Immunohistochemical study of tumor necrosis factor alpha (TNF-α) in the tumor microenvironment sample of Iraqi breast cancer

الكشف عن عامل الورم المتنخر الفا(TNF-α) في البيئة الداخلية لمرضى سرطان الثدي في العراق

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Abstract

Breast cancer is the most common malignant tumor and the leading cause of carcinoma death in women. The tumor microenvironment consist from cellular microenvironment, cytokines and chemokines. Its plays an important role in tumor development such as initiation, progression, metastasis and drug resistance. It is becoming increasingly apparent that the malignant tumor progression is maintained by dynamic interplay between tumor cells and many distinct cell types existing in the adjacent microenvironment. Objective of this study is to detect of TNF-α in tumor microenvironment in histological sections of breast cancer and compare it with that of benign breast lesions. This study included 32 patients with breast cancer and 21 patients with benign breast lesions, prognostic factors were registered including: age, histopathological subtype, degree of differentiation& stage of breast carcinoma. Results revealed positive expression of TNF-α in 20 cases, while 12 cases were negative out of 32 samples of breast cancer. In benign lesions 11 cases were positive, While 10cases were negative. Statistical analysis showed significant difference in rate of TNF-α expression between malignant breast samples and benign samples(P ≤0.05).

keywords: Microenvironment, TNF-α, Breast cancer, Immunohistochemistry

الملخص

ان سرطان الثدي هو من الاورام الخبيثة الاكثر شيوعا في النساء مسببا وفاة عدد من النساء المصابات به. البيئة الداخلية للاورام تتكون من مكونات خلوية، سايتوكينات وكيموكينات، وتلعب دورا مهما في تطور الورم مثل مرحلة بدء الورم, تقدم الورم, انتقال الورم الى اجزاء اخرى من الجسم ومقاومة الادوية. اصبح من الواضح ان وصول الورم الى مراحل متقدمة يكون بواسطة تفاعل ديناميكي بين الخلايا السرطانية والعديد من الخلايا المتجاورة الموجودة في البيئة الداخلية للورم. الهدف من هذه الدراسة هو الكشف عن عامل الورم المتنخر الفا TNF-α في البيئة الداخلية لمرضى سرطان الثدي في العراق ودراسة للمقارنة بين المرضى الذين يعانون من سرطان الثدي والمرضى الذين يعانون من آفات الثدي الحميدة. هذه الدراسة شملت 32 مريضا يعانون من سرطان الثدي و21 مريضا من الذين يعانون من آفات الثدي الحميدة وسجلت العوامل النديرة بما في ذلك: العمر, الانواع الفرعية النسيجية, ودرجة التمايزومرحلة الاصابة .لوحظ التعبير الايجابي لعامل الورم المتنخر الفا TNF-α في 20حالة , في حين كانت 12حالة من التعبير السلبي من مجموع 32 حالة مصابة بسرطان الثدي. في الحالات الحميدة كانت 11 حالة ايجابية التعبير, في حين ان 10حالة كانت سلبية التعبير. تعبيرTNF-α اظهر فرق معنوي بين عينات المصابة بسرطان الخبيث والحميد بقيمة P≤ 0.05 .

الكلمات المفتاحية: البيئة الداخلية للسرطان, عامل النمو المتنخرالفا, سرطان الثدي, الكيمياء المناعية النسيجية

Introduction

Breast cancer is the most common malignant tumor and the leading cause of carcinoma death in women [1].The term tumor microenvironment of metastasis (TMOM)coined by Robinson [2]. The tumor microenvironment consist from cellular microenvironment, cytokines and chemokines. It play an important role in the regulation of metastasis [3]. The communication between cancer cells and their microenvironments triggers cancer cells to break away from the original tumor and invade other areas of the body [4]. Cells migration and invasion play a vital role in the onset of metastasis. Tumor microenvironment also plays an important role in tumor development such as initiation, progression, metastasis and drug resistance [5].While the normal microenvironment can inhibit malignant cell growth, the modification that occur in the tumor microenvironment support cell proliferation.TNF-α is a 17 KDa polypeptide cytokine that was first described as a serum derived substance that causes tumor cell death [6].

بحث مستل من اطروحة الباحث الاول

TNF-α play important role in tumor progression in breast cancer Meng described high levels of TNF-α in malignant breast epithelial cells and suggested that TNF-α may promote tumor growth[7]. It is becoming increasingly apparent that the malignant tumor progression is maintained by dynamic interplay between tumor cells and many distinct cell types existing in the adjacent microenvironment [8].

