

RESEARCH ARTICLE

Immunohistochemical Expression of CD24 Protein as Cancer Stem Cells Marker in a Sample of Iraqi Women with Breast Carcinoma

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ABSTRACT

Breast cancer is composed of a heterogeneous group of tumors having numerous features, biology, and treatments. Cancer stem cells (CSCs) are a unique population of cancer cells featuring stem-cell like characteristics and, based on growing evidence, they may start the development of PC as is the situation with other neoplasms. CSCs can inherently be resistant to medical treatment and lead to tumor relapse. However, the CSCs that propagate the cancer cells and tumors resistant to medical therapy may differ. The growing knowledge for the mechanisms of cancer resistance and the development of therapeutic techniques will enhance clinical outcomes and prevent the challenge of chemotherapy resistance. CD24 is a tiny mucin-like glycosyl phosphatidylinositol (GPI) - joined cell surface protein included in cell adhesion.

Sixty patients of Iraqi women with breast carcinoma were included in this study. They were categorized into three groups, Group I (included 20 of newly diagnosed women patients with breast carcinoma), Group II (included 20 relapsed patients who underwent chemotherapy and recovered then after a while) and Group III (included 20 patients who showed resistance or no response to chemotherapy treatment). Clinicopathological parameters of each group of the study were studied and showed significant differences between groups, including age, grade, tumor size, histological type of tumor, and clinical stage. Histopathological parameters were also studied and showed different histopathological features of each group of the study, including high necrosis and severe hemorrhage and the tumor cells extended right from the luminal surface at the upper left towards the muscularis propria right to the lower right and there is a notable high variability in the spaces among the tumor mass longitudinal spaces. The percentages of immunohistochemical expression upon cell scoring data of CD24 positive cells for the groups I (43,55%), II (63,69%) and III with a higher percentage reaches to (78,89%) with significant ($p \leq 0.05$) differences between groups. In conclusion, CD24 marker could be targeted as cancer stem cells marker as they play a role in cancer resistance to chemotherapy and relapsing of the disease, so it could help in targeting therapy of breast cancer stem cells within the tumor tissue, preventing the recurrence of the tumor.

Keywords: Breast cancer stem cells, Cancer stem cells, CD24, IHC.

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INTRODUCTION

Breast cancer comprises a heterogeneous group of tumors having numerous features, biology, and treatments. The breast cancers are usually grouped into two important overarching groups: the carcinomas and the sarcomas. The first group "Carcinomas" is cancers that happen from the epithelial part of the breast. The epithelial part is composed of the cells lining the lobules and terminal ducts; according to normal conditions, these epithelial cells are in charge of making milk. The second group "sarcomas" is cancer that starts in tissues like bone or

muscle. Bone and soft tissue sarcomas are the main types of sarcoma. Soft tissue sarcomas can develop in soft tissues like fat, muscle, nerves, fibrous tissues, blood vessels, or deep skin tissues. They can be found in any part of the body.¹

World Health Organization (WHO),¹ reported that there are more than 18 million cancer cases globally and it was responsible for about 9.6 million deaths in 2018, and it was the cause of about 1 in 6 deaths worldwide. In addition, the latest WHO reports that breast cancer, worldwide, is the majority of prevalent malignancy around the world in 154 out of 185

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countries and often is the main cause of cancer related mortality in over 100 countries besides it is the majority of cancer among women for about 25% of the documented female cancers, with around 2.1 million new diagnosed cases in 2018.

CSCs are a unique population of cancer cells featuring stem-cell like characteristics and also, based on growing evidence, they may start the development of PC as is the situation with other neoplasms. Cancer stem cells appear to make daughter cells throughout the process of cell division that can occur following the ordinary tumor cells that represent the tumor's major element or self-renewal, keeping the full capability to divide and differentiate just like the parent stem cell can do. The chance of finding certain markers identifying PC CSCs had been researched. Many certain proteins are part of this group, including CD-133, CD-24, CD-44, CXCR-4, EpCAM, ALDH-1, Oct-4, ABCB-1, c-Met ABCG-2, and nestin.²

One of the major problems in treating breast carcinoma is the development of resistance to chemotherapy and recurrence of the disease in relapsed patients. Scientists showed that this may be attributed to the presence of cancer stem cells within the tumor mass as these types of tumor cells possess a different mechanism to resist the chemotherapy and survive to be the major cause to re-establish the tumor after chemotherapy courses. For this reason, the proper diagnosis and investigation of the presence of these types of cells within the tumor breast mass could help in the proper targeting and eliminating these cells via targeting their markers.

