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Circulating Tumor Cells in Breast Cancer: Correlation with Clinicopathological Parameters, Hormone Profile and MicroRNA Polymorphisms

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ABSTRACT

Objective: Circulating tumor cells are isolated tumor cells in the peripheral blood that serve as important prognostic indicators for many kind of tumors. The study was conducted to know the rate of detection of circulating tumor cells among breast cancer patients in comparison with benign breast diseases and control subjects and to know the association between CTC positivity and various clinicopathological parameters, hormonal profile and microRNA polymorphisms.

Material and Method: In the present case control study, we included 182 healthy controls, 108 cases of benign breast disease and 114 breast carcinoma cases. Various clinicopathological details of cases were recorded. Immunohistochemistry was performed for estrogen (ER) and progesterone receptors (PR) and Her-2 neu. Circulating tumor cells were analyzed using flow cytometry (EpCAM, CK, CD45). Genotypic frequency of micro RNA polymorphisms was determined by PCR-RFLP assay.

Results: Circulating tumor cell positivity was observed in 11/114 (9.64%) breast cancer cases but absent in benign and control groups, and was significantly associated with tumor size, histologic type, tumor grade, metastasis and skin infiltration ($p<0.05$). Circulating tumor cell positivity did not show any correlation with the immunohistochemical profile. No significant associations between pre-miRNA genetic variations miR-196a2 C/T (rs11614913), miR-146a G/C (rs2910164) and miR-499 T>C (rs3746444) polymorphisms and circulating tumor cell positivity were observed.

Conclusion: The flow cytometry protocol for detection and molecular characterization of circulating tumor cells is a time and cost-effective technique, suitable for routine clinical use. However, more elaborate studies are needed to establish the findings as our study was limited by small sample size.

Key Words: Breast cancer, Circulating tumor cells, Hormone receptors, microRNA, Flow cytometry

INTRODUCTION

Breast cancer is by far the most common cancer among women worldwide. According to the incidence of cancers, breast cancer ranks second in the world (1). According to the National Cancer Registry Program's (NCRP) recent report for 2008, the load of breast and cervical cancers together was 23.6-38.7% of total cancers in the Northeastern states of India, while in all the other states these two cancers contributed 35.2-57.7% of the total cancers (2). Different published reports of cancer registries in India indicate rising trends in breast cancer incidence (3).

The tumor cells shed into blood circulation from primary or metastatic cancers are referred to as circulating tumor cells (CTC). Although rare, CTC serves as a biomarker to evaluate the tumor genotypes during the course of treatment and progression of the disease. A proportion of CTC are capable of initiating a metastatic clone. CTC have been identified in a variety of epithelial cancers, predominantly breast, prostate, lung and colon. CTC are more likely to be detected in patients with metastatic disease, and they have also been reported in localized cancers (4,5).

For detection of CTC, a number of techniques are currently available, but none of these approaches constitute a desired optimal level to serve as a gold standard. Available techniques for CTC isolation and detection include either nucleic acid based detection (free DNA or RNA) (cell free circulating DNA, cfDNA) or intact CTC detection based on their physical properties (large cell size, differences in density, charge, migratory properties, granules etc.) or detection of CTC by directing antibodies against cell surface antigens (Cell Search System- FDA approved method, Isoflux and Flow cytometry). Among the cell

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surface antigens used with these technologies, the most widely used antibody is directed against epithelial cell adhesion molecule (EpCAM) (4-7).

CTC serve as important prognostic indicators. Various studies have concluded that CTC serve as independent prognostic markers in cancers of breast, prostate, lung and colorectum. The potential applications for CTC include isolation and identification of CTC (early diagnosis and prognosis), alteration in CTC levels to evaluate the response to new therapies (prognosis and prediction) and CTC phenotype and genotype (diagnosis, prognosis and direct therapy).

MATERIAL and METHODS

In the present case control study, we included 182 healthy controls, 108 cases of benign breast disease and 114 carcinoma breast cases. Healthy controls and diseased studied in the present work were of North Indian ethnicity and unrelated to each other. Patients were recruited (Dec 2010- Nov 2012) from the surgical oncology department; King George Medical University, Lucknow, India. Breast carcinoma patients included were those who had not received neoadjuvant chemotherapy yet. Controls were from healthy population and unrelated to diseased subjects. Informed consent in written was taken from all the study subjects. Approval from the institutional ethical committee was taken for the study protocols and the work done. The World Medical Association Declaration of Helsinki's norms were followed by the authors. Controls included fulfilled the criteria: no chronic disease, no history of present/ past malignancy or premalignant lesion. Cancer cases were frequency-matched to all the controls for characteristics like age, gender, and ethnicity.

