Molecular Predictors of the Outcome for Anthracycline - Based Adjuvant Chemotherapy in Egyptian High Risk Female Breast Cancer Patients

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Abstract: Anthracyclines represent one of the most important chemotherapeutics in breast cancer. However, they cause cardiac damage. Besides, some tumors might be anthracycline-resistant. The aim of the present work was to study the predictive value of estrogen receptors (ER) and progesterone receptors (PR) proteins. Furthermore, the predictive value of topoisomerase IIα (TOPOIIα) gene aberrations (amplification or deletion) and breast cancer 1, early onset (BRCA1) gene methylation for the outcome of 5-fluorouracil / Adriamycin / cyclophosphamide (FAC) adjuvant chemotherapy in Egyptian high risk female breast cancer patients. The present retrospective cohort study was conducted in Alexandria Main University Hospital, Egypt. It included fifty high risk female breast cancer patients (according to St Gallen guidelines 2007) with operable breast cancer. All of them have received FAC adjuvant chemotherapy between January 2007 and December 2007 and were followed for 2 years. Pretreatment breast tumor samples were obtained from formalin fixed/paraffin-embedded tissue blocks. Log rank survival analysis showed that TOPOIIα gene aberrations, methylated BRCA1 gene, negative ER protein and negative ER/PR proteins states were associated with significantly superior disease free survival (DFS) rates after FAC therapy. Cox regression analysis showed that ER protein and BRCA1 gene methylation states might be independent predictors for the outcome of FAC adjuvant chemotherapy while TOPOIIα gene state mightn't. However, if ER protein and BRCA1 gene methylation states can be used in tailoring chemotherapy or not, further studies have to be done on a bigger number of cases with longer follow-up period. Additionally, large-scale prospective studies will be needed to clearly define TOPOIIα gene and PR protein predictive values. Patients having BRCA1 gene methylation might be at risk of having distant metastasis. So, if proved by large-scale studies, such patients could be recommended for intensified follow-up and treatment.
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1. Introduction

Breast cancer is the most prevalent cancer among Egyptian women where it constitutes 29% of the National Cancer Institute cases (Omar, 2003). Breast cancer, like all malignancies, arises as a result of the accumulation of genetic alterations, especially deregulation of the expression of oncogenes and tumor suppressor genes (Omar, 2003). One of the most important adjuvant chemotherapeutics used in breast cancer are anthracycline-based combinations (Buzdar, 2006). A major component of anthracycline cytotoxicity is due to topoisomerase II (TOPOII) poisoning. Moreover, they intercalate into double-stranded deoxyribonucleic acid (dsDNA) and produce structural changes that interfere with DNA and ribonucleic acid (RNA) synthesis. Besides, anthracyclines generate reactive oxygen species (ROS) that damage DNA, messenger RNA (mRNA), proteins and lipids; the peroxidation of lipids may account for much of the cardiac toxicity characteristic of these drugs (Rubin, 2003).

Several predictive factors that might affect the outcome of chemotherapy in breast cancer were studied but none of them was proved to have enough predictive power (Tewari, 2008). Consequently, the choice of chemotherapeutic drugs in breast cancer is usually empiric and 30%–70% of patients with measurable disease fail to respond (Kennedy, 2004). In addition, financing cancer treatment is a major challenge especially for developing countries (Boutayeb, 2010). From here comes the importance of predictive factors in tailoring of chemotherapy.

Topoisomerases are enzymes that regulate the conformational changes in DNA topology. This occurs by catalyzing the breakage and rejoining of DNA strands during normal cellular processes like replication, transcription, recombination repair and chromatin assembly (Wang, 2002). DNA topoisomerases fall into two categories, type I and type II. Type I enzyme breaks single DNA strand at a time but type II enzyme, by contrast, breaks the dsDNA molecule (Rubin, 2003). In human cells, two distinct isoforms of TOPOII exist, termed α and β. Human *TOPOIIα* gene, located on chromosome 17q21-22, is approximately 30kb and contains 35 exons (Lang, 1998). Anthracyclines inhibit TOPOIIα protein action.

Consequently, there is growing evidence that TOPOIIα could be a possible predictor for anthracycline sensitivity (Tewari, 2008).

Breast cancer 1, early onset gene is 24 exons breast and ovarian tumor suppressor gene that is localized to chromosome 17q21.31. It encodes a 220 kDa nuclear protein (Atlas of genetics and cytogenetics in oncology and hematology). BRCA1 protein responds to DNA damage by participating in cellular pathways responsible for DNA repair, mRNA transcription, cell cycle regulation and protein ubiquitination (Kennedy, 2004).

DNA methylation is the most common covalent modification of human DNA and occurs almost exclusively at cytosine-phosphate-guanine (CpG) islands (Jovanovic, 2010 and Teodoridis, 2004). These CpG islands are frequently associated with the promoter regions of genes and methylation within these islands is associated with transcriptional inactivation of the associated gene (Jovanovic, 2010 and Teodoridis, 2004). Aberrant methylation of CpG islands is frequently observed in tumors compared to normal tissue. Additionally, it can vary widely between tumor types and within particular histological subtypes. The genes that are inactivated by DNA methylation events include genes involved in signal transduction cascade pathways, cell cycle regulation, angiogenesis, apoptosis and DNA repair (Teodoridis, 2004). While *BRCA1* gene germline mutations are uncommon, epigenetic alterations in *BRCA1* gene occur more frequently. Given the role of BRCA1 protein in DNA repair and cell cycle regulation, it was hypothesized that decreased expression of BRCA1 secondary to promoter methylation could affect sensitivity to chemotherapy (Collins, 2007).

