((Prevalence of Soluble Fas Protein in Breast Cancer Patients:correlation with the Clinico-Pathological Parameters))

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ABSTRACT

BACKGROUND:

Breast cancer is the most common cancer among the Iraqi population. Alteration in the expression of Fas and Fas ligand (FasL) compared to normal tissues are reported in the literature.

OBJECTIVE:

To investigate whether measuring this tumour marker in serum of breast cancer patients before and after treatment might also be useful markers in the diagnosis, screening and monitoring the malignant tumour progression and response to therapy.

METHODS:

Serum samples were obtained from (28) apparently healthy women (Control Group) with a mean age of 40.9 ± 7.6 years and (60) female patients complaining from primary breast cancer (Patients Group) with a mean age of 48.3 ± 8.9 years. They were divided according to their clinical end point into: Pre-Surgical Group, Post-Surgical Group and post- chemotherapy Group. Serum sFas level was measured using ELISA kits.

RESULTS:

The mean serum levels of sFas were significantly elevated ($P<0.05$) in breast cancer patients than controls. There was no significant influence of the studied personal and pathological characteristics upon biomarkers levels in any of the breast cancer subgroups ($P > 0.05$). sFas level was found an effective test ($P < 0.05$) in both pre- surgery and post- chemotherapy groups (accuracy is 87% and 90% respectively). At readings ≥ 300 pg/ml in both groups, sensitivity approached 85%. Fas was not found an effective test in the post surgery group ($P > 0.05$).

CONCLUSION:

It could be concluded that sFas is useful for monitoring the response of breast cancer patients to surgery and chemotherapy if the effect of systemic inflammatory reactions is excluded.

KEY WORDS: soluble fas,post-chemotherapy.

INTRODUCTION:

Breast cancer is the commonest type of female malignancy accounting for approximately one-third of the registered female cancer according to the latest Iraqi cancer registry. Early detection and screening, especially when combined with adequate therapy, offers the most immediate hope for a reduction in breast cancer mortality and morbidity[1].

Fas/Fas ligand (FasL) system: a major regulator of apoptosis, is involved in cancer cell death induced by the immune system and anticancer drugs. Fas is a cell-surface receptor that exists in two forms, transmembrane and soluble. The former induces apoptosis by ligation of FasL or agonistic anti-Fas antibody, whereas the latter inhibits Fas-mediated apoptosis by neutralizing its ligand[2].Fas is glycoprotein that is found on the cell surface of activated Human T and B lymphocytes and a variety of malignant Human lymphoid cell lines. is

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located on the long arm of chromosome 10 (10q24.1) [3,4].

Several studies have reported decreased levels of Fas in cancer cells, and the resultant resistance to Fas-mediated apoptosis may contribute to their escape from the immune system[5]. Fas-mediated apoptosis leads to the elimination of activated T-cells following an immune response. i.e killing a tumor[6]. Deregulation of Fas-mediated apoptosis is thought to play a role in the cancer progression, lymph node involvement and metastasis[7].

MATERIALS AND METHODS:

Settings: This study was conducted at three main medical facilities in Baghdad: The Main Training Center for Early Detection of Breast Tumours/Oncology Teaching Hospital, Al - Elweya Center for Early Detection of Breast Tumours and Al Amal Oncology Hospital. Eighty eight Iraqi women were enrolled in the study, including 28 apparently healthy women (used as a "Control Group I") and 60 female patients complaining from primary breast cancer (PBC) -"Patients Group II" diagnosed by using the triple assessment technique during the period ranging from November 2012 until March 2014.

In general, the age of the patients ranged between (30-65) years. Criteria for eligibility in the study included all patients diagnosed with PBC with no history of major liver, thyroid, endocrine diseases or other concurrent acute illness.

All patients were clinically interviewed and examined using the triple assessment technique, i.e.,clinical breast examination (CBE), mammography and /or ultrasonography , and fine needle aspiration cytology (FNAC).

