Journal of Medical and Pharmaceutical Sciences
Issue (3), Volume 1
September 2017
ISSN: 2518-5780

المجلة العربية للعلوم و نشر الأبحاث
Arab Journal of Sciences & Research Publishing
AJSRP

Estimation of Some Breast Cancer Tumor Markers Using Flow cytometry

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Abstract: Objective: The aim of this study was to estimate some tumor markers such as caspase-3, caspase-8 and annexin-v levels in blood and tissue of patients with breast cancer and establishing the relation between these apoptotic markers level and response to chemotherapy. Materials and Methods: A number of 150 females were divided into four groups, malignant neoplasm of breast cancer included 50 females, that were diagnosed into stage I, II, III, and IV and included inflammatory breast carcinoma, infiltrating ductal carcinoma, infiltrating lobular carcinoma, ductal carcinoma in situ and lobular carcinoma in situ, control group included 50 females with normal breast, benign neoplasm of breast cancer included 25 cases that contained a heterogeneous group of lesions including developmental abnormalities, epithelial and stromal proliferations and neoplasms and follow up group (after chemotherapy) include 25 cases that her surgery was done and taken chemotherapy. Flow cytometry was used to analyze cell cycle, caspase-3, caspase-8 and annexin-v among the study groups. **Results:** The results showed that caspase 3 and caspases-8 expressions were significantly decreased in malignant group compared with the control group (P = 0.0028 and P =0.008), respectively; caspase 3 and caspase-8 levels decreased significantly in malignant group compared with the follow up group (P =0.001and P =0.0105), respectively; caspase 3 and caspases-8 levels were decreased in benign group compared with the control group (P = 0.041and P =0.512). Annexin-v was highly expressed in both tissues and blood of malignant groups in viable stage than benign compared with the normal groups (P = 0.01 and P = 0.006) respectively. Annexin-v level was highly expressed in both tissues and blood of malignant groups in necrotic stage compared with the control and follow up groups (P = 0.0001 and P = 0.01). But in early and late apoptotic phase follow up group annexin-v level was significantly increased more than the malignant group with (P=0.0001 and P=0.0026) respectively. Sub G1 apoptosis level in malignant and benign group significantly decreased compared with the follow up group (P = 0.0001 and P = 0.003) respectively. Our data showed that S phase level was significantly increased among malignant group compared with the control and follow up groups (P = 0.0001), also S phase level was increased significantly among benign group more than the control and follow up groups (P= 0.9 and P=0.003) respectively; and S phase was significantly decreased in benign group compared with the malignant blood and tissue groups (P = 0.03 and P=0.05) respectively. **Conclusion:** Annexin-v, caspase-3 and caspase-8 level in blood and tissue may serve as markers for the diagnosis of breast cancer and the response to chemotherapy. Also, S phase of cell cycle is one of the significant factors in the development of breast cancer.

Keywords *Annexin-v, apoptosis, caspases, cell cycle, breast cancer*

1. Introduction:

Breast carcinoma is the most common type of cancer among women worldwide and accounts for the second highest cause of deaths related to cancer. Breast carcinoma is a risky disease but it could be very simple to treat if discovered in an early stage [1, 2]. The only way to decrease mortality and morbidity from breast carcinoma is to detect the disease before appearance of symptoms on the patient [3]. Caspases are a family of protease enzymes having lesions important roles in programmed cell death. They are the very important players in the initiation and execution of apoptosis. So it is reasonable to believe that decrease levels of caspases or impairment in caspase function may result in a decreased in apoptosis and cancer. By activation of initiator caspases, they produce a chain reaction and activating several other executioner caspases.

Executioner caspases degrade over 600 cellular components in order to produce apoptotic morphological changes [4, 5]. Caspase-8 is an important regulator of apoptosis, and an essential defense mechanism against hyper-proliferation and carcinogenesis [6]. Active caspase 8 can activate some effector caspases, as caspases 3 and 7 to carry out apoptosis [78 9] .The most important effector caspases is caspases-3 that plays an essential role in both the death receptor pathways that initiated by caspase-8, and the mitochondrial pathway involving caspase-9 [10, 11]. Caspases-3 cleaves a lot of cellular substrates including structural proteins and DNA repair enzymes. It also activates an endonuclease caspase activated DNAse, that leads to DNA fragmentation which is the most important feature of apoptosis [12, 13].

Occurrence of phosphatidylserine (PS) on the extracellular side of the plasma membrane is very important indicator of apoptotic cell transformation [14, 15]. Annexin-v is phospholipid binding protein with high affinity for PS in presence of calcium. Hence, this protein can be used as a sensitive indicator for PS exposure upon the cell membrane [16]. Flow cytometry is a widely used technique to detect cell cycle distributions, in which the DNA content of individual cells is quantified by its staining with fluorophores like propodeum iodide (PI) that is able to bind and label DNA and confirms that the cell is undergoing apoptosis and not necrosis [17]. DNA content of cells in G1 phase will give a specific signal, which increases during S phase and has a multiplied intensity after complete of S phase. Cells with doubled DNA content referred to the G2/M phase including both the G2 and the much shorter M phase [18].

2. Materials and Methods:

Patients Blood and tissue samples were collected from 150 females divided on the following groups: group A: malignant neoplasm of breast cancer includes 50 females, the mean age at diagnosis ± SD was (55.08 ± 10.6 years), this group was classified by the histopathology into four subgroups (stage I, II, III, and IV). Group B: benign neoplasm of breast cancer: analysis performed on 25 blood samples and 25 tissue samples from patients with age mean± SD (41 ± 14.1 years). Group C: normal control this group included: 50 females with normal breast, the age at diagnosis was mean± SD (55.08 ± 10.6 years) and served as

control. Group D: follow up analysis performed on 25 blood samples after chemotherapy. Tissue sections were obtained at the time of surgery from the tumor and normal part of the breast tissue and immediately stored in freezer.

Preparation of the samples for flow cytometry:

After collecting blood samples in EDTA tubes diluted 1:1 with phosphate buffer saline (PBS). 2ml ficoll added in a falcon tube then diluted blood carefully added over a ficoll. Centrifuged at 2000 rpm for 20 min. The upper layer (plasma) discarded using a clean pasteur pipette leaving the lymphocyte layer undisturbed at the interface then lymphocyte layer transferred to a clean centrifuge tube using a clean pasteur pipette after that 2 ml phosphate buffer solution added to the lymphocyte in the tube and the cells suspended. Centrifuged at 2000 rpm for10 min then the supernatant discarded and 2ml ethanol added into the pellet then kept in the refrigerator while working on them. Single cell suspensions from tissue sections are required for optimal staining of samples and the narrow bores of the sample injection needle and tubing on a flow cytometer will be easily clogged by aggregated cells and debris. Cell suspension was prepared by pressing with the plunger of a 3 ml syringe (or mashed between two glass slides) using phosphate buffer saline into petri dishes. Cell suspension passed through a cell strainer (or through the piece of nylon mesh) to eliminate clumps and debris then collected cell suspension was centrifuged at 2000 rpm for 10 min and supernatant discarded. 3ml PBS was added into the pellet then mixed well.

