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Fas Ligand as a Circulating Apoptosis Marker in Carcinoma Breast

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Abstract

***Background*:** Circulating apoptosis markers such as soluble Fas receptors and Fas ligand, are a new set of blood parameter that might affect tumor growth and aggressiveness as well as being able to monitor the effect of antitumor therapy. We evaluate serially by ELISA the levels of FasL in the serum of breast cancer patients at presentation, post surgery, and post chemotherapy and also correlate levels of apoptosis markers with tumor response.

***Methods*:** This prospective study undertaken on 24 patients of carcinoma breast of any stage in Department of General Surgery, IMS BHU in collaboration with the Department of Pathology, IMS, BHU between July 2012 to July 2014. Informed and written consent was obtained from all patients. Response Evaluation Criteria In Solid Tumors (RECIST) was used to determine objective tumor response for target lesions. Circulating levels of Fas Ligand in the serum of breast cancer patient was studied by ELISA technique.

Results: The mean age of the patients was 44.75 years (ranging from 25 to 75 years). In cases, the median Fas level at the time of presentation was 73.0 (35.76-90.0), at post surgery 58.0 (40.0-85.0) and at post-chemotherapy was 64.85 (26.75-89.5). As compared to control group, Fas level is significantly increased in patient of carcinoma breast at different time interval ($p=0.004$, 0.001, 0.010). In our study FasL levels were comparable in ER, PR and HER-2 positive and negative cases. There was no difference in serum levels of FasL among triple negative and non triple negative cases. Patients with lymph node positive status had a higher FasL level compared to those who had no lymph node status. The difference was greatest among patients who had 1–3 lymph nodes compared to N0 patients ($p<0.01$).

Conclusion: Our study indicates a considerable prognostic potential for FasL in breast cancer patients. Lack of these molecules is related to a significantly worse prognosis. This is the result of resistance of FasL deficient breast tumors to the mechanism of apoptosis.

Introduction

Biomarkers of breast cancer are necessary for prognosis and prediction to chemotherapy. Prognostic biomarkers provide information regarding outcome irrespective of therapy, while predictive biomarkers provide information regarding response to therapy [1]. Candidate prognostic biomarkers for breast cancers are growth factor receptors, steroid receptors, Ki-67, cyclins, urokinase, plasminogen activator, p53, p21, pro- and anti-apoptotic factors, BRCA1 and BRCA2 [2]. But currently, the predictive markers are

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Estrogen (ER) and Progesterone receptors (PR) responding to endocrine therapy, and HER-2 responding to trastuzumab.

This lead to search of new prognostic and predictive markers and the number of potential markers is constantly increasing due to proteomics and genomics studies [3].

The soluble markers of apoptosis may come from various stages of the overall apoptosis process. There are cell surface receptors which recognize the death signal and transmit it across the cell membrane. Members of the TNF family constitute most of the death receptors including Fas, Apo1, CD95 receptor, the TNF receptor 1 and death receptors DR3, DR4 and DR5 which are activated either by binding of specific ligands or stimulatory antibodies and leads to formation of death inducing signalling complex (DISC) by the association of intracellular proteins with the death domain [4].

The products of apoptosis are released into the circulation and may be assayed in serum or plasma. The soluble forms of apoptosis receptor sFas and its ligand FasL, circulating DNA and various cytokeratins are frequently measured markers among cancer patients. Circulating markers although lack organ and cell death specificity, have the advantage of being easily accessible and be available for serial measurements [5].

The Fas/FasL system is a major regulator of apoptosis. Fas a cell surface protein with a single transmembrane domain, belongs to the nerve growth factor/TNF receptor family while FasL is a type II membrane protein belonging to TNF family. FasL is expressed in activated T cells and cancer cells. Fas is expressed on the surface of cell membranes in a variety of normal tissue cells as well as malignant cells. Fas mediated apoptosis leads to elimination of activated T cell following an immune response which is killing a tumor [6].

Dysregulation of Fas mediated apoptosis is thought to play a role in cancer progression, lymph nodes involvement and metastasis.

It is confirmed that Fas/FasL systems induce apoptosis of activated immune cells and that the soluble isoforms of these proteins (sFas, sFasL) also inhibit their function. Elevated serum levels of sFas and sFasL have been reported in several types of cancer [7-9]. Although the precise role of sFas has not been explained, there are studies suggesting the role of sFas in cancer progression. sFas may inhibit Fas mediated apoptosis. sFas has been reported to play or role as inhibitor f Fas mediated apoptosis. It has been suggested that the Fas/FasL system is an important mechanism for tumor escape from the immune system. The clinical significance of circulating sFas and sFasL has not been defined yet.