The collected information included all data routinely recorded on the patient's file sheet questionnaire by the examining physician: age; marital status; history of lactation, contraceptive pills and/ or hormonal therapy; and family history of breast cancer. Data on tumour size and nodal status were obtained by examination of the tissue biopsies. Abdominal ultrasound and chest X-rays were carried out to exclude metastasis, and when indicated a skeletal survey was performed.

The clinico-pathologic data were obtained from patients' pathology reports. The collected data included tumor size, tumor pathological grade, axillary lymph node involvement, vascular invasion, status of Her2/neu Receptor , Estrogen Receptor (ER) and Progesterone Receptor (PR).

The clinical stage was determined by the oncologist according to the tumor-nodes-metastasis (TNM) classification system.

Patients Group II was further divided according to their clinical end point into:

I. Twenty patients who were recently detected and not yet operated upon neither receiving chemotherapy. Those were assigned as : "Pre-Surgical Group II".
II. Twenty patients who were subjected to mastectomy without receiving chemotherapy Those were assigned as: "Post-Surgical Group II".
III. Twenty patients who had adjuvant combination chemotherapy for at least six cycles. Those were assigned as: "Post-Chemotherapy Group II".

Blood sampling: 10 ml of venous blood was withdrawn from normal healthy female volunteers and from patients diagnosed with PBC before treatment, after surgery and after 6 cycles of chemotherapy by cubital venipuncture using 21 gauge needles in the sitting position. Immediately after withdrawing, blood samples were allowed to coagulate and centrifuged for 20 minutes at 3500 rpm. The separated serum samples were divided into two tubes and stored until assayed. After thawing, each serum sample was assayed only once.

Methodology: Soluble Fas (sFas):The level of soluble Fas in sera was determined using a ready for use Enzyme-Linked Immunosorbent Assay(ELISA) kit for the accurate quantitative measurement of Human sFas according to the producer's protocol. Briefly, prepared standards and diluted samples are added to the wells and incubated at 37°C for 90 minutes, discard contents of each well followed by addition of diluted biotin conjugate solution and incubation for 60 minutes at 37 °C. After washing, Avidin-Biotin-Peroxidase Complex is added and unbound conjugates are washed away with PBS or TBS buffer. Tetramethyl-benzidine (TMB) is then used to visualize the HRP enzymatic reaction. TMB is catalyzed by HRP to produce a blue color product that changes into yellow after adding acidic stop solution. The density of yellow coloration is directly proportional to the Human FAS amount of sample captured in plate. the absorbance was measured at 450 nm. sFas serum concentration was determined by referring to a standard curve. The

sensitivity of the assay was < 3 pg/mL (Abcam's , UK).

RESULTS:

The study sample included 28 subjects as Control. (Group I) 60 patients known to have PBC (Group II). All the enrolled patients were females with an age range between 30 to 65 years. The average ages for Group I and Group II were 40.9 ± 7.6 years and 48.3 ± 8.9 years respectively.

Group II were subdivided into three groups; Pre-Surgical, Post-Surgical and Post- Chemotherapy. Each group was composed of 20 patients.

The majority of the studied groups were married (90.0%) (table 1a), 70.0% of them were in their postmenopausal age .

85.0% of Group II PBC had a negative family history of breast cancer **.** 50.0% were diagnosed in stage II, and 37 % in stage III. Regarding the degree of differentiation of the tumour, 53% of lumps were Grade II, and 25% were Grade III. The tumor size was larger than 2 cm in around 81% of patients and nodal involvement was positive in 70% of patients (table 1b).

Estrogen receptor was positive in 83.3% of patients and progesterone receptor was positive in 85.% while Her2/neu receptor was positive only in 40% of patients (table 1b).

The level of s Fas significantly varied from group to another (small peak -566.9 Pg/ml - at before surgical group and larger peak -666.3 Pg/ml at after 6 cycles chemotherapy) (P < 0.05, table 2, figure 1).

Yields of post hoc multiple comparisons (findings with P < 0.05, table 3):

1- sFas was significantly lower (250.2 pg/ml) in control group compared to Pre surgery (566.9 pg/ml) and Post 6 cycles chemotherapy (666.3 pg/ml).

