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Analysis of Circulating Cell Free DNA (ccf-DNA) as a Biomarker in Breast Cancer- A Prospective Case Control Study.

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ABSTRACT

Serum ccf-DNA has recently emerged as a promising new indicator of the degree of the cell damage. Qualitative and quantitative testing of ccf-DNA can be applied for the management of malignant and benign neoplasia. The purpose of this study was to compare the serum ccf-DNA levels between breast cancer patients (pre and post neo-adjuvant chemotherapy) and healthy control. Also to analyze serum ccf-DNA in breast cancer patients and to correlate the changes with stages of cancer. 40 patients of histologically confirmed invasive breast carcinoma were taken as cases and 40 healthy individuals were taken as controls. The estimation of serum ccf-DNA before and after 2 cycles of neo adjuvant chemotherapy was done by spectrophotometric assay (Nano drop technique). The mean serum ccf-DNA concentration in cases were more than control group ($p<0.001$). The mean serum ccf-DNA level decreased significantly after neo-adjuvant chemotherapy ($p=0.019$). In stage II and III pre chemotherapy patients serum ccf-DNA were higher than post chemotherapy patients ($p<0.0001$). Serum ccf-DNA concentration can be used to judge the efficacy of neo-adjuvant chemotherapy. Depending on post chemotherapy changes of ccf-DNA levels, it may be used for the monitoring of chemotherapy response of the patients and also helpful to decrease mortality and morbidity of breast cancer patients. The change in the level of ccf-DNA has been associated with malignancy progression and tumour cell load. The serum ccf-DNA concentration may become a useful biomarker in breast cancer patient.

Keywords- Circulating Cell Free DNA, Breast Cancer, Neoadjuvant Chemotherapy, Spectrophotometry.

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INTRODUCTION

Breast cancer belongs to the most frequent and severe cancer types in women worldwide and its recurrence rates are very high (more than one million new cases per annum). Breast cancer is the most common malignancy and the second leading cause of death from cancer in women [1]. Widespread adoption of screening increases breast cancer incidence in a given population. The burden of breast cancer is growing worldwide and with it a more desperate need for better tools to detect, diagnose and monitor the disease is required. Serum tumor markers play an important role in patient management in malignancy [2-6].The most widely used serum markers in breast cancer are CA 15-3 and carcinoembryonic antigen (CEA). Less widely used markers include BR 27.29, tissue polypeptide antigen (TPA), tissue polypeptide specific antigen (TPS) and the shed form of HER-2 [7-9]. The potential uses of serum markers in breast cancer include aiding early diagnosis, determining prognosis, prospectively predicting response or resistance to specific therapies, surveillance after primary surgery and monitoring therapy in patients with advanced disease. Available prognostic factors for breast cancer include pathology criteria such as tumor size, tumor grade, and lymph node status [10],as well as newer biological factors such as hormone receptors, HER-2, urokinase plasminogen activator, and plasminogen activator inhibitor 1[11-12]. All of these factors require tumor tissue, thus necessitating either biopsy or surgery. Clearly, it would be desirable to have a circulating prognostic marker for breast cancer, particularly if it provided independent prognostic information. During the last few years, much research has been carried out to find new cancer biomarkers with the aim of reducing cancer mortality. However, most of the cancer biomarkers currently available are not sensitive or specific enough to be applied in routine clinical approaches.

The plasma/serum circulating cell-free DNA (ccf-DNA) can be utilized as a biomarker in various diseases. The ccf-DNA has the potential to provide biomarkers for certain cancers and disease states as well as identification of fetal DNA in maternal blood. Currently, significant advancements are being made in utilizing ccf-DNA as biomarkers for the early diagnosis, prognosis and monitoring of therapy for several cancer types and autoimmune diseases. The DNA is present in normal locations such as the nucleus and mitochondria or circulating free in the blood and body fluid. It can be utilized as a valuable biomarker. The ccf-DNA as a biomarker is easily accessible, reliable and reproducible. It is widely known that higher concentrations of free circulating DNA can be found, in most cases in the blood of patients with malignant diseases compared to healthy subjects. Several studies have been performed to establish whether a significant diagnostic or prognostic use could be found for circulating free DNA, both in quantity and quality, because of the noninvasive nature in which it can be obtained. Although much work has been done to determine the mechanism whereby these circulating DNA fragments are released into the blood [13]. The ccf-DNA is present in various body fluids including blood, urine, saliva, feces, synovial fluid, cerebrospinal fluid and peritoneal fluid [14]. In addition to assessing the quantities of ccf- DNA, qualitative features such as the circulating cell free DNA methylation level and fragment size distribution have been demonstrated to be useful diagnostic and prognostic markers in various pathologies.