The present study focused on CD24. CD24 is a tiny mucin-like glycosyl phosphatidylinositol (GPI) - joined cell surface protein included in cell adhesion. CD24 appears to be associated in integrin-associated cell to matrix regulation and adhesion of cellular morphology via stress fibers, processes of rounding, and nuclear condensation, and consequently protects cancer cells from apoptosis.³

The present study aims to evaluate the immunohistochemical expression of CD24 protein's role as cancer stem cell marker in a sample of Iraqi women patients with breast carcinoma to help in cancer targeting therapy.

METHODOLOGY

Samples Collection

The study included 60 patients, (Female) with ages ranging from 40 to 65 years. The samples were obtained from the histological unit in the Medical City Hospital, Department of Education Laboratories, Ministry of Health and environment in collaboration with Dr. Majed Al-Dari Clinic, Baghdad, Iraq. Sections of samples were taken from the Raji Al-Hadithy laboratories. Three groups of breast cancer were collected. Group I included 20 samples from newly diagnosed patients with Breast cancer, Group II included samples from relapsed patients, taking the chemotherapy treatment and the tumor relapsed or re-established again and Group III included 20 samples from patients who had resistance or no-response to chemotherapy.

Preparation of Avidin-Biotin Complex (ABC) Staining System Working Solution

Peroxide Block: Block for 5 minutes with PolyExcel H2O2.

Primary Antibody: It was prepared as mentioned by the manufacture instruction (PathSitu Biotechnology).

AB Enzyme Reagent: It was used only (biotinylated Horse radish peroxidase (HRP)), which is conjugated with secondary antibodies.

PolyExcel Target Binder: The tissue sections were covered with PolyExcel. Target Binder and incubated for 10 minutes at room temperature.

PolyExcel PolyHRP: The tissue sections were covered with PolyExcel. PolyHRP and incubated for 10 min at RT.

PolyExcel StunnDAB: Tissue sections were covered with StunnDAB working solution and incubate for 5 minutes at room temperature.

Peroxidase Substrate: It was prepared by mixing 1.6 mL of distilled water, 5 drops of 10x substrate buffer, 1 drop of 50x DAB chromogen, and 1 drop 50x peroxidase substrate.

1% of Hydrogen peroxide H2O2: To prepare 1% of H2O2 1-mL of H2O2 stock solution was mixed with 24 mL PBS.

Counter Stain: Hematoxylin ready to use.

Mounting Medium: Dibutylphthalate polystyrene xylene (DPX) mounting ready to use.

Fixing Reagent: Formalin solution (4%) was prepared by mixing 4 mL of stock formaldehyde (37%) with 96 mL PBS.

Method

Removal of the Wax: Following the elaboration of paraffin sections, all the elements were infiltrated with and enclosed by paraffin wax which is hydrophobic and impervious to aqueous reagents. The tissue and cell components mainly have no natural color and are invisible. The first step in performing an H&S stain is to resolve all the wax away with xylene (a hydrocarbon solvent).

Hydration of the Section: After comprehensive de-waxing, the slide was transferred through alteration a series of alcohol to take out the xylene from then the tissue washed in water. The section is now hydrated so that aqueous reagents will easily penetrate the tissue and cells elements.

Application of Hematoxylin Nuclear Stain: The tissue is now stained with a nuclear stain such as Harris Hematoxylin, which contains a dye (oxidized Hematoxylin or hematein) and a mordant or engaged agent (an aluminium salt) in solution. At first this stains the nuclei and few other elements a reddish-purple color.

Completing the Nuclear Stain by "Bluing": After washing in tap water, the section is "bluing" by handling with a weakly alkaline solution. This transforms the Hematoxylin to a dark blue color. Section ability now be washing and checked to see if the nuclei are duly stained, appear adequate disparity, and estimate the background stain level.

Removal of Excess Background Stain (Differentiate): On generality occasions when Harris Hematoxylin is used, a differential (de-staining) step is desired to take off non-specific background staining and to progress disparity. A poor acid alcohol was utilized. After this treatment, bluing and fully washing is still desired. Staining procedure that involves a de-staining or differentiation step is referred to as “regressive” stains.

Application of the Eosin Counterstain: The section is stained with an alcoholic solution of eosin. This colors several non-nuclear elements in different shades of pink.

Rinse, Dehydrate, Clear and Mounting (Apply Cover Glass): Pursuing the eosin stain, the slide is transferred through a series of alcohol to take off all water effects, then wash in many baths of xylene “clears” the renders and tissue it completely clear. A fluffy layer of polystyrene mountant is used, by a glass cover slip. If the stain and subsequent stages have been duly performed, the slide will detect all the necessary microscopic components and be steady for several years.