Immunohistochemistry for estrogen receptor (ER), progesterone receptor (PR) and her-2 neu were performed on representative blocks of paraffin embedded tumor tissue. 4µm thick sections were taken on poly-L-lysine coated slides and submitted for immunohistochemistry. Antigen retrieval was done using citrate buffer at pH 2.5 for hormone receptors and pH 6 for her-2 neu. The normal breast ducts served as internal positive control for ER/PR. Breast carcinoma with known her-2 neu overexpression served as an external positive control for her-2 neu staining. ER or PR were considered positive when more than 1% of tumor cell nuclei were immunoreactive.

For interpretation of Her-2 neu staining the following method was used (8):

Score 0 (Negative): No staining is observed or membrane staining is observed in less than 10% of the tumor cells

Score 1+ (Negative): A faint/barely perceptible membrane staining is detected in more than 10% of the tumor cells. The cells are only stained in part of their membrane

Score 2+ (Weakly positive): A weak to moderate complete membrane staining is observed in more than 10% of the tumor cells

Score 3+ (Strongly positive): A strong complete membrane staining is observed in more than 30% (formerly 10%) of the tumor cells

Score 3+ was considered as positive immunostaining for Her-2 neu.

Flow Cytometry

This was performed on Beckton-Dickinson Fluorescence Activated Cell Sorter (FACS). The samples were immunostained with EpCAM peridinin chlorophyll protein complex, CD45 fluorescein isothiocyanate, and pan cytokeratin (CK – 8/18/19-phycoerythrin (PE) (all from BD Biosciences, San Jose, CA) for 30 minutes at 4°C. BD FACS lyse buffer (BD Biosciences) was added for 15 minutes after staining to lyse RBCs. A total of 500,000 events were collected for analysis on a 2-laser, 6-color BD FACS Canto device using BD FACS Diva software (both from BD Biosciences). The data were exported as FCS 3.0 files and analyzed using Flowjo (Tree Star, Ashland, OR) analysis software.

Genotypic frequency of miRNA polymorphisms was determined by PCR-RFLP assay. Details of genotyping and statistical analysis for miRNA's have been given in our prior publication (9).

Statistical Analysis

The Statistical analysis was done by SPSS Software version 15.0 and graph pad prism version 5.01. We applied Chi-square and Fisher's exact test wherever required.

RESULTS

Characteristic Profile of Controls, Benign and Carcinoma Cases

The present study included 404 study subjects, out of which 114 were breast carcinoma cases, 108 benign breast disease and 182 controls. Benign or malignant cases were biopsy/ cytology-proven. Mean ages were 36, 33 and 64 years for controls, benign and malignant disease respectively. Most of the patients in control group (69%), benign breast disease group (62.28%) and breast carcinoma group (69.29%) were Hindus followed by Muslims. Premenopausal patients formed the majority in all study groups i.e. controls

(88.46%), benign (100%) and carcinoma cases (62.28%). Details are shown in Table I.

Clinico-Pathological Profile of Breast Cancer and CTC Positive Cases

Eleven out of 114 breast cancer cases were positive for CTC (9.64%), with no CTC positive case in either control or benign group. In the <40 years age group, 3/36 (8.33%) patients were found to be CTC positive while 8/78 (10.25%) were CTC positive in > 40 years age group. Out of all CTC positive cases (11), 72.72% (8/11) were above 40 years of age (Figure 1). CTC positivity in premenopausal vs postmenopausal group was found to be 5.63% vs 16.27% respectively. Most of the CTC positive cases (63.69%; 7/11) were postmenopausal (Figure 2). Neither age of patients nor menopausal status was found to have any association with CTC positivity (Table II).

None of the CTC positive cases belonged to T1 group (tumor size <2 cm). In the T2 group (tumor size 2- 5 cm), there were 2/61 (3.27%) cases while CTC positivity was very high (19.56%) in the tumors >5cm in size (T3). The difference was found to be statistically significant (p= 0.0049). 9/11 (81.82%) CTC positive cases belonged to T3 group while 2/11 (18.18%) belonged to T2 group (Figure 3).

Regarding histologic type, the number of cases of invasive ductal carcinoma (IDC) with CTC positivity was 9/111 (8.1%) compared to 2/3 (66.67%) for invasive lobular carcinoma, which was statistically significant (p= 0.0242). Out of all CTC positive cases, 81.82% belonged to IDC while 18.18% belonged to ILC (Figure 4, 5). CTC positivity when seen in relation to grade of tumor was highest for grade 3 (31.25%) followed by grade 2 (1.31%) and grade 1 (0) and the difference was found to be statistically significant (p<0.0001) (Figure 6).

