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RESEARCH

The Clinical Significance of Serum Soluble Fas and p53 Protein in Breast Cancer Patients: Comparison with Serum CA 15-3

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Abstract Serum sFas and p53 protein have been observed in breast cancer patients, but their clinical usefulness for diagnosis and therapy monitoring has not been clarified**.** The aim of this study was to compare the clinical utility of serum sFas and p53 protein with that of serum CA 15-3 as the most commonly used breast cancer tumor marker. Serum samples were taken from 35 normal healthy controls and 35 breast cancer patients before surgery, after 2 weeks of surgery and after six cycles of FAC chemotherapy. Serum sFas and p53 protein levels were measured using ELISA kits. Serum CA 15-3 levels were determined using IRMA kit. Mean Serum levels of sFas and CA 15-3 were significantly elevated while p53 protein was significantly declined in breast caner patients than controls. Serum p53 protein showed the greatest significant area under the ROC curve (84.3%) followed by sFas (80.5%), then CA 15-3 (78%). The sensitivity, specificity and cut-off value for diagnosing breast cancer patients were 84.2%, 82.6% and 2.88 U/ml for p53 protein, 83.3%, 68.2% and 497.3 pg/ml for sFas and 45.8%, 100% and 23 U/ml for CA15-3. Surgical removal of breast resulted in a significant decline in serum sFas level with no effect on serum p53 protein and CA 15-3 levels. Six cycles of chemotherapy resulted in a significant elevation in serum sFas level with no effect on serum p53 protein and CA 15-3 levels. sFas was significantly correlated with tumor grade. It could be concluded that although serum p53 protein is superior to sFas and CA15-3 for diagnosis of breast cancer patients, only sFas is useful for monitoring the response of breast cancer patients to surgery and chemotherapy if the effect of systemic inflammatory reactions is excluded.

Keywords Breast cancer · Apoptosis · Soluble Fas · p53 protein · CA 15-3 · Diagnosis · Surgery · FAC chemotherapy

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Introduction

Fas on membrane of many cells induce cell apoptosis by binding to the Fas ligand that is expressed predominantly in activated T cells. This interaction between Fas and Fas ligand is known to play an important role in the spontaneous death of cancer cells induced by the immune system. Another form of Fas, circulating soluble Fas (sFas), antagonizes the cell-surface Fas function and may offer a survival advantage to cells [1]. Although previous studies have demonstrated that sFas levels were increased in cancer patients from many sites including the breast [2], little has been published regarding the clinical utility of sFas in breast cancer.

p53 is a tumor suppressor gene known as the gardeian of the genome. The product of *p53* gene is a tumor suppressor protein (p53) with pro-apoptotic properties [3]. P53 protein is responsible for apoptosis induced by cellular response to external stress factors such as DNA damage, hypoxia and oncogene activation. Once the cell exposed to one of these stress factors, the p53 undergoes posttranscriptional modification including phosphorylation and acetylation by specific enzymes. The modified p53 starts transcription of genes encoding other proteins that activate and control apoptosis. Among these proteins is the key protein Bax. Cytochrome C, released from mitochondria by bax protein, takes part together with protein Apaf-1 in activation of caspase 9 which through other effective caspases initiates apoptosis [4, 5]. Another effect of p53 in initiating apoptosis is the stimulation of translocation of Fas from Golgi apparatus to the cell membrane which is a non-transacriptive effect of p53 [6]. Balogh et al. found that the positive rates of p53 protein in the sera of breast cancer patients were significantly higher than in the normal controls [7]. However, the clinical significance of p53 protein in breast cancer has not been clarified.

Cancer antigen 15-3 (CA15-3) is a circulating breast cancer-associated antigen. CA15-3 has low sensitivity in the diagnosis of early stages of breast cancer [8]. However it can be used for monitoring the response of breast cancer patients to therapy and in detecting recurrent disease [9].

The aim of this study was to elucidate the clinical significance of serum sFas and p53 protein in comparison with that of serum CA15-3 as the most commonly used breast tumor marker. This clinical significance includes their usefulness for diagnosis (including determination of their precise cut-off points and hence the sensitivity and specificity) and monitoring patients' response to surgery and chemotherapy.