RESULTS AND DISCUSSION

Clinicopathological Study

The paraffin embedded breast cancer samples of each group of the study were collected and examined under the consultancy Histopathological and oncologist consultant for the purpose of discriminating and comparing between different clinic pathological variables among the patients group depending on the information being following up the patients history files in Medical city hospital archives in Baghdad, the parameters being following up and measured among patients groups are age, grade, stage, tumor size and the histological type. The recorded clinical pathological features results are described in Table 1. All the patients included in the study were females having a mean age ranging from 40–65 years with a significant variation among groups. Group I (newly diagnosed breast cancer patients) shows a higher percentage of old women mean age than Groups II and III.⁴ Demonstrated that a sample of women with breast carcinoma showed a significant difference in age and other clinicopathological parameters measured from case report of the hospitals. Usually, there are two types of breast cancer depending on estrogen hormone, Estrogen and non-estrogen. According to the Iraqi Institute of Cancer Reports, estrogen dependent breast cancer is the most common cancer type in Iraq. Most women reach the menopause at the age of 50 years due to that estrogen secretions levels are quaintly dropping and reducing by age, results showed that in the group were the older patients regarding to age than the other groups of the study with a significant variations among groups (Table 1), usually this is due to the younger ages has more stem cells numbers in the intestinal mucosa and the latest may inter a different abnormal pathway to convert to cancer stem cells which have different mechanisms of resistance to chemotherapeutic agents. Most samples of group I, usually not subjected to chemotherapy treatment, recorded a higher

tumor mass size than other groups who submitted to a complete chemotherapy treatment protocol without response in group III and with a recurrence of the tumor after a time while after being subjected to a complete course of chemotherapy, so usually either slightly affected and regressed or complete regression of the tumor. According to the histological type, there are two types of Invasive Ductal Carcinoma (IDS) and Invasive Lobular Carcinoma (ILC) with variations in the number of cases within each group of the study.

Histopathological Sections Study

Histopathological parameters were measured for each study group after the preparation of H&E histological slides. Figure 1 showed the Histopathological analysis of group I, which demonstrates early signs of cancer represented by the cancer cells orientation and the condensation of chromatin in the rapidly dividing cells and the signs of hemorrhage and hyperplasia.^{5,6}

Histological sections of group II show the recurrence of the tumor mass from the core tissue of the lumen of the breast mass (Figure 2). After a complete course of chemotherapy, the tumor cells are characterized by this is all invasive ductal carcinoma. No DCIS, intraductal and invasive carcinoma with Glycogen rich.

The histological sections of group III (patients with high resistance to chemotherapy treatment courses) are shown in Figure 3. They are characterized by high necrosis and severe hemorrhage, and the tumor cells extended right from the luminal surface at the upper left towards the muscularis propria right to the lower right. There is a notable high variability in the spaces among the tumor mass longitudinal spaces, and the nuclei of the tumor cells are characterized by what is called chromatin granularity or clearing, indicating high proliferative state of the cells.⁷

Table 1: Clinicopathological parameters among Iraqi women patients for each group of the study

Variable	Group I (New diagnosis) (No. = 20)	Group II (Relapsed) (No. =20)	Group III (Resistant) (No. =20)
Patients age	56 ± 16.4 a	51 ± 8.8 b	41 ± 13.8 c
Grade			
I	4 ± 6.3a	2 ± 6.3b	2 ± 6.3c
II	9 ± 3.3a	8 ± 3.3b	8 ± 3.3c
III	7 ± 2.1a	10 ± 2.1b	10 ± 2.1c
Tumor size			
T1	4	2	-
T2	6	5	13
T3	10	13	7
Histologic type			
IDS	9	5	10
ILC	11	15	10
Clinical stage			
I	10	7	1
II	7	4	7
III	3	9	12