The aim of the present work was to reveal the possible predictive value of the routinely done breast cancer biomarkers; ER and PR proteins. Additionally, to assess the predictive value of two DNA-based biomarkers [*TOPOIIα* gene aberrations (amplification or deletion) and *BRCA1* gene methylation] for the outcome of anthracycline–based adjuvant chemotherapy in Egyptian high risk female breast cancer patients. The predictive value was assessed in terms of disease free survival (DFS), overall survival (OS) as well as time of relapse and type of relapse (local or distant). DFS was defined as the time between the date of randomization and the date of disease relapse and OS was defined as the time between randomization and death from any cause. Cardiotoxicity from anthracycline and mortality were recorded as well.

2. Subjects & Methods

The present retrospective cohort study was conducted in Alexandria Main University Hospital, Egypt. It included fifty high risk female breast cancer patients with operable breast cancer (Goldhirsch, 2007). These patients were either Node positive (1-3 involved nodes) with HER2/neu protein overexpression or just Node positive (≥ 4 involved nodes). Patients were randomly recruited from the Clinical Oncology Department. All of them have received anthracycline–based (FAC) adjuvant chemotherapy between January 2007 and December 2007. Pretreatment breast tumor samples were obtained from formalin fixed/paraffin-embedded tissue blocks, in accordance with the standard methods, from the archives of the Pathology Department. Before starting the treatment, all patients underwent a complete physical examination, laboratory and radiological investigations. Diagnosis was confirmed by a fine needle aspiration (FNA) or a core biopsy of the primary tumor. Modified radical mastectomy was the standard line of treatment. After surgery, all patients received 6 cycles of FAC and postmastectomy irradiation. Besides, hormone receptor (ER and/or PR) positive cases were assigned to receive tamoxifen for a period of 5 years. After finishing FAC-adjuvant chemotherapy, patients were followed every 2 months for a period of 2 years to detect local recurrence or distant metastasis. In addition, cardiotoxicity from anthracycline and mortality were recorded. During the follow-up period, patients were subjected to: thorough clinical examination, laboratory investigations, periodic radiological examination, ejection fraction and electrocardiogram (ECG) when needed. An informed consent was taken from every patient and the study was approved by the Institutional Ethics Committee. All breast cancer tumor samples were subjected to full histopathological examination. Human epidermal growth factor receptor 2 (HER2/neu) was determined by immunohistochemistry (IHC) (Rocha, 2009). HER2/neu protein score was classified into score 0, 1+, 2+ and 3+. Moreover, HER2/neu protein state was classified into negative (score 0, 1+) and positive (score 2+ and 3+) (Orlando, 2008). ER and PR states were determined by IHC as well (Tafjord, 2002). Furthermore, breast cancer tumor samples were subjected to the following assays:

2.1 Purification of genomic DNA from formalin-fixed / paraffin-embedded tissues

Genomic DNA was extracted using a spin column protocol [QIAamp DNA FFPE Tissue mini-kit (QIAGEN Inc. http://www.qiagen.com)] (Bailey, 2000). The total DNA yield was determined by spectrophotometer through absorbance at 260nm (A_{260}) using Tris-HCl buffer as a blank. The quality of DNA was assessed as well by measuring the absorbance at both 260nm and 280nm (A_{260}/A_{280} ratio) Ehli, 2008). Tumor samples having poor DNA-yield or extensively fragmented DNA were excluded from the analysis.

2.2 Determination of *TOPOIIα* gene copy number by semi-quantitative polymerase chain reaction (PCR) analysis [Modified]

Topoisomerase IIα gene state was analyzed using glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) gene co-amplification as an internal standard for quantification of gene aberrations (amplification or deletion). In order to avoid mispriming or other errors that might happen during PCR, Hot-Start PCR was used. Two pairs of primers (Operon Biotechnologies, Reagent Company Inc. of Midland, Texas. http://www.operon.com) were utilized to amplify *TOPOIIα* and *GAPDH* genes. *TOPOIIα* gene primers lead to an amplified fragment of 656 bp on DNA (Campain, 1995), while *GAPDH* gene primers lead to an amplified fragment of 104 bp on DNA (Komada, 1998). A total of ≈100 ng of genomic DNA was used in PCR amplification which was performed in a total volume of 25 µl using optimized primer volumes. Tubes were transferred to the thermal cycler (Whatman, Biometra, T personal. http://www.biometra.com). PCR was done using Gue′rin, 2003 protocol. For optimization of PCR, annealing temperature was modified to 50°C. Lymphocyte DNA from healthy individuals was used as a positive control. Blank control without DNA was included in each PCR assay as a negative control. Amplification level of *TOPOIIα* gene was expressed as the ratio of *TOPOIIα* and *GAPDH* genes for each sample. For determination of the cutoff value of the normal *TOPOIIα* gene state, lymphocyte DNA from 15 matched healthy controls was included in the assay and *TOPOIIα* gene / *GAPDH* gene ratio was determined. PCR products were analyzed using 1% agarose gel electrophoresis [Biometra Minicell Power Pack. http://www.biometra.com]. GeneRuler100bp DNA ladder served as a reference for DNA fragment size [Fermentas. http://www.fermentas.com]. PCR products were stained with ethidium bromide and were visualized under the UV Transilluminator [Biometra. http://www.biometra.com]. The gel image was captured by the use of Cleaver Gel Document System [http://www.Cleaverscientific.com]. Signals were quantified using the Gel pro-Analyser 3.1 software package (Figure 1a).