Subjects and Methods

Seventy females were enrolled in this case–control study. Females were divided into two groups: **Group I (breast cancer patient's group):** It included 35 female patients with breast invasive ductal carcinoma of clinical stages II and III [10]) (recently detected, not underwent surgery nor receiving chemotherapy). Their mean age was (43.73±12.2) years. Patients were recruited from the Departments of Experimental and Clinical Surgery and Cancer Management & Research of the Medical Research Institute, Alexandria University in the period from January 2010 to August 2010. **Group II (normal healthy control group):** It included 35 normal healthy female volunteers of comparable age (42.18±11.05), menstrual cycle and socioeconomic status as patients.

This research protocol was approved by the ethical committee of medical Research Institute. Each patient underwent full history recording, thorough clinical examination, routine laboratory investigations including complete blood count (CBC), mammography of breast and ultrasonography of abdomen and liver, radiological investigations including X-ray chest, CT scan and bone scan when needed and fine needle aspiration cytology (FNAC) of breast mass to establish the pathological diagnosis in the patients.

The clinicopathologic data were obtained from patients' pathology reports. The collected data included tumor size, tumor pathological grade, axillary lymph node involvement, vascular invasion and status of estrogen receptor (ER) and progesterone receptor (PR). Each breast cancer patient's clinical stage was determined by the oncologist according to the tumor-nodes-metastasis (TNM) classification system [10].

All 35 breast cancer patients were subjected to Modified Radical Mastectomy (MRM) surgery [11], then received adjuvant combination chemotherapy [5-Fluorouracil, Adriamycin and Cyclophosphamide (FAC)] [12] for 6 cycles. After 6 cycles of chemotherapy, breast cancer patients were evaluated clinically, laboratory and radiologically to estimate the clinical response. The patients were followed up clinically for 1 year for observation of local recurrence or metastasis.

Laboratory Investigations

Blood samples were collected once from normal healthy female volunteers and from breast cancer patients before surgery, after 2 weeks of surgery and after 6 cycles of chemotherapy. Immediately after withdrawing, blood samples were allowed to coagulate and centrifuged for 20 min at 3500 rpm. The separated serum samples were aliquoted, frozen at -80°C, and stored until assayed. After thawing, each serum aliquot was assayed only once. Determination of serum levels of sFas, p53 protein and CA 15-3 were carried out at Radiation Sciences Department, Medical Research Institute, Alexandria University.

Determination of Serum sFas and p53 Levels

The levels of sFas and p53 in sera were determined using ready-for-use enzyme linked immunosorbent assay (ELISA) kits for the quantitative detection of human sFas and p53 (eBioscience, UK) according the producer's protocol. Briefly, diluted serum was added to the corresponding sample well of the microwell plate. Diluted biotin-conjugated antibody solutions were added. After incubation and washing, diluted streptavidin-HRP solutions were added. Following

incubation and washing tetramethyl-benzidine (TMB) substrate solutions were added. After incubation at room temperature in dark, the reaction was stopped by adding stop solution (1 M phosphoric acid) and the absorbance was measured at 450 nm with a microplate reader. sFas and p53 serum concentrations were determined using standard curves. The sensitivity of sFas assay was 13.2 pg/ml and that of p53 was 0.33 U/ml.

Determination of Serum CA15-3

The level of serum CA 15-3 was determined using a ready-for-use Immunoradiometric assay (IRMA) kit (Diasource, Belgium) according the producer's protocol. Briefly, 50 µl of serum were added to a plastic tube coated with the capture antibody Mab1. The reaction tubes were incubated for 90 min at room temperature on a tube shaker (400 rpm). The content of each tube was decanted, then each tube was washed twice with 2 ml working wash solution. 50 µl of ^{125}Iodine-labeled anti-CA 15-3 antibody (Mab2) and the reaction tubes were incubated for 90 min at room temperature on a tube shaker (400 rpm). The content of each tube was decanted, then each tube was washed twice with 2 ml working wash solution. The bound radioactivity in each tube was counted in the gamma counter (Perkin Elmer, Finland) for 60 s. Computer assisted data reduction was used to simplify the calculations. The 5-parameter logistic function curve was used to calculate CA 15-3 level in each serum sample.