Flow cytometry:

Cell suspension from peripheral blood and tissue is prepared. The suspension of cells then mixed with fluorochrome labeled antibodies. Inside a flow cytometer cells in suspension are dragged into a stream created by a surrounding sheath of isotonic fluid that generates laminar flow allowing the cells to pass individually through an interrogation point. At this point a beam of monochromatic light, usually from a laser passes through the cells. Emitted light is stepping out in all directions and is collected through optics that direct the light to a group of filters and dichromic mirrors that isolate particular wavelength bands. The light signals are regulated by photomultiplier tubes and digitized for analysis by computer. The resulting data usually is displayed in histogram or two dimensional dot plot formats [19-21].

Assessment of caspase-3 activity by flowcytometry:

100 µl of sample added with 1ml cold PBS in centrifuge tube and leaved for 10 min at 37°C for fixation after that centrifuged for 5 min and the supernatant discarded. Cells stained with 5 µl anti caspase-3 antibody and incubated in the dark for 15 min at RT (25°C).After incubation cells washed and fixed with paraformaldehyde and intensity of the cleaved caspase-3 was measured by flow cytometry [22].

Assessment of caspase-8 activity by flowcytometry:

100 µl of sample were added with 1ml cold PBS in centrifuge tube then cell suspension centrifuged for 5 min at 2000 rpm and supernatant discarded. Cells stained with 10 µl anti-caspase-8

antibody and incubated for 30 min at RT in the dark. After incubation cells washed again with 1ml PBS to remove any unbound antibody .Centrifuged at 2000 rpm for 5 min and supernatant discarded then 10 µl immunoglobulin G (IgG) added for cell fixation and incubated again for 30 min at RT in the dark, after incubation cells washed again with 1ml PBS. Cell suspension centrifuged at 2000 rpm for 5 min and supernatant discarded and fixed with 250 µl paraformaldehyde and caspase-8 activity was measured by flowcytomer [23].

Annexin-v-fitc binding assay:

Working solution of propodeum iodide (PI) were prepared by diluting 5 µl of the 1 mg/ml PI stock solution in 45 µl 1X annexin binding buffer.100 µl of sample were added with 1ml cold PBS in centrifuge tube then centrifuged at 2000 rpm for 5 min and supernatant discarded. 100 µl annexin binding buffer were added to re-suspend the cells. Cells were stained with 5 µl PI (propodeum iodide) and 5 µl FITC-annexin-v (annexin-v combine with fluorescein isothiocyanate). Then the cells were incubated at room temperature for 15 min in the dark. After that, 400 µl 1X annexin binding buffer added and mixed gently. Then the stained cells were analyzed by flow cytometry. Calculated apoptotic cells were based on the percentage of annexin-v-positive cells. Cells with annexin-v-positive/PI-negative were considered as early apoptotic cells, whereas annexin-v-positive/PI-positive cells were considered as late apoptotic cells [24, 25].

DNA Content and cell cycle analysis:

Prepared cells suspension was centrifuged for 5 min at 300 rpm and supernatant decant thoroughly. The pellet was suspended in 5 ml of PBS and centrifuged at 300 rpm for 5 min. Pellet was suspended in 1 ml of PI staining solution and kept in the dark at room temperature for 30 min, or at 37°C for 10 min. The samples were put in the flow cytometer and measured the cell fluorescence. Maximum of PI excitation bound to DNA is at 536 nm, and emission at 617 nm [26, 27].

Statistical analyses:

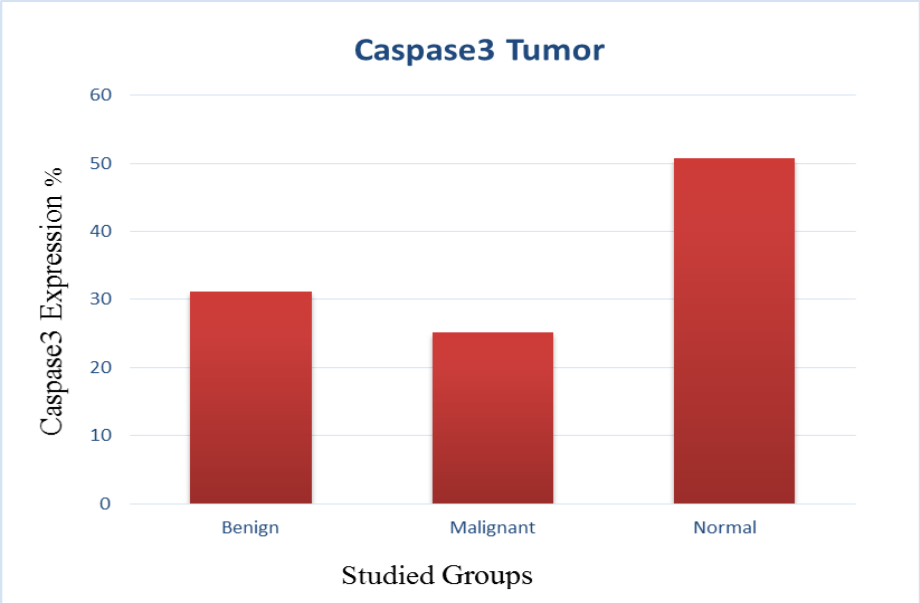
Mean values and standard deviation were calculated according to conventional methods. The data was collected and analyzed using statistical package for social sciences (SPSS). Level of Significance of all above mentioned statistical tests done, significance was fixed at 5% level (p value). P value > 0.05 indicates non-significant result. P value ≤ 0.05 indicates a significant result.

3. Results:

Caspase-3 activity in apoptotic pathway:

Results explained through figures (1, 2 and 3) showed that caspase-3 expression was decreased in both malignant tissue and blood groups with Mean± SD (25.62 ± 34.21) and (21.7±21.6) respectively compared with its level in normal control group and follow up groups with Mean± SD (50.7 ± 17.46) and (57.93 ± 24.02) respectively. Caspase-3 expression was decreased in benign blood and tissue groups Mean± SD (28.58 ± 12.31) and (31.23 ± 3.96) than in control and follow up groups Mean± SD (50.7 ± 17.46) and (57.93 ± 24.02) respectively. Caspase-3 level was increased in blood and tissues of benign

group with Mean± SD (28.58 ± 12.31) and (31.23 ± 3.96) than in malignant group with Mean± SD (21.7 ± 21.6) and (25.62 ± 34.21) respectively.