In this study we evaluate serially by ELISA the levels of FasL in the serum of breast cancer patients at presentation, post surgery, and post chemotherapy and also to correlate levels of apoptosis markers with tumor response in patients receiving neo-adjuvant chemotherapy.

Patients and Methods

This was a prospective study undertaken on 24 patients who were clinically presented to have carcinoma breast of any stage in a single surgical unit in Department of General Surgery, IMS BHU in collaboration with the Department of Pathology, IMS BHU between July 2012 and July 2014. Informed and written consent was obtained from all patients. Patients of carcinoma breast who have received neoadjuvant therapy or surgery previously were excluded in the study.

The diagnosis of carcinoma breast was based on the triple assessment of patients: clinical breast examination, imaging – ultrasound (<35 year), mammography (>35 year) and

Table 1: Median of Fas ligand level observed in our patients

Group	F1 [Median, (IQR)]	F2 [Median, (IQR)]	F3 [Median, (IQR)]
Case	73.0 (35.76-90.00)	58.0 (40.0-85.0)	64.85 (26.75-89.50)
Control	22.5 (12.5-37.5)	22.5 (12.5-37.5)	22.5 (12.5-37.5)
Mann-Whitney U test (p-value)	0.004	0.001	0.010

histopathology. In all the patient blood samples were taken in a plane vial at the time of presentation, at the time of surgery, and after two cycle of chemotherapy and sent to Department of Pathology for centrifugation. After centrifugation sample was stored at 0 – 8 degree centigrade in freezer compartment. The breast cancer tissue specimen obtained at surgery was sent for conventional histopathology as well as assay for ER, PR, and Her 2 neu status.

The response to chemotherapy was evaluated as per the RECIST criteria in patients who received neoadjuvent chemotherapy. Circulating levels of Fas Ligand in the serum of cancer breast patient was studied by the method of ELISA technique. Correlation was done between basal FasL levels and the tumor stage and grade. In patient who received Neo-adjuvant chemotherapy, level differences of Fas L level before and after chemotherapy with the RECIST criteria regarding response to chemotherapy was one.

Statistical analysis was done using SPSS version 16.0. For categorical variables Chi-square and Fischer's exact test was used. For comparing two groups of mean Student's t test was used. P- Value <0.05 is considered as statistically significant.

Results

The mean age of the patients was 44.75 years (ranging from 25 to 75 years). Out of all 24 patients, majority (50%) were in 4th and 5th decade of their life. The incidence of breast cancer was more in postmenopausal age group seen in 54.16% cases than in premenopausal (45.84%) women. Six patients out of 24 had a positive history of smoking (25%), rest were non-smokers. 10 out of 24 (40.8%) patients had used Oral Contraceptive pills (OCPs) at some stage of their life. Maximum number of patients had T4 lesions (46.9%) and none had T1 lesion. Lymph node spread was seen in 21 (87.5%) patients. Rest 12.5% had clinically negative nodes. 9 patients (37.5%) were in stage 3 followed by 62.5% in stage II. All patients had Infiltrating ductal carcinoma on histopathology. 13 out of 24 patients (54.17%) had grade II tumor (as per Bloom Richardson classification) followed by grade 3 (25%). Receptor status assessment was done in all 24 cases. 41.66% patients had ER + receptor status, 8 out of 24 patients was PR + (33.33%) & Her-2neu receptor was positive in 50% patients. Among 24 patients, 5 patients were triple negative (20%).

All patients received CAF (Cyclophosphamide, Adriamycin, and 5-Fluorouracil) regimen. All received 3 cycles of neoadjuvant chemotherapy. No cases included in this study had clinical complete response with disappearance of all target lesions, while 80.0% had partial response (as assessed clinically using RECIST criteria) and 20.0% had stable disease.

