Abnormal Antigens in Breast Cancer Tissues and Production of Monoclonal Antibodies Against one of these Antigens

By

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To my father, mother, wife, children, brothers and sisters, with appreciation to their endless support.
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**List of abbreviations**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>2D PAGE</td>
<td>Two Dimensional Poly Acrylamide Gel Electrophoresis</td>
</tr>
<tr>
<td>2DE</td>
<td>Two Dimensional Electrophoresis</td>
</tr>
<tr>
<td>4GlcNAc</td>
<td>Glucose Neuroaminic acid</td>
</tr>
<tr>
<td>AACR</td>
<td>American Association for Cancer Research</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
</tr>
<tr>
<td>CEA</td>
<td>Carcino Embryonic Antigen</td>
</tr>
<tr>
<td>CLDN</td>
<td>Claudin</td>
</tr>
<tr>
<td>Da</td>
<td>Dalton</td>
</tr>
<tr>
<td>DA 1</td>
<td>Distal Arthrogryposis type 1</td>
</tr>
<tr>
<td>DMSO</td>
<td>Di Methyl Sulpho Oxide</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme Linked Immuno Sorbent Assay</td>
</tr>
<tr>
<td>FCA</td>
<td>Freund’s Complete Adjuvant</td>
</tr>
<tr>
<td>FIA</td>
<td>Freund’s Incomplete Adjuvant</td>
</tr>
<tr>
<td>FNAC</td>
<td>Fine Needle Aspiration Cytology</td>
</tr>
<tr>
<td>HAT</td>
<td>Hypoxanthine Aminopterin Thymidine</td>
</tr>
<tr>
<td>HER-2</td>
<td>Human Epidermal growth factor Receptor 2</td>
</tr>
<tr>
<td>HIV</td>
<td>Human Immunodeficiency Virus</td>
</tr>
<tr>
<td>HMG</td>
<td>High Mobility Group Protein</td>
</tr>
<tr>
<td>HMGA1</td>
<td>High Mobility Group AT-hook 1</td>
</tr>
<tr>
<td>IUA</td>
<td>International University of Africa</td>
</tr>
<tr>
<td>LacNAc</td>
<td>Lactose Neuroaminic Acid</td>
</tr>
<tr>
<td>LC/MS</td>
<td>Liquid Chromatography/ Mass Spectroscopy</td>
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<tr>
<td>mAb</td>
<td>monoclonal Antibodies</td>
</tr>
<tr>
<td>MMTV</td>
<td>Mouse Mammary Tumor Virus</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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</tr>
<tr>
<td>NADPH</td>
<td>Nicotine amide Adenine Dinucleotide Phosphate Hydrogen</td>
</tr>
<tr>
<td>NEM4</td>
<td>Nemapaline Myopathy type 4</td>
</tr>
<tr>
<td>OFA</td>
<td>Oncofetal Antigen</td>
</tr>
<tr>
<td>PDB</td>
<td>Protein Database Bank</td>
</tr>
<tr>
<td>PEG</td>
<td>Poly Ethylene Glycol</td>
</tr>
<tr>
<td>pI</td>
<td>Isoelectric Point</td>
</tr>
<tr>
<td>rcTPM3</td>
<td>retro copy of Tropomyosin 3</td>
</tr>
<tr>
<td>RPMI</td>
<td>Roswell Park Memorial Institute medium</td>
</tr>
<tr>
<td>SAEC</td>
<td>Sudan Atomic Energy Commission</td>
</tr>
<tr>
<td>SPSS</td>
<td>Statistical Package for Social Sciences</td>
</tr>
<tr>
<td>SSE</td>
<td>Secondary Structure Elements</td>
</tr>
<tr>
<td>SWISSPROT</td>
<td>Switzerland Protein database</td>
</tr>
<tr>
<td>TNM</td>
<td>Tumor, Node, Metastasis cancer staging system</td>
</tr>
<tr>
<td>UDP</td>
<td>Uridine Di Phosphate</td>
</tr>
<tr>
<td>UNIPROT</td>
<td>United Europe Protein Database</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular Endothelial Growth Factor</td>
</tr>
<tr>
<td>Amino acids abbreviations</td>
<td>Description</td>
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<tr>
<td>---------------------------------------------------</td>
<td>-------------------</td>
</tr>
<tr>
<td>A, Ala</td>
<td>Alanine</td>
</tr>
<tr>
<td>C, Cys</td>
<td>Cysteine</td>
</tr>
<tr>
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<td>Aspartate</td>
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<td>Phenylalanine</td>
</tr>
<tr>
<td>G, Gly</td>
<td>Glycine</td>
</tr>
<tr>
<td>H, His</td>
<td>Histidine</td>
</tr>
<tr>
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<tr>
<td>K, Lys</td>
<td>Lysine</td>
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<td>L, Leu</td>
<td>Leucine</td>
</tr>
<tr>
<td>M, Met</td>
<td>Methionine</td>
</tr>
<tr>
<td>N, Asn</td>
<td>Asparagine</td>
</tr>
<tr>
<td>P, Pro</td>
<td>Proline</td>
</tr>
<tr>
<td>Q, Gln</td>
<td>Glutamine</td>
</tr>
<tr>
<td>R, Arg</td>
<td>Arginine</td>
</tr>
<tr>
<td>S, Ser</td>
<td>Serine</td>
</tr>
<tr>
<td>T, Thre</td>
<td>Threonine</td>
</tr>
<tr>
<td>V, Val</td>
<td>Valine</td>
</tr>
<tr>
<td>W, Trp</td>
<td>Tryptophan</td>
</tr>
<tr>
<td>Y, Tyr</td>
<td>Tyrosine</td>
</tr>
<tr>
<td>X</td>
<td>Any unknown amino acid</td>
</tr>
</tbody>
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ABSTRACT

Background
Breast cancer is associated with up regulation, down regulation of normal antigens or abnormal antigens. These antigens are very useful candidates as targets for the different breast cancer therapies and for vaccination trials.

Objectives
This study was done to characterize abnormal antigens, extract one of them and to produce monoclonal antibodies against the extracted antigen.

Methods
One hundred and twenty Sudanese female patients were included in this study after informed consent. The mean age was 47.2 years (16-80). Two tissue samples were obtained from each patient and they were confirmed as normal and cancerous breast tissues microscopically. 2D PAGE was used to analyze the protein content of samples. LC/MS and nr.fasta database search were used for separation and identification of the abnormal proteins.

Results
Three different patterns of 2D PAGE results were obtained, the first pattern involved detection of four abnormal proteins in 26.7% of the patient cancerous tissues while they were undetected in the normal tissues of the same patients. In the second 2D PAGE result pattern the cancerous and the normal tissues of 67.5% patients were identical and they did not contain the four abnormal proteins while the third 2D PAGE pattern involved the presence of two abnormal antigens (from the four) in the cancerous tissues of 5.8% of the patients and they were absent from the normal tissues of the same patients. The four abnormal proteins were identified as, human Thioredoxin (D60n...
mutant), X-ray crystal structure of human galectin-1, retrocopy of tropomyosin 3(rcTPM3) and beta-tropomyosin (isoform 2). The primary and the secondary structures were obtained from the SWISSPROT and the PDB databases. Beta-tropomyosin spot was extracted and used as antigen for monoclonal antibody production. Monoclonal antibody against beta-tropomyosin with a concentration of 0.35mg/ml and a G11 anti beta-tropomyosin hybridoma cell line were produced. The monoclonal antibody was with single band and approximately 30 KDa molecular weight. However, some tests were done to determine the specificity of the produced monoclonal antibody.

**Conclusions**

In conclusion, breast cancer tissues are associated with abnormal antigens, but with low incidence rate; this strongly favors the individualization of breast cancer diagnosis and treatment.
المقدمة

يتميز سرطان الثدي بزيادة في نقصان بعض البروتينات الطبيعية ووجود بروتينات محورة. هذه البروتينات المحورة يمكن استخدامها كمستقبلات للانواع المختلفة لادوية سرطان الثدي كما انها يمكن أن تستخدم لانتاج مصل لسرطان الثدي.

الأهداف

اجريت هذه الدراسة لعرض اكتشاف بروتينات محورة في انسجة ثدي سرطانية ومعرفة تركيبها واستخلاص اقدام هذه البروتينات وانتاج أجسام مضادة ضد احد هضمه البروتينات المحورة.

طرق البحث

شملت هذه الدراسة عشرون مريضة بسرطان الثدي. كان متوسط العمر 47.2 سنة (16-80).

