DETECTION OF CYTOKERATIN ANTIGEN ONE IN BREAST CANCER PATIENTS USING IMMUNOCHEMICAL TECHNIQUES

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ABSTRACT

Breast cancer ranks number one among female malignancies in Egypt, and it is usually diagnosed at advanced stage. Hence, new markers for early diagnosis and accurate prognosis are warranted to improve breast cancer care. Cytokeratins, belonging to the intermediate filament protein family, are particularly useful tool in oncology. The present study aimed to identify and characterize a cytokeratin 1 (CK1) antigen associated with breast cancer. SDS-PAGE and western blot techniques based on monoclonal antibody (CK_1) were used to identify CK1 antigen in serum of 75 Egyptian women associated with breast cancer, in addition to, 27 serum samples from healthy women. ELISA was developed for the detection of CK1 antigen in serum samples of breast cancer patients. The target CK1 antigen was identified at 68kDa. Serum CK1 antigen from breast cancer women was characterized as protein in nature using chemical and biological treatments. The 68-kDa CK1 antigen may be used as a diagnostic marker in breast cancer.

INTRODUCTION

Breast cancer is a daunting disease and constitutes a continuing medical health problem through the middle ages for millions of women worldwide. Breast cancer constitutes one of the old types of cancer described from ancient Egypt till now [Ekmektzoglou *et al.,* (2009)]. It is the most common cancer and the second principal cause of cancer deaths in women. In the Arab world, breast cancer is ranked as the most frequent tumor in young women below 50 years of age [Bener *et al.,* (2010)]. Today breast cancer control is significantly improved by the ability to diagnose the disease at a more favorable stage, such as when cells are (in situ), or when the invasive disease is too small to be palpable [Najjar and Easson, (2010)]. Early detection of breast cancer reduces the suffering and cost to society associated with the disease. When the cancer is discovered early, patients live longer, require less extensive treatment, and, in general,

fare much better than patients with more advanced cancer [Levenson *et al.,* (2007)]. Tumor markers are potentially useful in screening for cancer, monitoring the course of the disease, and detecting the relapse or recurrence after the treatment. Studies are directed to develop easily applicable, noninvasive, cheap and reliable tools with high specificity and sensitivity to a certain tumor [Şekeroğlu *et al.,* (2002)]. The most widely used serum markers in breast cancer are CA 15-3 and carcinoembryonic antigen (CEA). Because of the lack of sensitivity for early disease and lack of specificity, none of the available markers is of value for the detection of early breast cancer [Duffy, (2006)]. At present, evidence related to the most effective approach to breast cancer screening is incomplete, inconclusive, and inconsistent [Kearney and Murray, (2009)]. Cytokeratins, also known as keratins, are the type of the cytoplasmic intermediate filaments primarily existed in epithelial cells, and tumor cells derived from epithelia [Jiang et al., (2002)]. Cytokeratins are particularly useful tools in oncology [Barak *et al.,* (2004)], and they can be used for diagnostic and prognostic purposes in breast tumor cells [Doljak *et al.,* (2008); Mohammadizadeh *et al.,* (2009)], therefore the aim of the present study is the identification and characterization of a CK1 antigen from breast cancer patients using SDS-PAGE, western blot and ELISA techniques based on CK1 monoclonal antibody.

SUBJECTS AND METHODS

A total of 75 serum samples from Egyptian women with breast cancer were collected from oncology center, Mansoura University, Mansoura, Egypt with mean age 50.8 ± 12.6 years were included in this study. In addition, 27 serum samples from healthy women with mean age, 47.8 ± 6.3 years, were collected to be used as negative controls. Serum samples were stored at -20°C until analysis. The primary pathologic diagnosis was confirmed in H&E staining. All breast cancers tissues were invasive cancers with stages I–III. Clinical follow-up included history taking, physical examination, and laboratory tests, including: liver function test, complete blood count, chest radiography, abdominal and breast ultrasonography, mammography.