One of the recent issues is the integrity of circulating cell free DNA, e.g., the ratio of longer to shorter DNA fragments. In normal tissues, cell death occurs predominantly via apoptosis producing short, uniform fragments of 185-200 base pairs (bp) in length [15]. Therefore, in healthy individuals the main source of circulating cell free DNA is thought to be apoptotic cells. In contrast, necrotic cell death is a frequent event in solid tumor and DNA fragment released from tumor cells are variable in length [16].The ccf-DNA (circulating cell free DNA) present in small amounts in the serum/plasma of healthy individuals, is found in increased amounts in serum/plasma of patients with distinct disorders including cancer [17]. It is known that double stranded DNA fragments frequently occur in considerable quantities in the serum or plasma of cancer patients [18-19]. The quantification of this free DNA in the serum of patients with various types of cancer and healthy individuals showed that the DNA concentration in the normal controls had a mean of 13ng/ml, whereas in the cancer patients the mean was 180ng/ml. The presence of high levels of circulating DNA in blood of tumor patients has been suggested to be caused by apoptosis and necrosis of tumour cells or release of intact cells into the bloodstream and their subsequent lysis [16, 20, 21]. The circulating DNA is mostly released from degrading cells after cleavage by endonucleases that cut the chromatin into the basic nucleosomal elements [22]**.** The actual origin of circulating cell free DNA remains mainly unknown with cell lysis, necrotic death, apoptosis and active release as possible mechanisms of DNA release into the blood circulation [23]**.** It is believed that apoptotic cell death is the predominant source of circulating cell free DNA in healthy individuals producing short, uniform DNA fragments whereas the DNA released from tumour cells varies in size [15]. The ccf-DNA has been studied in a wide range of physiological and pathological conditions like pregnancy, trauma,

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inflammatory disorders and malignancy. The ccf-DNA is released from apoptotic or necrotic cells reflecting a differential DNA origin as well as from living cells through a mechanism of active release [14, 24]. Necrosis is common in solid malignant tumours and generates a spectrum of DNA fragments with variable size due to random digestion by DNAses. In contrast, cell death in normal blood nucleated cells occurs mostly via apoptosis that generates small and uniform DNA fragments [Figure 1].