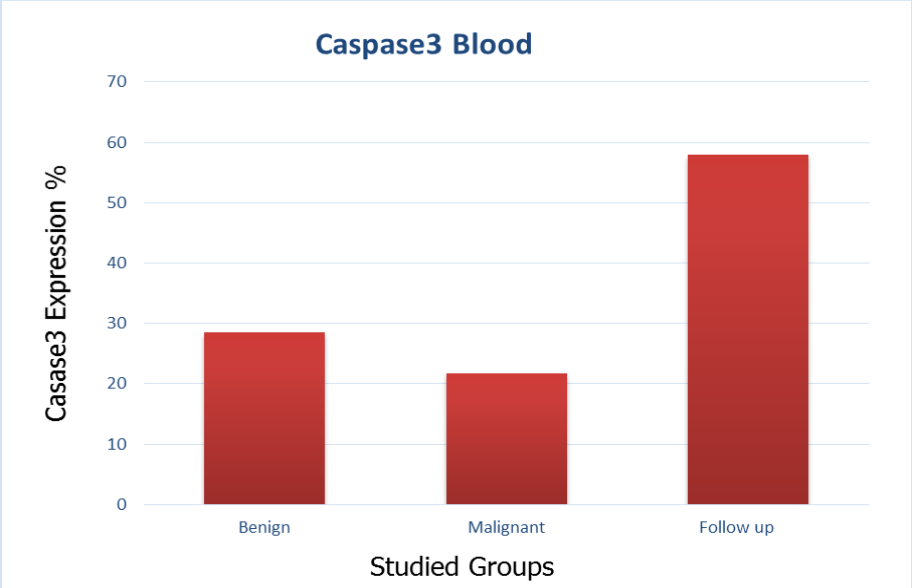
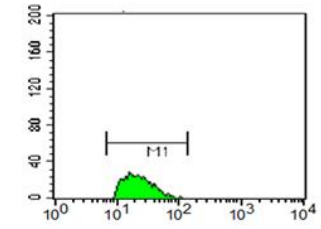
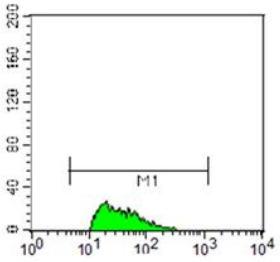


Figure 1: Caspase-3 activity in tissue groups

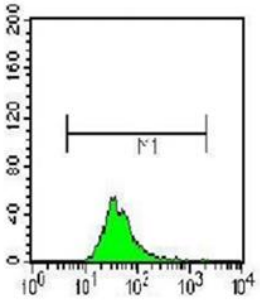
Figure 2: Caspase-3 activity in blood groups



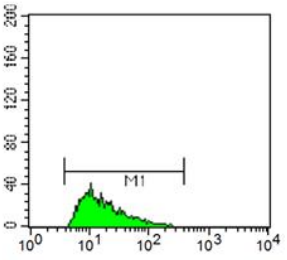
A: Active caspase3 malignant tumor cells.



B: Active caspase-3 malignant blood cell



C: Active caspase-3 normal cell



D: Active caspase-3 benign tumor cell.

Figure 3: Flow cytometric analysis of apoptotic cells for caspase-3 activity A and B: showing lower caspase-3 expression in both blood and tumor malignant cells than normal and benign cells (C and D) respectively.

Caspase-8 activity in apoptotic pathway:

Our results in figures (4, 5 and 6) explained that caspase-8 expression was decreased in blood and tissues among malignant groups with Mean± SD (28.62 ± 6.52) and (22.41 ± 11.25) than its level in control and follow up groups with Mean± SD (55.1 ± 9.11) and (70.05 ± 5.41) respectively, also concentration of caspase-8 decreased in blood and tissue benign groups with Mean± SD (39.48 ± 10.72) and (37.4 ± 7.23) than levels in control and follow up groups with Mean± SD (55.1 ± 9.11) and (70.05 ± 5.41) respectively. But level of caspase-8 increased in both blood and tissue benign groups with Mean± SD (39.48 ±10.72) and (37.4 ± 7.23) than its level in malignant groups with Mean± SD (28.62 ± 6.52) and (22.41 ± 11.25) respectively.

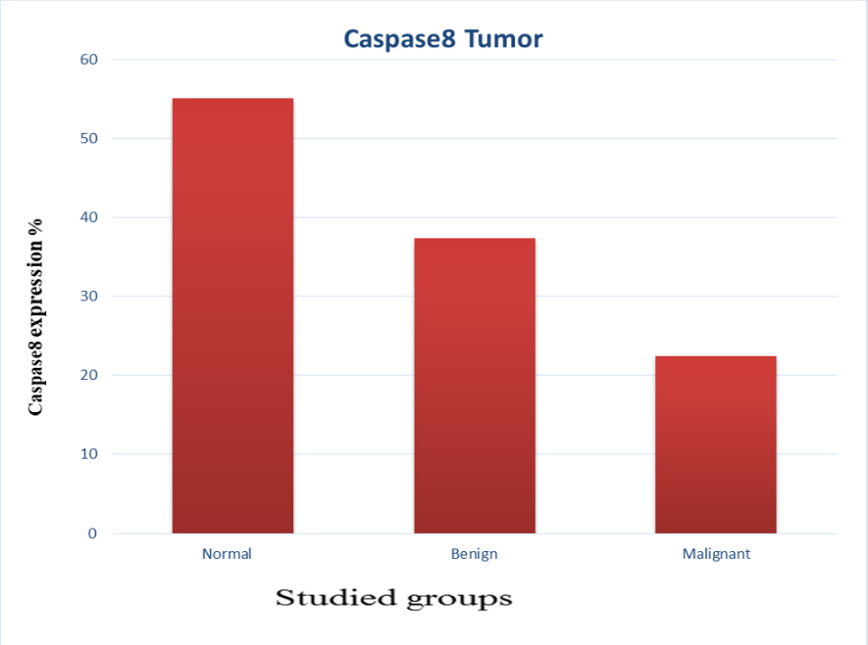


Figure 4: Caspase-8 activity in all tissue groups.