Data are Mean ± SD; different letters mean significant difference at p ≤ 0.05

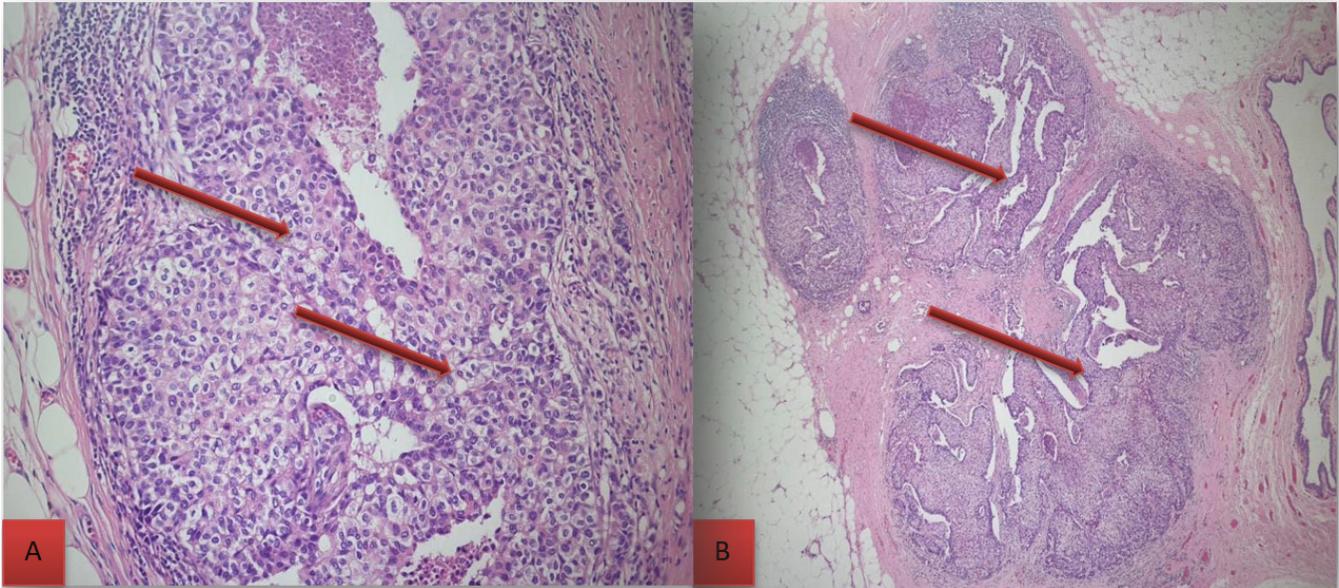


Figure 1: Histological sections for H&E stained breast tissue of group I (newly diagnosed patients) cancer cells Looks like micro papillary breast carcinoma like to be EMA (inverted polarity) Ductal carcinoma NST in DDX (Arrows), section shows signs of hemorrhage, hyperplasia and of chromatin condensation inside the cell's nucleus. A) 400X, B) 100X.

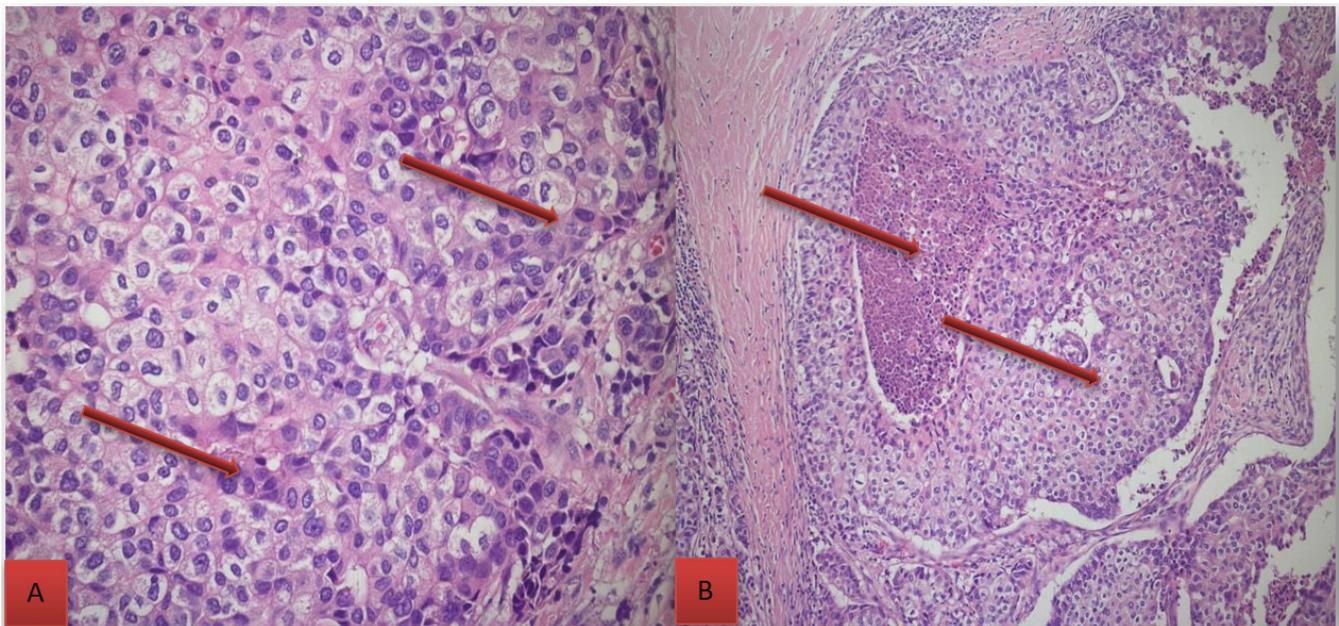


Figure 2: Histological sections Histological sections for H&E stained breast tissue of group II (relapsed patients) are all invasive ductal carcinoma. No DCIS, intraductal and invasive carcinoma with Glycogen rich. A) 400X, B) 100X.

Measurement of immunohistochemical expression of the selected specific markers within the tumor tissue (CD24 marker).