Identification of CK1 antigen by SDS-PAGE and western blot :

The CK1 antigen in serum samples of selected breast cancer patients were separated by SDS-PAGE as described by[Laemmli (1970)]. Then, the resolved samples were electrotransferred onto the nitrocellulose filter (0.45 μm Hoefer Scientific, CA, USA) in protein transfer unit (Hoefer Scientific, CA, USA) according to [Towbin *et al.,* (1979)], the nitrocellulose filter was blocked using a blocking buffer composed of 0.2 gm non fat milk (Sigma) was dissolved in 10 ml Tris Buffer saline (TBS), pH 7.4 and incubated with the CK1 monoclonal antibody diluted in PBS-T20 with constant shaking overnight. The blots were washed three times (15 min/wash) in TBS followed by two hours incubation with anti-mouse IgG alkaline phosphatase conjugate diluted in TBS. The blots were then washed three times with TBS. The reaction was visualized by incubating the nitrocellulose filter and soaked in premixed BCIP/NBT substrate and stopped by distilled water after color development within 10 min.

Detection of CK1 antigen using ELISA :

After optimization of ELISA conditions, according to the method of [Basta *et al.*, (1988)] with some modifications, polystyrene microtitre plates (Costar, USA) were coated with 50μl/well of diluted serum samples 1:400 in coating buffer pH, 9.6, to be tested for a CK1 antigen in breast cancer patients. The plates were incubated overnight at 4° C. After blocking, 50 μL/well of 1:200 diluted CK1 monoclonal antibody in PBS-Tween 20 (PBS-T20) then incubated at 37° C for 2 hour was added. Then, 50 μL/well anti-mouse IgG alkaline phosphatase (Whole molecule, Sigma) conjugate was diluted 1:500 in PBS-T20 containing 0.2 % BSA was incubated at 37° C for 1 hour. The plate was washed with PBS + 0.5% Tween 20 for 3 times after every step. The amount of coupled conjugate was determined by incubation with the substrate (one mg of p-Nitrophenyl phosphate (Sigma) was dissolved in 1 ml of prepared substrate buffer (glycin buffer), pH 10.4). The reaction stopped by using 3 M Sodium hydroxide (NaOH) and the absorbance read at 490 nm using ELISA reader (Σ960, Axion, Germany). The cut-off level of CK1 antigen was calculated as the mean optical densities (at 490 nm) of 8 serum samples from healthy individuals + 3 standard deviation (i.e. O.D + 3 S.D). O.D above the cut-off value was considered positive, while those equal or below it was considered negative.

Biochemical characterization of the CK1 antigen reactive epitope :

In order to identify physicochemical nature of target serum CK1 antigen, the serum CK1 antigen was subjected to different physicochemical treatments e.g. heat, chemicals, and proteolysis. Then, the reactivity of the CK1 antigen reactive epitope retested using ELISA according to [Attallah *et al,* (1999)]. First, the CK1 antigen 50 μg/well, was incubated at different temperature (37, 56 and 70°C) for one hour. In other experiment, it was incubated for one hour at room temperature with either 0.2 M HCl or 0.2 M NaOH. To determine the effect of deproteinizing agent, 50 μg/well of the CK1 antigen was incubated with the same volume of 40 % TCA at room temperature for 30 minutes. The mixture was centrifuged at 10,000 rpm for 15 minutes and then, the precipitate was washed twice using diethyl ether to remove the excess of TCA and the excess diethyl ether was removed by drying at 37°C. The precipitate was reconstituted with PBS to the original volume. A periodate oxidation was carried out 18 hours with (5, 10, and 20 mM) sodium meta-periodate (Sigma) at room temperature and the reaction was then inhibited by adding 20 μl of Glycerol (Sigma). The CK1 antigen (50 μg/well) was mixed with equal volumes of (zero, 20, 60 and 180 mM) β-Mercaptoethanol diluted in PBS incubated for 1 hour at 37°C. In the test with chymotrypsin enzyme, the CK1 antigen (50 μg/well) was incubated at 37 °C with α-Chymotrypsin (one mg/ml, Sigma) for different times (15, 30 and 45 minutes). Bovine serum albumin was tested in parallel as a control.