آخذت عينات من كل مريضة عينة من النسيج السرطاني واخرى من السليم وقد اكدت هذه العينة بالصفات العامة والمجهرية. حللت العينات لاستكشاف محتواها البروتيني باستخدام تقنية الرحلان الكهربائي ثنائية الاتجاه و لمعرفة البروتينات المحورة وخصائصها استخدمت تقنيات الفصل اللوني السائلة والمطيافية الضوئي، ثم البحث في موسوعة البروتينات العالمية.

النتائج

حصل على ثلاثة انواع من نتائج تقنية الرحلان الكهربائي ثنائية الاتجاه في النوع الأول احتوت الاسمية السرطانية على البروتينات محورة و كانت نسبة المرضى في هذا النوع 26.7%، فيما يخص النوع الثاني لم تحتوي الاسمية السرطانية على ما من البروتينات المحورة الأربعة و كانت نسبة المرضى الذين حملوا هذا النوع 67.5% و في النوع الثالث احتوت الاسمية السرطانية على بروتينين من الاربعة بروتينات المحورة و كانت نسبة المرضى الذين يتميزون بهذه الصورة 5.8%. تم التعرف على البروتينات المحورة الأربعة وهي الفيازويكسيب البشري (بروتين المحور 60ن)، التركيب البلوري السيني للفرقلاكتين-1 البشري، الصوره الراجعة للتروبوامابوزين 3 والبيتا تروبوامابوزين (النوع الثاني). استخلاص بروتين البيتا تروبوامابوزين (النوع الثاني) واستخدم في انتاج اجسام مضادة احادية المنشأ. انتجت الأجسام المضادة احادية المنشأ والخلايا الهجينة المنتجة لها. وجد ان الأجسام المضادة
لها حزمة رحلان كهربائي واحدة كما ان وزنها الجزئي يعادل تقريبا 30 كيلو دالتون. اجريت بعض الاختبارات لتحديد مدى حساسية الأجسام المضادة المنتجة.

الملخص

خلصت هذه الدراسة الى ان وجود بروتينات محورة في عينات نسج الثدي السرطانية بنسبة تردد قليلا يقوى الاتجاه نحو معالجة كل مريض بسرطان الثدي على حدة اعتمادا على نوع البروتينات المحورة التي يتميز بها كل مريض.
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Table.6 Second, after cloning, ELISA assay test results (Absorbance at 405nm).
CHAPTER ONE

Introduction

Breast cancer, abnormal antigens and monoclonal antibodies
CHAPTER ONE

Introduction

1.1-Epidemiology of breast cancer:

Breast cancer is the most common cancer among women in Africa accounting for about 20% of all cancer incidences [1]. Breast cancer has the second highest associated mortality world wide [2].

In Sudan, cancer is third killer disease after malaria and viral pneumonia [3]. An epidemiological study in East Africa region including Sudan discovered that, the breast cancer is in leading position amongst all female cancers but with a lower proportion of women below 30 years [4]. However the incidence and prevalence of cancer were determined in Sudan due to absence of cancer registry program.

1.2 Breast cancer risk factors

Many cases of breast cancer occur in women with no obvious risk factors. This means that women need to be alert in watching for possible changes in their breasts, performing self examinations, and scheduling clinical breast examinations and mammograms. It is likely that risk factors influence the development of breast cancer. Some of these include:

Age:

The risk of developing breast cancer increases as a woman ages, with the majority of breast cancer increases in women over the age 50. At the age 30, a woman's risk of developing breast cancer is one in 2525. By the age 50, her chances are one in 50, rising to one in 14 by the age 70 [5].
**Race:**

Although white women are more likely to develop breast cancer, black women are more likely to die from the disease. The reasons for this are unclear and probably involve both socioeconomic and biologic factors [6].

**A family history of breast cancer:**

Women who have a first degree relative (mother, sister, daughter) diagnosed with breast cancer are at increased risk of the disease. More than one first degree relative with breast cancer elevates that risk [7].

**Previous history of breast cancer:**

Women who have had breast cancer in one breast have three to four times the risk of breast cancer in their opposite breast [8].

**A genetic predisposition:**

Mutations to the BRCA1 or BRCA2 genes are associated with increased breast cancer risk. Screening tests are available to test for known mutations to these genes, but are recommended for every one and only following appropriate genetic counseling. Researchers estimate that breast cancers caused by these genes make up only 2% to 3% of all breast cancers.

BRCA1 is a gene located on chromosome 17 while BRCA2 is located on chromosome 13. Until recently, it was not clear what the function of these genes, but a related protein in yeast revealed their normal role: they participate in repairing radiation induced breaks in the double stranded DNA. It is thought that mutations in BRCA1 or BRCA2 might disable this mechanism, leading to more errors in DNA replication and ultimately to cancerous growth [9].
Estrogen exposure:

Estrogen is a hormone in women that controls the development of secondary sex characteristics (such as breast development). A woman's production of estrogen decreases at menopause. Doctors think that exposure to estrogen for a long time may increase breast cancer risk.

- A long menstrual history: woman who began menstruating before age 12 or went through menopause after 55 have a higher risk of breast cancer because their breast cells have been exposed to estrogen for longer periods of time.
- Late or no pregnancies: Women who have their first pregnancy after age 30 or who have never had a full – term pregnancy have risk of breast cancer. Pregnancy may protect against breast cancer because it pushes breast cells into their final phase of maturation.
- Estrogen in medication: Recent use of oral contraceptives or hormone replacement therapy (HRT), or long term use of (HRT), may increase a woman risk of breast cancer [10].

X-Ray and breast cancer:

It is clear that excessive X ray radiation increases the risk of breast cancer in women treated therapeutically with X-rays for acute and chronic breast conditions and in women with pulmonary tuberculosis whose artificial pneumothorax treatments were monitored by fluoroscopic chest X-ray [11].

A typical hyperplasia:

This is a condition characterized by abnormal, but not cancerous cells. A typical hyperplasia is a risk factor of breast cancer [12].
Life style factors:

As other types of cancer, studies continue to show that various habits may contribute to the development of breast cancer. These include:

- Obesity: According to a new study, being obese or even overweight increases a woman's risk of breast cancer.
- Lack of exercise: Exercise lowers hormone levels and boosts the immune system; lack of exercise contribute to obesity.
- Alcohol use: Drinking more than one alcoholic drink per day may raise the risk of breast cancer [10].

Radiation:

High doses of radiation may increase a woman's risk of breast cancer. An increased risk of breast cancer has been observed in long term survivors of atomic bombs and patients with lymphoma treated with radiation therapy to the chest [13].

1.3 Diagnosis and types of breast cancer:

Breast cancer is diagnosed clinically and confirmed by imaging, cytology and histopathology [14].

Bloom Richardson grading system is the most current cancer grading system used for breast cancer. It uses the degree of tubule formation, the mitotic index and the nuclear pleomorphism of tumor cells to grade the breast cancer [15]. Each of the three features is assigned a score ranging from 1 to 3. The scores are then added together to obtain score range from 3 to 9. The result of this scoring system is three grades, 3-5 grade I tumor and designed well-differentiated, 6-7 grade II tumor which is moderately- differentiated and 8-9 grade III tumor and this is the poorly- differentiated [15].
The famous system for breast cancer staging is the TNM system [16]. This system uses three criteria to judge the stage of the cancer: the tumor itself (T), the lymph nodes around the tumor (N), and if the tumor has spread to the rest of the body (M). There are five stages from stage 0 to stage IV. The stage provides a common way of describing the cancer. [16]

*Stage 0:* Ductal carcinoma in situ is cancer that has not spread past the ducts or lobules of the breast. It is called non-invasive cancer.

*Stage 1:* The tumor is small and has not spread to the lymph nodes (T1, N0, M0).

*Stage IIa:* Any one of these conditions:

- The tumor is less than 2cm and has spread to the axillary lymph nodes (T1, N1, M0).
- The tumor is between 2 and 5 cm but has not spread to the axillary lymph nodes (T2, N0, M0).
- There is no evidence of the tumor in the breast, but there is cancer in the axillary lymph nodes (T0, N1, M0).

*Stage IIb:* Any one of these conditions:

- The tumor is between 2 and 5 cm and has spread to the axillary lymph nodes (T2, N, M0).
- The tumor is larger than 5 cm but has not spread to the axillary lymph nodes (T3, N0, M0).

*Stage IIIa:* Any of these conditions:
• The tumor is smaller than 5cm and has spread to axillary lymph nodes that are attached to each other or other structures (T2, N2, M0).