Statistical Analysis

Statistical analysis was performed using SPSS 11.5 software package The Non-parametric Mann–Whitney *U*-test was used for studying differences between breast cancer patients group and control group regarding serum sFas, p53 and CA15.3. The non-parametric Kruskal-Wallis test was used to study the differences in serum parameters before and after surgery and chemotherapy. The Non-parametric Spearman's test was used to investigate correlations between serum parameters concentrations and age, tumor size, axillary lymph node status, clinical stage, tumor patholpgical grade, vascular invasion, PR and ER status. The diagnostic values of serum sFas, p53 and CA15-3 were compared using the Receiver Operating Characteristic (ROC) curve analysis. The cut-off point for each of serum sFas, p53 and CA15-3 was determined according to the best discrimination between patients and controls regarding optimal values of sensitivity and specificity using the ROC curve. P-values$<$0.05 were accepted as statistically significant.

Results

The Serum Levels of sFas in the Normal Healthy Volunteers and Breast Cancer Patients Before Surgery, After 2 Weeks of Surgery and After 6 Cycles of Chemotherapy

The mean serum level of sFas in breast cancer patients before surgery was significantly greater than that in normal healthy controls (946.81±329.44 pg/ml vs 496.22± 43.29 pg/ml; P=0.018). Surgical removal of the breast resulted in a significant reduction of serum level of sFas compared with its level before surgery (497.58±88.65 pg/ml vs 946.81±329.44 pg/ml; P=0.022). Six cycles of chemotherapy resulted in a significant elevation of serum level of sFas compared with its level after surgery (702.75±130.76 pg/ml vs 497.58±88.65 pg/ml; P=0.003), (Fig. 1).

The Serum Levels of p53 Protein in the Normal Healthy Volunteers and Breast Cancer Patients Before Surgery, After 2 Weeks of Surgery and After 6 Cycles of Chemotherapy

The mean serum level of p53 in breast cancer patients before surgery was signicantly lower than that in normal healthy controls (2.55±0.14 U/ml vs 3.85±0.55 U/ml; P=0.000). Surgical removal of the breast resulted in a nonsignificant reduction of serum level of sFas compared with its level before surgery (2.51±0.16 U/ml vs 2.55±0.14 U/ml; P= 0.661). Six cycles of chemotherapy resulted in a nonsignificant reduction of serum level of sFas compared with its level after surgery (2.48±0.16 U/ml vs 2.51±0.16 U/ml; P=0.444), (Fig. 2).

The serum levels of CA 15-3 in the normal healthy volunteers and breast cancer patients before surgery, after 2 weeks of surgery and after 6 cycles of chemotherapy

The mean serum level of CA 15-3 in breast cancer patients before surgery was signicantly higher than that in normal healthy controls (28.13±5.55 U/ml vs 13.76±4.7 U/ml; P=0.015). Surgical removal of the breast resulted in a nonsignificant reduction of serum level of CA 15-3 compared with its level before surgery (20.54±2.59 U/ml vs 28.13± 5.55 U/ml; P=0.711). Six cycles of chemotherapy resulted in a nonsignificant increase of serum level of CA 15-3 compared with its level after surgery (22.09±2.33 U/ml vs 20.54± 2.59 U/ml; P=0.117), (Fig. 3).

Comparison of the Diagnostic Value of Serum sFas, p53 Protein and CA 15-3 in Breast Cancer Patients Before Surgery Using the ROC Curve Analysis

The ROC curve analysis was used to compare the diagnostic value of sFas, p53 protein, and CA 15-3 depending on the