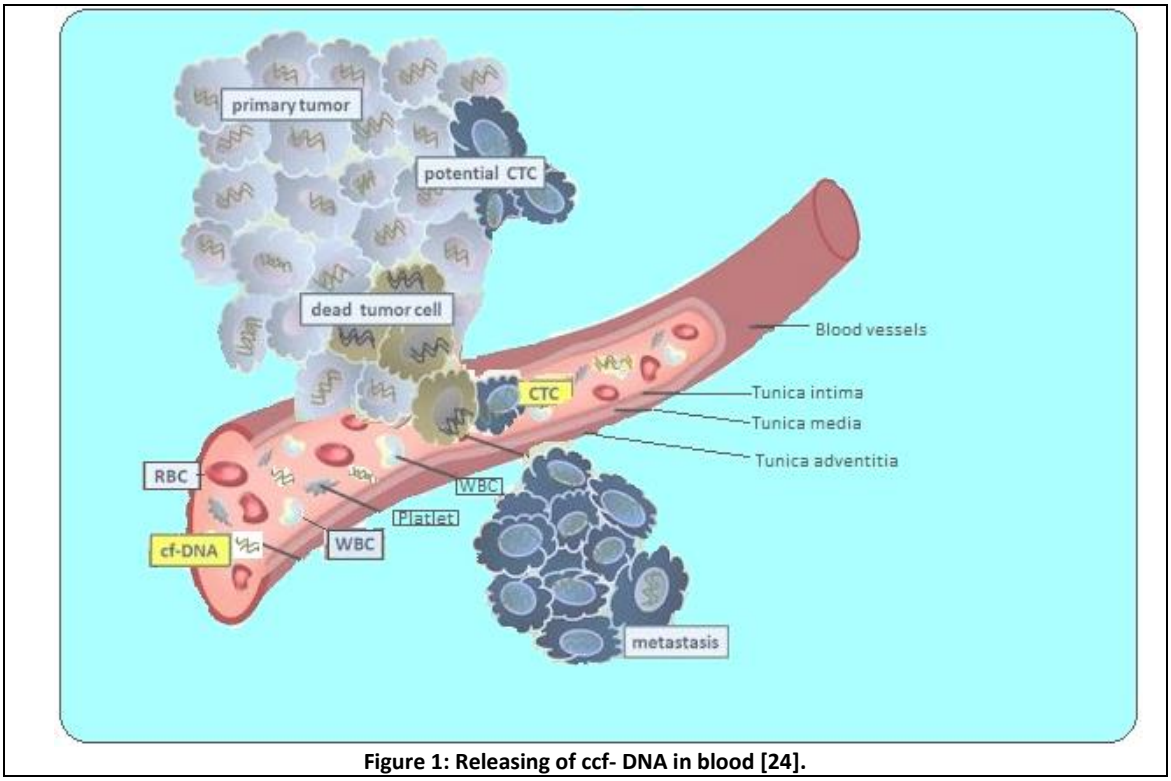

Figure 1: Releasing of ccf- DNA in blood [24].

In the present study, we investigated the concentration of serum ccf-DNA level during neo-adjuvant chemotherapy of breast cancer patient by spectrophotometric analysis (Nanodrop technique).

MATERIAL AND METHODS

Selection of patients

This prospective case control study was carried out in the Department of Biochemistry with collaboration of Department of Surgery, Institute of Medical Sciences, Banaras Hindu University Varanasi between October 2013 to July 2015.The level of significance is 5% and power of study is 90% of our variants. We selected 40 cases and 40 controls. The patients previously untreated but confirmed by histology as invasive breast carcinoma, admitted to General Surgery Department of Sir Sunderlal Hospital, Varanasi were taken as cases and 40 normal female patients of same age group and free of disease were taken as controls. Blood samples were collected, before and after two cycles of neo-adjuvant chemotherapy (combination of cyclophosphamide, adriamycin and 5FU given at 3 weekly interval). Ethical clearance was taken from Institute ethical committee (IEC, IMS, and BHU) before beginning the study and informed consent was taken before collection of blood sample in every case. After proper history and examination, patients underwent fine needle aspiration cytology for confirmation of diagnosis. In this study we excluded pregnant women, women on oral contraceptive pills for last three months, patients receiving HRT and patients with inflammatory breast diseases.

Sample collection

Under aseptic precaution 5 ml of venous blood sample was drawn from contra lateral hand of diseased breast cancer patients. The blood collected in clean and dry disposable tubes were allowed to stand

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for 30 minutes at room temperature for the retraction of clot and then centrifuged at 3000 rpm for 10 minutes to separate the serum. The serum samples were stored at -20^{0}C in the refrigerator for further analysis.

Procedure

The serum cell-free DNA Purification Kit (Norgen catalog no.-55500) provides a simple and direct procedure for DNA isolation. We first isolated the circulating cell free DNA from serum of breast cancer patients and then the level of cell free DNA was estimated.

The most common technique to determine DNA yield and purity is-"measurement of absorbance" by Nano Drop Spectrophotometer. Then we calculated sample purity ratio (260/280 nm and 260/230 nm) and ccf- DNA concentration.

Statistical analysis

All statistical analysis was done by using SPSS software. Differences between groups were assessed by using student's independent t–test and differences between pre and post chemo groups were assessed by using paired student's t test. The data were expressed as mean± SD, p value < 0.05 is considered as significant and mean ± SD, p value < 0.001 as highly significant.

