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Original Article

Changes of Cell-Free DNA Level in the Plasma of Breast Cancer Patients during Neoadjuvant Chemotherapy

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ABSTRACT

Background and Aim: Preoperative chemotherapy is increasingly given to shrink breast tumors prior to surgery. Plasma cell-free DNA (cfDNA) is elevated in cancer patients and decreases in response to effective treatments. Thus, cfDNA is considered as potential tumor marker for monitoring chemotherapy. **Methods:** In the present study, we investigated whether the plasma DNA concentrations in patients with breast cancer are altered during the course of CMF, CAF neoadjuvant chemotherapy. **Results:** In CR, PR to chemotherapy patients, the cfDNA concentration decreases from week 1 of first cycle of treatment. In contrast, it increases after 2 weeks in patients who show NC, PD, without reference to chemotherapy method. **Conclusion:** This study shows the possibility of using cfDNA changes as a useful biomarker with other molecular changes for personalized medicine of breast cancer.

Keywords: Breast cancer, Neoadjuvant chemotherapy, Plasma cell free DNA, CMF chemotherapy, CAF chemotherapy.

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INTRODUCTION

Breast Cancer (BC) is the most common cancer and the second cause of cancer-related death in women worldwide.[1] Chemotherapy is one among the most common effective treatment for breast cancer, alongside radiotherapy, hormone therapy, and targeted treatments. Pre-operative chemotherapy is given prior to surgery with the aim to reduce the tumor burden and to provide early information on the response to treatment.[2] Preoperative chemotherapy is now considered as the standard of care in breast cancer and has seen a rise in recent years with data from powered studies suggesting that the pathological complete response achieved following neoadjuvant chemotherapy might be a surrogate of good prognosis.[3]

Therapeutic options are increasing, but the response to treatments is not always efficient.[4] Therefore, it is very important to identify biomarkers for the prediction of treatment efficiency in facilitating the practice of personalized medicine in breast cancer. In clinical routine, the evaluation of serum markers as Carcinoembryogenic Antigen (CEA) or Cancer Antigen 15-3 (CA15-3) is still used for BC follow-up, but with a low specificity and sensibility.[4] Up-to-now, one of the most promising frontiers in this field is the liquid biopsy. Liquid biopsy provides the basic principle for a non-invasive method for the routinely monitoring of BC. Recently, the meta-analysis on the clinical utility of Circulating Tumor Cells (CTC) in early BC or in Metastatic BC (MBC) provides a solid rationale for their use in oncological settings. However, their routinely use is still compromised by the relatively high cost of the technique.[4]

Plasma free DNA is released into the blood circulation by cell lysis, necrosis, apoptosis, active release and by many other mechanisms.[5,6]

Cell free DNA is contained in small amounts in a healthy person's plasma or serum but is increased in patients with cancer and many other disorders. Thus, it is considered to be an important source of markers for genetic analysis in cancer patients and many others having precancerous disorders.[7-9]

CfDNA changes include quantitative and qualitative changes. Quantitative changes refer to changes of total cfDNA amounts, while qualitative changes include cfDNA strand integrity, tumor relative gene variation, methylation and mini-satellite changes.[10]

These quantitative changes result from the increased release of cfDNA from tumor cells and adjacent non-tumor cells.

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Concentrations of cfDNA are influenced by cancer-dependent variables such as tumor stage, size, location, and other risk and prognosis-related factors.[10] This change in quantitative level can be measured within a short time spending less cost and effort.

Both of plasma and serum are used as cell-free blood specimens for determination of the cfDNA. However, it has been addressed that plasma is more effective than serum as a source for measuring cfDNA.[10] The DNA concentrations in serum are 24-fold higher than those in plasma. These higher serum concentrations result from *in vitro* lysis of leukocytes during coagulation/fibrinolysis processes and do not correspond to actual cfDNA concentrations in the bloodstream of patients.[10]

Our previous study showed higher concentrations in BC patients with advanced tumor stage. And cfDNA concentrations were associated with tumor size (cutoff between 2 cm), lymph node status and tumor differentiation.[11]

But another study showed that it was not associated with estrogen/progesterone receptor status or HER-2/neu amplification status.[12]

Plasma cfDNA is elevated in cancer patients and decreased in response to effective treatments.[13] So circulating cell-free DNA is an important biomarker for early detection of cancer, residual disease, monitoring chemotherapy and other aspects of cancer management.[14]

Therefore, the present study was undertaken to investigate the changes of plasma DNA concentrations during the course of neoadjuvant chemotherapy in breast cancer patients.