Although CTC positivity was higher in node positive group (11.39%), we found 2 CTC positive cases (2/35; 5.71%) in node negative group as well but no association was found between CTC positivity and lymph node status. 81.82% of

Table I: Characteristic profile of study subjects

S. no	Variables	Status	Breast cancer cases (n=114) distribution no. (%)	Benign breast disease (n=108) distribution no. (%)	Control subjects (n=182) no. (%)
1	Age group	< or = 40 years	36 (31.57)	99 (91.66)	109 (59.89)
		>40 years	78 (68.43)	9 (8.34)	73(40.10)
2	Religion	Hindu	79 (69.29)	71 (62.28)	126 (69.23)
		Muslim	28 (24.56)	36 (31.57)	56(30.76)
		Sikh	4 (3.50)	1 (6.15)	0 (0)
		Christian	3 (2.65)	0 (0)	0 (0)
3	Menopausal status	Pre menopausal	71 (62.28)	108 (100.0)	161 (88.46)
		Post menopausal	43 (37.72)	0(0)	21(11.53)

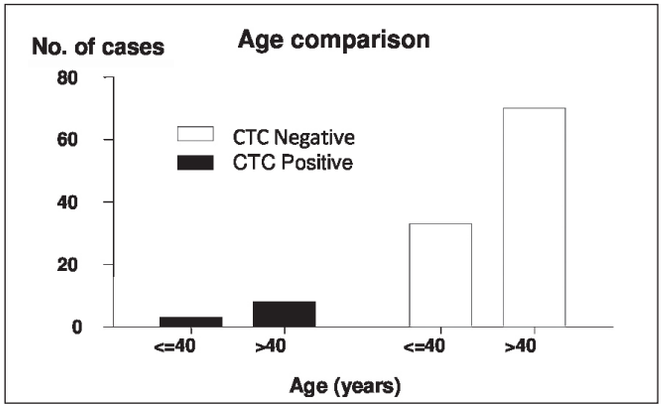

Figure 1: Comparison of age distribution in CTC positive and negative cases.

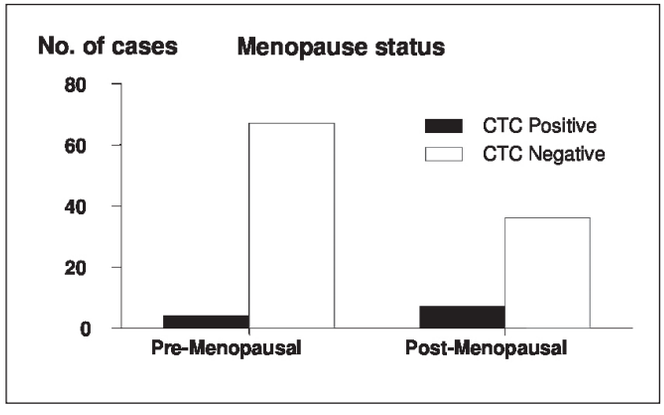

Figure 2: Comparison of menopausal status in CTC positive and negative cases.

Table II: Clinicopathological profile of breast carcinoma cases and circulating tumor cells (CTC) positive cases

S. no.	Variables	Status	Breast cancer cases (114) distribution. no. (%)	CTC positive cases (11) distribution no. (%)
1.	Age	< or = 40 years	36 (31.57)	3/11 (27.78)
		>40 years	78 (68.43)	8/11 (72.72)
2.	Religion	Hindu	79 (69.29)	8/11 (72.72)
		Muslim	28 (24.56)	3/11 (27.28)
		Sikh	4 (3.50)	0/11 (0)
		Christian	3 (2.65)	0/11 (0)
3.	Menopausal status	Pre menopausal	71 (62.28)	4/11 (36.36)
		Post menopausal	43 (37.72)	7/11 (63.64)
4.	Tumor size	< or = 2 cm	7(6.14)	0/11(0)
		2-5 cm	61(53.50)	2/11(18.18)
		>5 cm	46 (40.35)	9/11(81.82)
5.	Tumor type	IDC	111(97.36)	9/11(81.82)
		ILC	3(2.64)	2/11(18.18)
6.	In situ component	Absent	66 (57.89)	7/11(63.64)
		Present	48 (42.11)	4/11(36.36)
7.	MRB grade	I	6 (5.26)	0/11(0)
		II	76 (66.66)	1/11(9.09)
		III	32 (28.08)	10/11(90.91)
8.	Lymph node	Absent	35 (30.70)	2/11(18.18)
		Present	79 (69.3)	9/11(81.82)
9.	Skin infiltration	Absent	98 (85.96)	3/11(27.27)
		Present	16 (14.04)	8/11(72.73)
10.	Metastasis	Absent	102 (89.47)	2/11(18.18)
		Present	12 (10.53)	9/11(81.82)
11.	Intratumoral and peritumoral lymphocytes	Absent	61 (53.50)	6/11(54.54)
		Present	53 (46.50)	5/11(45.46)

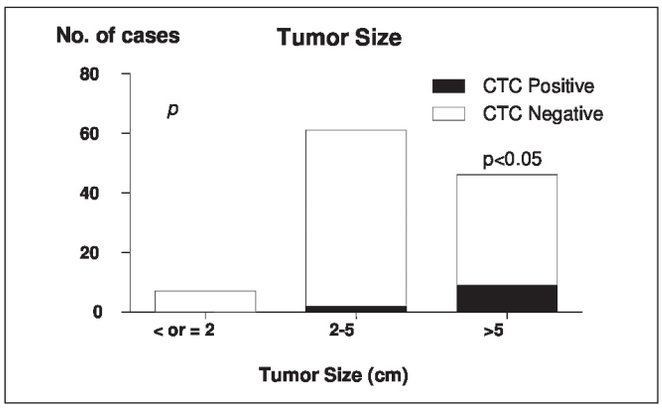

Figure 3: Comparison of tumor size in CTC positive and negative cases.