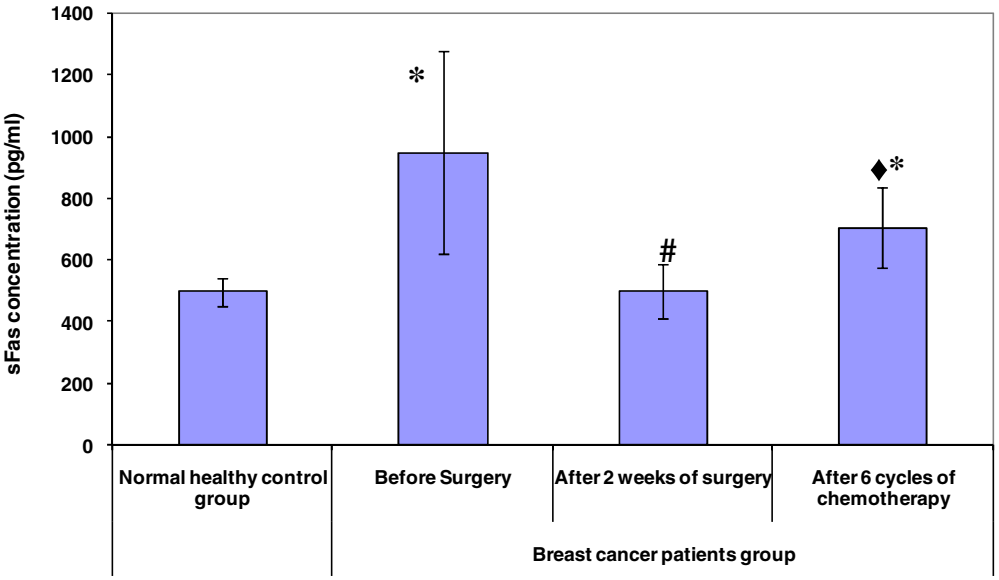

Fig. 1 M±SE serum levels of sFas in the normal healthy controls and breast cancer patients before surgery; after 2 weeks of surgery and after 6 cycles of chemotherapy. *: Significance was compared with control group. #: Significance was compared with breast cancer patients group before surgery. ♦: Significance was compared with breast cancer patients group after 2 weeks of surgery. Significance was considered at the level of P-value<0.05

area under the ROC curve (AUC). The higher AUC corresponds to a better diagnostic test. Serum p53 protein showed significant AUC (84.3%; P=0.000) with sensitivity (84.2%) and specificity (82.6%) at a cut-off (2.88 U/ml). Serum sFas showed significant AUC (80.5%; P=0.000) with sensitivity (83.3%) and specificity (68.2%) at a cut-off (497.33 pg/ml). Serum CA 15-3 showed significant AUC (78.0%; P=0.001) with sensitivity (45.8%) and specificity (100%) at a cut-off (23.0 U/ml), (Figs. 4 and 5) and (Table 1).

Correlations of Serum sFas, P53 Protein and CA 15-3 with Age and Clinicopathological Data of Breast Cancer Patients Before Surgery

Serum sFas showed a significant direct correlation with tumor pathological grade (r=0.494; P=0.019). Serum CA 15-3 showed significant direct correlations with tumor size (r=0.458; P=0.032) and patient's clinical stage (r=0.571; P=0.009). The rest of the correlations of serum sFas, p53