MATERIALS AND METHODS

Between January, 2018 and October, 2019, primary breast cancer patients (*n*=57) received preoperative chemotherapy and operated were admitted in Breast Oncology institute in Pyongyang Maternity Hospital. Among them, 35 patients were given preoperative CMF chemotherapy (cyclophosphamide 600 mg/m^2, methotrexate 40 mg/m^2, 5-fluouracil 600mg/m^2 given intravenously on day 1 of therapy were repeated four 21-day cycles) and 22 were given preoperative CAF chemotherapy (cyclophosphamide 500 mg/m^2, doxorubicin 50 mg/m^2, 5-fluouracil 500 mg/m^2 given intravenously on day 1 of therapy were repeated four 21-day cycles). Table 1 shows the patient characteristics.

The efficacy of combination of preoperative CMF and CAF chemotherapy was evaluated according to the tumor shrinkage. Plasma free DNA was isolated using AxyPrepTM Multi source Genomic DNA Miniprep Kit. Plasma free DNA was measured using Nano-drop (Eppendorf-GERMANY) for nucleic acid concentration measurement.

Statistical Analysis of Data

Data were analyzed using SPSS 16.0. Results were expressed as the mean and SE. Two sample *t*-test was used to compare cfDNA levels and chemotherapeutic effect. P value ≤0.05 was considered to be statistically significant.

RESULTS

The relationship between preoperative CMF chemotherapeutic effect and plasma free DNA concentration in breast cancer patients

Table 2 shows the relationship between plasma free DNA concentration and CMF chemotherapy was studied in 35 breast cancer patients whose tumor shrinkage could be evaluated after preoperative CMF chemotherapy.

First, we analyzed the relationship between preoperative CMF chemotherapy and plasma free DNA concentration and as shown in Table 2, before therapy and after 1 week of first cycle in Complete Response (CR), Partial Response (PR) group (843.6±164.5, 477.5±82.7 ng/mL) and No Change (NC), Progressive Disease (PD) group (959.0±171.7, 722.9±111.7 ng/mL). There was no significant difference in the cell free DNA concentration (p=0.640, p=0.107) and moreover after 2 weeks and 3 weeks of first cycle, second, third, and fourth cycle of therapy the CR, PR group's plasma free DNA concentration (267.8.5±31.6, 275.7±36.0, 128.3±87.8, 99.2±15.4, 92.0±9.1 ng/mL) was significantly low (p<0.001) compared to NC, PD group's concentration (1762.4±187.9, 3005.8±276.8, 2663.0±207.0, 1917.1±151.5, 1500.9±120.0 ng/mL).

Next, we analyzed the relationship between preoperative CMF chemotherapeutic efficacy and plasma free DNA concentration according to the cycle of therapy and as shown in Table 2 in the group that show CR, PR to preoperative CMF chemotherapy, the plasma free DNA concentration was significantly lowered after treatment in the following times; first week of first cycle (477.5±82.7 ng/mL, p=0.007), second week (267.8.5±31.6 ng/mL, p=0.001), third week (275.7±36.0 ng/mL, p=0.001), after second cycle (128.3±22.7 ng/mL, p=0.001), after third cycle (99.2±15.4 ng/mL, p<0.001), after fourth cycle (92.0±9.1 ng/mL, p<0.001).