• The tumor is larger than 5cm and has spread to the axillary lymph nodes, which may or not be attached to each other or to other structures (T3, N1 or N2, M0).

**Stage IIIb:** The tumor has spread to the chest wall or caused swelling or ulceration of the breast or is diagnosed as inflammatory breast cancer. It may or not have spread to the lymph nodes under the arm, but has not spread to other part of the body (T4, N0, N1, N2, M0).

**Stage IIIc:** A tumor of any size has not spread to distant parts of the body, but has spread to the lymph nodes above the clavicle, or both the nodes inside the breast or under the arm (any T, N3, M0).

**Stage IV:** The tumor can be any size and has spread to distant sites in the body, usually the bones, lung, liver, or brain (any T, any N, M1)[16].

1.4 Treatment of breast cancer

Even though the doctor will tailor the treatment for breast cancer to patient's disease and personal situation, there are some general steps in the logic of treating the disease. Primarily, the initial therapy for early stage disease is aimed at eliminating any visible tumor. Therefore doctors will recommend surgery to remove the tumor with or without radiation therapy.

The next step in the management of early disease is to reduce the risk of the disease recurring and to eliminate any cancer cells that may remain. If a tumor is of a certain size or lymph nodes are involved, the doctor may recommend additional therapy,
chemotherapy or hormonal therapy. If the cancer recurs, the patient may choose additional surgery, depending on where the cancer is found, or a variety of treatments designed to fight distant metastases.

When planning the treatment for a patient's breast cancer, the doctor will consider many factors, including:

- The stage and grade of the tumor.
- The tumor's hormone status (ER, PR).
- The patient's age and general health.
- The patient's menopausal status.
- The presence of known mutations to breast cancer genes.
- Factors that may signify aggressive tumor, such as HER-2/neu amplifications [17].

**Surgery:**

Generally, the smaller the tumor, the more surgical options the patient has. The general types of surgery include the following:

- A lumpectomy remove the tumor and small disease free margin of tissue around the tumor. Follow up radiation therapy is given to the disease site.

- A partial mastectomy removes the tumor, an area of normal tissue, and part of the lining over the chest muscle where the tumor was. This surgery is similar to a lumpectomy. It is also called a segmental mastectomy and requires follow up radiation.

- A modified radical mastectomy removes the breast, some of the underarm lymph nodes, and the lining over the chest muscles.
For invasive breast cancer, the combination of lumpectomy or partial mastectomy, underarm lymph node removal, and radiation therapy has been proven in clinical trials to be as effective as a modified radical mastectomy in treating small tumors.

- A total mastectomy removes the entire breast, leaving the underarm lymph nodes intact. This surgery is also called simple mastectomy.

- A radical mastectomy removes the breast, chest muscles, all lymph nodes under the arm, and additional fat and skin. This surgery is also called the Halsted radical mastectomy, named after the physician who devised it. It used to be the standard surgical treatment for breast cancer, but it is an uncommon procedure today.

Women are encouraged to talk with their doctors about which surgical procedure is right for them. More aggressive surgery is not always better and may result in additional complications.

Women who undergo a total mastectomy may wish to consider breast reconstruction, which is surgery to rebuild the breast. Reconstruction may be done with tissue from another part of the body, or with synthetic implants. The woman may choose to have this done at the time of mastectomy, or at some point in the future.

Because both lumpectomies and partial mastectomies leave a good portion of the breast intact, it is important that the surgeon make sure that none of the cancer has spread. Therefore, the surgeon, also has to evaluate the lymph nodes under the arm to make sure there is no sign of cancer cells. The surgeon can perform one of two procedures:

- Axillary lymph node dissection, in which the surgeon has to remove a number of lymph nodes from under the arm and have them examined by a pathologist for cancer cells.
• Sentinel lymph nodes sampling is a newer procedure in which the surgeon finds and remove the sentinel (first) lymph node that receive drainage from the breast and examine it for cancer cells. If it is cancer free, research has shown that there is a good possibility that the subsequent nodes are free of cancer. To identify the sentinel lymph node, the surgeon injects either a dye or a radioactive tracer into the area around the person's primary breast tumor. The dye or tracer will travel to the lymph nodes, arriving at the sentinel node first. The surgeon can find the node when it turns color (if the dye is used) or emits radiation (if the tracer is used) [18].

**Radiation therapy:**

Radiation is given regularly for a number of weeks following a lumpectomy or partial mastectomy in order to eliminate remaining cancer cells near the tumor site. Radiation therapy is also recommended for many women after a mastectomy depending upon the size of their tumor, number of involved lymph nodes under the arm, and width of the margin of resection obtained by the surgeon. Some times radiation is given before the surgery to shrink a large tumour and make it easier to remove.

Radiation therapy is very effective in reducing the chance of breast returning in both the breast and the chest wall. The most common type of radiation used in breast cancer is given from a source outside the body and is called external beam radiation therapy. Radiation may also be given by pellets implanted in the affected area (brachytherapy).

Radiation therapy can cause side effects, including fatigue, swelling, and skin changes. Most women report a good or excellent long term cosmetic result of their
treated breast. A small amount of lung beneath the chest can be treated by the radiation, and the risk of pneumonitis, or a radiation related pneumonia, is very rare.

In the past with older equipment and techniques of radiation, women treated for left sided breast cancers had small increase in the long term risk of heart disease. Modern techniques are now able to spare most of the heart from the radiation, so current studies generally do not show higher rates of dying from heart disease in women with breast cancer treated with radiation compared with women treated without radiation. While radiation is thought to be a risk factor of cancer after long periods of time, less than one in 500 survivors will develop a different type of cancer, other than a breast cancer, within the area that was treated [19].

**Chemotherapy:**

Chemotherapy destroys cancer cells that have migrated from the original site of the tumor. It may be given orally or intravenously, and is usually given in cycles. Chemotherapy generally does not require a hospital stay; women are usually treated in an outpatient setting.

An oncologist may administer chemotherapy before surgery (neoadjuvant therapy) to shrink a large tumor, or after surgery (Adjuvant therapy). The goal is to remove the entire tumor during surgery.

Different chemotherapy drugs are useful for different cancers, and research has shown that combinations of certain drugs are more effective than individual drugs. Some common regimens for breast cancer include combinations of two or three of the following chemotherapy drugs: cyclophosphamide, methotrexate, 5-fluorouracil,
doxorubicin, epirubicin, paclitaxel, and docetaxel. Women in clinical trials may be offered new drugs or new combinations of existing drugs.

Chemotherapy drugs are powerful and affect both healthy and cancerous cells in the body. Normal cells that grow quickly, such as those lining the gastrointestinal tract or hair follicles, may be damaged or killed along with cancer cells. Side effects can include fatigue, nausea, and vomiting, lowered white blood cell count, and a corresponding increased risk of infection, mouth sores, hair loss, and premature menopause. Most of these side effects go away once treatment is stopped and are not long term [20].

**Hormonal therapy:**

Hormonal therapy is useful to manage tumors that test positive for either estrogen or progesterone receptors. These tumors use hormones to fuel their growth. Blocking the hormones receptor limit the growth of these types of tumors.

Tamoxifen is a drug that researchers have studied. It has been shown effective in reducing the risk of recurrence in the treated breast, the risk of developing cancer in the opposite breast, and the risk of developing cancer in women with no history of the disease but who are at higher than average risk of developing breast cancer. Tamoxifen's side effects include hot flashes, a small increased risk of uterine cancer, and an increase in the risk of blood clots.

Newer drugs, such as raloxifene, are showing promise as hormonal agents. They may prove to be as effective as tamoxifen without side effects, but still need to be compared in clinical trials.

Aromatase inhibitors are emerging as the preferred treatment for women with advanced, hormone sensitive cancers. They work by blocking the aromatase enzyme,
which is necessary for the production of estrogen. They are also being studied as an adjuvant therapy in early breast cancer [21].

**Adjuvant therapy:**

Adjuvant means “in addition to” and these therapies are given in addition to surgery or surgery plus radiation to decrease the risk of the breast cancer returning. Adjuvant therapies include radiation therapy, chemotherapy, and hormonal therapies. They are intended to eliminate any breast cancer cells lingering in the body. If it is determined that the tumor uses estrogen or progesterone to grow (hormone receptor positive), then hormonal treatment may be used alone or together with chemotherapy [18].

**Immunotherapy:**

Cancer treatment is enhanced first by understanding, and then utilizing the immune system activity. Cancer may develop when the immune system breaks down or is not functioning.

Treatments such as interferon and colony stimulating factors are used now, either alone, or in conjunction with other modalities such as surgery, radiation and chemotherapy [22].

