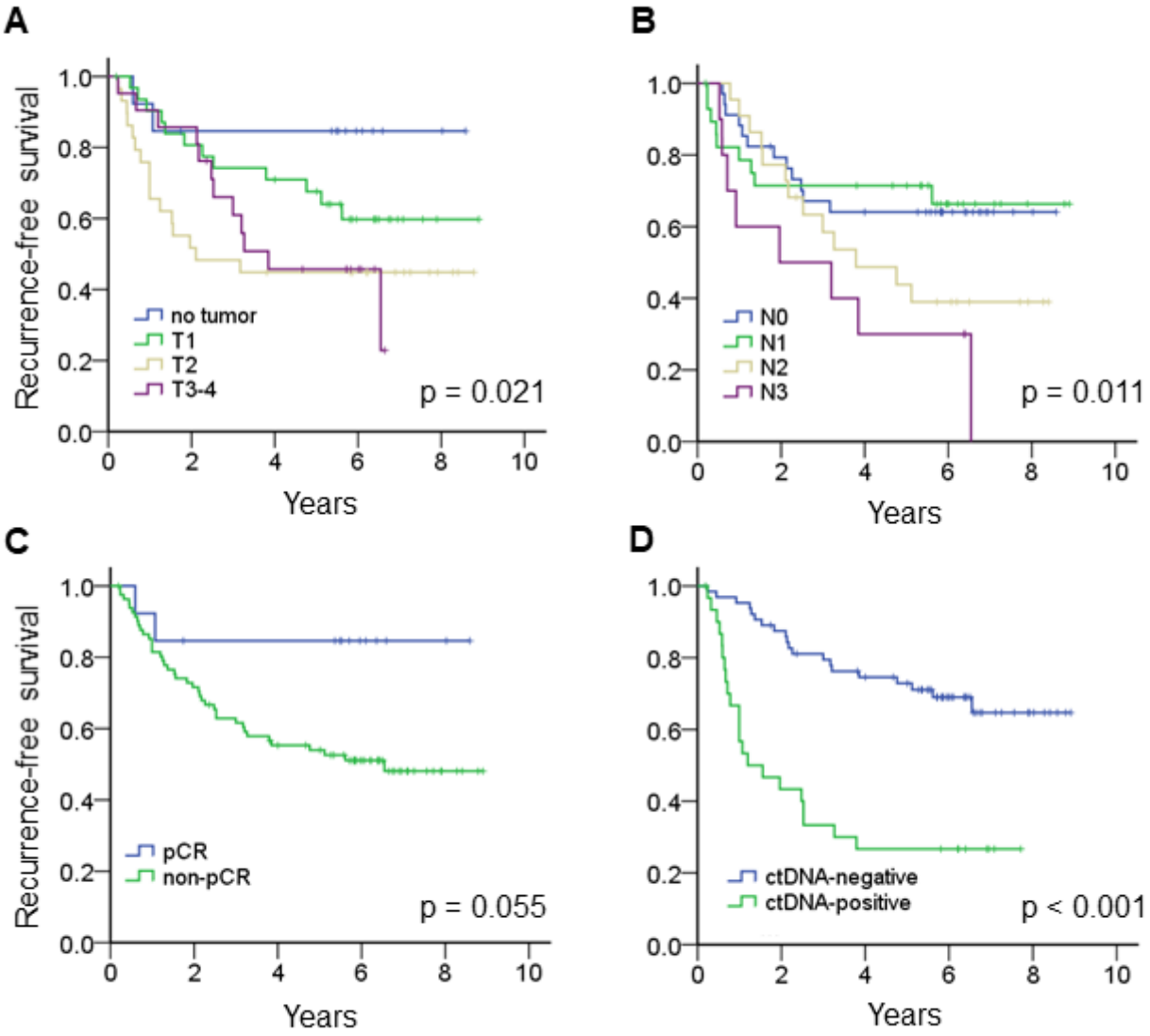

Figure 2

Kaplan-Meier analysis estimated the recurrence-free survival of the entire cohort according to (A) the tumor size after NAT (p = 0.021), (B) N classification after NAT (p = 0.011), (C) pCR (p = 0.055) and (D) ctDNA after NAT (p < 0.001).