2.3 Determination of *BRCA1* gene methylation state by methylation specific PCR (MSP) analysis. [Modified]

DNA samples (1 to 2 µg) were modified with sodium bisulfite which converts the unmethylated cytosines but not the methylated ones to uracil using the EzWayTM DNA Methylation Detection Kit (KOMA BIOTECH Inc. http://www.komabiotech.com) (Tiwari, 2009). Two primer sets (Operon Biotechnologies, Reagent Company Inc. of Midland, Texas. http://www.operon.com) designed to distinguish between methylated and unmethylated *BRCA1* gene sequences were used to carryout two separate PCRs. Unmethylated *BRCA1* gene primers lead to an amplified fragment of 86 bp on DNA (Esteller, 2000). Moreover, methylated *BRCA1* gene primers lead to an amplified fragment of 75 bp on DNA (Esteller, 2000). A total of ≈100 ng of genomic DNA was used in PCR amplification performed in a total volume of 25 µl using the optimized primer volumes and Hot-Start PCR. Tubes were transferred to the thermal cycler (Whatman, Biometra, T personal. http://www.biometra.com). PCR was carried out using Bagadi, 2008 protocol. For optimization of PCR, annealing temperature was modified to 50°C. Methylated DNA from a tumor with hypermethylation and lymphocytes DNA from healthy individuals were used as positive controls for the methylated and unmethylated reactions, respectively. Blank control without DNA was included in each PCR assay as a negative control. Control primer specific for cytosine / uracil (C/U) converted sequence of human MutS homolog 2 (*hMSH2*) gene served as a control for the efficiency of the chemical modification. PCR products were analyzed using 2% agarose gel electrophoresis [Biometra Minicell Power Pack. http://www.biometra.com]. PCR products were stained with ethidium bromide and visualized under the UV Transilluminator [Biometra. http://www.biometra.com]. GeneRuler100bp DNA ladder served as a reference for DNA fragment size [Fermentas. http://www.fermentas.com]. The gel image was captured by the use of Cleaver Gel Document System [http://www.Cleaverscientific.com] (Figure 1b).

Statistical analysis

Demographic data including diagnosis details, tumor characteristics, chemotherapy scheme, toxicity and chemotherapy outcome were entered prospectively into a database. Continuous data were expressed as mean (for normally distributed data) or median (for abnormally distributed data) and range between parentheses. Qualitative data were presented as absolute numbers and percentages. Survival curves were obtained by the Kaplan–Meier method. Differences between the survival curves were investigated using the log-rank univariate analysis. A regression analysis based on the Cox proportional hazards model was conducted using selected covariates to reveal the possible independent predictors for DFS and OS. Mann-whitney test was done to assess the possible effect of the studied biomarkers on the time of relapse. Chi-square test was conducted to assess the possible relation between the studied biomarkers and the type of relapse. In addition, Chi-square test was used to assess the possible relation between HER2/neu protein and *TOPOIIα* gene. Data were analyzed using SPSS software package version 18.0. All statistical tests were two sided and probability values of $p<0.05$

were regarded as significant. Kaplan-Meier curves were plotted in Harvard Graphics.

3. Results and Discussion

Although the role of TOPOIIα, BRCA1, ER and PR in breast cancer was previously studied in published articles, there is still however lack of information in Egyptian patients. The current patients' age ranged from 27-72 years (mean=46.56±10.05 years). Moreover, 10 patients (20%) were less than 40 years while 40 patients (80%) were equal to or more than 40 years. Twenty one patients (42%) were postmenopausal while 29 (58%) were premenopausal. Nine patients (18%) had a positive family history for breast cancer while 41 (82%) had a negative family history. Regarding tumor histological type, 43 patients (86%) had invasive ductal carcinoma (IDC) tumor, 4 (8%) had invasive lobular carcinoma (ILC), 1 (2%) had IDC with lobular carcinoma *In Situ* (LCIS), 1 (2%) had a mixed IDC with ILC, and 1 patient (2%) had malignant phyllodes tumor (MPT). As for tumor stage, 6 patients (12%) had stage IIA breast cancer, 19 (38%) had stage IIB breast cancer, 14 (28%) had stage IIIA breast cancer and 11 patients (22%) had stage IIIB breast cancer. Regarding tumor grade, 4 patients (8%) had grade 1 breast cancer, 30 (60%) had grade 2 breast cancer and 16 patients (32%) had grade 3 breast cancer. The DFS time of the study cohort ranged from 3-24 months (mean=17.2±7.38 months). By the end of the study period, 22 patients (44%) didn't have relapse and 28 (56%) had relapse. Of the latter group, 17 patients (34%) had local recurrence while 11 (22%) had distant metastasis. Furthermore, by the end of the study period, 3 patients (6%) were documented to have cardiotoxicity from FAC chemotherapy. The OS time of the study cohort ranged from 10-24 months (median=24±0.25 months) and by the end of the study period, 38 cases (76%) were alive and 12 cases (24%) were dead (Supplementary Table 1).