Immunotherapy may be used to:

- Stop, control, or suppress processes that permit cancer growth.
- Make cancer cells more recognizable, and therefore more susceptible to destruction by the immune system.
- Boost the killing power of the immune system cells, such as T-cells, NK – cells and macrophages.
• Alter cancer cells’ growth patterns to promote behavior like that of healthy cells.
• Block or reverse the processes that changes a normal cell or a precancerous cell into a cancerous cell.
• Prevent breast cancer cells from spreading to other parts of the body [23].

1.5 Breast cancer antigens:
Breast cancer tissues are characterized by over expression of normal antigens, down regulated antigens and presence of new antigens which are not found in normal tissues.

Over expressed antigens:
Some breast cancer tissues are characterized by over expression of specific antigens like estrogen receptor, progesterone receptors, Her2/new [24], HMGA1 (High Mobility Group AT-hook 1) antigen [25], VEGF (Vascular Endothelial Growth Factor) [26].

Estrogen Receptor:
Estrogen receptor refers to a group of receptors that are activated by the estrogen hormone [27]. Two types of estrogen receptor exist: ER which is a member of the nuclear hormone family of intracellular receptors and the estrogen G protein coupled receptor GPR30 (GPER), which is a G-protein coupled receptor. The estrogen receptor that is involved in the breast cancer is the ER type [27]. The main function of the estrogen receptor is as a DNA binding transcription factor that regulates gene expression. However, the estrogen receptor has additional functions independent of DNA binding [28].

Estrogen receptors are over-expressed in around 70% of breast cancer cases, referred to as ER positive. Two hypotheses have been proposed to explain why this causes tumorigenesis, and the available evidence suggests that both mechanisms contribute:
Firstly, binding of estrogen to the ER stimulates proliferation of mammary cells, with the resulting increase in cell division and DNA replication leading to mutations. Secondly, estrogen metabolism produces genotoxic waste. The result of both processes is disruption of cell cycle, apoptosis and DNA repair and therefore tumor formation [29].

There are two types of ER, ERα is certainly associated with more differentiated tumors, while evidence that ERβ is involved is controversial. Different versions of the ESR1 gene have been identified (with single-nucleotide polymorphisms) and are associated with different risks of developing breast cancer [30].

**Progesterone Receptors:**

The progesterone receptor (PR) also known as NR3C3 (nuclear receptor subfamily 3, group C, member 3), is an intracellular steroid receptor that specifically binds progesterone [31]. It has two main forms, A and B, that differ in their molecular weight and mode of action. PR A antagonize the action of estrogen by inhibiting the epithelial cells proliferation, while the PR B contributes to epithelial cells proliferation in presence or absence of estrogen [32]. It is well known that progesterone receptors are elevated in most of ER+ breast cancer patients [33].

**HER-2/neu:**

HER2/neu (also known as ErbB-2, ERBB2) stands for "Human Epidermal growth factor Receptor 2" [34]. HER2 is a cell membrane surface-bound receptor tyrosine kinase and is normally involved in the signal transduction pathways leading to cell growth and differentiation [35]. HER-2/neu is found in abundance in about 30% of breast cancers and is most likely directly related to uncontrolled cancer growth. It has been studied as an immune target in breast cancer [36].
**High Mobility Group Protein (HMG):**

HMG is a protein encoded by High mobility group AT-hook 1 (HMGA1) gene [37]. It is a non-histone protein involved in many cellular processes, including regulation of inducible gene transcription, integration of retroviruses into chromosomes, and the metastatic progression of cancer cells including breast cancer cells [38-39].

**Down regulated antigens:**

**Claudins (CLDN)**

Claudins are a family of proteins that are the most important components of the tight junctions [40]. Claudins are 24 small transmembrane proteins. They are very similar in their structure, although this conservation is not observed on the genetic level [41]. The major function of the claudins is to control the paracellular transport and signal transduction [42]. Some of the claudins are down regulated in breast cancer including CLDN 1, 4 and 7 [43].

**Abnormal antigens:**

Some breast cancer tissues present abnormal antigens, which are not presented in the normal breast tissues, some of them are well studied, while others stay not well studied [44].

**14C5 antigen:**

14C5 antigen is one of the well studied abnormal antigens in breast ductal carcinoma. 14C5 antigen plays a role in substrate adhesion and subsequently also in invasion of breast cancer cells. The 14C5 antigen is a protein composed of two polypeptide chains with molecular weight of 50 and 90 kd.
14C5 monoclonal antibodies are produced and they are able to inhibit cell substrate adhesion and invasion in vitro of breast cancer cells [45].

**Oncofetal Antigen (OFA):**

Oncofetal antigen (OFA) is 44 KDa glycoprotein expressed during early to mid gestation fetal development and re-expressed as a surface antigen by tumor cells soon after transformation. The antigen is detectable on all types of human tumors tested, but it is undetectable on normal cells [46].

**UDP-N-acetyl-D-galactosamine-polypeptide N-acetylgalactosaminyl transferases (ppGalNAc-T6):**

ppGalNAc-T6 is a transferase enzyme responsible for the transfer of GalNAc from UDP-GalNAc to normal cellular proteins (mucins). Addition of the GalNAc to the normal protein activates sequential addition of glucose residues by glycosyltransferases, which also participate in changing the normal cellular antigen structure. ppGalNAc-T6 is present in breast cancer tissues and is sporadically found in non-malignant breast diseases (sclerosing adenosis and fibroadenoma with focal hyperplasia), while it is totally absent from normal breast tissues. The presence of this enzyme in the cancerous breast tissues leads to the glycosylation of normal structural proteins and so production of many abnormal antigenic structures in breast cancer tissues [47]. Targeting immune response to glycosylated proteins (mucins) by ppGalNAc-T6 represent a promising approach for development of therapeutic vaccines against cancers [47].

**Survivin:**

Human survivin is a cytoplasmic protein, with a molecular weight of 16.5kDa. It consists of 142 amino acids, and its gene is located on chromosome 17[48]. Survivin is a unique
inhibitor of apoptosis usually expressed in the embryonic lung and fetal organs in the developmental stages but undetectable in normal adult tissues. However, survivin seems to be selectively expressed in transformed cells and in most human cancers, including breast cancer [49].

1.6 Monoclonal antibodies and their importance in breast cancer diagnosis and treatment:

Monoclonal antibodies (mAb) are monospecific antibodies that are identical because they are produced by one type of a single parent immune cell. Given almost any substance, it is possible to create monoclonal antibodies that specifically bind to that substance; they can then serve to detect or purify that substance [50].

The monoclonal antibodies are produced by the hybridoma technique, which involves fusion of a plasma cell with a myeloma cell to produce a hybridoma cell using poly ethylene glycol (PEG) as a fusing agent and Hypoxanthin Aminoptern Thymidine (HAT) medium as selective media. The HAT media permits hybridoma cells to grow, while inhibiting myeloma cells and plasma cells.

The HAT media inhibits the DNA synthesis salvage pathway through inhibiting the Hypoxanthine Guanine Phosphoribosyle transferase enzyme (HGPRT). The myeloma cells lack the denovo DNA synthesis and depend totally on the salvage pathway; thus the myeloma cells are not capable of growing in HAT medium. The denovo and the salvage pathways of DNA synthesis are active in the plasma cells; the HAT media can not kill the plasma cells. However the life span of the plasma cells is short. Only the hybridoma cells can grow in the HAT media because they posses the denovo pathway from the plasma cells and the unlimited life span from the myeloma cells [51].
The hybridoma cells possess the functions of the plasma and myeloma cells; production of antibody and rapid, unlimited division respectively. The hybridoma cells are cloned to obtain a hybridoma cell line which originated from one cell. There are different techniques to prepare a hybridoma cell line originated from one cell. e.g. limiting dilution growing in soft agar cloning and flow cytometry [52].

Once monoclonal antibodies for a given substance have been produced, they can be used to detect the presence of this substance. The Western blot test and immuno dot blot tests detect the protein on a membrane. They are also very useful in immunohistochemistry which detect antigen in fixed tissue sections, immunofluorescence test which detect the substance in a frozen tissue section or in live cells and immunoassays [50].

The use of monoclonal antibodies is one way to fight breast cancer, since they can be directed against antigens located on the surface of tumor cells. Trastuzumab and bevacizumab are the examples of the use of monoclonal antibodies in the treatment of breast cancer [53].