Subjects and methods

Total sample Fifty three patient (32 paraffin embedded blocks with breast cancer and 21 paraffin embedded blocks with benign breast lesion) were collected from Iraqis female patients with breast carcinomas, age ranging from 29 to70 years and to whom either mastectomy or lumpectomy were done and attended from Teaching Laboratory-Medical City and Private Laboratory, Baghdad, between the years 2009 to 2012. The clinicopathological records for each patient, which included: age, histologic tumor grade and stage were obtained from pathological reports Of the patients and confirmed by an experienced pathologist. Hematoxylin and eosin (H and E) stained slides were prepared from the formalin fixed paraffin embedded blocks and examined by a pathologist for histopathological diagnosis and determining the degree of differentiation of the tumor.

Antibody used in study:

 Monoclonal mouse Anti-Human TNF-α was used in the present study from Santa Cruz Biotechnology Company USA.

preparation of reagents

Dilution of primary antibodies

Dilution of primary antibodies was done by using sterile PBS in a concentration according to each data sheet of monoclonal antibodies. Antibody was tested with several runs as a technical control staining in order to reach the optimum positive run. TNF-α was diluted into 1/50 times.

Dilution of DAB solution

DAB was prepared by mixing 1ml of (DAB Buffer) with 20µl of (DAB chromogen) in dark tube, and then kept in a dark place and using immediately after prepared.

Principle of the Test

The labeled streptavidin-biotin (LSAB) system is sensitive and versatile IHC procedure which permits simultaneous processing of numerous specimens with mouse primary antibodies. Endogenous peroxidase activity is quenched by incubating the specimen with 3%hydrogen peroxide. The specimen is then incubated with the appropriate diluted mouse primary antibody, followed by incubations with a biotinylated link antibody (contaning anti-mouse immunoglobulins) and peroxidase- labelled streptavidin. Staining is completed after an incubation with the Substrate Chromogen (DAB) incubation with 3-3 diamino benzidine (DAB) Substrate- Chromogen which results in a brown colored precipitate at the antigen site [9].

Immunohistochemical staining procedure for detection of Tumor necrosis factor alpha(TNF-α)

1.Slides baking: the slides of paraffin section of breast tissue were placed in a $45°$ angled position in a hot air oven at $60°$c over night.

2. Deparaffinization: the slides were immersed in xylene for 15 minutes two times at room temperature.

3.Rehydration: the slides were immersed sequentially in the following solutions at room temperature starting with:

-Twice in absolute ethanol for 5 minutes in each concentration 95% -90% -80%- 70%. And in Distilled water for 5 minutes.

4.Ag retrieval.

5.Enough drops of hydrogen peroxide block were added to slides and incubated in humid chamber at $37°$C for 10 minutes ,then socked 2 times in buffer (5 minutes for each).

6.Enough drops of protein block were added to slides and incubated in $37°$ C for 10 minutes ,Then washed 2 times in buffer (5 minutes for each),finally drained and blotted gently.

7.Diluted primary antibody was applied to each slide, incubated in humid chamber at $4°$ C overnight .Early in the next day the sildes were washed in buffer 4 times(5 minutes for each), finally drained and blotted gently as before.

8. Enough drops of secondary antibody (link antibody yellow drops) regent were added and incubated in humid chamber for 20 minutes at 37˚C. After that, the slides were washed 4 times in buffer (5 minutes for each), finally drained and blotted gently.

 9.Streptavidin-HRP antibodies (red drops) was applied on tissue and incubated for 20 minutes at 37˚C. After that, the slides were washed 4 times in buffer (5 minutes for each), finally drained and blotted gently.

10.Diluted DAB was applied on tissue (this process was done in dark room) and incubated in humid chamber at 37˚C.Then slides washed carefully in tap water for 5 minutes.

11.The Slides were bathed in hematoxylin counterstain for 1-2 minutes then they were rinsed with tap water for 10 minutes.

12.Dehydration: the slides were dehydrated by immersing them in ethanol and xylene containing jars as follows (70%-80%-90%-95%).And twice in absolute ethanol for 1 minute each.

Xylene for 1 minutes.

Fresh xylene for 5 minutes.