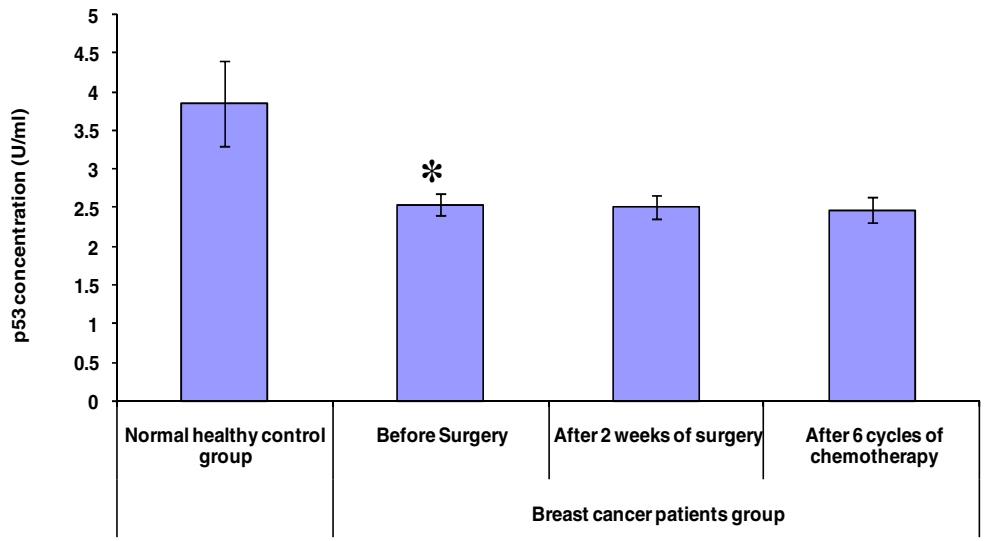

Fig. 2 M±SE serum levels of p53 in the normal healthy controls and breast cancer patients before surgery; after 2 weeks of surgery and after 6 cycles of chemotherapy. *: Significance was compared with control group. Significance was considered at the level of P-value<0.05

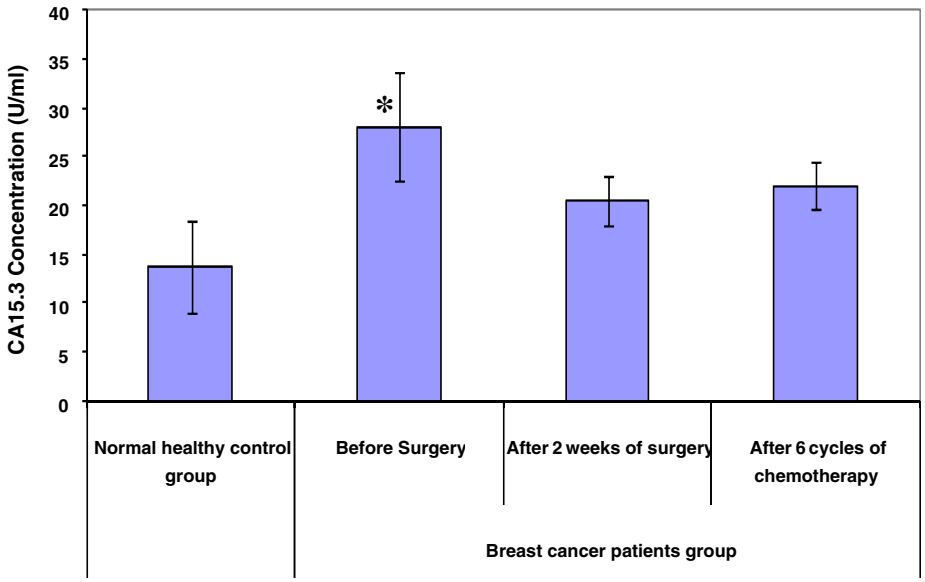

Fig. 3 M±SE serum levels of CA15-3 in the normal healthy controls and breast cancer patients before surgery; after 2 weeks of surgery and after 6 cycles of chemotherapy. *: Significance was compared with control group. Significance was considered at the level of P-value<0.05

protein and CA15-3 with age and other clinicopathological data were nonsignificant.

Discussion

Over the last several years, researches on the role of apoptosis in malignancy in general and in breast cancer in particular had increased [13, 14]. Apoptotic markers are now being investigated to have a role in detecting the progression of cancer and its response to various chemotherapeutic agents [15].

In the present study, the serum level of sFas was significantly higher in breast cancer patients before surgery than in the normal healthy controls. It was suggested that cancer cells can escape Fas-mediated apoptosis by different ways. First, the loss of cell-surface sFas can render cancer cells resistant to FasL-mediated apoptosis by immune cells. Second, neutralization of FasL by sFas can prevent ligation [16]. Our results support the results reported by El-Sarha et al. [17] and Sheen-