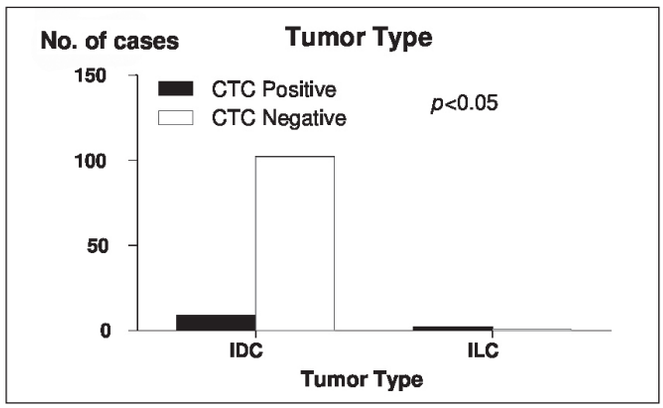

Figure 4: Distribution of tumor type among CTC positive and negative cases.

all CTC positive cases had lymph node metastasis (Figure 7). Positive CTC cases were 50% in patients with skin infiltration by the tumor compared to only 3.06% in those without skin infiltration, the difference being statistically significant (p<0.0001). Among all CTC positive cases, 72.73% had skin infiltration by tumor (Figure 8).

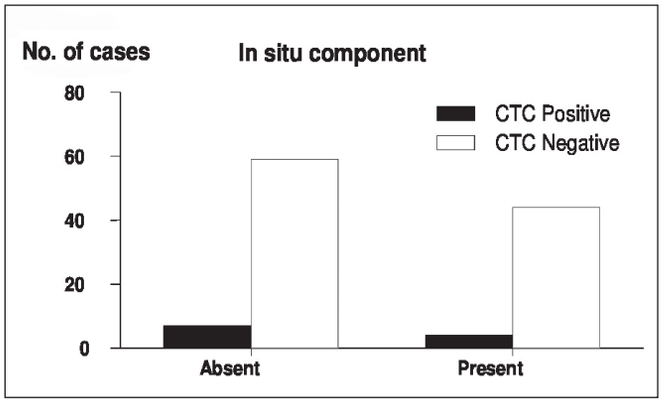

Figure 5: Comparison of in situ component among CTC positive and negative cases.

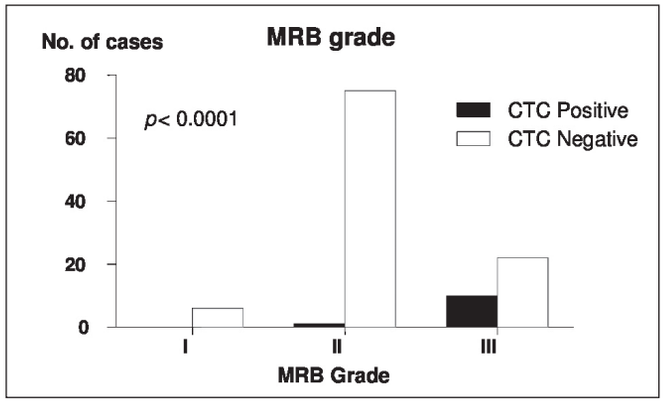

Figure 6: Distribution of MRB grade among CTC positive and negative cases.

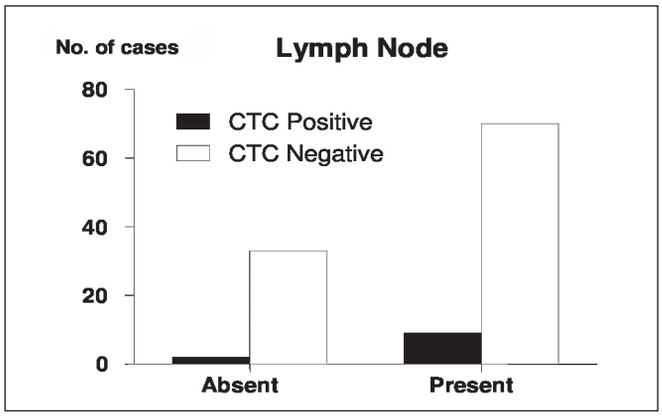

Figure 7: Comparison of lymph node status among CTC positive and negative cases.