3.1 *TOPOIIα* Gene

Topoisomerase IIα gene ratio ranged from 0.5-3.11 (mean=1.37±0.89) in the patients group, while in the control group, the range was 0.85-1.95 (mean=1.40±0.36). The cutoff value for *TOPOIIα* gene aberrations was chosen as mean±2SD [95% confidence interval (CI)]. Consequently, the cutoff value for *TOPOIIα* gene amplification was 2.12 and that for *TOPOIIα* gene deletion was 0.67 (Supplementary Table 2).

In the current study, *TOPOIIα* gene aberrations were associated with a statistically significantly superior 2-year DFS rate (52.5%) to that of normal *TOPOIIα* gene state (10%) (log rank p=0.016*). Besides, *TOPOIIα* gene amplification was associated with the best 2-year DFS rate (61.5%) to be followed by *TOPOIIα* gene deletion (48.1%) and finally normal *TOPOIIα* gene state (10%) (log rank p=0.04*) (Table 1, Figures 2a, 3b). On the other hand, Cox regression analysis suggested that *TOPOIIα* gene aberrations couldn't be considered as an independent predictor for DFS after FAC adjuvant chemotherapy (p=0.19) (Table 1). Although statistically insignificant, *TOPOIIα* gene aberrations were accompanied by a superior 2-year OS rate (80%) to normal *TOPOIIα* gene state (60%) (log rank p=0.26). In addition, although statistically insignificant, *TOPOIIα* gene amplification was associated with the best 2-year OS rate (92.3%) to be followed by *TOPOIIα* gene deletion (74.1%) and finally normal *TOPOIIα* gene state (60%) (log rank p=0.25) (Table 1).

Published studies reported that *TOPOIIα* gene aberrations could be a predictive marker for the outcome of anthracycline - containing therapies (Konecny, 2010 and Press, 2011). In the current study, the relation between *TOPOIIα* gene amplification and superior 2-year DFS and OS rates could be explained by being TOPOIIα protein a target for anthracycline (Tewari, 2008), so it is expected that *TOPOIIα* gene amplification could be accompanied by sensitivity to these drugs. Additionally, the relation between *TOPOIIα* gene deletion and superior 2-year DFS and OS rates could be explained by the study hypothesis of Di Leo, 2008 where they proposed that deletions might involve one of the two alleles of the *TOPOIIα* gene and that the wild-type allele might compensate in the presence of conditions requiring a high level of TOPOIIα protein activity. This compensation mechanism could happen for *TOPOIIα* gene as its promoter seems to be controlled at different levels including transcription and translation phases. In contrary to the current results, Moretti**,** 2009, reported that topoisomerase IIα cannot be considered as a clinically valuable biomarker in guiding prescription of anthracyclines possibly due to disease heterogeneity that dictate prediction by tumor profiles, rather than any single biomarker. This contradiction could also be related to different cutoffs used for defining *TOPOIIα* gene aberrations and different TOPOIIα assays used by different studies (Slamon, 2009). Varying doses and schedules of anthracycline-containing combinations used by several studies could be other contributing factors (Buzdar, 2006).

Among the relapsed patients, *TOPOIIα* gene state (normal versus aberrations) had no significant effect on the time of relapse (p=0.82). Moreover, it had no significant relation to the type of relapse (p=0.70) (Table 2). In contrast, Usha**,** 2008, proposed that breast cancer patients having *TOPOIIα* gene aberrations, especially gene deletion, might have unfavorable prognosis regardless the type of chemotherapy. Furthermore, they reported that *TOPOIIα* gene deletion might be a marker for another genetic event, most likely involving a nearby gene, whose altered expression confers an adverse

prognosis. The latter study was based on 5-year follow-up period besides the use of anthracyclines versus non-anthracyclines treated groups, which might have given a better insight about the prognostic impact of *TOPOIIα* gene.

In the current study, there was a significant relation between *TOPOIIα* gene state (normal versus aberrations) and HER2/neu protein state (positive versus negative) (p=0.048*). Meanwhile, the probability of the co-existence of positive HER2/neu protein state and *TOPOIIα* gene aberrations state was 4.88 (95% CI=0.92-25.97) (Table 3). The latter result was supported by published reports (Orlando, 2008 and Konecny, 2010). Such relation was explained by the hypothesis that *HER2/neu* gene could be the "driver" of the amplification event on chromosome 17q12–21. Furthermore, this amplicon might be variable in size, frequently extending telomerically and/or centromerically from *HER2/neu* gene to include other genes. In addition, the amplicon could be sometimes flanked by deletions of the immediately adjacent regions of DNA. The result of these amplicon-related phenomena is that a number of flanking genes are frequently either co-amplified or deleted in breast cancers containing *HER2/neu* gene amplification. One such gene might be the *TOPOIIα* gene (Slamon, 2009).