In addition, as shown in Table 2 the plasma free DNA concentration tends to be decreased at week 1 of first cycle of therapy (722.9±111.7 ng/mL, p=0.058) in the group that show NC, PD to preoperative CMF chemotherapy, and week 2 (1762.4 ±187.9 ng/mL, p=0.004), week 3 (3005.8±276.8 ng/mL, p<0.001), after second cycle (2663.0 ±207.0 ng/mL, p<0.001), after third cycle (1917.1±151.5 ng/mL, p<0.001), after fourth cycle (1500.9±120.0 ng/mL, p=0.002), it was significantly high.

Figure 1 shows the transition curve of plasma free DNA concentration according to therapy cycle and efficacy of preoperative CMF chemotherapy in breast cancer.

Table 1: Patient characteristics.

Classification	Treatment Method		*p* value
	CMF(*n*=35)	CAF(*n*=22)	
Age			
≤39 yr	9	5	0.966
40~49 yr	14	9	
≥50 yr	12	8	
Clinical T stage[a]			
T2	4	3	0.932
T3	20	13	
T4	11	6	
Clinical N stage[a]			
N1	8	5	0.649
N2	15	7	
N3	12	10	
Clinical TNM stage[b]			
IIb	2	2	0.627
III	33	20	
Therapy Efficiency			
CR, PR	15	10	0.847
NC, PD	20	12	

a: There was none in T1 stage and in N0 stage.b: In clinical TNM stage the patients with I, IIa, IV stage were not included in the study.**CR:** Complete response; **PR:** Partial response; **NC:** No change; **PD:** Progressive disease.

Table 2: Relationship between preoperative CMF chemotherapy and plasma free DNA concentration.

Classification	Plasma free DNA concentration (ng/mL) $\bar{X}$ ± SE (95% CI)		P1	P2	P3
	CR, PR (n=20)	NC, PD (n=15)			
Before Treatment	843.6±164.5	959.0±171.7	0.640	-	-
Week 1 of First Cycle	477.5±82.7	722.9±111.7	0.107	0.007	0.058
Week 2	267.8±31.6	1 762.4±187.9	<0.001	0.001	0.004
Week 3	275.7±36.0	3 005.8±276.8	<0.001	0.001	<0.001
Second Cycle	128.3±22.7	2 663.0±207.0	<0.001	0.001	<0.001
Third Cycle	99.2±15.4	1 917.1±151.5	<0.001	<0.001	<0.001
Fourth Cycle	92.0±9.1	1 500.9±120.0	<0.001	<0.001	0.002

P1: Comparison of CR, PR and NC, PD groups preoperative CMF chemotherapy; **P2:** Comparison of before and after treatment according to cycle of preoperative CMF chemotherapy in CR, PR group; **P3:** Comparison of before and after treatment according to cycle of preoperative CMF chemotherapy in NC, PD group.**CR:** Complete response; **PR:** Partial response; **NC:** No change; **PD:** Progressive disease.

As shown in figure 1 in the group that show CR, PR to preoperative CMF chemotherapy, plasma free DNA concentration started to decrease from week 1 of first cycle of treatment and by the fourth cycle of treatment the results were the lowest and in the group that show NC, PD the results decreased at week 1 of first cycle of treatment but started to increase from week 2 and reached its peak at week 3 and started to decrease after second cycle until fourth cycle of treatment but it was significantly higher than before treatment.

The relationship between preoperative CAF chemotherapeutic effect and plasma free DNA concentration in breast cancer patients

In 22 breast cancer patients whose tumor shrinkage could be evaluated after preoperative CAF chemotherapy and we studied

Table 3: Relationship between preoperative CAF chemotherapy and plasma free DNA concentration.