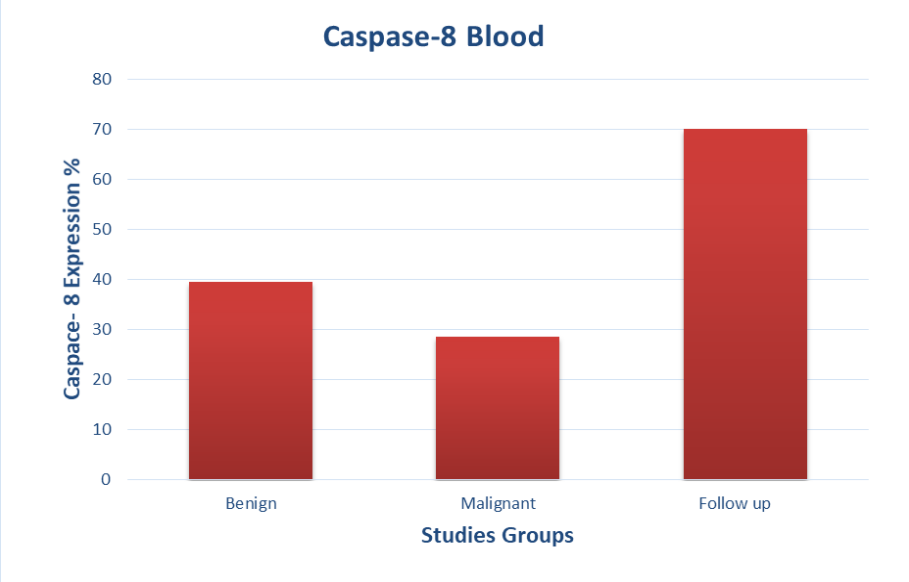


Figure 5: Caspase-8 activity of all blood groups.

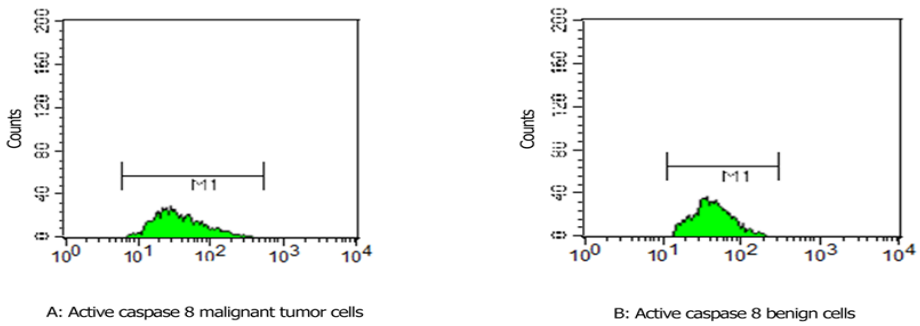


Figure 6: Flow cytometric analysis of apoptotic cells for caspase-8 activity. Apoptotic cells count in malignant breast cells (A) was lower than that in benign breast tumor (B).

Detection of apoptosis by annexin-v-fitc binding assay:

Results in figure (7, 8 and 9) showed that annexin-v found to be increased in blood and tissue samples of malignant group in necrotic stage with Mean± SD (3.91 ± 2.29) and (3.70 ± 3.67) respectively, more than the control and follow up groups with Mean± SD (1.42 ± 0.31) and (1.22 ± 0.62) respectively, also an increase in viable stage for malignant group in both blood and tissue was observed with Mean± SD(71.268 ± 34.49) and (74.3 ± 47.11) respectively , more than the control and follow up groups with Mean± SD (45.94 ± 14.07) and (24.65 ± 4.28.9) respectively. On the other hand, annexin-v was decreased through late apoptosis in malignant group in blood and tissue with Mean± SD (10.57 ± 9.33) and (10.12 ± 2.76) than in control and follow up groups with Mean± SD (12.16 ± 2.18) and (30.51 ± 25.5), and also decreased in malignant groups in early apoptosis with Mean± SD (16.23± 31.13) and (19.26 ± 13.1) compared with the control and follow up groups, Mean± SD (40.16 ± 21.50) and (45.63 ± 2.45) respectively.

Annexin-v is highly expressed in blood and tissue malignant groups with Mean± SD (3.70 ± 3.67) and (3.91 ± 2.29) for necrotic stage than in both benign groups with Mean± SD (0.81 ± 0.05) and (0.06 ± 0.0280), also increased in viable stage for malignant blood and tissue groups with Mean± SD (74.3 ± 47.11) and (71.268 ± 34.49) than in both blood and tissue benign groups with Mean± SD (50.03 ± 9.97) and (53.22 ± 5.48) respectively. Annexin-v expression increased in blood and tissue malignant groups with Mean± SD (10.57 ± 9.33) and (10.12 ± 2.76) than in benign blood and tissue groups through late apoptosis with Mean± SD (7.92 ± 0.24) and (3.85 ± 0.9) respectively. But expression of annexin-v decreased through early apoptosis in blood and tissue malignant groups with Mean± SD (19.26 ± 13.1) and (16.23 ± 31.13) than in benign blood and tissue groups with Mean± SD (28.4 ± 4.5) and (28.91 ± 9.36) respectively.

Annexin-v more expressed in control and follow up groups with Mean± SD (1.42 ± 0.31) and (1.22 ± 0.62) than both blood and tissue benign groups with Mean± SD (0.06 ± 0.028) and (0.81 ± 0.05) for necrotic stage, also increased in late apoptotic stage for control and follow up groups with Mean± SD (12.16 ± 2.18) and (30.51 ± 25.5) than in both benign groups with Mean± SD(7.92± 0.24) and (3.85 ± 0.9) respectively, and also increased through early apoptosis in control and follow up groups with Mean± SD (40.16 ± 21.50)and (45.63 ± 2.45) than in blood and tissue benign with Mean± SD(28.4 ± 4.5)and (28.91 ± 9.36). On the other hand, annexin-v decreased in viable stage for control and follow up groups with Mean± SD (45.94 ± 14.07) and (24.65 ± 4.28.9) than in benign blood and tissue groups with Mean± SD (53.22 ± 5.48) and (50.03 ± 9.97) respectively. So annexin-v binding of PS can be used as a reliable marker for detecting of stages of apoptosis on blood and tissue breast cancer samples.