3.2 *BRCA1* Gene

The present study showed that methylated *BRCA1* gene state was associated with superior 2-year DFS rate (63.3%) to unmethylated *BRCA1* gene state (15%) (log rank p=0.003*) (Table 1, Figure 2c). By Cox regression analysis, *BRCA1* gene methylation was suggested to be an independent predictor for better DFS after FAC adjuvant chemotherapy (p=0.045*). Moreover, methylated *BRCA1* gene state decreased the risk of poor DFS after FAC treatment by 0.444 times (95% CI=0.201-0.982) (Table 1). Methylated *BRCA1* gene 2-year OS rate (80%) was slightly better than unmethylated *BRCA1* gene state (70%) as well (log rank p=0.51) (Table 1). An interesting finding in this study was the presence of two cases showing mixed methylated and unmethylated alleles. The mixed state in the latter cases could probably be explained by the presence of infiltrating lymphocytes and/or non malignant epithelial cells in the primary tumors (Karray-Chouayekh, 2009) (Figure 1b). Although *BRCA1* gene mutation or dysfunction increases the risk of breast cancer (Kennedy, 2004 and Collins, 2007), the current findings point to the possible importance of *BRCA1* gene methylation in tailoring of chemotherapy. In accordance to these results, overall preclinical and clinical studies have shown that decreased expression of BRCA1 due to promoter methylation might increase the sensitivity to DNA-damaging chemotherapeutic agents (Collins, 2007 and Mullan, 2006). The same might be applied to anthracyclines possibly due to inhibition of apoptosis by BRCA1 protein through three mechanisms: First, activating the synthetic (S) - phase and gap 2/mitosis (G_2/M) - phase DNA damage checkpoints (Kennedy, 2004), second, playing an important role in repairing DNA damage by homologous recombination (HR), non-homologous end joining (NHEJ) and nucleotide excision repair (NER) (Kennedy, 2004) and third, stabilizing 53 kDa protein (p53) to redirect p53 transcriptional activation to target genes involved in cell cycle arrest and DNA repair at the expense of apoptosis (Mullan, 2006). Consequently, it is expected that in absence of BRCA1 protein, by gene mutation or dysfunction, anthracyclines might be able to exert greater effect. In contrary to the current results, Yuan, 2009, reported that primary breast cancer patients having unmethylated *BRCA1* gene are likely to be more prone to achieve a better response to anthracycline-based neoadjuvant chemotherapy than those having methylated *BRCA1* gene. This contradiction could probably be explained by the racial variations between patients of the latter study and those of the current study. Additionally, the current high risk breast cancer patients might have unique molecular profile that could explain their sensitivity to anthracyclines.

Among the relapsed cases, *BRCA1* gene methylation had no significant effect on the time of relapse (p=0.28). On the other hand, there was a significant relation between *BRCA1* gene methylation state and the type of relapse (p=0.034*). Moreover, methylated *BRCA1* gene state was found to increase the risk of distant metastasis by 5.68 times (95% CI=1.07-29.99) (Table 2, Supplementary Table 3). This important finding points to the suggestion that *BRCA1* gene methylation might be a risk factor for the occurrence of distant metastasis. Consequently, if proved by large-scale study, such patients would be recommended for intensified follow-up and treatment. In this context, Chen, 2009, suggested that inactivation of the *BRCA1* gene by CpG methylation might be involved in the progression of breast cancer and could affect the clinical course and response to treatment of such patients. That current finding could partially be explained by the hypothesis that dysfunction of *BRCA1* gene might promote loss of phosphatase and tensin homolog (*PTEN*) expression leading to cell transformation, proliferation, migration, angiogenesis, genomic instability; maintained stem cell compartments; and finally poor prognosis in breast cancer (Chen, 2009).

3.3 ER&PR Proteins

Negative ER state, in the current study, was associated with a statistically significantly superior 2-year DFS rate (62.5%) to positive ER state (11.1%) (log rank p=0.000*) (Table 1, Figure 2d). Moreover, in spite of being statistically insignificant, negative ER state had a slightly better 2-year OS rate than positive ER

state (78.1% and 72.2% respectively) (log rank p=0.62) (Table 1). Cox regression analysis revealed that negative ER protein state might be an independent predictor for better DFS after FAC adjuvant chemotherapy (p=0.006*) (Table 1). Although negative ER state is associated with poor prognosis in breast cancer (Donegan, 1997), it was found to decrease the risk of poor DFS after FAC treatment by 0.323 times (95% CI=0.145-0.718) (Table 1). The current results were in accordance to published reports (Miyoshi, 2010 and Dowsett, 2008). Dowsett, 2008, hypothesized that loss of ER could result in good response to chemotherapy partly due to the higher proliferation rate in ER negative tumors. Moreover, in vitro studies suggested that ER signaling might increase the levels of B-cell lymphoma-2 (bcl-2) protein thus induce anthracycline resistance (Teixeira, 1995).

Although being statistically insignificant, negative PR state had a slightly superior 2-year DFS rate (48.3%) and 2-year OS rate (79.3%) to positive PR state (DFS rate 38.1% and OS rate 71.4%) (log rank p=0.28 and 0.44 respectively) (Table 1). In the adjuvant setting, PR expression was reported to have a strong prognostic, with a little predictive, value (Dowsett, 2008).