2- sFas was significantly higher in Pre surgery (566.9 pg/ml) compared to Post surgery level (361.8 pg/ml).

3- sFas significantly increased doubles after 6 cycles chemotherapy (666.3 pg/ml) compared to Post surgery (361.8 pg/ml).

Validity of breast cancer biomarker: sFas was found as an effective test (P < 0.05, table 4) in both Pre surgical and Post chemotherapy conditions (with an accuracy equivalent to 87% and 90% respectively). At readings ≥ 300 pg/ml in both groups, sensitivity approached 85% and specificity 61%. sFas was not found an effective test for Post surgical group (P > 0.05, table 4).

There was no significant influence of the studied personal and the pathological characteristics of the tumour upon the biomarkers levels in any of the breast cancer subgroups (P > 0.05, tables 5-7)

Table 1a: Distribution of the study sample according to Age, Menopausal status & Family History of BC.

Table 1a

	Control		Pre-Surgical		Post-Surgical		Post-Chemotherapy		All Cancer Patients	
Variable	N=28	%	N=20	%	N=20	%	N=20	%	N=60	%
Age Group										
• 30-39 year	12	42	2	10	2	10	6	30	10	17
• 40-49 year	12	42	5	25	10	50	8	40	23	38
• 50-59 year	3	11	7	35	8	40	4	20	19	32
• Over 60	1	5	6	30	0	0	2	10	8	13
Total	28	100	20	100	20	100	20	100	60	100
Marital status										
• Unmarried	5	18.0	2	10.0	2	10.0	2	10.0	6	10.0
• Married	23	82.0	18	90.0	18	90.0	18	90.0	54	90.0
Total	28	100	20	100	20	100	20	100	60	100
Menopause										
• Premenopausal	7	25.0	2	10.0	7	35.0	9	45.0	18	30.0
• Postmenopausal	21	75.0	18	90.0	13	65.0	11	55.0	42	70.0
Total	28	100	20	100	20	100	20	100	60	100
Family History										

•	Positive	1	1.0	3	15.0	1	5.0	5	25.0	9	15.0
•	Negative	27	99.0	17	85.0	19	95.0	15	75.0	51	85.0
Total		28	100	20	100	20	100	20	100	60	100

Table (1b): Distribution of the study sample according to the Pathological Characteristics of the Tumour in Group II PBC.

	Pre-Surgical		Post-Surgical		Post- chemotherapy		All Cancer Patients	
Variable	N=20	%	N=20	%	N=20	%	N=60	%
Stage								
• I	1	5.0	3	15.0	1	5.0	5	8.3
• II	10	50.0	8	40.0	12	60.0	30	50.0
• III	7	35.0	8	40.0	7	35.0	22	36.7
• IV	2	10.0	1	5.0	0	0.0	3	5.0
Total	20	100	20	100	20	100	60	100
Grade								
• I	3	15.0	5	25.0	5	25.0	13	21.7
• II	12	60.0	10	50.0	10	50.0	32	53.3
• III	5	25.0	5	25.0	5	25.0	15	25.0
Total	20	100	20	100	20	100	60	100
Tumor size								
• Up to 2 cm	2	10.0	4	21.1	5	25.0	11	18.6
• 2.1- 5 cm	11	55.0	10	52.6	13	65.0	34	57.6
• > 5 cm	7	35.0	5	26.3	2	10.0	14	23.7
Total	20	100	20	100	20	100	60	100
Nodal Status								
• Positive	19	95.0	10	50.0	13	65.0	42	70.0
• Negative	1	5.0	10	50.0	7	35.0	18	30.0
Total	20	100	20	100	20	100	60	100
Estrogen Receptor								
Positive	18	90.0	13	63.2	19	95.0	50	83.3
Negative	2	10.0	7	36.8	1	5.0	10	16.7
Total	20	100	20	100	20	100	60	100
Progesterone Receptor								
Positive	18	90.0	14	68.4	19	95.0	51	85
Negative	2	10.0	6	31.6	1	5.0	9	15
Total	20	100	20	100	20	100	60	100
Her2neu Receptor								
Positive	5	25.0	8	42.1	10	50.0	24	40
Negative	15	75.0	12	57.9	10	50.0	36	60
Total	20	100	20	100	20	100	60	100