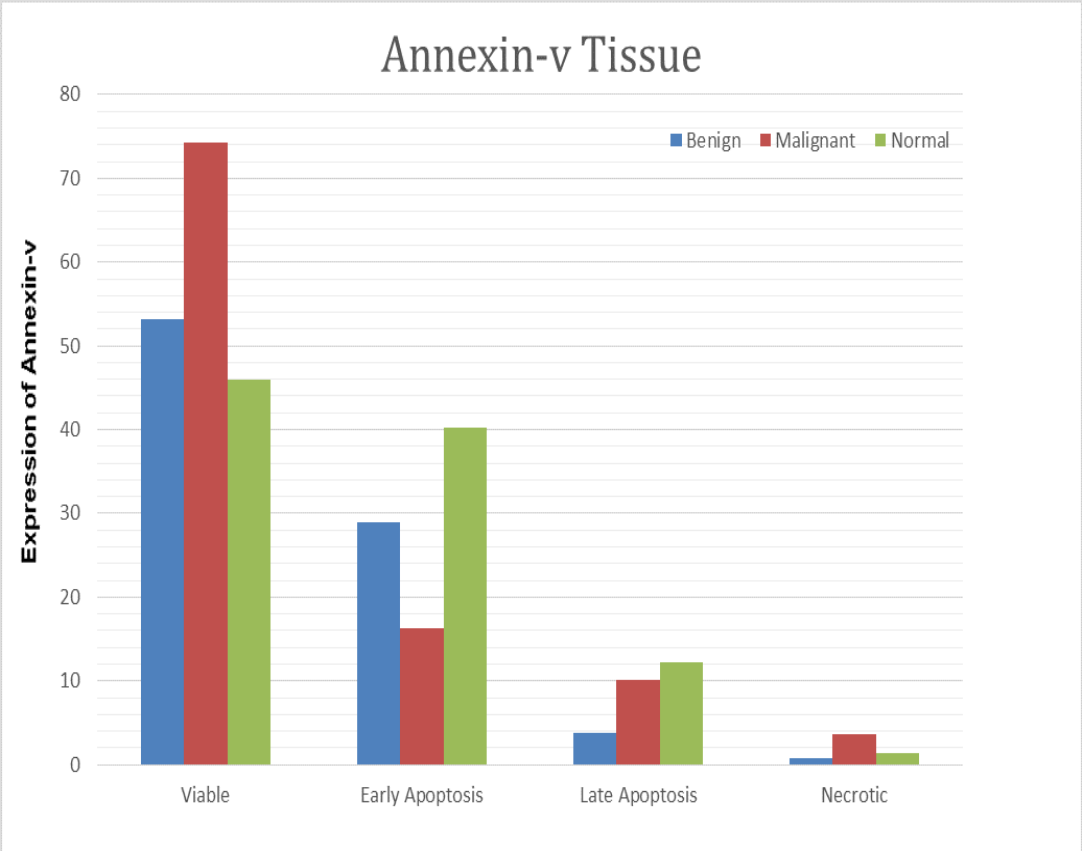


Figure 7: Annexin-v expression for quantitative measurements of PS exposure on malignant group vs. control and benign tissue groups.

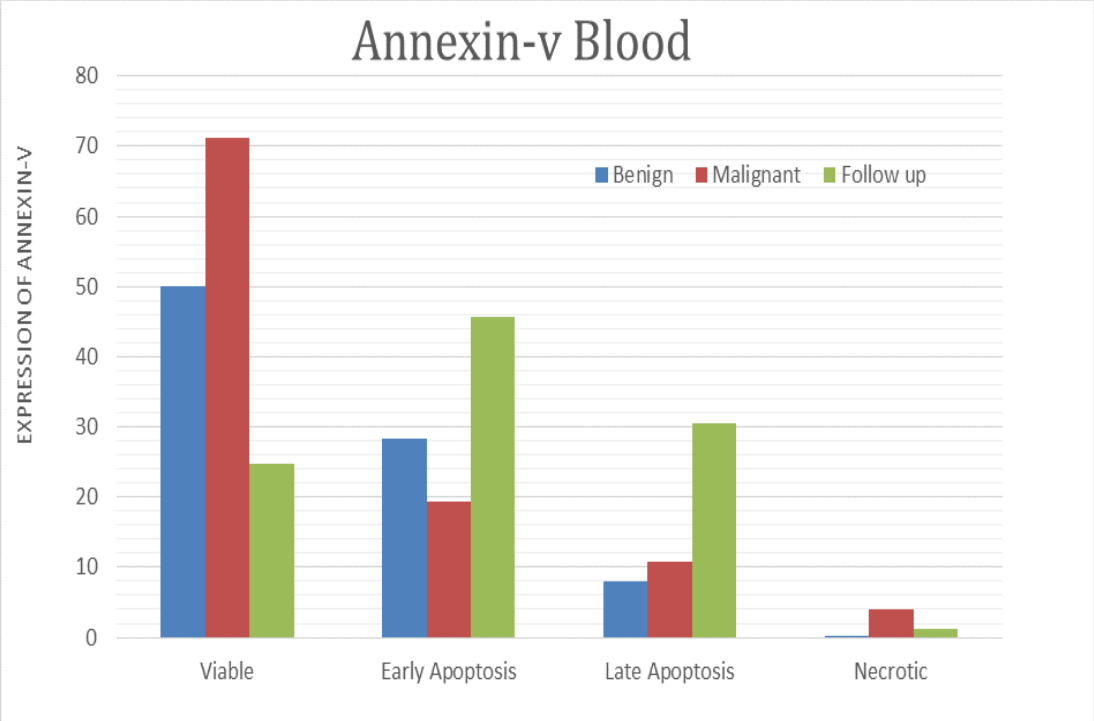


Figure 8: Annexin-v expression for quantitative measurements of PS exposure on all blood groups.