Two-year DFS rate of negative ER/PR state was significantly better than positive ER and/or PR state (59.1% and 32.1% respectively) (log rank p=0.047*) (Table 1, Figure 2e). On the other hand, by Cox regression analysis, ER/PR state mightn't be an independent predictor for the outcome of FAC therapy (p=0.49) (Table 1). Two-year OS rate of combined ER/PR state categories didn't show much difference (negative ER/PR cases 77.3% and positive ER and/or PR cases 75%) (log rank p=0.78) (Table 1). In this context, negative ER/PR tumors were reported to exhibit a remarkable sensitivity to anthracycline-based chemotherapy as compared to those with any single factor (Wei, 2007). The current results might reflect a specific breast cancer molecular subtype which could be anthracycline sensitive.

Within the relapsed cases, none of ER, PR or ER/PR states had significant effect on the time of relapse (p=0.94, 0.15 and 0.42 respectively). There was a statistically insignificant relation between the latter markers and the type of relapse as well (p= 0.82, 0.93 and 0.70 respectively) (Table 2). Caudle, 2010, reported that patients having negative ER/PR state could be at risk for tumor progression on anthracycline and/or taxane-based neoadjuvant chemotherapy. The latter study failed to meet the goal of identifying patients at risk for tumor progression on standard chemotherapy because the studied variables were also associated with response to chemotherapy. One limitation regarding ER and PR state in the present study was that IHC results of the patients' records mostly didn't show ER and PR scoring system that might be able to give more details about the effect of hormone receptors on the outcome of FAC therapy.

4. Conclusions

In conclusion, *BRCA1* gene methylation and ER protein states might be considered as predictors for the outcome of FAC adjuvant chemotherapy in Egyptian high risk female breast cancer patients while *TOPOIIα* gene mightn't. However, if *BRCA1* gene and ER protein can be used in tailoring chemotherapy or not, further studies have to be done on a bigger number of cases with longer follow-up period. Besides, large-scale prospective studies will be needed to clearly define *TOPOIIα* gene and PR protein predictive values. Patients having *BRCA1* gene methylation might be at risk of having distant metastasis. When proved by large-scale studies, such patients would be recommended for intensified follow-up and treatment. Further studies are required to verify the possible predictors of breast cancer progression for patients on anthracyclines therapy.

Table 1. Univariate (Log Rank) and multivariate (Cox proportional hazards regression) analysis for the studied biomarkers according to: A. disease free survival (DFS) and B. overall survival (OS)

Biomarker	Log Rank Test	Cox Proportional Hazards Regression[a]	
	p value[b]	*p* value	Exp(B)[c]
A. DFS			
TOPOIIα Gene State	0.016*	0.19	-
TOPOIIα Gene Ratio Categories	0.04*	-	-
BRCA1 Gene State	0.003*	0.045*	0.444 (95% CI[d]=0.201-0.982)
ER Protein State	0.000*	0.006*	0.323 (95% CI= 0.145-0.718)
PR Protein State	0.28	-	-
ER/PR Protein State	0.047*	0.49	-
B. OS			
TOPOIIα Gene State	0.26	-	-
TOPOIIα Gene Ratio Categories	0.25	-	-
BRCA1 Gene State	0.51	-	-
ER Protein State	0.62	-	-
PR Protein State	0.44	-	-
ER/PR Protein State	0.78	-	-

a. The significant parameters in log rank univariate analysis were included in Cox proportional hazards regression analysis to reveal the possible independent predictors for DFS. By log rank test, except for tumor type and HER2/neu protein score (log rank p=0.002* and 0.001* respectively), none of the clinicopathological characteristics of patients were significantly related to DFS, so only tumor type and HER2/neu protein score were included in the regression analysis. (Supplementary Table 4) By Cox regression analysis, neither tumor type nor HER2/neu protein score were independent predictors for DFS (p=0.54 and 0.81 respectively).
b. All statistical tests are two sided and probability (p) values of <0.05 are regarded as significant (*).
c. Exp(B): Exponential (B).
d. CI: Confidence interval.

Table 2. Relation between the studied biomarkers and each of relapse time and type.

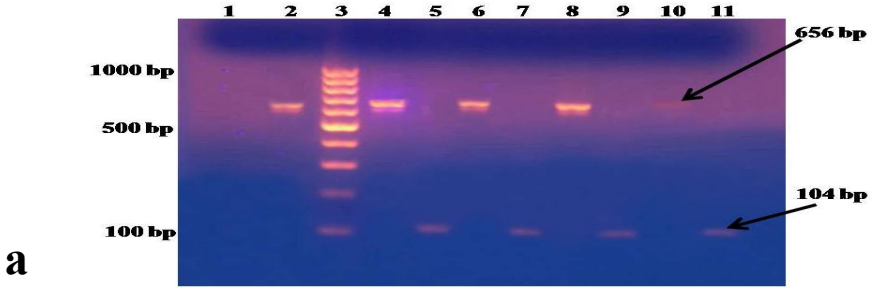
Biomarker	Relapse Time		Relapse Type		
	Mann-Whitney U	p value[a]	χ2 value	p value	RR[b]
TOPOIIα Gene State	81.00	0.82	0.148	0.70	-
BRCA1 Gene State	71.00	0.28	4.504	0.034*	5.68 (95% CI[c]=1.07-29.99)
ER Protein State	94.50	0.94	0.05	0.82	-
PR Protein State	66.50	0.15	0.007	0.93	-
ER/PR Proteins State	69.50	0.42	0.148	0.70	-

a. All statistical tests are two sided and probability (p) values of <0.05 are regarded as significant (*).
b. RR: Relative Risk c. CI: Confidence interval