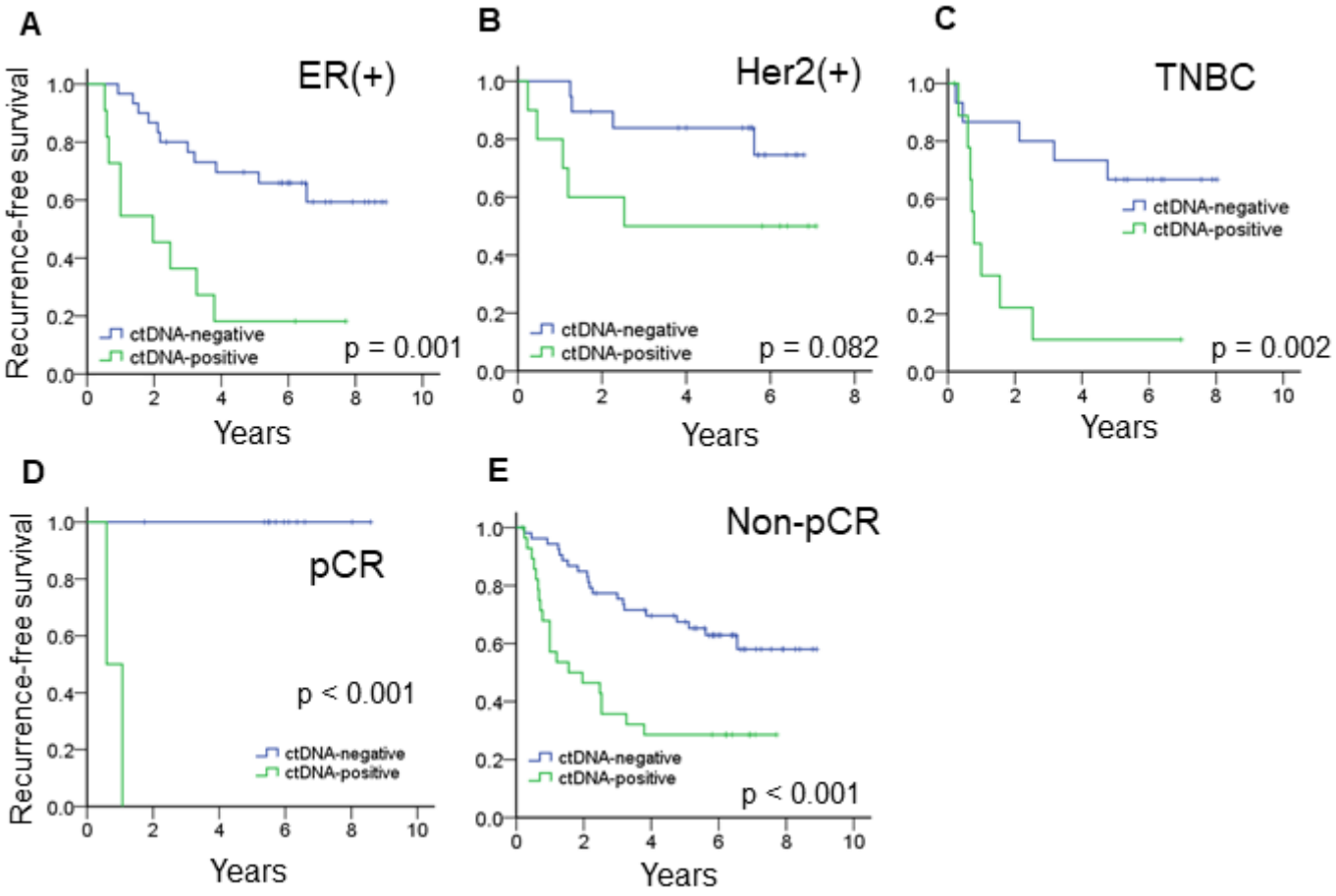

Figure 3

The prognostic impact of ctDNA after NAT in patients with (A) ER(+) breast cancer, (B) Her2(+) breast cancer and (C) TNBC. ctDNA after NAT predicted RFS in (D) pCR and (E) non-pCR patients.

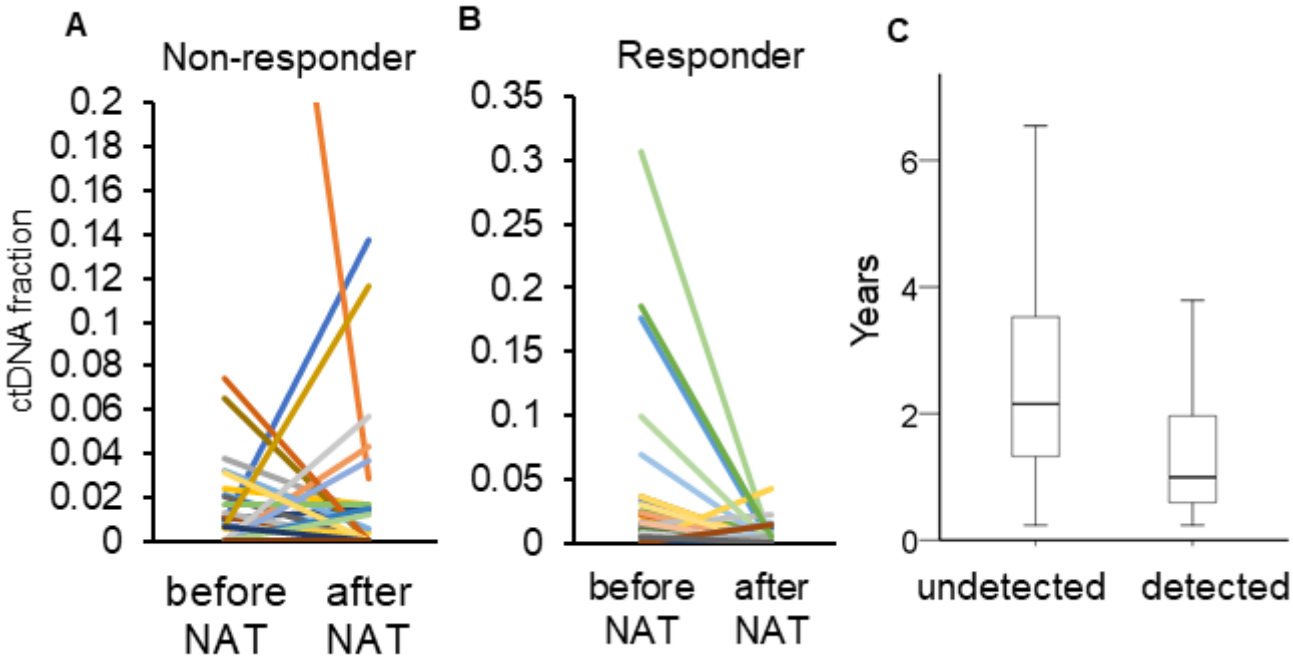

Figure 4

(A and B) Changes in the fraction of ctDNA in patients who did and did not respond. (C) The duration of RFS in patients with (detected) and without (undetected) ctDNA after NAT.

Supplementary Files

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