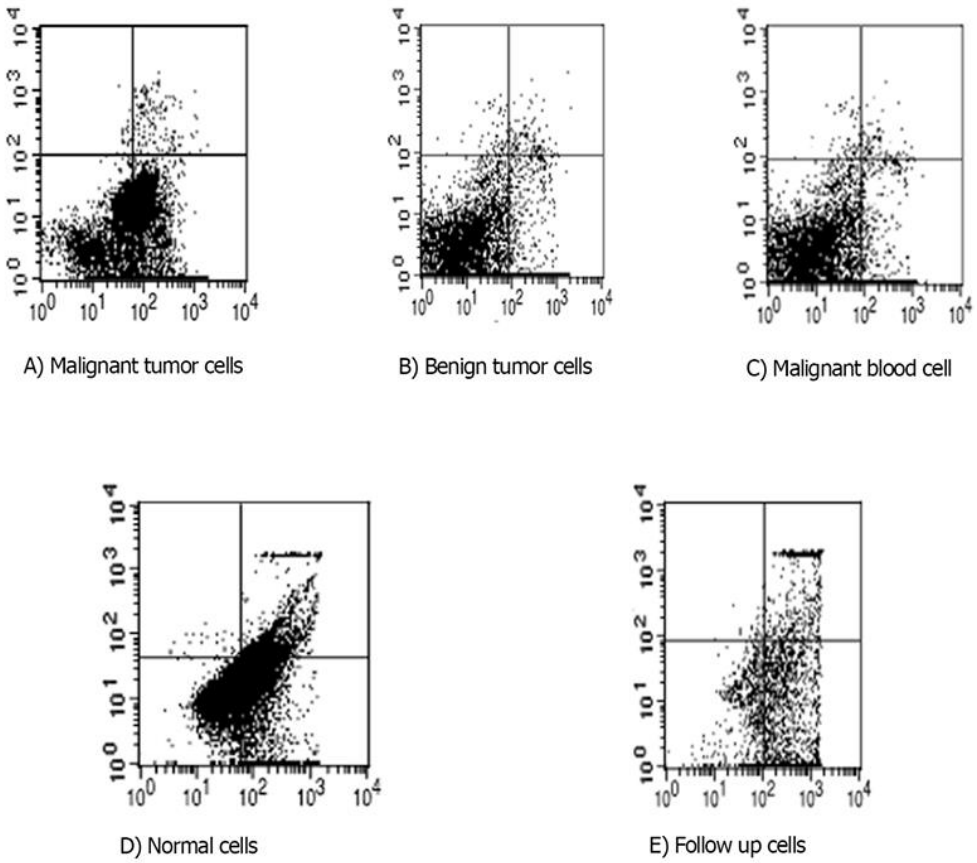


Figure 9: Flow cytometric expression of annexin-v in human breast cells A and C: showing the high annexin-v expression in viable (LL) and necrotic phase (UL) malignant breast cells B: showing the high annexin-v expression in early apoptotic (LR) benign breast tumor cells D: showing the high annexin-v expression in late apoptotic (UR) normal breast cells E: showing the high annexin-v expression in late apoptotic (UR) and early apoptotic (LR) follow up breast cells.

DNA content and cell cycle analysis:

Figure (10, 11 and 12) showed that Sub G1 apoptosis level decreased significantly in blood and tissues of malignant groups with Mean± SD (26.6 ± 14.3) and (27.68 ± 14.3) respectively compared with the control and follow up groups with Mean± SD (56.9 ± 6.1) and (59.9 ± 3.7) respectively. Also G0/G1 phase level decreased significantly in malignant blood and tissue groups with Mean± SD (25.04 ± 11.3) and (24.1 ±13.7) than control and follow up groups with Mean± SD (38.15 ± 22.1) and (39.6 ±5.65). But S phase and G2/M levels significantly increased in blood and tissues of malignant groups with Mean± SD (27.33 ±11.17&12.15 ±5.89) and (31.97 ±19.24 &10.37±6.82) respectively more than its levels among

control and follow up groups with Mean± SD (12.32 ± 6.91& 3.54 ± 1.8) and (1.48 ± 0.37&0.86 ± 0.52) respectively. Sub G1 apoptosis increased significantly in blood and tissues of benign groups with Mean± SD (43.3 ± 5.9) and (45.7 ± 5.4) compared with both malignant groups with Mean± SD (26.6 ± 14.3) and (27.68 ± 14.3). Also G0/G1 phase level increased with Mean± SD (30.2± 1.8) and (42.3 ± 10.3) in both blood and tissues of benign groups compared with malignant group blood and tissue with Mean± SD (25.04 ± 11.3) and (24.1 ± 13.7) respectively.

But S phase level increased significantly in malignant group blood and tissues with Mean± SD (27.33 ± 11.17) and (31.97 ± 19.24) more than its levels in both blood and tissues of benign groups with Mean± SD (12.57 ± 5.7) and (12.61 ± 4.98), also G2/M phase increased in blood and tissues of malignant groups with Mean± SD (12.15 ± 5.89) and (10.37 ± 6.82) than in benign blood and tissue groups with Mean± SD (3.72 ± 0.23) and (5.67 ± 1.54) respectively. In control and follow up groups Sub G1 phase of apoptosis level increased with Mean± SD (56.9 ± 6.1) and (59.9 ± 3.7) than in benign blood and tissue groups with Mean± SD (43.3 ± 5.9) and (45.7 ± 5.4), but G0/G1 phase level increased in benign group with Mean± SD (42.3 ± 10.3) than control group with Mean± SD (38.15 ± 22.1). In G2/M phase benign blood and tissue groups increased with Mean± SD (3.72 ± 0.23) and (5.67 ± 1.54) than in control and follow up groups with Mean± SD (3.54 ± 1.8) and (0.86 ± 0.52).Where S phase increased in blood and tissue benign groups with Mean± SD (12.57 ± 5.7) and (12.61 ± 4.98) than in control and follow up groups with Mean± SD (12.32 ± 6.91) and (1.48 ± 0.37).

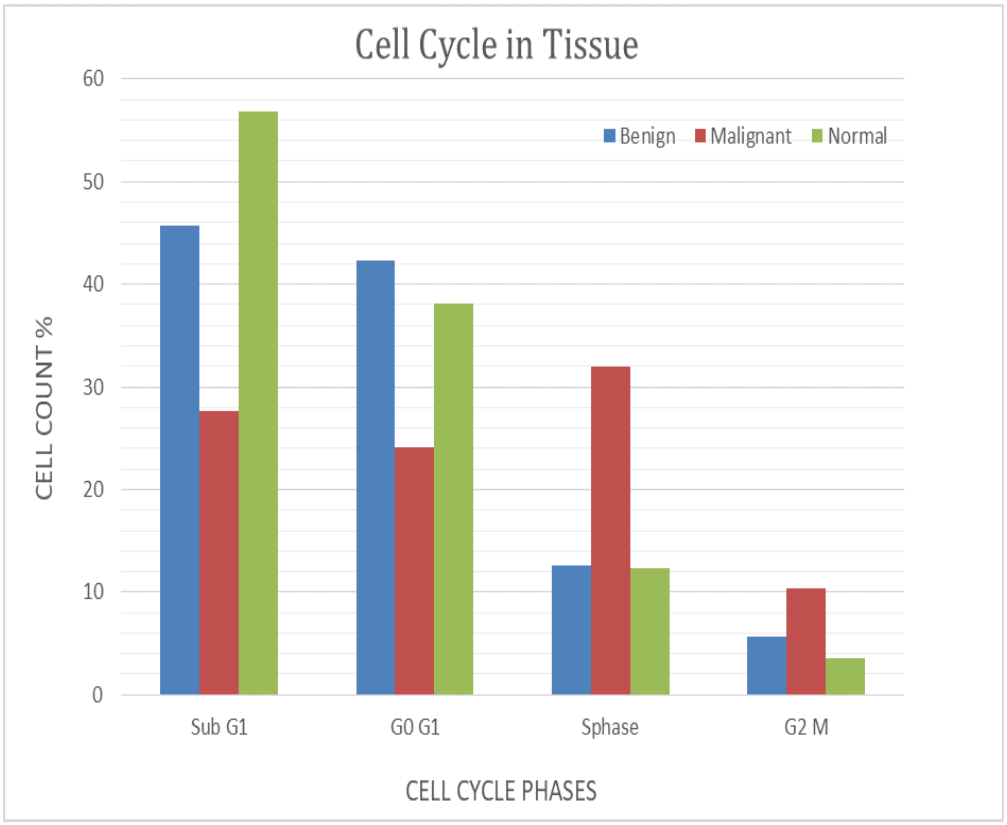


Figure 10: Cell cycle phases in propodeum iodide stained cells for all tissue groups.