Table 2: Descriptive statistics for sFas according to the study groups

	Level (Pg/ml)					
Descriptive Statistics	Control	Pre-Surgical	Post-Surgical	Post- Chemotherapy	P value	Statistical of significance
Mean	250.2	566.9	361.8	666.3	< 0.05	S
Median	214.5	602.0	271.0	648.0		
SD	134.1	245.5	196.6	314.9		
95%CI; lower	198.2	451.9	269.8	519.0		
upper	302.2	681.8	453.9	813.7		
Minimum	57	143	143	205		
Maximum	602	973	895	1398		

S : Significant , P : Probability value .

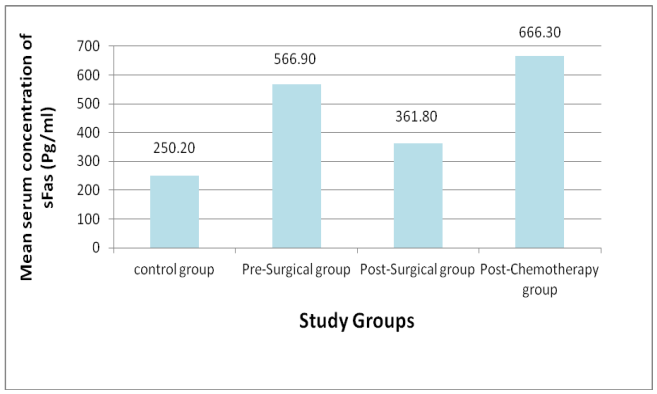

Figure 1: Mean levels of sFas in each study groups.

Table 3: P values for Post hoc multiple comparisons (using Tukey HSD test) of levels for sFas.

Variables & Study Groups	Study Group			
	Control Group	Pre-Surgical	Post-Surgical	Post- Chemotherapy
	P value	P value	P value	P value
sFas				
Control	---	0.000	0.332	0.000
Pre-Surgical	0.000	---	0.025	0.503
Post- Surgical	0.332	0.025	---	0.000
Post-CT*	0.000	0.503	0.000	---

Table 4: Validity for serum levels of sFas at selected cut-off points in the study groups.

Study Groups	Cut-off Point*	P value	AUC	Sensitivity	Specificity
Pre-Surgical	300.0	< 0.001	0.870	0.850	0.607
Post- Surgical	246.0	0.076	0.688	0.700	0.571
Post-Chemotherapy	300.0	< 0.001	0.903	0.850	0.607

Table 5: Descriptive statistics for sFas in breast cancer patients- Pre surgical group according to the study variables.

Variables	Mean	SD	N	P value	Statistical of significant
Age Group				>0.05	N.S
30-45 year	748.5	207.2	2		
46-65 year	546.7	246.1	18		
Menopause				>0.05	N.S
Premenopausal	748.5	207.2	2		
Postmenopausal	546.7	246.1	18		
Marital status				>0.05	N.S
Unmarried	500.0	144.2	2		
Married	574.3	256.1	18		
Family History				>0.05	N.S

Negative	610.7	238.1	17		
Positive	318.3	100.8	3		
Stage				>0.05	N.S
I	205.0	.	1		
II	581.9	243.2	10		
III	537.4	233.3	7		
IV	775.5	245.4	2		
Grade				>0.05	N.S
I	525.3	399.5	3		
II	577.0	198.3	12		
III	567.4	311.1	5		
Tumor size (cm)				>0.05	N.S
Up to 2	589.0	543.1	2		
2.1- 5	594.7	202.1	11		
> 5	516.7	262.9	7		
Nodal Status				>0.05	N.S
Negative	205.0	.	1		
Positive	585.9	236.6	19		
Estrogen R				>0.05	N.S
Negative	372.5	324.6	2		
Positive	588.4	237.2	18		
Progesterone R				>0.05	N.S
Negative	372.5	324.6	2		
Positive	588.4	237.2	18		
Her2neu R				>0.05	N.S
Negative	592.9	228.9	15		
Positive	488.8	304.6	5		

Table 6: Descriptive statistics for sFas in breast cancer patients- Post surgical group according to the study variables.