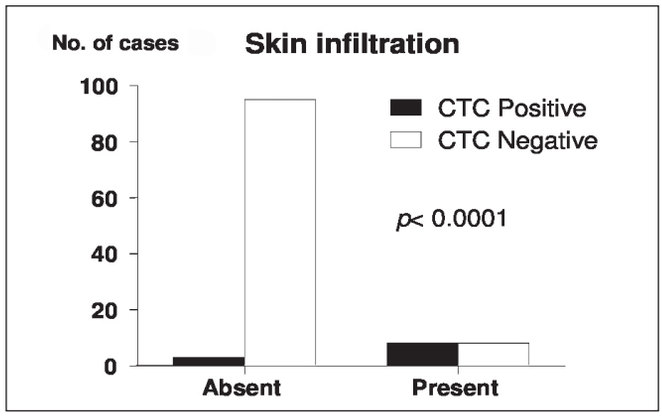

Figure 8: Comparison of skin infiltration among CTC positive and negative cases.

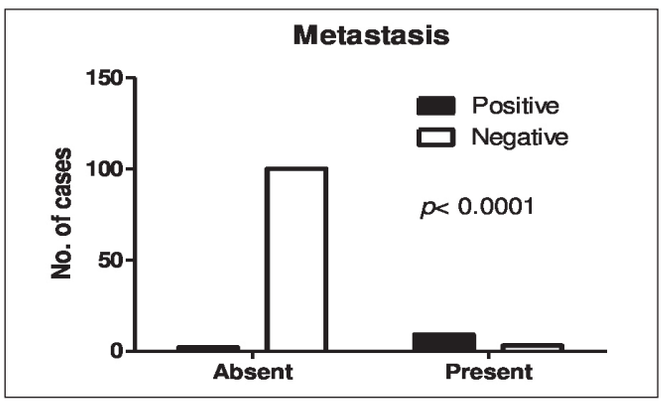

Figure 9: Comparison of metastatic status among CTC positive and negative cases.

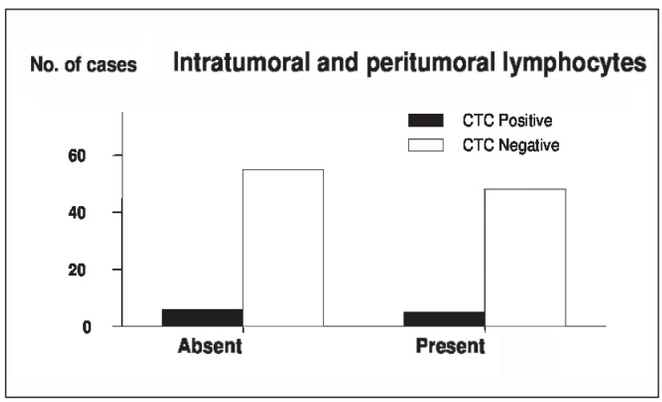

Figure 10: Distribution of tumoral lymphocytes among CTC positive and negative cases.

75% of CTC positive cases were observed in the metastatic breast cancer group while CTC positivity was 1.96% in the non metastatic group, which was statistically significant (p< 0.0001). 81.82% of all CTC positive cases had distant metastasis (Figure 9). There was not much difference in CTC positivity between breast cancer cases with and without intratumoral and peritumoral lymphocytes (9.43 vs 9.83) (Figure 10). Details are shown in Table III.

Table III: Distribution of breast cancer cases and CTC positive/negative cases

S. no.	Variables	Status	Breast cancer cases distribution. No. (%)	CTC positive cases distribution in relation to breast cancer cases distribution. No. (%)	CTC negative cases distribution in relation to breast cancer cases distribution. No. (%)	p value	Odds ratio (95% CI)	Statistical analysis
1	Age	< or = 40 years	36 (31.57)	3/36 (8.33)	33/36 (91.67)	1	-	Fischer's exact test
		>40 years	78 (68.43)	8/78 (10.25)	70/78 (89.74)			
2	Menopausal status	Pre-menopausal	71 (62.28)	4/71 (5.63)	67/71 (94.37)	0.0988	-	Fischer's exact test
		Post-menopausal	43 (37.72)	7/43 (16.27)	36/43 (83.72)			
3	Tumor size	< or = 2 cm	7(6.14)	0/7 (0)	7/7 (100)	**0.0049***	-	Chi-square test for p-trend
		2-5 cm	61(53.50)	2/61(3.27)	59/61 (96.72)			
		>5 cm	46 (40.35)	9/46 (19.56)	37/46 (80.43)			
4	Tumor type	IDC	111(97.36)	9/111(8.1)	102/111 (91.89)	**0.0242***	0.04 (0.003-0.53)	Fischer's exact test
		ILC	3(2.64)	2/3(66.66)	1/3 (33.33)			
5	In situ component	Absent	66 (57.89)	7/66 (10.6)	59/66 (89.39)	0.7582	-	Fischer's exact test
		Present	48 (42.11)	4/48 (8.33)	44/48 (91.67)		-	
6	MRB grade	I	6 (5.26)	0/6 (0)	6/6 (100)	**< 0.0001***	-	Chi-square test for trend
		II	76 (66.66)	1/76 (1.31)	75/76 (98.68)			
		III	32 (28.08)	10/32 (31.25)	22/32 (68.75)			
7	Lymph node	Absent	35 (30.70)	2/35 (5.71)	33/35 (94.29)	0.4988	-	Fischer's exact test
		Present	79 (69.3)	9/79 (11.39)	70/79 (88.61)			
8	Skin infiltration	Absent	98 (85.96)	3/98 (3.06)	95/98 (96.94)	**< 0.0001***	0.03158 (0.006 to-0.14)	Fischer's exact test
		Present	16 (14.04)	8/16 (50)	8/16 (50)			
9	Metastasis	Absent	102 (89.47)	2/102 (1.96)	100/102 (98.04)	**< 0.0001***	0.006 (0.0009 -0.045)	Fischer's exact test
		Present	12 (10.53)	9/12 (75)	3/12 (25)			
10	Intratumoral and peritumoral lymphocytes	Absent	61 (53.50)	6/61 (9.83)	55/61 (90.16)	1	-	Fischer's exact test
		Present	53 (46.50)	5/53 (9.43)	48/53 (90.57)		-	