In this study, all slides collected from the three groups of patients were stained immunohistochemical (IHC), and the expression of the CD24 marker was detected and compared for the three groups of patients, and then microscopically examined. For each sectioned slide, the proportion of each CD marker was measured in five fields. The brownish color of positive tumor cells stained with DAB stain indicates the presence of CD24 marker, whereas the deep or light brownish

color indicates the approximate strength or percentage of marker expression within the IHC stained tumor mass slides. The percentage of accurately expressed cancer stem cells within the tumor mass was calculated by counting the positive CD24 marker cells and then comparing their numbers to the total tumor cells in the field to evaluate and calculate the percentage of each marker in all study groups. The American Society for Clinical Pathology (ASCP) has approved the procedure, which involves dividing the number of positive cells for the specified marker in five selected microscopic fields by the total number of positive and negative cells. Figures 4 and 5

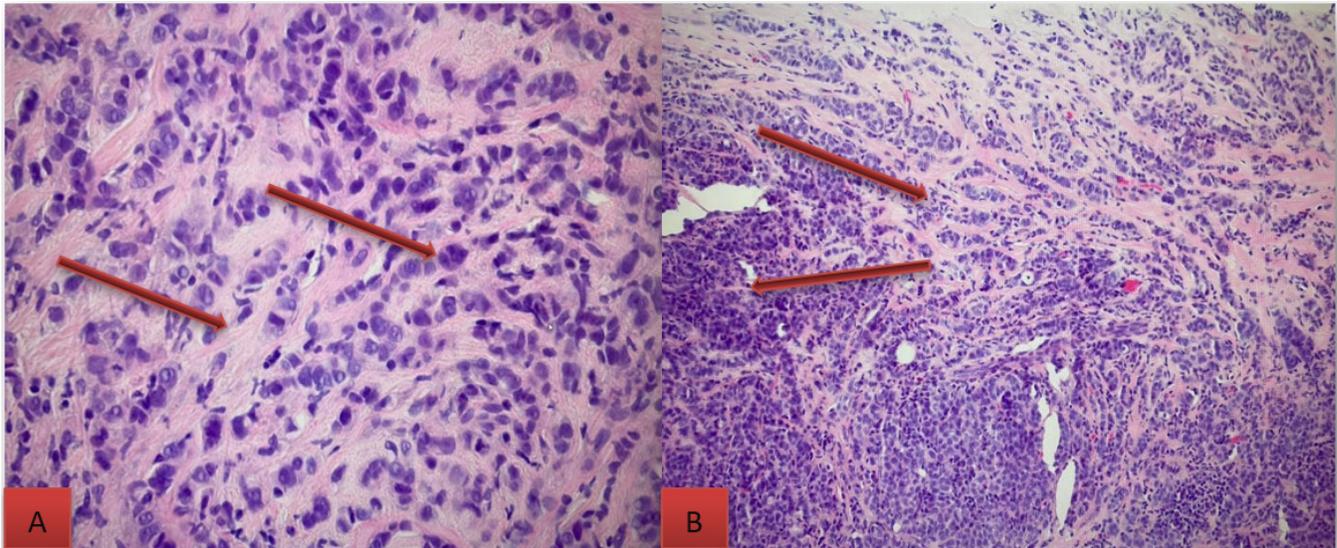


Figure 3: Histological sections for H&E stained breast tissue of group III (resistant patients), Invasive lobular carcinoma E cadherin to rule out NST carcinoma with lobular feature after a complete course of treatment with no response to the applied chemotherapy, (H&E, X400).