RESULTS

The observations regarding the estimation of serum ccf- DNA was done at the time of presentation and after 2 cycles of neo-adjuvant chemotherapy. The changes in the level of serum ccf-DNA following 2 cycles of chemotherapy have been correlated with staging of tumor and prognosis after chemotherapy in the tumor patients. The mean serum ccf-DNA level in carcinoma breast patients was 16.37 ± 4.78 ng/µl and in control group was 4.62 ± 1.49 ng/µl ($p < 0.001$). There was an increase in the level of serum ccf-DNA seen in cases as compared to control [Figure 2]. We also compared pre chemotherapy serum ccf-DNA (mean-16.37 ± 4.78 ng/µl) with post chemotherapy serum ccf-DNA (mean- 7.61 ± 3.63 ng/µl). Overall, there was a higher activity of serum ccf-DNA seen in pre chemotherapy patients ($p < 0.001$).Both the results were highly significant [Figure 3]. In our study, out of 40 patients, 17 had stage II and 23 had stage III breast cancer. There was minimal difference in ccf-DNA levels as per the stage distribution. Breast carcinoma stage II pre chemotherapy patients serum ccf DNA level (mean 16.15 ± 5.20 ng/µl) is higher than post chemotherapy patients (mean 8.40 ± 3.45 ng/µl).The result is highly significant ($p < 0.001$). In stage III breast carcinoma pre chemotherapy patient serum ccf DNA level mean is 16.54±4.55 ng/µl and in post chemotherapy patients mean 7.03 ± 3.72 ng/µl. It is observed that in post chemotherapy patients ccf DNA level decreases. It is found to be statistically highly significant ($p < 0.001$; Figure 4).

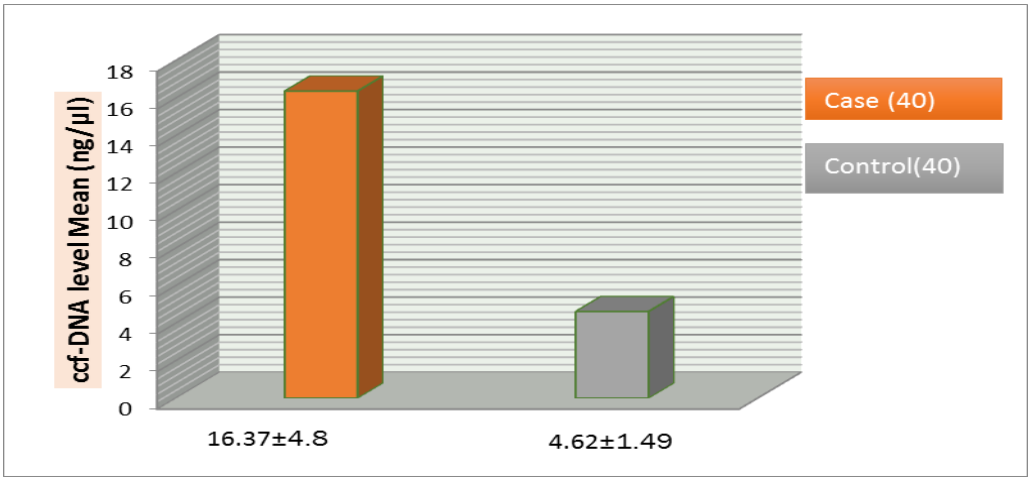

Figure 2: Comparison of serum ccf-DNA level between Breast cancer patients cases (Pre chemotherapy) and controls (by unpaired t test).

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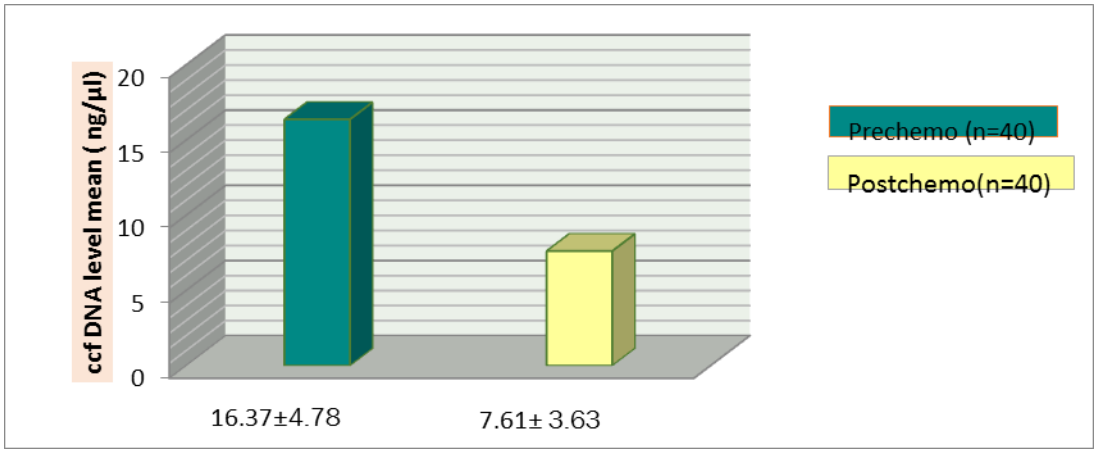

Figure 3: Comparison of serum ccf-DNA level between Breast cancer patients of pre chemotherapy and post chemotherapy (by paired t test).