In our study we found that median FasL levels were significantly higher in breast cancer patients compared to age matched controls (Table 1). However the levels of FasL did not significantly vary with the age of the subjects. The FasL levels were also comparable in estrogen receptor positive and negative patients as well as progesterone

Table 2: Level of fas ligand according to lymph node status

Lymphnode	F1 [Median, (IQR)]			F2 [Median, (IQR)]			F3 [Median, (IQR)]		
(a) 0	34.3 (15.6-75.3)			40.0 (20.57-40.0)			42.0 (13.4-60.8)		
(b) 1-3	86.0 (61.0-95.0)			79.6 (55.5-129.5)			84.1 (47.0-101.2)		
(c) >3	40.0 (11.5-76.6)			42.0 (19.9-65.0)			35.0 (24.6-79.0)		
Kruskal-Wallis Test									
	a vs b	a vs c	b vs c	a vs b	a vs c	b vs c	a vs b	a vs c	b vs c
p-value	0.001	0.002	0.041	0.001	0.003	0.091	0.001	0.029	0.195

receptor positive and negative patients. Among HER 2 neu positive and negative patients also the FasL serum levels were comparable. There was no difference in serum levels of FasL among triple negative and non triple negative cases. The only significant difference was found on the lymph node status of the breast cancer patients. Patients with lymph node positive status had a higher FasL level compared to those who had no lymph node status. The difference was greatest among patients who had 1–3 lymph nodes compared to N0 patients ($p<0.01$) (Table 2).

Discussion

Breast Cancer is the most common site-specific cancer in women and is the leading cause of death from cancer for women aged 20-59 years. More so, it has a very varied clinical, pathologic, molecular features and treatment modalities. Recently, microarray expression patterns and immunohistochemical (IHC) signatures are used to distinguish breast cancer subtypes and likely reflect important differences in pathogenesis and etiology, and also determine the type of therapy.

Biomarkers of breast cancer are necessary for prognosis and prediction of response chemotherapy. Prognostic biomarkers provide information regarding outcome irrespective therapy, while predictive biomarkers provide information regarding response to therapy [10].

Patients suffering from breast carcinoma have a poor prognosis because of the lack of effective treatment strategies. Detection and identification of prognostic markers for predicting therapeutic response is very essential for breast cancer treatment.

The soluble markers of apoptosis may come from various stages of the overall apoptosis process. There are cell surface receptors which recognize the death signal and transmit it across the cell membrane. Members of the TNF family constitute most of the death receptors. These include Fas, Apo1, CD95 receptor, the TNF receptor 1 and death receptors DR3, DR4 and DR5. These receptors are activated by binding of specific ligands or stimulatory antibodies. The result is formation of death inducing signalling complex (DISC) by the association of intracellular proteins with the death domain.

The products of apoptosis are released into the circulation and may be assayed in serum or plasma. Among cancer patients the frequently measured markers are the soluble forms of apoptosis receptor sFas and its ligand FasL.

Our study was a prospective, observational study on 24 newly diagnosed cases of carcinoma breast who presented in surgery

outpatient department, Sir Sunderlal hospital, BHU during the study period from July 2012 to June 2014. The observation regards to patients demographic data, clinical, pathological and hormone receptor status of carcinoma breast and their correlation with Her2neu over expression and fas ligand expression.

In our study, age of the patients of carcinoma breast ranged from 25 -75 years with mean age of presentation being 44.75 years. Mean age of presentation in our group is less than western population [11]. Three patients belonged to age group of more than 60 years. In Indian women diagnosis of breast cancer is late and about 70% of patients diagnosed, belong to clinically advanced disease [11]. 37.5 % of patients were <40 years. This shows that young patients are more commonly involve in our study. Hindu's were more commonly affected than Muslim which is due to high population ratio in this part of country. Most of the patients having carcinoma breast are housewives. In our study most of the patient did not have any co-morbidity.

Majority of the patients in our study having a tumor size in between 2 – 5 cm consisting of 54.17% of total population in this study (13/24) and 10 patients having tumor size of more than 5 cm (41.67%). There was only one patient with tumor size less than 2 cm. Reason for the late presentation of carcinoma breast is lack of awareness in Indian population. In Indian women diagnosis of breast cancer is late and about 70% of patients diagnosed, belong to clinically advanced disease [11]. Also, Indian women have biologically aggressive breast cancer, as shown by lower incidence of estrogen receptor-positive, progesterone receptor- positive tumor and higher incidence of Her-2Neu positivity.

In our study clinically negative axilla (N0) was found in only 3 patients whereas 87.50% patients have mobile nodes in axilla (N1). This again reflects the lack of awareness in Indian population.

Our study also shows that stage 2 breast cancer was found in 15 (62.50%) patients and only 9 out of 24 patients having stage 2 diseases (37.50%). Stage 1 breast cancer was not reported in any of patient. This again reflects the aggressive behavior of disease as well as lack of awareness in Indian population.