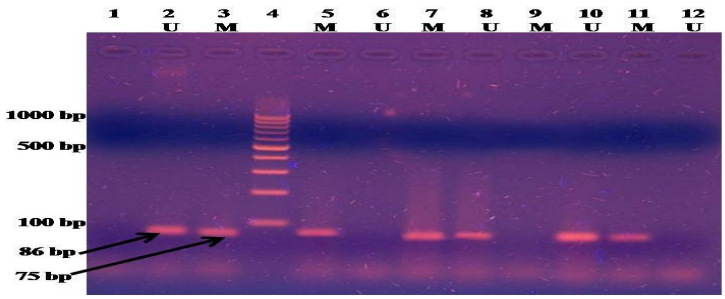
Table 3. TOPOIIα gene state in relation to HER2/neu protein state

HER2/neu Protein State		*TOPOIIα* Gene State		Total
		Normal	Aberrations	
Negative	No.	8	18	26
	% within HER2/neu	30.8	69.2	100.0
Positive	No.	2	22	24
	% within HER2/neu	8.3	91.7	100.0
Total	No.	10	40	50
	% within HER2/neu	20.0	80.0	100.0
χ^2	3.926			
p[a]	0.048*			
RR[b]	4.88 (95% CI[c]=0.92-25.97)			

a. All statistical tests are two sided and probability (p) value of <0.05 is regarded as significant (*).
b. RR: Relative risk c. CI: Confidence interval.



a



b

Figure. 1 a. Semiquantitative PCR analysis of *TOPOIIα* gene. Lanes 1 and 2 show the negative and the positive controls for PCR respectively. Lane 3 shows the 100bp DNA ladder. Each case is represented with two lanes, one for *TOPOIIα* gene (656 bp) and the other for the co-amplified internal standard *GAPDH* gene (104bp). Two cases with *TOPOIIα* gene amplification are shown in lanes (4,5) and (8,9). A case with normal *TOPOIIα* gene state is shown in lanes (6,7) and a case of *TOPOIIα* gene deletion is shown in lanes (10,11). b. MSP analysis of *BRCA1* gene. Lane 1\ shows the negative control for PCR. Lanes 2\ and 3\ show the positive controls for unmethylated and methylated *BRCA1* gene respectively. Lane 4\ shows the 100bp DNA ladder. Each case is represented with two lanes, one for the methylated *BRCA1* gene amplified fragment (75bp) and the other for the unmethylated *BRCA1* gene amplified fragment (86bp). Two cases with methylated *BRCA1* gene are shown in lanes (5\,6\) and (11\,12\). A case with unmethylated *BRCA1* gene is shown in lanes (9\,10\). A case with mixed unmethylated and methylated *BRCA1* gene alleles is shown in lanes (7\,8\)