Classification	Plasma free DNA concentration(ng/mL) $\bar{X}$ ± SE (95% CI)		P1	P2	P3
	CR, PR (*n*=12)	NC, PD (*n*=10)			
Before treatment	920.3±222.0	1 079.5±201.3	0.601	-	-
Week 1 of First Cycle	414.6±114.6	707.3±130.1	0.114	0.037	0.121
Week 2	232.0±25.2	1 846.5±386.7	0.002	0.011	0.042
Week 3	164.4±33.7	2 966.8±554.9	<0.001	0.005	0.001
Second Cycle	145.2±44.0	2 734.9±611.0	0.001	0.006	0.007
Third Cycle	107.4±13.2	1 932.8±284.0	<0.001	0.004	0.003
Fourth Cycle	103.6±15.3	1 518.5±208.6	<0.001	0.004	0.042

P1: Comparison of CR, PR and NC, PD groups preoperative CMF chemotherapy; **P2:** Comparison of before and after treatment according to cycle of preoperative CMF chemotherapy in CR, PR group; **P3:** Comparison of before and after treatment according to cycle of preoperative CMF chemotherapy in NC, PD group.**CR:** Complete response; **PR:** Partial response; **NC:** No change; **PD:** Progressive disease.

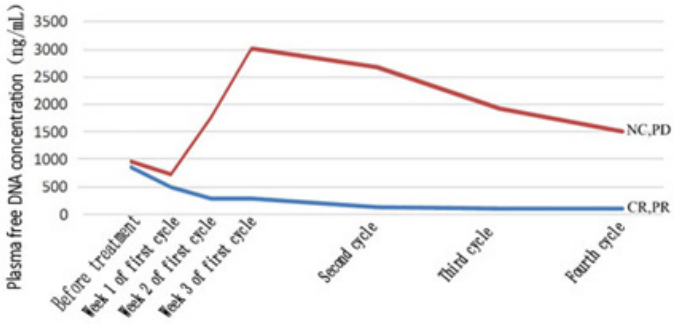

Figure 1: Transition curve of plasma free DNA concentration according to therapy cycle and efficiency of preoperative CMF chemotherapy in breast cancer.

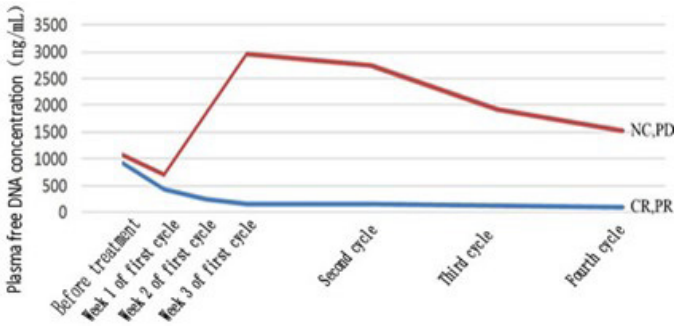

Figure 2: Transition curve of plasma free DNA concentration according to therapy cycle and efficiency of preoperative CAF chemotherapy in breast cancer.

the relationship between plasma free DNA concentration and CAF chemotherapy.

First we analyzed the relationship between preoperative CAF chemotherapy and plasma free DNA concentration and as shown in Table 3, before therapy and after 1 week of first cycle in CR, PR group (920.3±222.0, 414.6±114.6 ng/mL) and NC, PD group (1079.5±201.3, 707.3±130.1 ng/mL), there was no significant difference in the cell free DNA concentration (p=0.601, p=0.114) and moreover after 2 weeks and 3 weeks of first cycle, second, third, and fourth cycle of therapy the plasma free DNA concentration of CR, PR group (232.0±25.2, 164.4±33.7, 145.2±44.0, 107.4±13.2, 103.6±15.3 ng/mL) was significantly low compared to NC,PD group's concentration (1846.5±386.7, 2966.8±554.9, 2734.9±611.0, 1932.8±284.0, 1518.5±208.6) (p=0.002, p<0.001, p=0.001, p<0.001, p<0.001).

Next, we analyzed the relationship between preoperative CAF chemotherapeutic efficacy and plasma free DNA concentration according to the cycle of therapy and as shown in Table 3 in the group that show CR, PR to preoperative CAF chemotherapy, the plasma free DNA concentration was significantly low compared to preoperative state (920.3±222.0 ng/mL) in the following times; 1 week of first cycle (414.6±114.6 ng/mL, p=0.037), second week (232.0±25.2 ng/mL, p=0.011), third week (164.4±33.7 ng/mL, p=0.005), after second cycle (145.2±44.0 ng/mL, p=0.006), after third cycle (107.4±13.2, p=0.004), after fourth cycle (103.6±15.3, p=0.004).