Variables	Mean	SD	N	P value	Statistical of significance
Age Group				>0.05	N.S
30-45 year	340.4	159.7	8		
46-65 year	376.2	223.5	12		
Menopause				>0.05	N.S
Premenopausal	359.7	162.1	7		
Postmenopausal	363.0	219.2	13		
Marital status				>0.05	N.S
Unmarried	303.5	50.2	2		
Married	368.3	206.4	18		
Family History				>0.05	N.S
Negative	370.1	198.4	19		
Positive	205.0	.	1		
Stage				>0.05	N.S
I	358.3	213.4	3		
II	312.9	114.1	8		
III	406.9	271.2	8		
IV	404.0	.	1		
Grade				>0.05	N.S
I	365.2	193.4	5		
II	330.0	151.1	10		
III	422.2	296.1	5		
Tumor size (cm)				>0.05	N.S
Up to 2	435.5	242.2	4		

2.1- 5	340.9	205.7	10		
> 5	296.8	134.5	5		
Nodal Status				>0.05	N.S
Negative	323.6	153.6	10		
Positive	400.1	234.0	10		
Estrogen R				>0.05	N.S
Negative	252.3	73.8	7		
Positive	395.7	207.5	13		
Progesterone R				>0.05	N.S
Negative	249.7	80.5	6		
Positive	385.8	201.7	14		
Her2neu R				>0.05	N.S
Negative	364.8	225.3	12		
Positive	312.6	104.8	8		

Table 7: Descriptive statistics for sFas in breast cancer patients- Post Chemotherapy group according to the study variables.

Variables	Mean	SD	N	P value	Statistical of significance
Age Group				>0.05	N.S
30-45 year	643.6	291.2	11		
46-65 year	694.1	357.6	9		
Menopause				>0.05	N.S
Premenopausal	576.4	278.5	9		
Postmenopausal	739.9	336.2	11		
Marital status				>0.05	N.S
Unmarried	550.0	487.9	2		
Married	679.3	308.3	18		
Family History				>0.05	N.S
Negative	604.2	332.1	15		
Positive	852.8	164.2	5		
Stage				>0.05	N.S
I	1053	.	1		
II	743	322.5	12		
III	479.7	214.3	7		
IV	---	---	0		
Grade				>0.05	N.S
I	666.0	347.7	5		
II	644.2	367.8	10		
III	711.0	204.5	5		
Tumor size (cm)				>0.05	N.S
Up to 2	773.0	417.7	5		
2.1- 5	629.0	287.2	13		
> 5	642.5	345.8	2		
Nodal Status				>0.05	N.S
Negative	625.0	265.5	7		
Positive	688.6	346.7	13		
Estrogen R				>0.05	N.S
Negative	903.0	.	1		
Positive	653.9	318.4	19		
Progesterone R				>0.05	N.S
Negative	903.0	.	1		
Positive	653.9	318.4	19		
Her2neu R				>0.05	N.S
Negative	695.3	255.4	10		
Positive	637.4	377.1	10		

DISCUSSION:

It is well known that breast cancer is by far the most frequent cancer among women worldwide. According to WHO mortality estimates, cancer is the fourth ranked cause of death in the Eastern Mediterranean Region (EMR), after cardiovascular diseases, infectious / parasitic diseases and injuries[8].In Iraq, it is considered the most common type of malignancy among women accounting for about one third of the registered female cancers[9]. The present study showed that breast cancer occurs most frequently during the fourth and fifth decades of women's life followed by the sixth/ These results were compatible to what has been reported in other studies, conducted in Iraq; documenting that the malignant breast lesions occur mostly during the fifth decade followed by the sixth[10] . In addition the current study showed that the mean age of breast cancer patients was 48.3 years. This is in agreement with the result published by many studies in Iraq and other Arabian countries in addition to Islamic republic of Iran[11]. The Iraqi cancer board (2004) registered the mean age of Iraqi women, diagnosed with breast cancer was 49 years; close to what was reported from Iran (48 years); and comparable to those figures displayed by local investigators including the Iraqi National Breast Cancer Research Center[12].