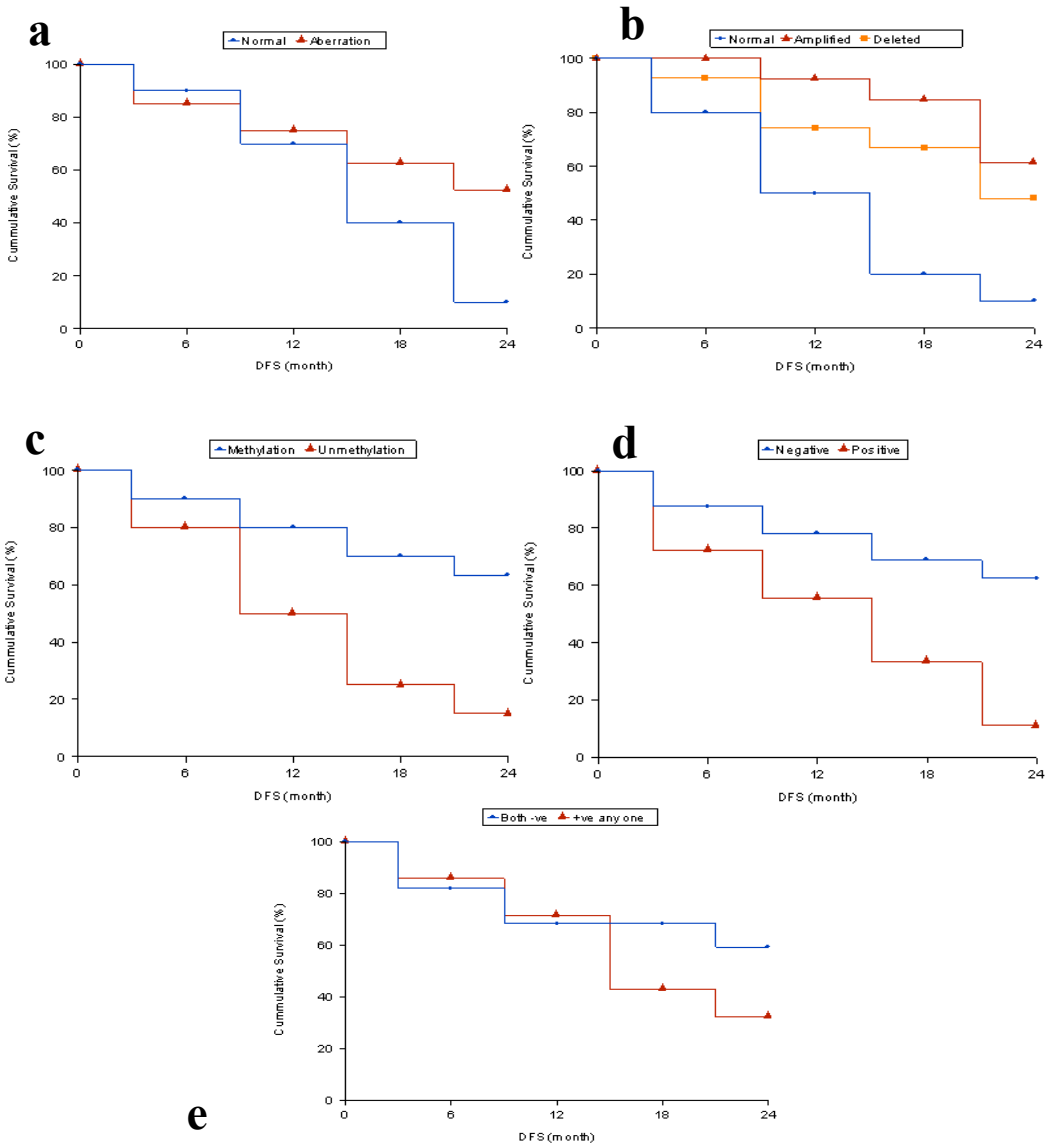


Figure. 2 Kaplan-Meier disease free survival (DFS) curve for FAC-treated breast cancer cases in Alexandria, Egypt, according to: a. *TOPOIIα* gene state, b. *TOPOIIα* gene ratio categories, c. *BRCA1* gene methylation state, d. ER state and e. ER/PR state. Kaplan-Meier curves were plotted in Harvard Graphics.

Supplementary Table 1. Clinicopathological features of the studied patients

Variable	No.	%
Age		
<40 years	10	20.0
≥40 years	40	80.0
Range Mean±SD[a]	27 – 72 46.56±10.05	
Menopausal State		
Postmenopausal	21	42.0
Premenopausal	29	58.0
Family History		
Positive	9	18.0
Negative	41	82.0
Tumor Type		
IDC[b]	43	86.0
IDC & LCIS[c]	1	2.0
ILC[d]	4	8.0
Mixed IDC & ILC	1	2.0
MPT[e]	1	2.0
Clinicopathololological Stage		
IIA	6	12.0
IIB	19	38.0
IIIA	14	28.0
IIIB	11	22.0
Histological Grade		
1	4	8.0
2	30	60.0
3	16	32.0
FAC Chemotherapy[f]		
6 cycles	50	100.0
Locoregional Radiotherapy		
Yes	50	100.0
Hormonal Therapy		
Yes	28	56.0
No	22	44.0
Chemotherapy Toxicity		
Yes	3	6.0
No	47	94.0
DFS[g]		
Range Mean±SD	3–24 17.20±7.38	
Relapse		
No	22	44.0
Yes	28	56.0
Relapse Type		
Local	17	34.0
Distant	11	22.0
OS[h]		
Range Median±IQR[i]	10–24 24±0.25	
Mortality		
Survive	38	76.0
Die	12	24.0

a. SD: standard deviation, b. IDC: invasive ductal carcinoma, c. LCIS: lobular carcinoma in situ, d. ILC: invasive lobular carcinoma, e. MPT: malignant phyllodes tumor, f. FAC: 5-Fluorouracil/Adriamycin/Cyclophosphamide, g. DFS: disease free survival, h. OS: overall survival, i. IQR: interquartile range.

Supplementary Table 2. *TOPOIIα* gene ratio in the patients and control groups

TOPOIIα gene ratio	Range	Mean±SD[a]
Control	0.85-1.95	1.40±0.36
Patients	0.5–3.11	1.37±0.89

a. SD: Standard deviation.

Supplementary Table 3. *BRCA1* gene methylation in relation to relapse type

BRCA1 Gene State		Relapse Type		Total
		Distant	Local	
Methylated	**No.**	7	4	11
	% Within *BRCA1*	63.6	36.4	100.0
Unmethylated	**No.**	4	13	17
	% Within *BRCA1*	23.5	76.5	100.0
Total	**No.**	11	17	28
	% Within *BRCA1*	39.3	60.7	100.0
χ^2 p[a] RR[b]	4.504 0.034* 5.68 (95% CI[c]=1.07-29.99)			

a. All statistical tests are two sided and probability (p) value of <0.05 is regarded as significant (*).
b. RR: relative risk
c. CI: Confidence interval.

Supplementary Table 4. Log rank univariate disease-free survival analysis for the clinicopathological features of the studied group

Variable	Log Rank p value[a]
Age	0.688
Menopausal State	0.139
Family History	0.122
Tumor Type	0.002*
Tumor Stage	0.464
Tumor Grade	0.139
HER2/neu Protein Score	0.001*

a. All statistical tests are two sided and probability (p) value of <0.05 is regarded as significant (*)

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Conflict of interest

None

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