RESULTS

Identification of CK1 antigen by sodium dodocyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and western blot:

Serum samples from women with breast cancer and healthy women, were analyzed by 12 % one dimensional (SDS-PAGE) under reducing conditions and staining with coomassie blue. The coomassie blue stained separated polypeptides have a wide range of molecular weights ranged from 205 kDa to 29 kDa Fig. (1). The separated proteins were electrophoretically transferred to nitrocellulose (NC) paper. CK1 monoclonal antibody was used as a primary antibody and anti-mouse IgG alkaline phosphatase was used as a conjugate (secondary antibody labeled enzyme). The BCIP/NBT system was used as enzyme substrate. An intense sharp band of cytokeratin antigen was observed in serum samples from breast cancer patients at 68 kDa but no reaction was observed in serum samples from healthy women Fig. (2).

Standardization of ELISA technique for detection of cytokeratin antigen in serum samples from breast cancer patients :

ELISA is the method most routinely used because of its efficacy, eases of use, its low cost and availability and is a biochemical technique used mainly in immunology to detect an antigen sample using antibody. The cut-off level of CK1 above or below at which the tested sample is considered positive or negative respectively, was calculated as the mean optical densities (at 490 nm) of serum samples from healthy individuals + 3 standard deviation (i.e. O.D + 3 S.D), using sera of 8 healthy individuals and 8 samples from breast cancer patients showing optical densities above the cutoff level, it was set at 0.26.
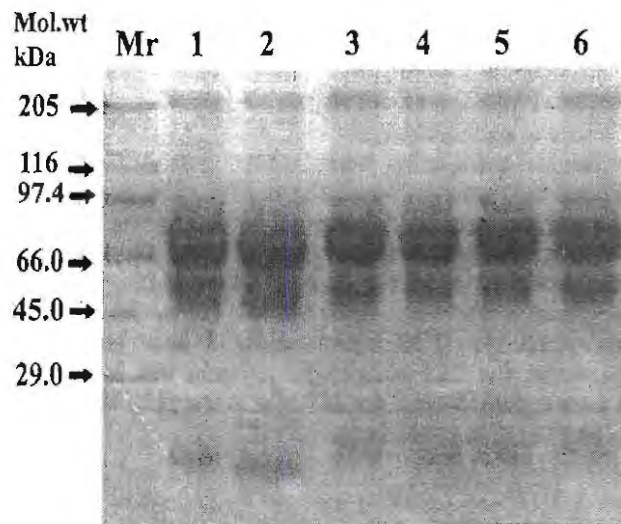

Figure (1). Coomassie blue stained SDS-PAGE showing the polypeptide pattern of serum samples from breast cancer patients and healthy women.