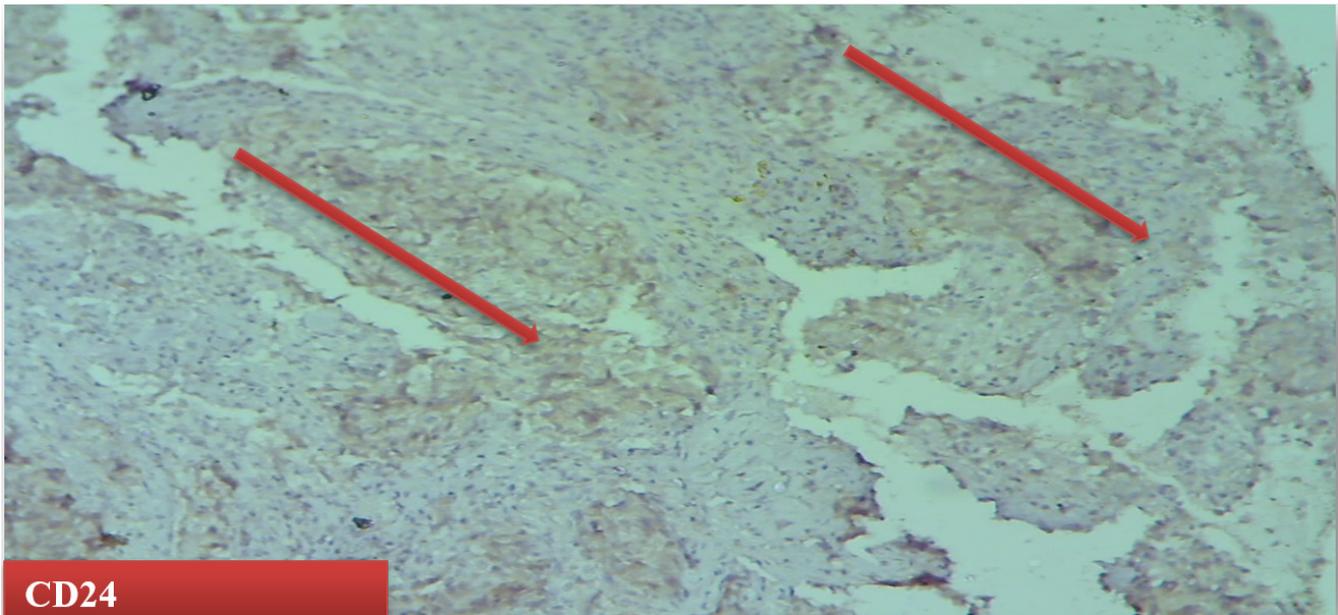


Figure 4: Immunohistochemical expression of each CD24 for the group I of newly diagnosed patients showing the positive expressed cancer stem cells with stained with the DABI stain as a brown color (arrows), and the negative cancer cells stained with the counter hematoxylin stain the violet to blue color (arrows), magnification power 400X.

show the results of CD24 marker. The positive CD24 cells within the tumor mass of Group I appear brownish colored, indicating their positivity to the CD24 marker and calculated as cancer stem cells within the tumor mass. In contrast, the negative CD24 cells appear deep blue to violet color, indicating their reactivity to each measured marker and calculated as cancer stem cells within the tumor mass.

Results show that the highest expression of both CD24 marker was recorded in Group III (resistance patients) who had no response to chemotherapy as recorded after following up patients history (Figure 6).

Similar results were observed by were demonstrated that the putative stem cell marker CD24 was significantly associated

with worse survival based on the obtained data. In particular, CD24 may play a role in tumor genesis and cancer progression. However, further large-scale studies are needed to confirm these findings.^{8,9}

Percentage of IHC Expression of CD24 Marker Upon Cell Scoring

The percentages of positivity for each CD within each group are shown in Figure 7. The results show significant differences between groups. Group I (newly diagnosed patients) demonstrates a mild to moderate expression to CD 24 compared to other groups, while the highest expressed CD markers shown in group III was compared to the other groups. Group II (relapsed patients) reveals higher expression of CD