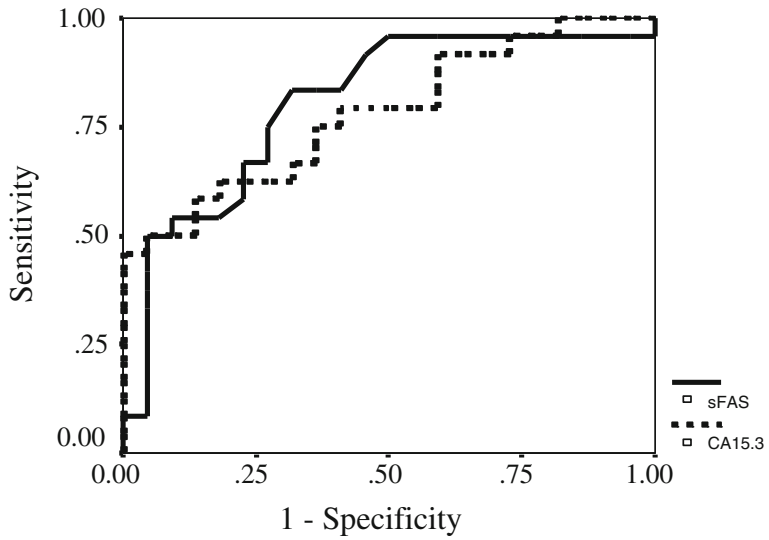

Fig. 4 Graphical representation of the ROC curves for serum sFas and CA 15.3 in breast cancer patients before surgery

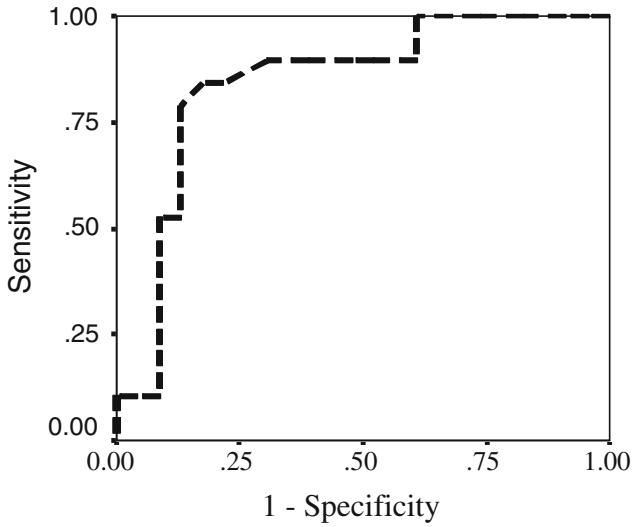

Fig. 5 Graphical representation of the ROC curve for serum p53 protein in breast cancer patients before surgery

Chen et al.[2] who found significantly elevated serum sFas in breast cancer patients before surgery than in breast benign tumor control group.

The results of the present study showed a significant correlation between serum sFas level and tumor pathological grade. Our results are in line with the results of Ohbu et al. [18] who reported that the rate of apoptosis was higher in well-differentiated esophageal tumors than in poorly-differentiated tumors. Also our results are in accordance with the results of Habibagahi et al. [16] who found a higher concentration of sFas in the sera of head and neck carcinoma patients who had higher grades.

In the present study, after 2 weeks of surgical removal of breast, the serum sFas level showed a significant decline compared with its level before surgery. This post-surgery decrease in sFas levels suggests that sFas may be produced by, or be closely linked with breast tumor cells. Moreover, after surgery the mean sFas level reached its level in the control group which means that tumor resection was complete and successful and, hence, sFas can be used for monitoring the efficacy of breast cancer surgery. In this regard, our results are in accordance with the results of Pignataro et al. [19] who have found a significant decrease in serum concentration of sFas 2 weeks after surgery of laryngeal carcinoma.

In the present study, six cycles of FAC chemotherapy resulted in a significant elevation in sFas level compared with its level after 2 weeks of surgery. As it is mentioned in many of the literature [20, 21], an increase in sFas level after chemotherapy is an indicator of chemotherapy resistance. However, after completing six cycles of chemotherapy, the patients included in the present study were followed up clinically, radiologically and laboratory for observation of any cancer recurrence or metastasis. Although, all of our patients were free of any cancer recurrence or metastasis, after 6 cycles of chemotherapy 8 out of the 35 (23%) breast cancer patients showed elevated serum sFas levels concomitant with systemic inflammation. It has been suggested that sFas decreases neutrophil apoptosis in patients postoperatively [22]. Paunel-Gorgulu et al. [23] demonstrated that elevated serum sFas inhibits neutrophil apoptosis associated with increased systemic inflammation.Also, it was reported that neutrophils may cause tissue damage by the secretion of reactive oxygen species (ROS) and proteolytic enzymes, of which neutrophil elastase (PMNE) is themost abundant [24, 25]. This means that serum sFas can be used to monitor the response of breast cancer patients to chemotherapy if the effect of the inflammatory reactions is ruled out. In this regard, our results confirm those of Nadal et al. [26] who reported that an increment of sFas/sFasL ratio after oxaliplatin-5-fluorouracil combination treatment could be an excellent marker of chemosensitivity in colorectal cancer, while a decreased ratio after treatment can be a predictor of chemoresistance.