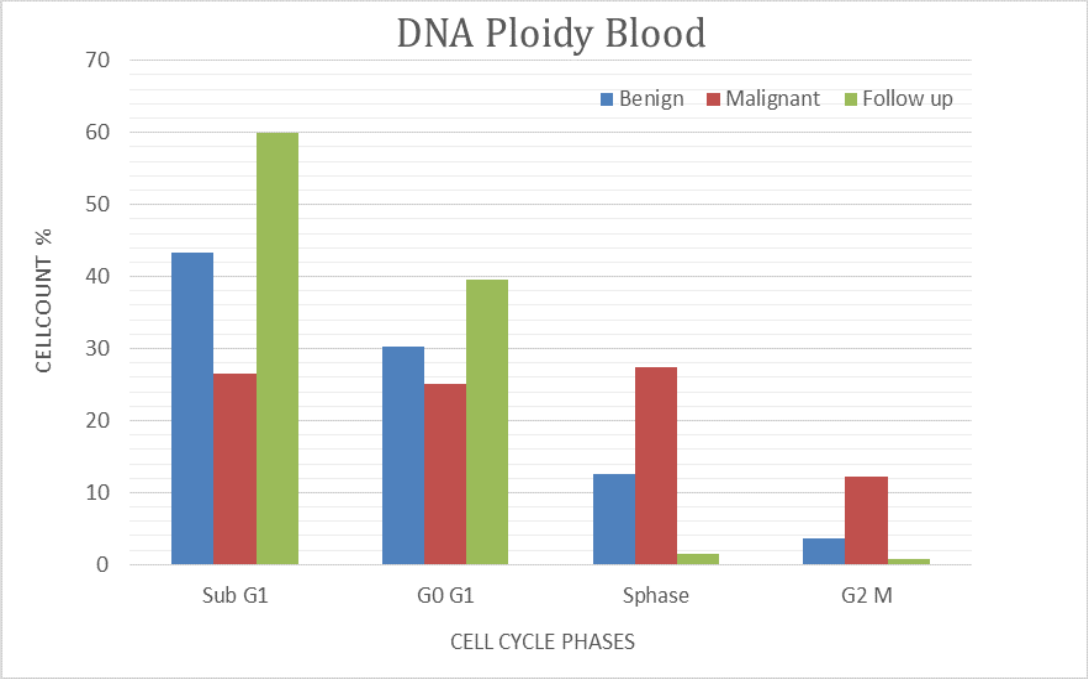


Figure 11: Cell cycle phases in propodeum iodide stained cells for all blood groups.

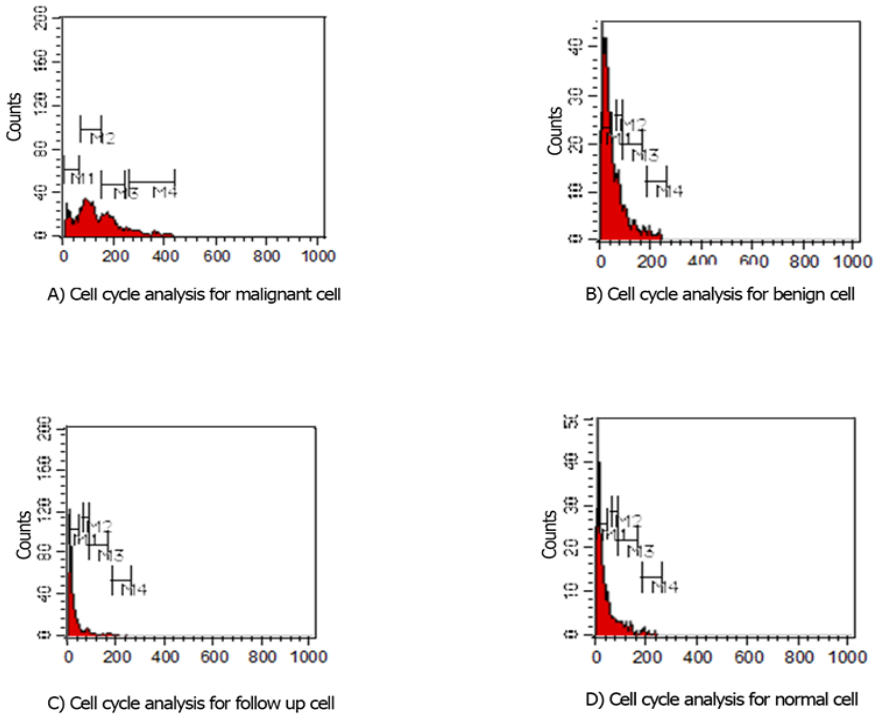


Figure 12: Flow cytometric analysis of cell cycle phases. A: malignant breast cells showing that higher S phase and lower Sub GI phase than (B, C and D). Control and follow up cells (C and D) showing lower G2/M phase than (A and B) malignant and benign cells.

4. Discussion:

Apoptosis is a very essential type of cell death with specific biochemical and genetic pathways that have an evident role in the development and homeostasis in normal tissues [28].Because an excess or loss of level of apoptosis is important factor of disease, so detection of apoptosis could contribute to focus pathological sites, study disease progression, support diagnosis and measure efficacy of therapy [29]. The most important feature of early apoptotic cell is an exposure of phosphatidyl serine (PS) on the outer layer of the plasma membrane, activation of caspases, DNA fragmentation and chromatin condensation, and cells in late apoptotic phase are characterized by shrinkage of the cell, fragmentation of the cell and apoptotic body formation.

Annexin-v is part of a protein family that connected to negatively charged phospholipids in presence of calcium so, detection of early apoptosis and cell death were carried out using annexin-v with propodeum iodide staining and flow cytometry [30]. Our findings showed that the level of annexin-v was increased more than other apoptotic parameters due to high proliferation of tumor cells than benign and normal cells as shown in Fig(7). Annexin-v expression was significantly increased in malignant group tissues and blood in the necrotic stage more than the control and follow up groups, also annexin-v expression was significantly increased in malignant group tissues and blood in viable stage more than the control and follow up groups. But annexin-v expression was significantly decreased in malignant group tissues and blood in early and late apoptosis compared with the control and follow up groups. Malignant blood and tissue groups were significantly increased in necrotic stages than in blood and tissue benign groups. Annexin-v was significantly high expressed in malignant blood and tissue groups for viable stages than in the blood and tissue benign groups. Annexin-v more significantly increased in blood and tissue malignant groups for late apoptosis than in blood and tissue benign groups.