And as shown in Table 3 in the group that show NC, PD to preoperative CAF chemotherapy, the plasma free DNA concentration decreased at week 1 of first cycle of therapy (722.9±111.7 ng/mL, p=0.058) after treatment (1079.5±201.3 ng/mL) but no significant difference was observed and at week 2 (1846.5±386.7ng/mL, p=0.042), week 3 (2966.8±554.9 ng/mL, p=0.001), after second cycle (2734.9±611.0 ng/mL, p=0.007), after third cycle (1932.8±284.0ng/mL, p=0.003), after fourth cycle (1518.5±208.6 ng/mL, p=0.042), it was significantly high.

Figure 2 shows the transition curve of plasma free DNA concentration according to therapy cycle and efficacy of preoperative CAF chemotherapy in breast cancer. As shown in Figure 2 in the group that show CR, PR to preoperative CAF chemotherapy, plasma free DNA concentration started to decrease from week 1 of first cycle of treatment and by the fourth cycle of treatment the results were the lowest and in the group that show NC, PD the results decreased in week 1 of first cycle of treatment but started to increase from week 2 and reached its

peak in week 3 and started to decrease after second cycle until fourth cycle of treatment but it was significantly higher than before treatment.

DISCUSSION

Breast cancer is the most common cancer and the second cause of cancer-related death in women.[1] In most breast cancer patients, chemotherapy is the most important treatment option and the chemotherapy field has made great progress recently by the development of many anticancer drugs. However, the response to treatment is not always efficient.[4]

The study of free DNA has opened a new prospect of noninvasive diagnosis and treatment and offers great help in early diagnosis, evaluation of therapeutic efficacy, metastatic status, prognosis and recurrence in breast cancer patients.[4,10] As plasma free DNA concentration changes with chemotherapy, we carried out a research among the breast cancer patients in our country to find out the relationship between plasma free DNA concentration and efficacy of preoperative CMF or CAF chemotherapy to evaluate the response to drugs and make a basic data to establish a personalized treatment strategy. The previous data concerning the relationship between plasma free DNA concentration and efficacy of preoperative CMF chemotherapy could not be found. On the previous study concerning the cultured MCF-7 breast cancer cells, it is reported that the combined use of Doxorubicin-Cyclophosphamid- 5-fluouracil is not only related to nonapoptotic cell death but also the combination of apoptosis. As a result, total DNA concentration increased during the adjuvant chemotherapy and this confirms that DNA concentration changes according to the different drugs used in the adjuvant chemotherapy.[15]

However, researchers found out that the non-apoptotic DNA fragments are the superior biomarkers for the evaluation of adjuvant chemotherapeutic efficacy in breast cancer and that the total DNA concentration decreases with preoperative chemotherapy.[16] According to our study in the patients received preoperative CMF chemotherapy, there was no significant difference in plasma free DNA concentration before treatment in the CR, PR group and NC, PD group. However, the plasma free DNA concentration of CR, PR group was significantly lower than NC, PD group ($p<0.001$, respectively) on week 1 of first treatment cycle but on week 2, week 3 of first cycle and second, third and fourth cycle (Table 2). In addition, among the patients received preoperative CAF chemotherapy there was no significant difference in plasma free DNA concentration before treatment in the CR, PR group and NC, PD group and the plasma free DNA concentration of CR, PR group was significantly lower than NC, PD group ($p=0.002$, $p<0.001$, $p=0.001$, $p<0.001$, $p<0.001$, respectively) at week 1 of first treatment cycle but at week 2, week 3 of first cycle and second, third and fourth cycle (Table 3).

This tells us that the plasma free DNA concentration changes differently in groups showing CR, PR and in groups showing NC, PD for some reason during cell destruction by drugs. From the fact that during cleavage stoppage by preoperative CAF, CMF chemotherapy the plasma free DNA concentration significantly decreased at week 2 in spite of no change of tumor shrinkage in CR, PR group, it is concerned that the total plasma free DNA concentration might be decreased by decrease of non-apoptotic DNA or activation of some phagocytosis mechanism for the plasma free DNA emerging during cell destruction.