Infiltrating ductal carcinoma was the histopathology seen in all operative specimens; lobular carcinoma was not seen in any of the patient. Invasive ductal carcinoma is the most common malignancies of the breast, accounting for 80% and 15% of all invasive breast cancer [12]. In our study, grade 2 breast cancers was found in 13 patients (54.17%) while grade 1 and grade 3 tumor were found in 20.83% and 25% of samples respectively. Five patients out of 24 sample size having more than 3 positive axillary lymph node (20.83%), 20.83% of the patients having no lymph node metastasis whereas 58.34 % had 1-3 positive axillary lymph nodes.

Roughly two-third of breast cancers test positive for hormone receptors. Estrogen-receptor-positive (or ER+) or progesterone-receptor-positive (PR+) cancer cells may receive signals from estrogen or progesterone respectively that could promote their growth. Therefore, from therapeutic point of view, it is essential to test the presence of hormone receptors because this result will help to decide whether the cancer is likely to respond to hormonal therapy or other treatments. Interventions using hormonal therapy include either (1) lower the amount of estrogen in body or (2) block estrogen from supporting the growth and function of breast cells if they have hormone receptors. However this therapy is unlikely to work in hormone-

receptor-negative cancer.

In our study about 41.66% patients of the above mentioned sample size have positive estrogen receptor whereas 58.34% have negative receptor status. 33.34% (8/24) of the patients were positive for progesterone receptor and 66.66 % were negative. 50 % of examined pathological specimen shows over expression of Her2neu whereas 50% of the sample size does not show any over expression for her 2neu.

We found that FasL levels were significantly higher in breast cancer patients compared to age matched controls. However the levels of FasL did not significantly vary with the age of the subjects. The FasL levels were also similar in estrogen receptor positive and negative patients as well as progesterone receptor positive and negative patients. Among HER 2 neu positive and negative patients also the FasL serum levels were similar. The levels were similar in pre and postmenopausal women. There was no difference in serum levels of FasL among triple negative and non triple negative cases.

In a study conducted by Blok *et al*., [13] no associations were found for histological subtype or tumor stage. Authors observed significantly higher FAS expression in grade 3 tumors compared to grade 1 and 2. Additionally, ER-negative tumors showed twice the average expression of FAS compared to ER-positive tumors ($p<0.05$). For HER2 showed no statistical differences. Combining ER, PR and HER2, it was shown that triple negative tumors showed significantly higher FAS-expression (average of 49% positive tumor cells) compared to the other subtypes ($p<0.001$), especially ER-positive subtypes.

The only significant difference was found on the lymph node status of the breast cancer patients. Patients with lymph node positive status had a higher FasL level compared to those who had no lymph node status. The difference was greatest among patients who had 1–3 lymph nodes compared to N0 patients ($p<0.01$). In a study conducted by Bębenek *et al*., [14] found significant associations were noted between Fas expression and lymph node involvement ($p<0.0001$) or the number of recurrences ($p=0.02$) and between the presence of FasL and the histological grade of tumor ($p=0.007$).

Results of various authors suggest that the phenotype of Fas-deficient primary breast tumour is more aggressive and reflects a worst prognosis [15]. Mottolese *et al*., [16] revealed that the disease free survival was longer with Fas-positive tumors compared to the ones with Fas- negative breast cancer tissues. These levels were confirmed by Reimer *et al*., [17] who found that the FasL: Fas ratio>1 was related to significantly shorter disease free survival.

The above data has found correlation in our study on breast cancer. We have found significant association between the lack of Fas expression and lymph node involvement. In a study conducted by Bębenek *et al*., [18] concluded that the Fas/FasL-dependent mechanisms of spread may be different for various target tissues with reduced Fas expression in breast cancer patients in whom malignant cells infiltrated through the perilymphatic fat. Consequently it can be inferred that Fas deficient tumors have poor prognosis and Fas positive tumors have a better prognosis. In a review conducted by Bębenek *et al*., [19] described the expression of the Fas/Fas-ligand system has potential prognostic application in view of current knowledge, and consequently should be considered as an additional prognostic factor in breast cancer patients.

In conclusion, our study indicates a considerable prognostic potential for FasL in

breast cancer patients. Lack of these molecules is related to a significantly worse prognosis. This is the result of resistance of FasL deficient breast tumors to the mechanism of apoptosis.

Authors Contribution

RKS: did the literature search and collection of data

RNM: prepared the draft manuscript

SKT: Helped in preparation of draft manuscript

SK: Helped in acquisition of data and analysis

RK: Conceived and designed the study and edited the final version

All authors have read and approved the manuscript

Ethical consideration

The study was approved by the institute Ethics committee and consent was obtained from all participants.

Conflicts of interest

The authors declare that there are no Conflicts of interest

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