The monoclonal antibodies can be used either alone to kill cancer cells, or as carriers of other substances used either for treatment or diagnostic purposes. For example, chemotherapeutic agents can be attached to monoclonal antibodies to deliver high concentrations of these toxic substances directly to the tumor cells. In theory, this approach is less toxic and more effective than conventional chemotherapy because it reduces the delivery of harmful agents to normal tissues [54].

During the diagnostic process, monoclonal antibodies can be used to carry radioactive substances to cancer cells within the body, thus pinpointing the location of
metastases that were previously undetected by other methods. Satoh T and his colleagues used radio labeled monoclonal antibodies against the Carcinoembryonic Antigen (CEA) to detect metastasis of colorectal cancer [55].

1.7 Objectives:

**General objectives:**

To identify and characterize abnormal antigens in cancerous breast tissues in comparison to normal breast tissues.

**Specific objectives:**

1- Determine the identified abnormal antigens molecular weight, pI and orders

2- Extract one of the abnormal antigens.

3- Produce monoclonal antibodies against the extracted abnormal antigen.

1.8 Rationale:

This study is directed towards extraction and characterization of a new abnormal antigen in breast cancer tissues from Sudanese patients so as to cope with the international efforts to study the abnormal antigens which can, in the future, lead to new effective cancer therapies. The ultimate goal is to control the breast cancer by production of effective vaccine. Another aim of this study is to produce monoclonal antibodies against the extracted abnormal antigen which can be used in the early diagnosis and treatment of breast cancer. Also this study can participate in the transfer of the proteomic techniques to the Sudan, which lead to the development of the Sudan in the area of proteomic research.
CHAPTER TWO

Material and Methods
CHAPTER TWO

Material and Methods

The study area and population

This study was done within the period of October 2006 to June 2008. Samples were collected from Khartoum Teaching Hospital, Elengaz Surgical complex and Ibrahim Malik Teaching Hospital. All the hospitals are located in Khartoum locality of Khartoum state. Breast tissue samples were obtained from 120 patients after informed consent, diagnosed clinically as breast cancer patients and confirmed by Fine Needle Aspiration Cytology (FNAC). Two tissue samples were obtained from each patient, one from the normal tissue and the other from the cancerous tissue. The samples were confirmed by a histopathologist according to Bloom Richardson’s grading system. All patients were at early stage with neither nodal involvement nor metastasis to any other organs. Tissue samples were directly transferred to liquid nitrogen and stored.

Sample preparation

Tissue samples were crushed using liquid nitrogen cooled mortar and pestle. Proteins were extracted using SIGMA ProteoPrep® sample extraction kit (PROTTOT- 1KT) according to manufacturer instructions (Annex 1). The protein concentration was determined using BioSystems modified Bradford protein assay kit using Bovine Serum Albumin (BSA) as standard. The supernatant was kept at -70°C refrigerator.
Two Dimensional Poly Acrylamide Gel Electrophoresis (2D PAGE)

450 µg of each sample were loaded on 10cm pharmalyte pH gradient strips pH 3-10, which are prepared locally in capillary tubes (Labnet, cat. No. E2110-2D-CT, USA). Also some samples were loaded on 18cm Immobiline pH gradient strips (Amersham IPG, pH 3-10, 18 cm, UK). The two types of loaded strips were rehydrated for 12 hours at room temperature. Isoelectric Focusing was performed with 50µA per strip at 20°C with 4 step voltage modes, Step and Hold (500V, 0.5 kV/h), gradient (1000 V, 0.8 kV/h), gradient (8000 V, 13.5 kV/h) and step and hold (8000 V, 12.2 kV/h). Separation of the second dimension was performed in 12% SDS/polyacrylamide gels (10x10 cm and 20x20cm) using the Enduro 2D PAGE system (Labnet, USA) and Multiphore™ (Amersham, UK) respectively. Proteins in the analytical gels were stained with Coomassie stain. The results of the locally produced strips and the amersham strips were comparable. The Coomassie stain was prepared using Coomassie Brilliant Blue G-250 stock following the manufacturer’s protocol.

The stained gels were examined by image scanner II (Amersham, UK) using LabScan5 software (Amersham, UK). Certain defined spots were digested by Trypsin enzyme using Ettan™ spot handling workstation (Amersham, UK) for identification and one spot was used for monoclonal antibody production.

Liquid Chromatography Mass Spectroscopy (LC/MS) and protein identification

A total of 5µl of protein digest were loaded onto a reversed-phase (RP) C18 column for LC/MS analysis. The HPLC system used was Finnigan Surveyor™ MS pump with a flow splitter, column 0.18×100 mm C18 (Thermo Electron, USA) and flow rate of 200µl/min while the mobile phases were A: water with 0.1% formic Acid and B:
acetonitrile with 0.1% formic acid and gradients were 2-60% B in 20 min, 65-80% B in 5 min, hold 5 min and 80-2% B in 2 min. LC/MS used was Finnigan LTQ linear ion trap mass spectrometer (Thermo Electron, USA) and the conditions were as follows: ionization mode was nanospray, positive ion, 200°C capillary temperature, 1.8 KV spray needle voltage, 400-1.600 m/z Mass range. Scan sequence was Full-scan mass spectrometry and Acquisition modes were normal, data dependent and dynamic exclusion.

Protein identification was performed using the Turbo SEQUEST algorithm in the BioWorks™3.1SR1 software package (Thermo Electron, USA) and nr.fasta database (www.ionsource.com).

Hybridoma technique

The spot of the interest was solublized in 1ml of 0.5M phosphate buffered saline and the protein concentration was determined using the BioSystem modified Bradford assay. 30 µg of the antigen was dissolved in 0.5ml Freund’s Complete Adjuvant (FCA) and injected subcutaneously in multi sites of 2 Balb/C mice. After 3 weeks intervals the mice, again, were injected subcutaneously in multi sites with 30 µg of the antigen in 0.5ml Freund’s Incomplete Adjuvant (FIA) and tested for antibodies.

Test for antibodies

The mouse was wormed to dilate the tail vein. The tail vein was punctured with sterile hypodermic needle and the blood was absorbed onto a filter paper to form a blood spot. The blood spot was punched into a test tube and eluted in 150 µl ELISA assay buffer, this gives approximately 50 times dilution. The diluted blood spot was serially
diluted to set a titration curve then tested for antibody using Enzyme Linked Immuno Sorbent Assay (ELISA). Five 96 well ELISA plates and 100 polystyrene test tubes were coated with 100 µl of beta-tropomyosin spot with a concentration of 5 µg/ml, incubated overnight at 4°C. For the detection of antibody sensitivity 50 polystyrene test tubes were coated with casein and bovine serum albumin (BSA). 100µl of the harvested blood was added to some the coated polystyrene test tubes and incubated for one hour at 37°C. Then 100µl of rabbit anti mouse IgG bound to alkaline phosphatase and incubated for one hour at 37°C. After the incubation period substrate was added (picric acid) and the absorbance was measured at 405nm. The coated ELISA plates were used in the cloning step.

**Fusion and cloning**

The mouse with highly positive ELISA test was sacrificed and the spleen was taken out and a cell suspension was made to 50×10⁶ total number of cells. Myeloma (sp2) cell line was donated from the SAEC and was grown to 50×10⁶ total number of cells with 90% viability. The count and the viability of cells were determined using inverted microscope and 0.4% trypan blue staining.

The immunized spleen lymphocytes and the myeloma cells were mixed and centrifuged at 800g for 5 minutes. 0.8 ml of PEG were added slowly over one minute with gentle mixing at 37°C, then mixed for 90 seconds, diluted with 10ml RPMI 1640 over 5 minutes, centrifuged at 800g for 5 minutes, resuspended in 10mls culture flask in complete medium which contained 20% fetal calf serum and incubated overnight at 37°C. In the second day, 5mls were removed from the culture flask and 5mls of 2x Hypoxanthine Aminopterin Thymidine (HAT) was added. The dilution process was repeated on day 4, 6, 8, 10.and 12. ELISA screening tests were done at day 4, 8 and 12.
After day 12 and by the day 18 the hybridoma cells in the culture flask were transferred to complete medium.