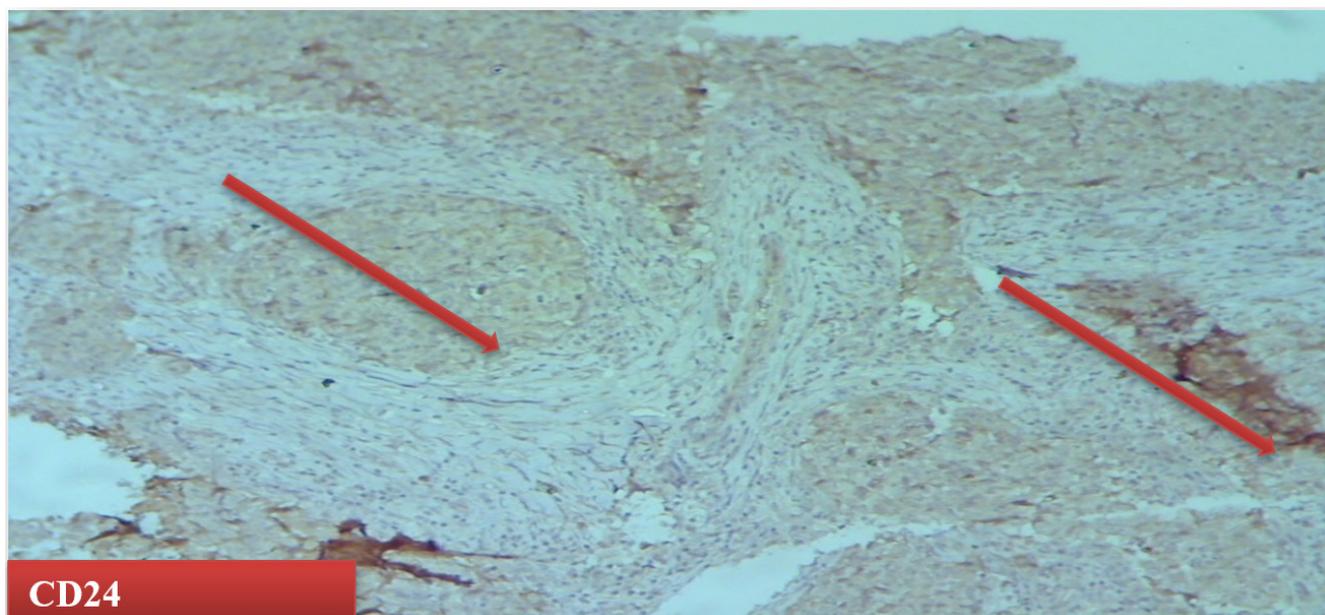


Figure 5: Immunohistochemical expression of each CD24 for the group II of relapsed patients showing the positive moderately high expressed cancer stem cells within the breast tumor mass which stained with the deep DABI stain as a brown color (arrows), and the negative cancer cells stained with the counter hematoxylin stain the violet to blue color (arrows), magnification power 400X.

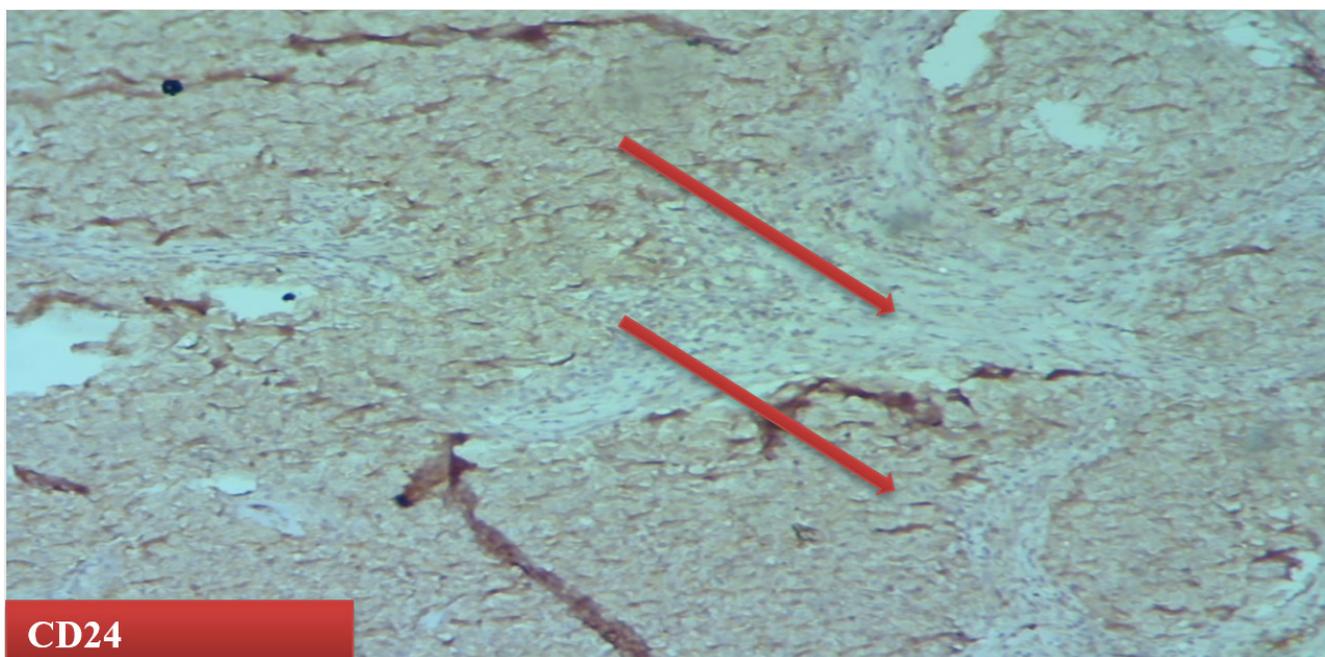


Figure 6: Immunohistochemical expression of each CD24 for the group III of resistance patients showing the positive high expressed cancer stem cells with stained with the deep DABI stain as a brown color (arrows), and the negative cancer cells stained with the counter hematoxylin stain the violet to blue color (arrows), magnification power 400X.

markers than the newly diagnosed group, with a significant difference among groups. After pioneering studies,¹⁰ who showed the in vitro study in human breast cancer line for CD24 marker cells were recognized as cancer stem cells marker for basal/mesenchymal cell lines MCF 7 and MDA-MB-231. This was subsequently supported by other researches¹¹ that these cells are rich in a multiple genes involved in cancer invasion and resistance to chemotherapy. Later on, a large body of evidence demonstrated that this phenotypic

character may not be expressed in all breast cancers, emphasizing the need to identify other breast CSC markers to discriminate these cells from other types of cells in the tumor tissue.