Serum samples from breast cancer patients at 20 µg/lane were loaded per well and electrophoresed under 200 volts for 45 minutes. **Lanes (1-3):** 3 serum samples from healthy women. **Lanes (4-6):** 3 serum samples from breast cancer patients. **Molecular weight marker (Mr.) includes:** Myosin (205.0 kDa), ß- galactosidase (116.0 kDa), phosphorylase B (97.4 kDa), BSA (Bovine serum albumin) (66.0 kDa), ovalbumin (45.0 kDa), and carbonic anhydrase (29.0 kDa).
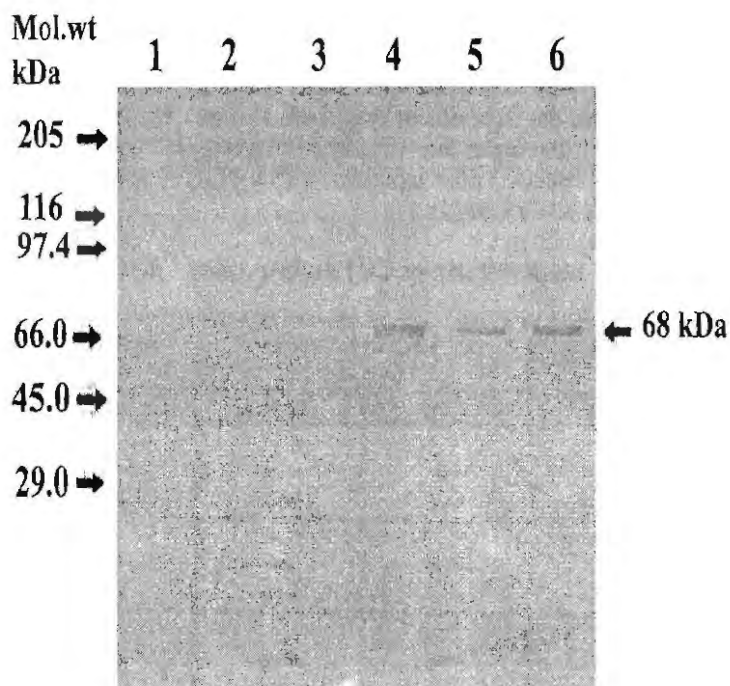
Figure (2). Immunoblots of serum samples from breast cancer patients and healthy women:

Serum samples from breast cancer patients at 20 µg/lane were resolved in 12 % SDS-PAGE and electroblotted on nitrocellulose. The CK1 monoclonal antibody identified a CK1 antigen in breast cancer patient samples at 68 kDa. No specific reaction was observed with serum samples from healthy women. Lanes (1-3): 3 serum samples from healthy women. Lanes (4-6): 3 serum samples from breast cancer patients. Molecular weight marker (Mr.) includes: Myosin (205.0 kDa), ß- galactosidase (116.0 kDa), phosphorylase B (97.4 kDa), BSA (Bovine serum albumin) (66.0 kDa), ovalbumin (45.0 kDa), and carbonic anhydrase (29.0 kDa).

Partial Characterization of serum CK1 antigen reactive epitope:

The partial characterization of the nature of the CK1 antigen recognized by CK1 monoclonal antibody was carried out by exposing the CK1 antigen to various physicochemical changes e.g. heat, chemicals, and α-chymotrypsin enzyme. Then the epitope reactivity against CK1 monoclonal antibody was tested using ELISA and BSA was used as a negative control. The results showed that the reactivity of the CK1 antigen was lost after exposure to 56°C and higher degrees of temperature. Also the reactivity of the CK1 antigen was lost after exposure to acid, alkali but it was maintained after periodate and after treatment with β–Mercaptoethanol concentrations (20 and 60 mM), while it was lost above concentration 60 mM β-Mercaptoethanol. CK1 antigen precipitated with TCA showed high reactivity against CK1 monoclonal antibody in contrast to the supernatant that show no reactivity. The CK1 antigen was treated with constant concentration of α-chymotrypsin enzyme, the reactivity was maintained at (15, 30 minutes) after treatment with α-chymotrypsin, while it was lost at 45 minutes after treatment Table (1).

Table (1). Partial characterization of CK1 antigen using ELISA*.