Regarding the cloning step, the number of hybridoma cells in the culture flask was counted and the viability was adjusted at 90%. The hybridoma cells were diluted to obtain a final concentration of 10 cells per ml (one cell per every 100 µl). 10 ml of the diluted hybridoma cells were distributed into one culture plate of 96 wells and incubated at 37°C. The incubated cells were followed daily and tested for antibody every four days. The positive wells from one cell were transferred to 24 well culture plate, incubated at 37°C and growth was followed and tested. Positive wells were transferred to 6 well culture plates, followed and tested. From the 6 well culture plates, the positive wells were transferred to 10 ml culture flask, then to 35 ml culture flasks. The cells of 35 ml culture flask were frozen after addition of Di Methylene Sulpho Oxide (DMSO) in 2 cryo tube. Each cryo tube contained 5 million hybridoma cells, 0.5 ml DMSO and 0.5 ml cells mixture. The two cryotubes were firstly transferred to -20°C refrigerator for one day then transferred to -70°C refrigerator for 24 hours and finally to liquid nitrogen.

**Antibody harvest and purification**

The cells of the 35 ml culture flasks were centrifuged at 2000 g for 15 minutes, the cells were re suspended in 30 ml RPMI medium and the supernatant (antibody containing) was kept frozen. The monoclonal antibody was purified following SIGMA antibody purification kit protocol (Annex 2). PAGE (the same method as described above excluding the isoelectric focusing step) was done for the purified monoclonal antibodies so as to determine the molecular weight and to check the purity. ELISA tests for the purpose of determining the specificity were done using the casein and BSA coated tubes.
All the monoclonal antibodies production methods were extracted from the practical guide of monoclonal antibody production prepared by Eryl Liddell and A Cryer [56].

**Statistical analysis**

The general information of the patients was analyzed using the Statistical Package for the Social Sciences computer program (SPSS) version 16.
CHAPTER THREE

Results
CHAPTER THREE

Results

The total number of the patients involved in this study was 120 female patients. The mean age of the patients was 47.2 (16 to 80). Two age groups were with equal, highest, incidence rate (≥ 44) and (45-64) and the Khartoum state had the highest incidence rate (Table.1).

Depending on the histopathology reports, the tissue samples were diagnosed as invasive ductal and lobular carcinomas with three grades. Most of the patients (112, 93.3%) were diagnosed as invasive ductal carcinoma, while 8 patients (6.7%) were diagnosed as lobular carcinoma (Table.2).

2D PAGE results:

Three different patterns of 2D PAGE results were obtained. In the first 2D result four abnormal antigens were detected in the cancerous breast tissues and undetected in the normal breast tissues of the same patient. The number of patients with the first 2D result were 32 patients (26.7% of all patients) (Fig.1 and Fig.4).

The second 2D result reflected that the cancerous and normal breast tissues of 81 patients (67.5% of all patients) were the same; there was no abnormal antigens in the cancerous breast tissues (Fig.2 and Fig.4).

The last 2D result involves presence of two abnormal antigens of the first 2DPAGE result. Seven patients (5.8% of all patients) carried the last 2D result pattern (Fig.3 and Fig.4).

All the normal breast tissues of all patients were missing all the four abnormal antigens (Fig.4).
### Table 1: Age groups and geographical distribution of the patients with breast cancer.

<table>
<thead>
<tr>
<th>Geographical distribution of patients</th>
<th>Age groups (years)</th>
</tr>
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<tbody>
<tr>
<td>Khartoum state</td>
<td>≥ 44</td>
</tr>
<tr>
<td>Central states</td>
<td>45-64</td>
</tr>
<tr>
<td>Northern states</td>
<td>≤ 65</td>
</tr>
<tr>
<td>Western states</td>
<td></td>
</tr>
<tr>
<td>Eastern states</td>
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</table>

<table>
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<tr>
<th></th>
<th>Khartoum</th>
<th>Central</th>
<th>Northern</th>
<th>Western</th>
<th>Eastern</th>
<th>≥ 44</th>
<th>45-64</th>
<th>≤ 65</th>
</tr>
</thead>
<tbody>
<tr>
<td>No of patients</td>
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<td>19</td>
<td>13</td>
<td>15</td>
<td>12</td>
<td>49</td>
<td>50</td>
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<tr>
<td>Total</td>
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<td></td>
<td></td>
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<td></td>
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<td></td>
</tr>
<tr>
<td>Mean age</td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>47.2</td>
</tr>
</tbody>
</table>


Table 2: Types and grades of breast cancer among the Sudanese patients.

<table>
<thead>
<tr>
<th>Type of cancer</th>
<th>Ductal carcinoma</th>
<th>Lobular carcinoma</th>
</tr>
</thead>
<tbody>
<tr>
<td>Grade</td>
<td>I</td>
<td>II</td>
</tr>
<tr>
<td>Number of patients</td>
<td>28</td>
<td>51</td>
</tr>
<tr>
<td>Total</td>
<td>112</td>
<td></td>
</tr>
</tbody>
</table>
Fig. 3: Representative 2D PAGE result of cancerous breast tissues containing the four abnormal antigens.

*control is Fig. 4
Fig. 2: Representative 2D PAGE result of cancerous breast tissues missing the four abnormal antigens.

*Control is Fig. 4
Fig.3: Representative 2D PAGE result of cancerous breast tissues missing two of the four abnormal antigens.

*control is Fig.4
Fig. 4: Representative 2D PAGE result of all normal breast tissues from Sudanese breast cancer patients

(kDa)  pH 3  10

200  150  100  85  70  60  50  40  30  25  20  15  10

4→
3→
1→ 2→
Identification of the abnormal antigens:

LC/MS results:

Each trypsin digestion products of each spot were introduced in the LC/MS machine. For each digestion site a spectrum was generated and the amino acid sequence of the digestion product was determined by the soft ware attached to the machine.

Spot 1:

In spot 1, there were two trypsin digestion sites, i.e., there were two polypeptides produced by the action of the enzyme. The precursor mass of the first digestion site was 604.26 and its amino acid sequence was CMPTFQFF (Fig.5), While the precursor mass of the second digestion product was 612.35 and its amino acid sequence was CM*PTFQFF (Fig.6).

Spot 2:

Spot 2 digestion produced two polypeptides and two spectra were generated, the first one of precursor mass 824.96 and amino acid sequence of FNAHGDANTIVCNS (Fig.7) and the second polypeptide was of precursor mass 893.92 and amino acid sequence of LNLEAINYMAADGDF (Fig.8).

Spot 3:

In spot 3, also, two polypeptides were obtained by the action of the trypsin. The polypeptide was with a precursor mass of 732.04 and had the amino acid sequence of CTKEEHLCTQ (Fig.9). The second polypeptide was with a molecular weight of 973.45 and had the amino acid sequence of ALKDEEKMLEQLQL (Fig.10).
Spot 4:

Spot 4 was digested by trypsin and three polypeptide fragments were produced. The polypeptide was with a precursor mass of 673.19 and amino acid sequence of QLEEEQQALQ (Fig.11). The precursor mass of the second polypeptide was 801.49 and its amino acid sequence was GTEDEVEKSYSEV (Fig.12). The third digest product molecular weight was 817.3 with amino acid sequence of CKQLEEEQQALQ (Fig.13).
Fig. 5 Spectrum of the trypsin digest of precursor mass 604.26 and its amino acid sequence of spot 1.
Fig. 6 Spectrum of the trypsin digest of precursor mass 612.35 and its amino acid sequence of spot 1.
Fig. 7 Spectrum of the trypsin digest of precursor mass 824.96 and its amino acid sequence of spot 2.
Fig. 8 Spectrum of the trypsin digest of precursor mass 893.92 and its amino acid sequence of spot 2.
Fig. 9 Spectrum of the trypsin digest of precursor mass 732.04 and its amino acid sequence of spot 3.
Fig. 10 Spectrum of the trypsin digest of precursor mass 973.45 and its amino acid sequence of spot 3.
Fig. 11 Spectrum of the trypsin digest of precursor mass 673.19 and its amino acid sequence of spot 4.
Fig. 12 Spectrum of the trypsin digest of precursor mass 801.49 and its amino acid sequence of spot 4.
Fig. 13 Spectrum of the trypsin digest of precursor mass 817.30 and its amino acid sequence of spot 4.
nr.fasta and Protein Databases search results:

By inserting the spectra of each spot in the nr.fasta database the spots were identified as: Spot 1 was human Thioredoxin (D60n mutant), with 105 amino acids, the average mass of 11737.5 Da and the Isoelectric point (pI) was 4.95 (Fig.14). The secondary structure elements (SSE) were obtained from the PDB (Fig.15). However the tertiary structure is not mentioned in the PDB. Spot 1 was detected in 39 patients.

Spot 2 was X-ray crystal structure of human galectin-1, with 134 amino acids, average mass of 14595.4 Da and the pI was 5.34 (Fig.16). The SSE were shown in (Fig.17), also the tertiary structure of X-ray crystal structure of human galectin-1 is not mentioned. Spot 2 was detected in the cancerous tissues of 39 patients.