The results of the present study showed that serum p53 level was significantly lower in breast cancer patients compared with normal controls. However, Kolomecki et al. [5] found a significantly higher level of plasma p53 protein in patients with benign and malignant thyroid tumors compared with normal healthy controls. Also, Balogh et al. [7] found that the positive rates of p53 protein in breast cancer patients were significantly higher than normal healthy control. As p53 is a tumor suppressor protein with pro-apoptotic properties, it is logic to have lower level of p53 in breast cancer patients compared with normal controls which is the finding of the present study. However, no significant change

Table 1 The Area under the ROC curves, sensitivity, and specificity for serum sFas, p53 protein and CA 15-3 in breast cancer patients before surgery

Variables	Area under the curve (%)	p-value *	Cut-off	Sensitivity (%)	Specificity (%)
p53 protein (U/ml)	84.3	0.000	2.88	84.2	82.6
sFas (pg/ml)	80.5	0.000	497.33	83.3	68.2
CA15.3 (U/ml)	78.0	0.001	23.00	45.8	100

* Significance was considered at p-value <0.05

was observed in serum p53 levels after 2 weeks of surgery and after 6 cycles of chemotherapy. This means that p53 protein has no role in monitoring the response of breast cancer patients to surgery and chemotherapy.

The results of the present study revealed that serum CA15-3 level was significantly greater in breast cancer patients than normal controls which are in line with Hewala et al. [27]. At the same time, the present results showed that serum CA 15-3 was significantly correlated with tumor size and clinical stage. This means that tumor tissue itself might be the source of serum CA 15-3. These results supported the study of Gion et al. [28]. However, no significant change was observed in serum CA15-3 levels after 2 weeks of surgery and after 6 cycles of chemotherapy. This means that serum CA15-3 protein has no role in monitoring the response of breast cancer patients to surgery and chemotherapy. Although, many studied [9, 29] reported that serum CA15-3 a role in monitoring the response of breast cancer patients to therapy, the absence of this role of CA15-3 in the present study may be due to the small sample size included in this study.

The significant elevation in the serum levels of sFas, and CA 15-3 and the significant decline in the serum level of p53 in breast cancer patients before surgery compared to normal controls suggest the possibility of using anyone of these parameters for diagnosis of breast cancer to differentiate the breast cancer patient from the normal healthy controls. This directed us to compare the diagnostic values of these parameters to determine which of them has the highest and the lowest diagnostic value. This comparison also involved determination of the precise cut-off value and the corresponding sensitivity and specificity for each parameter. This comparison was carried out using the ROC curve analysis in such a way that the greater area under the ROC curve corresponds to a better diagnostic test. Serum p53 protein showed the greatest significant area under the curve (84.3%) followed by sFas (80.5%), then CA 15-3 (78%). The sensitivity, specificity and cut-off value for diagnosing breast cancer patients were 84.2%, 82.6% and 2.88 U/ml for p53 protein, 83.3%, 68.2% and 497.3 pg/ml for sFas and 45.8%, 100% and 23 U/ml for CA15-3. These results suggest that serum p53 protein is superior to sFas and CA15-3 for diagnosis of breast cancer patients. Although serum sFas and p53 protein have been observed in breast cancer patients, to the best of our knowledge, this is the first study that compares the diagnostic values of serum sFas and p53 protein with those of serum CA 15-3 with determination of the precise cut-off value, sensitivity and specificity of each protein in breast cancer patients.

In conclusion, although serum p53 protein is superior to serum sFas and CA15-3 for diagnosis of breast cancer patients, only sFas is useful for monitoring the response of breast cancer patients to surgery and chemotherapy if the effect of systemic inflammatory reactions is excluded.

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