13.one to two drops of DPX mounting medium was applied to the xylene wet sections and covered with cover slips and left to dry for 30 minutes.

Evaluation of Immunohistochemistry results

 Positive reading was indicated when the cells display a brown cytoplasmic pigmentation staining , while negative reading was indicated for absence of immunostaining.

Immunohistochemical scoring of TNF-α:

Cut off values for all the antibodies used in the study were done with the help of pathologist. The scoring was done under light microscope to evaluate the immunostaining of the antibodies; Positively stained cells were counted at 5 representative fields (40x).

TNF-α expression was seen in the cytoplasm of breast cancer cell and benign cells and the scoring of positive tumor cell was considered as follows[10] (Fox et al ., 1996):

$1+ = < 25\%$ -- $2+ = 25\text{-}75\%$ -- $3+ = $ more than 75%

Statistical Analysis:

 Chi-square test and mean ± S.D. were used for the clinicopathological studies. ANOVA test and P value were used for IHC studies , all the statistical analyses analysis of variance (ANOVA) using SAS computer program version 7.5. Differences in results were considered significant at probability value equal or less than 0.05[11] (SAS.2004) and Microsoft Excel.

Results

Clinic pathological features

A total of (53), 32 Iraqi females patients newly diagnosed with breast cancer were involved in this study, their mean age was (48.45±11.6) years with a range of 29 to 70 years compared with 21 patient (with benign breast lesions: 15, fibroadenomas and 6 fibroadenosis; with a range of 19 to 49 years).

Regarding the malignant lesion ,the age incidence in 7 cases below forty years were (21.87%).13 patients(40.62%)were between (40-49)years .4 cases(12.5%)were between (50-59)years old and 8 cases were above(60) years old about(25%) Table (1). In Benign Breast Iesion.13 cases (86.66)were less than 40 years;2 cases(13.335) were 40-49 years and no cases aged more than 49 years, Table (2).

The invasive breast carcinoma Not otherwise specified (NOS) type were graded according to the Nottingham histological grading (WHO and modified Bloom–Richardson grading system), 20 csaes(62.5%) were grade II and 12 cases (37.5%)cases were grade III.

 Table (1) reveals the stages of breast cancer for 32 patients according to TNM system, 16 cases (50%)were stages(I and II), 13 cases(40.6%) were stage III and 3 cases (9.37%) were stage IV

Table(1): Clinicopathological characteristics of breast cancer patients

parameter	No	Malignant breast(32)
Age(year)		%
<40	7	21.875
40-49	13	40.625
50-59	4	12.5
≥60	8	25
	32	100
Tumor Type		
Invasive ductal carcinoma	32	100
Histological grade		
Grade II	20	62.5
Grade III		37.5
Staging system		
Stage I and II	16	50
Stage III	13	40.62
Stage IV	3	9.37

Table(2): Clinicopathological data of the Benign Breast Lesion

parameter	Fiberoadenoma(15)		Fiberoadenosis (6)	
Age(year)	No	%	No	%
<40	13	86.66	4	66.66
40-49	2	13.33	2	33.33
50-59	0	0	0	0
≥60	0	0	0	0

IHC Results

In this study immunohistochemistry technique was used to detect the expression of TNF-α in the breast tissue, among breast cancer groups and benign tumor groups.

IHC expression of TNF-α

 TNF-α is expressed in the cytoplasm of the tumor cells. Negative expression were observed in 12(37.5%) out of 32 samples it scored 0 found in 12 out of 32 samples(37.5%), score (1+) found in 3(9.37%) out of 32 samples, score(2+) found in 13 (40.62%) out of 32 samples and score (3+) found in 4(12.5%) out of 32 samples. While the benign breast lesions revealed positive expression in (52.38%) of lesions figure (1). Statistical analysis of TNF-α showed significant difference between malignant s breast samples and benign samples (p ≤0.05) figure (1) . Brown stained cytoplasm which indicates the positive expression of TNF-α figure (2) and negative expression showed no cytoplasm staining figure (3).
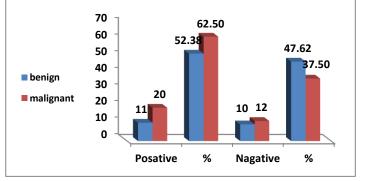

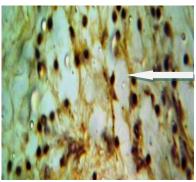**Fig. (1): Immunohistochemical staining of TNF- α of the breast samples (Malignant and Benign).**