Spot 3 was rcTPM3 (retro copy of tropomyosin 3), with 247 amino acids, average mass of 28824.2 Da and pI of 4.77 (Fig.18).The tertiary structure of rcTPM3 was obtained from the SWISSPROT database (Fig.19). Spot 3 was detected in the cancerous tissues of 32 patients.

Spot 4 was identified as beta-tropomyosin (Iso form 2). Bet-tropomyosin was composed of 257 amino acids with average molecular weight of 29942.3 Da and pI of 4.70 (Fig.20). The tertiary structure of beta-tropomyosin is shown in (Fig.21), it was obtained from the SWISSPROT database. Spot 4 was detected in the cancerous tissues of 32 patients.

All the protein identities including their names, swissprot database accession numbers, molecular weights, isoelectric points, the percentages of the trypsin digest masses of each protein total mass and the ideal number of peptides of each trypsin digestion products as tabulated in the ne.fasta database were shown in [Table.3].
Fig. 14 nr.fasta database search result of spot 1.

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Protein Coverage

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Protein Coverage Totals

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<td>% by AA's</td>
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Fig. 15 Secondary structure elements of human thioredoxin (D60n mutant)
Fig. 16 nr.fasta database search result of spot 2.

![Database Search Result](image)

**Reference:** p143242377p001162A Chain A: X-Ray Crystal Structure of Human GHRP1

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**Protein Coverage:**

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**Protein Coverage Totals:**

- % by Mass: 88.49
- % by Position: 51
- % by AA's: 52.91

[Update]
Fig. 17 Secondary structure elements of X-ray crystal structure of human galectin-1.
Fig. 18 nr.fasta database search result of spot 3.
Fig. 19 The tertiary structure of rcTPM3.
Fig. 20 nr.fasta database search result of spot 4.
Fig. 21 The tertiary structure of beta-tropomyosin.
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**Results of monoclonal antibody production.**

Two mice were immunized with beta-tropomyosin-like spot red colour lable marked and green colour lable marked mice (Annex.3 and Annex.4). One mouse is not immunized and is marked with white colour lable as a control mouse (Annex.5). The green colour lable marked mouse had the highest response, the blood sample give a positive result up to 516000 time dilution (516000 titer) [Table.4], so it was sacrificed and its spleen was removed (Annex.6). The red colour labled mouse was saved. The spleen cells were scratched and fused with the sp2 myeloma cell line and cultured in 10ml culture flask. ELISA tests were done to test production of antibodies on day 4, day 8 and day 12. The absorbance was increasing from 0.49 to 0.57 and finally the absorbance was 0.74. The total number of hybridoma cells was counted and it was $4.5 \times 10^6$ cell. The viability was 90%. In order to do the cloning, by the limiting dilution method, 10ml of hybridoma cells with a concentration of 10cells/ml (one cell/ 100µl) was prepared and distributed in 96well culture plate, 100µl per each well. The 96 well cell culture plate was tested for antibody productivity using ELISA plate. Two tests were done to follow the production of antibody and to decide which well should be expanded to obtain a cell line originated from one cell per well. The first ELISA assay test was done after 4 days of cloning. 6 wells were found containing one cell and they were producing antibody A12, B11, F3, G11, H4 and H9 [Table.5].The second ELISA assay test was done 8 days after cloning. By comparing ELISA tests, it was clear that only B11 and G11 were increasing their antibody productivity while the productivity in the other wells was decreasing [Table.6]. Depending on the two ELISA results, a decision was taken to expand B11 and G11.
The expansion of B11 was failed due to contamination. G11 was expanded to 24 well culture plate, 6 well culture plate, 10ml culture flask and finally to 35ml culture flask. The antibody harvested and finally the G11 cell line was frozen with Di Methyl Sulpho Oxide (DMSO) in -20ºC, -70 ºC refrigerators and finally in liquid nitrogen.

The harvested antibody was purified and the final antibody concentration was 0.35mg/ml and the volume of the harvest was 25 ml. Specificity ELISA tests were negative when casein and BSA coated tubes were used. The PAGE results has shown single band for the monoclonal antibody with a molecular weight approximately equivalent to 30 KDa [Fig.22].
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K \equiv 1000
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Table 6 Second after cloning ELISA assay test results (Absorbance at 405nm).

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Fig. 4 PAGE result for the purified anti beta-tropomyosin monoclonal antibody
CHAPTER FOUR

Discussion, Conclusions, Recommendations, References and Annexes
CHAPTER FOUR

Discussion

The general description of the patients can not be compared to the local, regional or international data, because the number of patients is limited. However, this study showed that the mean age group of having breast cancer was 47.2 compared to 40 in USA and Europe [57-2]. The most affected age groups by breast cancer were 0-44 and 45-64, this finding is, to some extent, similar to the international data [2]. Most of the patients were diagnosed as invasive ductal carcinoma (93.3%), while the international findings show that the ductal carcinomas represent approximately 70% of the breast cancers [58]. Khartoum state was the most affected area; this is because Khartoum is capital of Sudan and all the quality services are located in it.

This study detected four abnormal proteins in the cancerous breast tissues and they were absent from the normal tissues of the same patients. Two of them were synthetic proteins, synthesized by X-ray crystallography; they are not expected to be found in human tissues. They were thioredoxin (D60n mutant) and X-ray crystal structure of human galectin-1. The other two abnormal proteins were rcTPM3 and the isoform2 of beta-tropomyosin.

**Human Thioredoxin (D60n mutant):**

This study detected the presence of the thioredoxin (D60n mutant) in 32.5% from the study population. The thioredoxin (D60n mutant) is a synthetic protein synthesized by X-ray crystallography for the native thioredoxin. The thioredoxin (D60n mutant) is characterized by replacement of Aspartate 60 to Asparagine. This mutation causes the dimerization of the thioredoxin. The dimer is stabilized by disulphide bonds between
cysteine residues [59]. However, thioredoxins are a family of small redox proteins that share a highly conserved Trp-Cys-Gly-Pro-Cys-Lys active site [60]. The active site cysteines of thioredoxins undergo reversible oxidation and reduction catalyzed by thioredoxin reductase, an enzyme that use NADPH as its source of reducing equivalents [61-62]. Human thiredoxin can be exported out of the cell where it has additional functions, including the ability to stimulate normal and cancer cell growth [59]. Thioredoxins has been shown to play a major role in a variety of human diseases like diabetes mellitus, goiter and cancer. Over expression of thioredoxin protect cancer cells from spontaneous and drug induced apoptosis. Clinically thioredoxin is over expressed in a number of human primary tumors where it is associated with increased cell proliferation, decreased apoptosis and decreased patient survival. In contrast cells expressing the inactive thioredoxin mutant are more sensitive to spontaneous and drug induced apoptosis [63]. Cells stably transfected with a redox inactive thioredoxin mutant showed decreased cell growth, decreased colony formation in soft agar and inhibited tumor formation in vivo [64]. Presence of the thioredoxin (D60n mutant) is not an artifact because it is not detected in the normal tissues of the same patients beside that the cancerous and normal tissues were treated equally. The presence of this synthetic protein in cancerous tissues can be due to X-ray investigation which is a routine investigation, environmental factor like the UV of the sun light or it can be a compensation mechanism of the human bodies to resist breast cancer since the elevation of the normal thioredoxin leads to drug and apoptosis resistance in breast cancer.
Galectin-1 and X-ray crystal structure of human galectin-1:

Galectin-1 (gal-1), a member of the mammalian beta-galactoside binding proteins, preferentially recognizes Galbeta1-4GlcNAc (LacNAc) sequences of oligosaccharides associated with several cell surface glycoconjugates [65]. Galectin-1 exist as a homodimer and can facilitate intramolecular and intermolecular cross bridging. Such cross bridging may facilitate cellular signaling through growth factor receptors, or mediate cell-cell adhesion [66]. Galectin-1 is upregulated in several cancers and its expression is often higher in more differentiated tumors [67-69]. High level expression of galectin-1 is found in lymphatic organs, which feature high rate of apoptosis. Further more, it is known that galectin-1 can initiate T cell apoptosis [65]. Other studies concluded that galectin-1 is a negative growth regulator for certain types of tumors and activated T cell [70]. X-ray crystal structure of human galectin-1 is synthesized by X-ray crystallography, amino acid number 16 is converted to unknown amino acid (X). The X-ray induced single site mutation revealed the presence of many alterations in the wild type (galectin-1) conformation and function [70]. The mutant galectin-1 has no capability to undergo the induction of apoptosis and the negative regulation of cell growth leading to the clinical picture complications of the cancer patients. However, this study detected this mutated protein in 32.5% of the study population. The presence of this mutated protein is not due to an artifact. It can be due to nuclear techniques investigations or any other environmental factor.
The retro copy of tropomyosin 3 (rcTPM3):

This study reported the detection of rcTPM3 in 26.7% of the study population cancerous tissues. rcTPM3 is a retro copy of tropomyosin3, which is produced by a mechanism known as retro positioning. Retro positioning is a mechanism responsible for generating new intronless gene copies (retrocopies) by reverse transcription of mRNAs derived from source genes (parental genes), followed by reintegration of the resulting gene in the genome [71]. Retro position was commonly thought to generate non functional gene copies that accumulate disablement such as premature stop codons and frame shift mutations [72]. Some studies have shown that retroposition has generated a significant number of new functional genes (retro genes) in mammalian and invertebrate animals genomes [73]. Detection of rcTPM3 in the cancerous tissues may be due to HIV infection or MMTV infection, since the gene is obtained by reverstranscriptase. There is some evidence showing the correlation between HIV and MMTV infection and breast cancer incidence [74-77].