Moreover, the previous study stated that the plasma free DNA is released from the proliferating tumor cells during tumor genesis and plasma free DNA release could be decreased if tumor growth is inhibited by anticancer drugs.

This suggests that when breast cancer patients receive preoperative CMF, CAF chemotherapy, the plasma free DNA concentration is significantly lower in CR, PR group than in NC, PD group.

Moreover, we observed the changes of plasma free DNA concentration according to treatment cycle in both groups that show CR, PR and NC, PD to preoperative CMF, CAF chemotherapy. According to our study, in the group which show CR, PR to preoperative CMF chemotherapy, the plasma free DNA concentration at week 1, after 2 weeks, 3 weeks, second cycle, third cycle and fourth cycle were significantly lower compared to before treatment ($p=0.007$, $p=0.001$, $p=0.001$, $p=0.001$, $p<0.001$, $p<0.001$ respectively) (Table 2).

In NC and PD groups, the plasma free DNA concentration tends to be decreased at week 1 of first cycle of therapy and after 2 weeks, 3 weeks, after second cycle, after third cycle, after fourth cycle, it was significantly high ($p=0.004$, $p<0.001$, $p<0.001$, $p<0.001$, $p=0.002$, respectively) (Table 2). And in CR and PR groups, the plasma free DNA concentration at week 1, after 2 weeks, 3 weeks, second cycle, third cycle and fourth cycle were significantly lowered after treatment ($p=0.037$, $p=0.011$, $p=0.005$, $p=0.006$, $p=0.004$, $p=0.004$ respectively) (Table 3).

In the NC and PD groups, the plasma free DNA concentration tends to be decreased at week 1 of first cycle of therapy and after 2 weeks, 3 weeks, after second cycle, after third cycle, after fourth cycle, it was significantly high ($p=0.042$, $p=0.001$, $p=0.007$, $p=0.003$, $p=0.042$) (Table 3).

We compared the changes in plasma free DNA concentration of all the groups and in the CR, PR group, it was started to decrease from week 1 of first cycle of treatment and was the lowest in fourth cycle and in the NC, PD group, it was decreased compared to before treatment at week 1 of first cycle but increased from week 2 and became the highest at week 3 and then it was started to decrease from second cycle until fourth cycle but was still significantly higher than before treatment (Figures 1 and 2).

This suggests that if the plasma free DNA concentration becomes lower than before treatment at week 2 of first cycle after carrying out preoperative CMF, CAF chemotherapy, we can estimate that the chemotherapy is taking effect and chemotherapy should be continued. However, when plasma free DNA concentration is higher than before treatment after 2 weeks then it should be considered that the chemotherapy is not effective and at this time the preoperative chemotherapy should be discontinued and the treatment strategy should be changed.

We would like to point out that this study has a certain amount of significance in the accurate and convenient prediction of the chemotherapeutic effect by analyzing plasma free DNA concentration in breast cancer patients who are undergoing personalized treatment.

CONCLUSION

First, in CR, PR group, the plasma free DNA concentration decreased significantly from week 1 of first cycle of treatment. Second, in NC, PD group, the plasma free DNA concentration increased from week 2 and reached the peak in week 3 and started to decrease thereafter but still remained higher than before treatment at the end of the therapy.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

ABBREVIATIONS

BC: Breast Cancer; **CEA:** Carcinoembryogenic Antigen; **CA15-3:** Cancer Antigen 15-3**; CTC:** Circulating Tumor Cells; **MBC:** Metastatic BC; **cfDNA:** Cell-Free DNA; **CMF Chemotherapy:** Cyclophosphamide, Methotrexate, 5-fluouracil; **CAF Chemotherapy:** Cyclophosphamide, Doxorubicin, 5-fluouracil; **CR:** Complete Response; **PR:** Partial Response; **NC:** No Change; **PD:** Progressive Disease.

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