DISCUSSIONS

Worldwide more than one million new cases of breast cancer are diagnosed every year and it is the leading cause of cancer death among women. It is the most commonly occurring tumor in women, and the second most common tumor, after lung cancer, in both genders [25].There is marked increase in the incidence of newly diagnosed case of breast cancer and the greatest increase has been seen in Asian countries which peaks among women in their forties [26]. It has a varied clinical, pathological, molecular features and treatment modalities. The most widely used serum markers in breast cancer are CA 15-3 and carcinoembryonic antigen (CEA). Less widely used markers include BR 27.29, tissue polypeptide antigen (TPA), tissue polypeptide specific antigen (TPS) and the shed form of HER-2 [7-9]. Biomarkers of breast cancer are necessary for prognosis, follow up, prediction of response to chemotherapy and as possible therapeutic targets. Prognostic biomarkers provide information regarding outcome irrespective of therapy, while predictive biomarkers provide information regarding response to therapy.

The DNA is present in normal locations such as the nucleus and mitochondria or circulating free in the blood and body fluid. Most of the nucleic acids (DNA and RNA) in the body are located within cells, but a fair amount of extracellular nucleic acids can also be found circulating in the bloodstream also called circulating nucleic acid [13,24,27,28,29,30]. The ccf-DNA is present in various body fluids including blood, urine, saliva, feces, synovial fluid, cerebrospinal fluid and peritoneal fluid [14]. The ccf-DNA is present in small amounts in the serum/plasma of healthy individuals but increased amount is present in serum/plasma of patients with distinct disorders including cancer [17]. The actual origin of circulating cell free DNA remains unknown but the cell lysis, necrotic death, apoptosis and active release are the possible mechanisms of DNA release into the blood circulation [23]**.** It is believed that apoptotic cell death is the predominant source of circulating cell free DNA in healthy individuals producing short, uniform DNA fragments, whereas the DNA released from tumour cells varies in size [15]. The ccf-DNA as a biomarker is easily accessible, reliable and reproducible.

In present study, we analysed serum circulating cell free DNA level as a biomarker in breast cancer patients on chemotherapy. This marker was increased in most of the patients of breast cancer as compared to control and significant change was also observed in pre and post chemotherapy patients. In pre chemotherapy patients ccf-DNA level was higher than the post chemotherapy patients.The comparison between mean levels of ccf-DNA in breast cancer patients of pre chemotherapy was 16.37 ng/µl and post chemotherapy was 7.61 ng/µl which was also highly significant (p value < 0.001). The decreased level of ccf-DNA after chemotherapy shows that breast cancer cells respond to therapy. The ccf- DNA levels in stage II breast carcinoma patients before chemotherapy (mean 16.15 ng/µl) was more as compared to post chemotherapy patients and stage III pre chemotherapy ccf-DNA level (mean 16.54 ng/µl) was also higher than post chemotherapy (mean 7.03 ng/µl). In both stages the results were highly significant (p < 0.001). This could be attributed to the fact that in cancer patients, ccf-DNA is released from apoptotic and necrotic cells as well as from living cells through a mechanism of active release [14] and very low amounts of ccf-DNA have been found in healthy individuals, although some authors consider it undetectable in plasma [31].

On the basis of the findings mentioned earlier, we concluded that serum ccf-DNA is an important biomarker in breast carcinoma and can be used for early diagnosis to determine the prognosis of disease process and for monitoring the response of chemotherapy. The present study is also useful to correlate staging of the cancer with the biomarkers and for monitoring the chemotherapeutic response of the patients and also helpful to decrease mortality and morbidity of breast cancer patients. The lacunae of our study are that it requires long term monitoring after treatment and genomic study is also required.

The present study is a preliminary one and requires further detailed study with large number of cases, controls and multicentric trials to validate the utility of this biomarker in the detection and the treatment of carcinoma breast and other malignancies.

CONCLUSION

On the basis of the findings mentioned earlier it was concluded that serum ccf DNA is an important biomarker in breast carcinoma and can be used for early diagnosis to determine the prognosis of disease process and for monitoring the response of chemotherapy. The present study is also helpful to correlate staging of the cancer with the biomarkers and for monitoring the chemotherapy response of the patients and also helpful to decrease mortality and morbidity of breast cancer patients.

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