Treatment	68 kDa cytokeratin antigen		Bovine Serum Albumin (BSA)	
	Untreated	Treated	Untreated	Treated
Heat:				
a) 37 °C	+ve	+ve	-ve	-ve
b) 56 ° C and above	+ve	-ve	-ve	-ve
Acid and Alkali:				
a) 0.2 M HCl	+ve	-ve	-ve	-ve
b) 0.2 M NaOH	+ve	-ve	-ve	-ve
TCA treatment:				
a) Precipitate	+ve	+ve	-ve	-ve
b) Supernatant	-ve	-ve	-ve	-ve
Periodate oxidation	+ve	+ve	-ve	-ve
β-Mercaptoethanol				
a) Zero, 20, 60 mM	+ve	+ve	-ve	-ve
b) 180 mM	+ve	-ve	-ve	-ve
α-chymotrypsin:				
a) 15, 30 minutes	+ve	+ve	-ve	-ve
b) 45 minutes	+ve	-ve	-ve	-ve

*The cut-off level was 0.26 of ELISA. -Ve: Negative i.e OD value lower than the cut-off value (reactivity lost). +Ve: Positive i.e OD value above cut-off value (reactivity maintained).

DISCUSSION

Breast cancer is a complex disease that still imposes a significant health care burden on women worldwide [**Arciero** *et al.,* **(2003)**]. It is the most common cancer affecting women worldwide and the leading cause of cancer death globally in women [**Nielsen** *et al.,* **(2008)**]. In Egypt, breast cancer ranks number one among female malignancies [**Vieira** *et al.,* **(2008)**], and it is usually diagnosed at an advanced stage [**Boulos** *et al.,* **(2005)**]. Early diagnosis remains the best method of improving the odds of curing breast cancer [**Bénard and Turcotte, (2005)**], yet the diagnosis of breast cancer is hampered by the lack of an adequate detection method [Gast *et al.,* (2009)]. Serum tumor markers have several potential applications in clinical oncology. They include screening, diagnosis and prognosis [**Buccheri and Ferrigno, (2001)**]. CEA and CA 15-3 are the most thoroughly investigated serum tumor markers in breast cancer. It is generally agreed that tumor markers in breast cancer patients are not a tool for primary diagnosis, because of their low sensitivity and specificity [**Ebeling** *et al.,* **(2002)**]. Hence, new markers for early diagnosis, accurate prognosis and prediction of response to treatment are warranted to improve breast cancer care [Gast *et al.,* (2009)]. So that, the aim of the present study was identification and characterization of CK1 antigen associated with breast cancer. Cytokeratins, belonging to the intermediate filament protein family. At present, more than 20 different cytokeratins are known and are divided into types I and II. Cytokeratins 1–8 constitute the type II group (53–68 kDa, neutral to basic protein components), while cytokeratins 9–20 constitute the type I group (40–56 kDa, acidic proteins) [**Barak** *et al.,* **(2004)**]. Cytokeratins expression profiles are remarkably tissue-specific and are significantly different in a variety of cancer cells [**Bambang** *et al.,* **(2009)**]. Keratins have acquired great importance in tumor biology. They have been used effectively as markers for epithelial carcinomas, such as breast carcinomas. Keratin has been used as a differential marker in breast tumors [**Malati, (2007)**]. CK 8 was determined by its molecular weight (55 kDa) in breast cancer cells [**Hembrough and Gonias, (1996)**]. Also, CK19 was expressed and released by breast cancer cell lines [**Alix-Panabières** *et al.,* **(2009)**]. In this study, the presence of a CK1 antigen was investigated in serum samples obtained from breast cancer patients using SDS-PAGE **Fig. (2)** under reducing conditions and western blot technique based on the CK1 monoclonal antibody. A single immunoreactive band at 68 kDa was identified in selected serum samples from breast cancer patients using CK1 monoclonal antibody. Cytokeratin 1 was a single protein isolated at 68 kDa using SDS-PAGE and western blot [**Joseph** *et al.,* **(1999)**], and it is a member of the basic-neutral subfamily of cytokeratins. In vivo, basic-neutral cytokeratin 1 is coexpressed and paired with the acidic cytokeratin 10 to form a heterodimer. The amino and carboxy termini of cytokeratin 1 and 10 are glycine-rich, hydrophobic [**Shariat-Madar and Schmaier, (1999)**]. Cytokeratins represent important structural components of the epithelial cytoskeleton and thus constitute major protein markers for cellular differentiation [**Moll** *et al.,* **(1982)**]. In the present study **Fig. (2)**, the reactivity of 68 kDa CK1 antigen towards CK1 monoclonal antibody was lost at higher temperatures this indicating a conformational nature of the identified antigen which is protein moiety. As the same as, cytokeratin 19 fragment that is referred to as CYFRA 21-1, radioactivity bound of CYFRA 21-1 decreased by increasing the temperature from 4°C to room temperature