Beta-tropomyosin (isoform 2):

There are three isoforms of beta-tropomyosin; isoform 1 with 284 amino acids [78], isoform 2 with 284 amino acids [79] and isoform 3 with 248 amino acids [80]. Beta-tropomyosin binds to actin filaments in muscle and non muscle cells. It plays a central role in association with the troponin complex, in the calcium dependant regulation of vertebrate striated muscle contraction. In non muscle cells, beta-tropomyosin is implicated in stabilizing cytoskeleton actin filaments [81]. There are two diseases associated with defects in beta-tropomyosin, Nemaline Myopathy type 4 (NEM4) and the distal arthrogryposis type 1 (DA 1). NEM4 is a form of congenital myopathy
characterized by abnormal thread or rod like structures in muscle fibers on histology [82].
DA 1 is a form of inherited multiple congenital contractures [83]. Up regulation of
tropomyosin isoform 2 was previously reported in highly metastatic breast cancer cell
lines [84]. Down regulation of the beta- tropomyosin isoforms expression has been
reported previously in primary breast carcinoma cell lines [85] and in primary breast
tumors [86]. The tropomyosin alpha forms were detected in normal human mammary
epithelial cell lines [86]. This study reported the presence of isoform 2 of beta-
tropomyosin in the cancerous tissues of 26.7% from the study population and it was
undetectable in all the normal tissues of the patients. The detected beta-tropomyosin is
either a new isoform or a truncated form because it contain 257 amino acids while the
other forms contain 284, 284 and 248 amino acids. The probability that this protein is a
new isoform rather than a truncated form was shown by Ben Yousef and Francomano
when they sequenced the gene of human beta-tropomyosin and found that it is composed
of 257 nucleotides [87].

**Monoclonal antibody production:**

Monoclonal antibodies have been produced with low concentration 0.35mg/ml
compared to ideal situation 1-5mg/ml [56]. The low productivity may be due to that
growth factors and feeder cells were not used due to financial and political issues. Two
tests were done in order to determine specificity of the produced monoclonal antibody.
The monoclonal antibody has shown some specificity since they did not bind the casein
and the BSA. The number of peptides and the molecular weight of the monoclonal
antibody has been determined. However the full determination of the specificity requires
western blot analysis and immunohistochemistry tests [88]. The western blot analysis was
not done due to that the amount of the beta-tropomyosin of the 2D PAGE gels were not enough for the immunization, ELISA and the specificity determination tests. Regarding the immunohistochemistry tests, the tissues were not enough because the 2D PAGE analysis was done 2-4 times for the positive tissues so as to obtain enough antigen concentration for immunization and ELISA tests.
CHAPTER FOUR

Conclusions

1- Breast cancer tissues are characterized by abnormal proteins with the presence in one third of the study population.

2- The abnormal proteins were Thioredoxin (D60n mutant), X-ray crystal structure of human galectin-1, rcTPM3 and beta- tropomyosin (isoform 2).

3- The presence of the abnormal proteins in one third of the study population strongly directs towards individualization of diagnosis and treatment of breast cancer patients.

4- Monoclonal antibodies against beta-tropomyosin has been produced. The specificity tests have shown that it does not react non specifically, but the western blot and immunohistochemistry tests were not done.

5- The number of bands and the molecular weight of the monoclonal antibody were determined.
CHAPTER FOUR

Recommendations

Further studies in multi centers are needed with:

1- Large number of patients to verify the presence of the abnormal antigens in the studied population.

2- Involvement of different stages and grades of breast cancer.

3- Adequate amount of the tissue (10 grams at least).

4- Full antibody characterization should be done.

5- Clinical research to find the association of presence of the abnormal antigens and the aggressiveness of the tumour, response to therapy or prognosis.
CHAPTER FOUR

References


34- Coussens L. Tyrosine kinase receptor with extensive homology to EGF receptor shares chromosomal location with neu oncogene. Science 1985; 230: 1132–1139.


73- Marques AC, Dupanloup I, Vinckenbosch N, Reymond A, Kaessmman H.


82- Donner K, Ollikainen M, Ridanpaa M, Christen H, Wallgren- Pettersson C.


86- Raval GN, Bharadwaj S, Levine EA, Willingham MC, Geary RL, Kute T, Prasad GL.


CHAPTER FOUR

Annexes

Annex 1 ProteoPrep® sample extraction kit protocol.

1- Suspend 3 grams of sample in 2ml of reagent 1, 2, 3 or 4 and sonicate on ice
   ( 4 ×15 seconds ) using ultra sonic probe.

2- Centrifuge the suspension at 15000 × g for 30 minutes at 15˚C.

3- Decant the supernatant into a clean tube and discard the insoluble pellet.

4- Reduce the supernatant by adding 50 µl of TBP ( Tributylphosphine ).

5- Alkylate the solution by adding 60 µl of iodoacetamide.

6- Centrifuge at 20000 × g for five minutes at room temperature.

7- Determine the concentration of the protein and Load on Iso Electric focusing gels.
Annex 2 Protein A antibody purification kit protocol (SIGMA PURE1A).

1- To each 10 ml of culture supernatant, add one ml of binding buffer and mix well.

2- Fill a 10 ml syringe with 10 ml HEPES buffer and connect it to the top of the desalting cartridge. Regenerate the cartridge by passing the buffer through the cartridge at an approximate flow rate 1 ml/min.

3- Fill a 5 ml syringe with five ml of regeneration buffer and connect it to the top of the protein A cartridge. Regenerate the cartridge by passing the buffer through the cartridge at an approximate flow rate of 1 ml/min.

4- Fill a 10 ml syringe with 4 ml of binding buffer. Equilibrate the protein A cartridge by passing the buffer through the cartridge at an approximate flow rate of 1 ml/min.

5- Fill a 10 ml syringe with the sample-binding buffer mixture. Load the protein A cartridge by passing the sample – binding buffer mixture through the cartridge at an approximate flow rate of 0.5 ml/min.

6- After the sample is loaded on the protein A cartridge, rinse the 10 ml syringe with water, refill it with 6 ml of binding buffer and wash the protein A cartridge by passing the buffer through the cartridge at an approximate flow rate of 1 ml/min.

7- Attach the female end of the protein A cartridge to the male Leur lock fitting of desalting cartridge.

8- Fill the 5 ml syringe with 5 ml of elution buffer. Attach the syringe to the protein A cartridge. Elute the cartridges by passing the elution buffer through the cartridges at approximate flow rate of 0.5 ml/min. The elute contains the purified immunoglobulin at a physiological pH.
9- Detach the two cartridges. A- Regenerate the protein A cartridge by passing 5 ml of regeneration buffer through the cartridge. B- Regenerate the desalting cartridge by passing 10 ml of HEPES buffer through the cartridge. The cartridges are now ready for another affinity chromatographic purification.

10- For the long term storage of the cartridges, wash the cartridges with 10 ml phosphate buffered saline containing 0.02% sodium azide. Cap the cartridges with the end closures provided and store at 2-8°C.
Annex 3 Immunized mouse (red color label marked)
Annex.4 Immunized mouse (Green color label marked).
Annex 5 Control mouse (white color label marked).
Annex 6 The spleen of the sacrificed mouse.
Annex.7 Published papers


Annex.8 Information Entry in SWISSPROT database

4 results for author:"Ahamed M.E." in UniProtKB

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