CTC: Circulating tumor cells), **OR:** Odds Ratio, **CI:** Confidence Interval, p-value<0.05 was considered significant. *refers to significant p-value given in bold. Sample size is too small and confidence intervals are therefore very wide.

Hormone Receptor Status of Breast Cancer and CTC Positive Cases

Out of all the CTC positive cases, 72.72% were ER negative, 54.54% were PR negative, 63.64% were her-2 neu negative while 72.72% were triple negative as depicted in Table IV.

Most of the CTC positive breast cancer cases were estrogen receptor (ER) negative (16.32% vs. 4.61% in CTC positivity in ER negative vs ER positive groups). However, the difference was not statistically significant (p= 0.053). CTC positivity did not show much difference in progesterone receptor (PR) negative vs PR positive groups (9.83% vs. 9.43%) (Table V).

In the Her-2 neu positive group, CTC were detected in 4/55 patients (7.27%) compared to 7/59 (11.86%) in the her-2 neu negative group. In the triple negative tumors (ER, PR, Her-2 neu negative), CTC positivity was observed in 8 out of 32 cases (25%). The details are depicted in Table V.

Table IV: Hormone receptor status of breast carcinoma cases and circulating tumor cells (CTC) positive cases

S. no.	Hormone receptor	Status	Breast cancer cases (114) distribution no. (%)	CTC positive cases (11) distribution in no. (%)
1.	ER	Negative	49 (42.98)	8/11 (72.72)
		Positive	65 (57.02)	3/11 (27.28)
2.	PR	Negative	61 (53.50)	6/11 (54.54)
		Positive	53 (46.50)	5/11 (45.46)
3.	Her-2/Neu	Negative	59 (51.75)	7/11 (63.64)
		Positive	55 (62.25)	4/11 (36.36)
4.	ER/PR/Her-2	Triple negative	32 (28.07)	8/11 (72.72)

CTC: Circulating tumor cells.

Table V: Distribution of breast cancer cases and CTC positive/negative cases in relation to hormone receptor status

S. no.	Hormone receptor	Status	Breast cancer cases distribution no. (%)	CTC positive cases distribution in relation to breast cancer cases distribution no. (%)	CTC negative cases distribution in relation to breast cancer cases distribution no. (%)	p value	Statistical analysis
1	ER	ER Negative	49 (42.98)	8/49 (16.32)	41/49 (83.67)	0.0532	Fischer's exact test
		ER Positive	65 (57.02)	3/65 (4.61)	62/65 (95.38)		
2	PR	PR Negative	61 (53.50)	6/61 (9.83)	55/61 (90.16)	1	
		PR Positive	53 (46.50)	5/53 (9.43)	48/53 (90.57)		
3	Her-2/Neu	Her-2/Neu Negative	59 (51.75)	7/59 (11.86)	52/59 (88.14)	0.5309	
		Her-2/Neu Positive	55 (62.25)	4/55 (7.27)	51/55 (92.73)		
4	ER/PR/Her-2	Triple negative	32 (28.07)	8/32 (25.0)	24/32 (75)	-	-

CTC: Circulating tumor cells, **ER:** Estrogen receptor, **PR:** Progesterone receptor, **OR:** Odds Ratio, **CI:** Confidence Interval, p-value<0.05 was considered significant. Sample size is too small and confidence intervals are therefore very wide.

In nutshell, CTC positivity was observed to be significantly associated with tumor size, histologic type, tumor grade, metastasis and skin infiltration.