[Sarwar *et al.,* (1994)]. Also, this study showed that Table (1), 68 kDa CK1 antigen was sensitive to acid and alkali, the epitope reactivity of 68 kDa CK1 antigen treated with acid or alkali was lost towards the monoclonal antibody. However, the reactivity towards 68 kDa CK1 antigen was maintained when the antigen was treated with oxidizing agents like sodium m-periodate which indicate that the antigen does not contains carbohydrates. The 68 kDa antigen was precipitated by 40% TCA, and the precipitated fraction showed high reactivity as the untreated antigen did. On the other hand, the soluble fraction did not show any reactivity with 68 kDa CK1 antigen, indicating the protein nature of the identified antigen epitope. Chymotrypsin is a proteolytic enzyme selective for peptide bond with aromatic or large hydrophobic side chain (tyrosine, tryptophan, phenylalanine, leucine, and methionine) on the carboxyl side of this bond [Kossiakoff and Spencer, (1980)]. In the present study, the 68 kDa CK1 antigen was treated with constant concentration of α-chymotrypsin enzyme. By increasing the incubation time with α-chymotrypsin, the reactivity was decreased and was completely lost at 45 min incubation. This suggests that CK1 antigen have a number of acidic groups. 2-mercaptoethanol is the most commonly used reducing reagent to break disulfide bonds [Katoh *et al,* (2004)], and keratins have a large amount of the sulphur-rich amino acid, cysteine [(Bulaj, (2005)]. In this study, the reactivity of 68 kDa CK1 antigen towards CK1 monoclonal antibody was lost at higher concentrations of reducing agents such as mercaptoethanol, it was lost above concentration 60 mM β-Mercaptoethanol.

In conclusion: 68 kDa CK1 antigen was identified and characterized as a protein, and 68 kDa cytokeratin antigen may be helpful in diagnosis of breast cancer.

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تعيين السيتوكراتين ١ في مرضى مصابين بسرطان الثدى باستخدام تقنيات الكيمياء الحيوية