On the other hand, American and European studies usually record significantly higher ages for their breast cancer patients at the time of presentations reaching 66 years ([13]. In South Africa it has been observed that breast cancer occurs at earlier ages in Blacks (mean 49 years) than in Whites[14]. In general the age-standardized incidence of breast cancer is lower in developing countries than in developed countries, and incidence rates vary widely between and within countries[15]. No significant relationship was noted between menopausal status and the frequency of breast cancer in this report. has been authorized that the increased incidence of breast cancer in women aged below 50 years could be due to active ovarian function and estrogen hormone secretion while the increased incidence in those aged above 50 years could attribute to an imbalance in the adrenal estrogen[16].

This study showed that about 15% of patients diagnosed with breast cancer have a family history of this disease. The rate was rather consistent to what was displayed in an earlier survey from Iraq (Alwan, 2010). In the current study, merely **57.**6% of breast cancer patients sought medical advice at the time when their tumours measured from 2-5 cm in diameter. Al-Janabi (2003) [18], reported lower percentage of patients presenting at stage III and stage IV.

Only 21.7% of malignant tumours in our study were well differentiated while the majority (78.3%) were in Grade II and III (53.3% and 25.0% respectively). Al- Anbari (2009) [19], reported higher rates of patients diagnosed at higher grades (48% in grade II and 41% in grade III), and 50% of her patients presented with tumors measuring less than 2 cm.

The progression of breast cancer is often linked to changes in the expressions of PR, ER, and Her-2/neu receptor status[20]. The hormonal imbalance may cause a multifold cell division. It may be possible that the functional sites of these proteins may be altered and the extra cellular subunit of these proteins might be interacting with α domain of these receptors which may increase the chance of metastasis in breast cancer by stimulating RAS protein Pathway. The present study found that these receptors (PR, ER, and Her-2/neu) were positive in 42% of premonopausal patients, while 53% of postmenopausal women's cancer was ER, PR positive and Her-2/neu negative and the remaining 5% were triple negative (ER, PR and Her-2/neu -ve). Results of this study were in accordance with another study that found a significant association between the development of breast cancer and the positivity of ER, PR and Her-2/neu receptor[21].

The results of the present study showed that 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[24] . Our results support the results reported by El-Sarha & Sheen-Chen et al., 2009 [25]& Bhatia et al., 2006[26] they found significantly elevated serum sFas in breast cancer patients before surgery.

In the present study, after 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.,2003[27] who have found a significant decrease in serum concentration of sFas two weeks after surgery of laryngeal carcinoma.

In the present work, we were able to demonstrate that six cycles of chemotherapy resulted in a significant elevation in sFas level compared with its level after surgery. It has been mentioned in the literature Ugurel et al.,2001[28]& Shimizu et al.,2005[29], that 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, 8 out of the 20 (40%) breast cancer patients showed elevated serum sFas levels concomitant with systemic inflammation. It has been suggested that sFas decreases neutrophil apoptosis in patients postoperatively[30]. Paunel-Gorgulu et al., 2011 [31]demonstrated that elevated serum sFas inhibits neutrophil apoptosis associated with increased systemic inflammation. 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 the most abundant Donnelly 1995[32,33]. That indicates that serum sFas can be used to monitor the response of breast cancer patients to chemotherapy if the effect of the inflammatory reactions could be ruled out. In this regard, our results support the findings displayed by Nadal et al., 2005[34] who reported that an increment of sFas/sFasL ratio after chemotherapy treatment could be an excellent marker of chemosensitivity in colorectal cancer, while a decreased ratio after treatment could be a predictor of chemoresistance.

CONCLUSION:

It could be concluded that 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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