Pre-miRNA Genetic Variations (miR-196a2 C/T (rs11614913), miR-146a G/C (rs2910164) and miR-499 T>C (rs3746444) Polymorphisms and Circulating Tumor Cell (CTC) Status

In the present study, we did not find any significant associations between pre-miRNA genetic variations miR-196a2 C/T (rs11614913), miR-146a G/C (rs2910164) and miR-499 T>C (rs3746444) polymorphisms and Circulating tumor cells (CTC) positivity in susceptibility to breast cancer (data not shown). Due to very low sample size, there were not significant cases in each group, so we were not able to analyze the association between the pre-miRNA genetic variations (miR-196a2 C/T (rs11614913), miR-146a G/C (rs2910164) and miR-499 T>C (rs3746444) polymorphisms and Circulating tumor cells (CTCs) unlike our previous work in which we could find associations between miR and breast cancer risk (9).

DISCUSSION

According to GLOBOCAN 2012 (WHO), breast cancer is the second most common cancer in the world and, by far, the most frequent cancer among women with an estimated 1.67 million new cancer cases diagnosed in 2012 (25% of all cancers). Breast cancer ranks as the fifth cause of death from cancer overall (522,000 deaths) and is the most frequent cause of cancer death in women in less developed regions (324,000 deaths, 14.3% of total). An estimated 70218 women died in India due to breast cancer, which is highest than any other country in the world (10). Thus early diagnosis by adequate screening of the breast lump

is of prime importance to safeguard the health of women globally and particularly for our country.

Many patients continue to die of the disease especially in developing countries like India, including those diagnosed at an early stage despite advances in early detection and treatment. It is believed that after the completion of primary therapy, minimal residual disease ultimately leads to disease relapse and distant metastases. Circulating tumor cells are isolated epithelial cells with similar characteristics to the tumor cells of the primary site that have been identified in the peripheral blood of many solid cancers like breast, prostate and colon. The greatest challenge lies in the detection of these rare cells (1 in 10^6 to 1 in 10^7 of all nucleated cells) from among numerous hematopoietic cells (5,6).

A variety of methods have been developed for detection of CTC. To increase the chances of detecting these cells, we require techniques that utilize different methods to increase the concentration of CTC in blood, namely, differential centrifugation, Ficoll enrichment and cell separation by immunomagnetic technique. Another limitation is the loss of malignant cells on account of their fragility. The positive detection of CTC has been used in a number of techniques like immunohistochemistry, immunofluorescence, Fluorescent in situ hybridization (FISH), flow cytometry, southern blot, Northern Blot, Polymerase chain reaction (PCR), Real time PCR, etc (4-7).

Out of this exhaustive list, the Cell Search System is the most commonly utilized, commercially available technique, which is FDA approved. It is a semi quantitative device for detection of CTC based on expression of epithelial cell adhesion molecule (EpCAM) with antibody coated magnetic beads as an enrichment media. CTC are defined as cytokeratin +/CD 45 – nucleated cells (4-7). Cristofanilli et al. (11) used cell search system to detect CTC and theirs' was the first study to establish a threshold of 5 CTC per 7.5ml blood for differentiating between patients with favourable and unfavourable prognosis.

Flow cytometry has also been applied for detection of CTC in patients with metastatic cancers by Riethdorf et al. (12), however, they found it to be less sensitive. Cruz et al. (13) comparatively evaluated different cytokeratin types (CK 7, CK 20, pan CK, CK8/CK18, CK 8 and CK 18) by flowcytometry for identification of best combination of DNA/ CK staining for detecting scarce circulating breast cancer cells. They observed that CK 18 was the brightest and more sensitive staining for breast cancer cells by flow cytometry. The advantage of this method is that a special machine is not required, so it can be of great utility in resource poor settings especially in developing countries like India.

Hristozova et al. (14) described a sensitive and reliable multicolor flow cytometry protocol for CTC detection by using an electronic threshold during data acquisition.

There is an ongoing debate as to which is better: morphologic or molecular detection of CTC. Slide based counting has the advantage of being highly specific, but many authors believe that this method has low sensitivity compared to quantitative mRNA techniques.

The importance of detecting CTC is more if it can be done in early stage cancers when metastasis has not taken place so that appropriate therapeutic remedy can be provided to patient. Lucci et al. (15) studied the prognostic value of CTC in early stage breast cancer: 73 patients had $\geq$ 1 CTC, 29 patients had $\geq$ 2 CTC while 16 had $\geq$ 3 CTC per 7.5 ml blood. They did not observe any correlation between primary tumor features and CTC detection. However, presence of CTC was associated with significant short progression free survival. On the contrary, in the present study, CTC positivity significantly correlated with tumor size, histologic type, tumor grade, metastasis and skin infiltration. 66.66% (2/3) of ILC cases as compared to 8.1% (9/111) cases of IDC were positive for CTC, this could be due to absence of E-Cadherin which leads to early dissemination of cancer cells into the blood stream.