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يعتبر سرطان الثدي من اخطر الأمراض التي تصيب النساء في العــالم والــسبب الرئيــسي للوفــاة بالسرطان بين النساء على مستوى العالم و في مصر يعتبر سرطان الثدي من أكثــر الأمــراض انتــشارا بــين السيدات. يعتبر سرطان الثدي مرض معقد و مركب حيث يصاحبه مشاكل صحية و نفــسية عديــدة للــسيدات. عوامل خطر الاصابة بسرطان الثدي عديدة وتشمل التقدم في العمر والتعــرض للاشــعاع والتــاريخ العــائلي لسرطان الثدي. ولذلك فأن الكشف المبكر لهذا النوع من الأورام ضروري وهام للتغلب على انتــشار المــرض حيث ان التشخيص المبكر والدقيق لسرطان الثدي هو مفتاح العلاج الفعال والنــاجح. دلالات الأورام يمكــن أن تكون مفيدة في الكشف عن السرطان والكشف عن تكرار المرض بعد العلاج. ومع ذلك، قلة الحساسية للمــرض في مرحلة مبكرة إلى جانب قلة الخصوصية يحول دون استخدام جميع الدلالات الموجــودة للتــشخيص المبكــر لسرطان الثدي. وبالتالي، هناك حاجة لدلالات جديدة قادرة على الكشف عن سرطان الثدي في مرحلة مبكرة قبل انتشار الخلايا السرطانية. ينتمي السيتوكراتين إلى عائلة بروتينات الخيط الوسطي. هناك ٢٠ نوع مــن عديــدة البيبتيد التي تنقسم الــى نــوع ١ الحمــضية (CK9 – CK20) والنــوع ٢ القاعديــة مــن (CK8 – CK1) سيتوكيراتين. وهي تتراوح في الوزن الجزيئي بين ٤٠ كيلو دالتون و٦٨ كيلــو دالتــون. القيمة التشخيــصية للتعرف على السيتوكراتين تكمن في التشخيص المبكر والسريع في تعيين مدى كفاءة الاستجابة للعــلاج فــي سرطانات الخلية الطلائية و على الرغم من إن الهدف الرئيسي هو ضبط عملية العلاج و تقييم مدى الاســتجابة للعلاج و مدى انتشار الورم بالجسم إلا انه يساعد في الكشف عن أنواع مختلفة من سرطانات الثدي. ولذلك كان الهدف من الدراسة الحالية التعرف علي السيتوكراتين في السيرم لسيدات عندهم سرطان الثدى باستخدام تقنيــات كيمياء حيوية حديثة ومنها طريقة الجل الالكتروفيروسي وطريقة الشغط المناعي والاليزا. ولقد قمنــا فــي هــذه الدراسه بتجميع ٧٥ عينه سيرم لاشخاص مصابين بسرطان الثدي بالاضافه الي ٢٧ عينــة ســيرم لاشــخاص اصحاء استخدمت كمجموعه ضابطه. وقد تم التعرف علي سيتوكيراتين ١ انتيجين باستخدام تقنية الشغط المناعي الغربي و تقنية الادمصاص المناعى الانزيمى. تم فصل بروتينات الأمصال للمرضي المصابين بسرطان الثــدي باستخدام تقنية الفصل الألكتروفورريسي على جل عديد الأكريلاميد (SDS-PAGE) ثم التعرف علــي هــذه البروتينات. تم التعرف علي أنتيجين سيتوكيراتين ١ في عينات هذه المرضي باستخدام تقنية الــشغط المنــاعى الغربي (western blot) كما تم تعيين الوزن الجزيئي لأنتيجين سيتوكيراتين ١عند ٦٨ كيلودالتون في العينــات المصابه وعدم ظهور اي الحزم المفصوله bands عند هذا الوزن في العينات الغير مصابه.

كما ثبت ان السيتوكيراتين ١ له طبيعة بروتينية حيث انه لا يفقد فاعليته الا بالتسخين لمدة ساعة عند ٥٦ درجة منوية أو أكثر ويفقد نشاطه عند معاملته بهيدروكسيد الصوديوم و حمض الهيــدروكلوريك وقــد وجــد أن هــذا الانتيجين يترسب عند معاملته بثلاثى كلور حمض الخليك "40% TCA' وعند أعادة ذوبان الراسب لــوحظ أنــه يتفاعل بدرجة عالية مع الجسم المضاد الخاص بالسيتوكيراتين ١ أنتيجين. اما مع الرشيح فلــم يكــن هنــاك أي

تفاعل وذلك عند قياس درجة تفاعله مع الجسم المضاد الخاص به بطريقة الأمصاص المناعي "ELISA". وأنـه ايضا لا يتأثر بالعوامل المؤكسدة كالبيرايودات الصوديوم. ومع ذلك فقد تأثر بالتركيزات العالية مـن العوامـل المختزلة مثل"Mercaptoethanol –β". كما انه يفقد فاعليته بأستخدام الفاكيموتريبسين.

وللخلاصة أنه تم فصل و توصيف أنتيجين سيتوكيراتين ١ عينات امصال المرضى المصابين بـسرطان الثـدي والذي يمكن أن يكون له أهمية تطبيقية في مجال تشخيص الإصابة بسرطان الثدي.