On the other hand in early apoptotic stage annexin-v was highly significantly decreased in both blood and tissue malignant groups than in blood and tissue benign groups. In control group annexin-v highly significant increase in necrotic and late apoptotic stages than its expression in benign group, but annexin-v expression not significantly decreased in viable stage in control group than in benign group. Annexin-v expression was not significantly decreased in early apoptosis in benign group than in control group. Annexin-v was more expression not significantly in benign group through viable stage than follow up group. Expression of annexin-v decreased significantly in benign group through early apoptotic stage than follow up group, but expression of annexin-v decreased insignificantly in benign group through late apoptotic stage more than the follow up group. In necrotic stage annexin-v was significantly decreased in benign group more than in follow up group. These data agree with [31] who suggest accelerating cell turnover along the continuum of breast cancer and in the transition from normal epithelium to hyperplasia and from pre-invasive lesions to invasive carcinoma and the net growth of epithelial cells results from a growth imbalance in favor of proliferation. In the transition from hyperplasia to pre-invasive

lesions there is an imbalance in favor of apoptosis. Also after chemotherapy our results agree with [32] who showed that there was significantly increased in uptake of 99mTc-EC-annexin-v after chemotherapy and irradiation treatment. Indicate that apoptosis can be quantified using 99mTc-EC-annexin-v and that it is feasible to use 99mTc-EC-annexin-v to image tumor apoptosis.

Caspase-3 is very important effector caspases and it is familiar as good indicator for cells undergoing apoptosis in both normal tissue and tumor cells [33]. Caspase-3 expression was significantly decreased in malignant tissue group than in control group. Caspase-3 expression was not significantly decreased in both blood and tissue malignant groups than benign groups. On the other hand caspase-3 concentration increased significantly in follow up group than in malignant group. Also caspase-3 expression was significantly increased in control and follow up groups than in benign group. My study found that breast carcinoma contains low amount of active caspase-3 and these results found great concept with [34] which demonstrated that survivin regulates the G2/M phase of the cell cycle by associating with mitotic spindle microtubules, and it directly inhibits caspase-3 and caspase-7 activity, also survivin and caspase-3 expression correlate with higher histologic grade and high proliferation, but not with outcome, in breast carcinoma patients.

This study also found an agreement with [35] which his results indicated that activation of apical caspase-2 after chemotherapy results in the activation of caspase-3 and -7 without the participation of mitochondria. Caspase-9 can be activated directly via caspase-2 or alternatively. After cytochrome c release from mitochondria, then caspase-9 activation can also lead to caspase-3 and -7 activations. It seems that there is also a parallel pathway involving mitochondria that can cooperate in taxane induced cell death in breast tumor cells. My data after chemotherapy agree with [36] which demonstrated that caspase-3 and-7 levels in serum are useful as a predictive indicator of response to chemotherapy in both locally advanced and metastatic breast carcinoma.

Procaspase-8 is activated by interactions between the DED (death effector domain) on FADD (FAS associated death domain) and similar DED sequences on caspase-8 [37]. Active caspase-8 can activate caspase-3 directly, or it can cleave Bid, that facilitates release of cytochrome c from mitochondrial so it is very important initiator caspase involved in the process of apoptosis in breast tumor cells [38]. Expression of caspase-8 is significantly decreased in malignant tissue group than control group and not significantly decreased in benign group than control group. Also caspase-8 expression decreased significantly in both blood and tissue malignant groups than in benign groups. On the other hand caspase-8 expression more significantly increased in follow up group than in malignant group. Caspase-8 level increased in malignant breast cells than benign and normal insignificantly in follow up group than in benign group.

These results due to a non-receptor tyrosine kinase (c-Src) that is localized in intracellular membranes of the cells that overexpressed and highly activated cells and it is a proto-oncogene that has been strongly implicated in the development, growth, progression, and metastasis of breast cells and also

leads to in activation of caspase-8. This study found great concept with [39, 40] which demonstrated that dasatinib a highly potent inhibitor of c-Src, which could be attributed to activation of both caspase-9 and-8 and arrest of the cell cycle at G0/G1 cycle and treatment of breast carcinoma.

Cell cycle was measured by flowcytometry and the results showed that sub G1 apoptosis and G0/G1 phase levels decreased significantly in malignant group than in control group. But S phase and G2/M phase levels were significantly increased in malignant group than those levels in control group. In benign group Sub G1phase level decreased significantly more than its level in the control group, but G0/G1 phase level highly increased significantly in benign group than in control group. G2/M phase level increased significantly in benign group than in control group.

While S phase level was increased insignificantly in benign group than its level in control group. Sub G1phase level for both blood and tissue benign groups increased significantly more than in blood and tissues of malignant groups levels, also G0/G1 phase level highly increased significantly in benign group compared with malignant groups in blood and tissue. Passing through S phase, there was a markedly significant decreased in benign group than in blood and tissue malignant groups, G2/M phase levels showed observed high significant increase in blood and tissues of malignant groups compared with the benign group.

In follow up group, Sub G1 phase level increased significantly higher than its level in both malignant and benign groups, also G0/G1 phase level increased significantly in follow up group compared with malignant and benign groups, while G2/M and S phase levels decreased significantly compared with the follow up group and malignant groups. Also G2/M and S phase levels decreased significantly compared with the follow up group and benign groups. This study found in agreement with [41] which found that there was significantly greater proportion of cells in S and G2 + M phases of the cycle in breast tumor specimens than in the S and G2 + M phases of the cycle in benign and control samples of breast tissue. Also my study found agree with [42] indicated that the algal sulfated polysaccharide (ASPE) induces G1-phase arrest and apoptosis in human breast tumor cells by induction of Bax transcripts, inhibition of Bcl-2 and cleavage of caspase-3 protein. ASPE may serve as a potential therapeutic agent for breast carcinoma. Also this study after chemotherapy found great concept with [43] that showed a significant accumulation of cells in the G2/M phase of the cell cycle (arrest of cell cycle) after incubation with the effective levels of the taxanes (chemotherapy).

5. Conclusion

Breast cancer is a perilous disease, but it could be treated if diagnosed in its early stages. This study conclude that, blood sample is the most suitable sample from the breast cancer patients to evaluate some apoptotic markers like (Annexin-V and Caspace-3 & 8) by Flow Cytometry which consider as a good diagnostic and prognostic factor during follow-up of breast cancer patients.

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