Breast cancer stem cells possess many transmembrane proteins that participate in the process of pumping the drugs outside of the cells, as well as these cells proliferate relatively slowly as compared to the surrounding breast cancer cells around the tissue. Breast cancer stem cells can resist

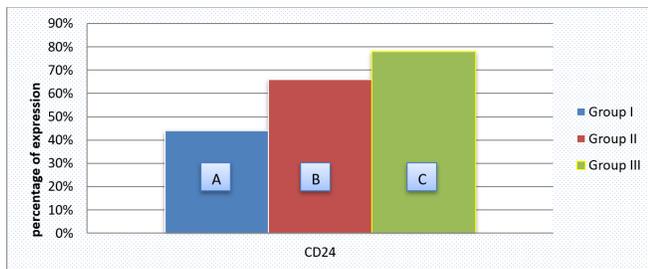


Figure 7: Percentage of immunohistochemical expression upon cell scoring data of CD24 positive cell for the groups I (newly diagnosed patients), II (relapsed patients) and III (resistant patients) showing significant differences between groups, (data are Mean \pm SD) Different letters mean significant differences between groups at ($p \leq 0.05$).

chemotherapy, causing tumor relapse down the regulation of their DNA repair mechanisms.

NF-kappa¹² showed that non-cell-autonomously regulates cancer stem cell populations in the basal-like breast cancer subtype. Nat Commun 4, 2299 The Breast Cancer Surveillance Consortium (BCSCs), in addition, express Oct4, a pluripotency marker in NSCs, a feat that allows these cells to be able to differentiate and create heterogeneous tumors. On the other hand, the phenomenon is also discovered in breast cancer. For example, specific human basal epithelial cells automatically dedifferentiate to stem-like cells. This kind of occurrence is much more increased in malignant tissue. Interleukin 6 (IL-6) has also been known to produce CSCs through non-tumorigenic cancer cells. This signifies a dynamic equilibrium between differentiated cells and CSCs. Chemotherapy and radiotherapy have been presented to enrich BCSCs, verifying the resistant nature of these cells according to the tumor bulk. This is also caused by activating self-renewal pathways Hedgehog, Notch and Wnt, have also been included in cell fate decisions. As example, Notch induces expression of surviving, which deregulates various cell cycle check points and inhibits radiation or drug-induced apoptosis. Also, the cycling D1 significantly governs cell survival and cell cycle regulator, which is a target of the Wnt and Notch and pathways. In addition, cycling D1 is usually required for differentiation and self-renewal of BCSCs and stimulates tumorigenicity. There's a direct relationship between cancer stage and BCSCs survival, metastasis, reduced and relapse survival. The BCSCs resistance to classic therapies requires therapeutics to straight target these cells. These kinds of therapies can target cell surface markers of BCSCs, affecting the significant signaling pathways or following the resistance mechanisms of drug onboard these cells.¹³ The recurrence of breast cancer is an important clinical manifestation and presents the major cause of deaths by breast cancer. Around 10% of patients having breast cancer will make isolated breast cancer recurrence following adjuvant treatment. Many researchers have attempted to estimate some sort of pattern concerning breast cancer recurrence. This has involved research in several breast cancer subtypes in which breast cancers are characterized by the existence of receptors including HER2/ErbB2 receptor (HER2), progesterone receptor, and estrogen receptor or through the absence of all

of them that known as triple-negative breast cancers. From sides, it is found that ER-negative breast cancers are related to higher risk of recurrence throughout first 5 years after diagnosis, when compared with ER-positive breast cancers. Then, the risk of recurrence frequently rises in ER-positive breast cancers for the upcoming 10 years, and during 15 years after diagnosis, the risk seems to become the same for both subtypes. For case of ductal carcinoma in situ, it is often considered that the ER-negative/PR-negative although HER2-positive cancers have a higher risk of recurrence when compared with ERpositive/PRpositive/HER2negative cancers. Besides the basic classification of breast cancers described recently, there are several sub-classifications of breast cancers, including HER2 enriched, basal, luminal A and luminal B. The presence of these kinds of subtypes, which often at time overlapping however, the majority of the time so specific presents a difficult task for selecting suitable therapy. This has resulted in the suggestion of customized therapy that meets individual patients' requirements.^{14,15} Usually, CD24 is extremely expressed in bladder cancers, breast cancers, renal cancers, prostate cancers, ovarian cancers, non-small cell carcinomas, and other human cancers. It's included in cell metastasis and adhesion. This signifies that CD-24 might be a considerable marker in tumor diagnosis and prognosis. Functionally, it can be an alternative ligand for P-selecting, where their interaction facilitates the passageway of tumor cells within the blood stream throughout metastasis. It raises the adhesion and proliferation of tumor cells to lamina, collagen, and fibronectin. CD24 metastatic correlations increase its significance as a prognostic factor and a new CSCs marker.¹⁶

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