Molecular methods in which mRNA of tumor cells is amplified can also be used to detect CTC and have greater sensitivity. Multimarker assay can be used instead of single probe assay to further improve sensitivity. Disadvantages of amplification-based tests are the false positivity, heterogeneity in expression levels of particular target transcripts as well as false negative (6).

Pukazhendhi and Glück (4) reviewed 81 manuscripts on CTC in breast cancer and categorized them into those in discovery datasets, prognostic factor in metastatic breast cancer, predicting clinical utility in early breast cancer. Based on this, they commented that the current diagnostic modalities for CTC mainly focus on epithelial markers, however measurement of circulating DNA is the best approach.

Giordano et al. (16) studied the clinical impact of CTC in various molecular subtypes of breast cancer. Baseline CTC detection had good prognostic value in all breast cancer subtypes except Her 2 neu positive cancer. Guiliano et al. (17) observed the effect of different first line systemic

treatment on the prognostic value of CTC in 492 advanced breast cancer patients. A pre treatment level more than or equal to 5 CTC/7.5 ml blood was associated with an increased baseline number of metastatic sites compared to those with less than 5 CTC/7.5 ml (p=0.0077). They had 4 different treatment groups, out of which groups with endocrine treatment and CT alone, high CTC was associated with worse prognosis while the groups receiving either her 2 neu targeted treatment or biological agent, did not maintain the negative prognostic value of high CTC at baseline.

Krishnamurthy et al. (18) evaluated the presence of CTC in peripheral blood and its correlation between various clinicopathological characteristics and hormone receptor profile. CTC were found in 13 out of 43 T1 tumors while in T2 tumors 12/38 were CTC positive. There was no correlation between detection of CTC and standard prognostic factors contrary to our findings of significant association of CTC with tumor size, histologic type, tumor grade, metastasis and skin infiltration. In our study, 31.25% of grade 3 tumors were positive for CTC, followed by 1.31% of grade 2 tumors, signifying that higher the grade, more the positivity for CTC.

Turker et al. (19) determined the effectiveness of CTC in 22 metastatic and 12 Early stage breast cancer cases for prediction of progression free survival (PFS) and overall survival (OS) as an adjunct to standard treatment care in breast cancer management. CTC was positive in 3 (13.6%) patients before chemotherapy (CT) and 6 (27.3%) patients during CT in the metastatic subgroup whereas positive in only one patient in early stage subgroup before and during CT. CTC positivity was confirmed as a prospective marker in this study even with small patient group.

Franken et al. (20) undertook a study to explore whether the presence of CTC at the time of diagnosis was associated with recurrence free survival (RFS) and breast cancer related death (BRD) in 404 breast cancer patients. Patients were stratified into unfavorable (CTC $\geq$ 1) and favorable (CTC =0 in 30 ml peripheral blood). They concluded that CTC in breast cancer patients before undergoing surgery with curative intent is associated with an increased risk of BRD.

Peeters et al. (21) explored potential differences in the detection and prognostic significance of CTCs in MBC according to immunohistochemical subtypes of breast cancer. They did not observe any significant differences in the absolute CTC counts (P=0.120) or in CTC positivity rates according to ≥1 and ≥5 CTCs per 7.5 ml blood detection thresholds (P=0.165 and P=0.651, respectively) between immunohistochemical subtypes. Very high CTC counts, defined as ≥80 CTCs per 7.5 ml, were observed more frequently in patients with Luminal A and triple negative (TN) breast cancer (P=0.024). In the total study population, the presence of ≥5 CTCs was the single most significant prognostic factor for both PFS and OS in multivariate analysis (P<0.001).

Rack et al. (22) analyzed CTC in 2026 patients with early breast cancer before adjuvant chemotherapy and in 1492 patients after CT using Cell Search System. Before CT, CTC were detected in 21.5% of patients (435/2036), out of which node negative versus node positive patients with CTC were 19.6% vs. 22.4% (p<0.001), similar to the current study where the node status significantly correlated with CTC positivity. However, no association was found with tumor size, tumor grade or hormone receptor status which is contrary to our results as we found a significant association with tumor size and tumor grade.

This study had many limitations. The sample size is too low and confidence intervals are very wide, and the power of the study is too low to reach to any significant conclusion. The study strongly needs to be validated and replicated in a bigger sample size.

In conclusion, the flow cytometry protocol for detection and molecular characterization of CTCs is a time and cost-effective technique, suitable for routine clinical use. However, more elaborate studies are needed to establish the role of flowcytometry in detection of circulating tumor cells as a prognostic marker. One added advantage of flow cytometric immunophenotyping is that panels can be expanded to get additional information. Estrogen and Progesterone receptors and *Her2neu* status in metastatic breast carcinomas or *BRAF* mutation status (using of mutation-specific antibodies) can be very useful in the current approach towards personalized treatment.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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