Fig. (2): Immunohistochemical staning of TNF-α in breast cancer sections shown positive expression TNF-α (brown). Immunohistochemical by peroxidase/DAB(brown) counterstained with haematoxylin (blue). (40X).
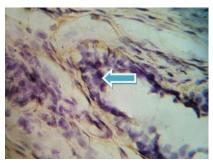

Fig.(3): Immunohistochemical staning of TNF-α in breast cancer sections shown negative (no expression) Immunohistochemical by peroxidase/DAB(brown) counterstained with haematoxylin (blue). (40X).
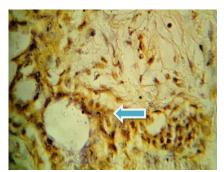

Fig. (4): Immunohistochemical staning of TNF-α in benign breast lesions sections shown positive expression TNF-α (brown). Immunohistochemical by peroxidase/DAB(brown) counterstained with haematoxylin (blue). (40X).

Discussion

The relation between age and breast cancer

The present results on Iraqi patients revealed that high age frequency of cancer occurred between (40-49) years old 40.6% .This is due to several causes such as environmental factors,the nutrition ,poor health education, radiation exposure previous breast disease, and the exposure to a high dose of depleted uranium for long time. Iraqi community is suffering from depleted uranium pollution because the wars that happening and these increased incidence of various cancers and birth defects[12]. The present results agree with many studies in Iraq performed on breast cancer and revealed that 40% of the cancer patients were in the peak of age frequency of 40-49 years [13]. Hamadee [14], revealed a mean age of mean age of 46.6 years and that 36% of the cancer patients were in the peak age frequency of 40-46 years.

The risk of breast cancer is higher in middle age and elderly women than in young women .This risk increase as a woman ages , rising sharply after the age of 40. This incidence of breast cancer mean age is lower than that of American and European countries when the mean age 62 years[15].

Histological grade:

Although grading system could be variable because of its subjective evaluation, its regarded as an important parameter in the prognosis[16]. Grading of the malignant cases was assessed according to American Joint Committee on Cancer(AJCC). In this study, 68.7% were grade II and 31.2% were grade III. Result were agrees with Al-Alwan [17], who found 56.6% moderately differentiated and 39.9% poorly differentiated.

Immunohistochemical Evaluation of TNF-α

Breast carcinomas need a blood vessels for tumor grows to greater and it requires new vessel growth for adequate oxygen and nutrient delivery and for removal of waste products[18]. TNF-α has been suggested to be a promoter of tumor angiogenesis in breast carcinomas[19]. It has confirmed by other studies which have demonstrated that the increased level of TNF-α is correlated with angiogenesis and cancer development [20].

Showing the leading role of TNF-α in tumor angiogenesis and progression in many different cancers [21]. Some studies note that TNF-α which is pro angiogenesis factor is able to initiate cellular apoptosis and it is possible that these apoptotic pathways are deactivate in tumor cells [22].

TNF-α is a double dealer .On one hand TNF-α could be an endogenous tumor promoter because TNF-α stimulates cancer cells growth, proliferation, invasion, metastasis and tumor angiogenesis. On the other hand TNF-α could be a cancer killer[23].

The tumor promoting functions of TNF-α may be mediated by its ability to induce pro- angiogenic functions to promote the expression of matrix metalloproteinase (MMP) and endothelial adhesion molecules and to cause DNA damage via reactive oxygen. The overall effect of which is promotion of tumor related processes[24].

In the present immunohistochemistry –based study, we examined the expression of TNF-α in invasive ductal carcinoma. Our results relatively agreed with the results obtained from Leek [25] who showed that the most of the positive TNF-α stained was associated with malignant cells and tumor epithelial. Our results demonstrate that these significant association between TNF-α level and breast cancer agree with study of Soria [26] who indicated that TNF-α is elevated in breast cancer patients.

Our data indicate that TNF-α expression in breast cancer higher than in benign breast lesion, this finding agreed with Tunon [27].

Conclusions

Immunohistochemical staining with TNF-α is considered to be useful technique for detection the modified of microenvionment of breast cancer. TNF-α overexpression was observed in(62.50%) of the malignant sample, while the expressed of the benign sample was observed in (52.3%).There were a significant differences in the presences of TNF-α between patients with breast cancer and patients with benign breast lesions (p≤0. 05).

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