Expression of GSTP1, TOP2α and ALDH1A in triple negative breast cancer patients receiving neoadjuvant chemotherapy

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Abstract: Background: Breast cancer is the most common cancer in females worldwide. In Egypt, the disease accounts for 37% of women's cancer with an incidence rate of 49.6/100,000. Neoadjuvant chemotherapy (NAC) for primary breast cancer enhances the operability of patients with advanced tumors previously considered inoperable, as well as making breast-conserving surgery more feasible especially in cases with large tumor size. Although the standard NAC regimen consists of a sequential taxane and anthracycline based regimen, the predictive value of GSTP1, TOP2α and ALDH1A proteins for this standard regimen is not fully investigated. **Aim of the work:** To investigate the ability of GSTP1, TOP2α and ALDH1A protein expression to predict response to neoadjuvant chemotherapy (AC- Taxol) in triple negative breast cancer. **Materials and Methods:** GSTP1, TOP2α and ALDH1A proteins expression was assessed immunohistochemically in a series of 30 cases of triple negative breast cancer and their utility to detect response to neoadjuvant chemotherapy was evaluated. **Results:** GSTP1 protein expression showed significant direct association with tumor grade, stage and lymph node metastasis (p= 0.039, < 0.001, 0.001 respectively), TOP2α expression level was inversely associated with advanced tumor stage, nodal metastasis, but not tumor grade (p= <0.001, 0.028, 0.116 respectively). ALDH1A expression levels were directly associated with advanced tumor stage, lymph node metastasis, but not with tumor grade (p= <0.001, 0.014, 0.552 respectively). Both GSTP1 and ALDH1A expression levels were inversely correlated to TOP2 α (r = -0.733, p = <0.001& r = -0.720, p = <0.001 respectively), however they were directly correlated to each other (r = +0.626, P = <0.001). GSTP1 expression level had 100% sensitivity and 90.9% specificity with AUC of 0.959 in predicating overall response versus no response to NAC, however adding ALDH1A and TOP2 α expression to GSTP1 had 94.7% sensitivity, 100% specificity and AUC of 0.976. **Conclusion:** GSTP1 protein expression has the potential to serve as an independent marker for identifying patients with triple negative breast cancer that are unlikely to benefit from neoadjuvant chemotherapy, however combining TOP2 α and ALDH1A with GSTP1 seems to increase its predicatively.

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Key words: GSTP1, TOP2α, ALDH1A, immunohistochemistry, neoadjuvant chemotherapy (NAC), triple negative breast cancer (TNBC).

1. Introduction:

Breast cancer is the most common female malignancy worldwide. It is reported that over 508 000 women died in 2011 due to breast cancer[(1)]. In Egypt, the disease accounts for 37% of female cancer with an incidence rate of 49.6/100,000[(2)]. It is often advanced at the time of diagnosis, with a high mortality rate. Since the behavior of the disease is generally governed by its molecular subtype, there have been speculations that Egyptian women suffer aggressive subtypes more frequently[(3)].

Triple negative breast cancers (TNBCs) are characterized by the lack of expression of estrogen receptors (ER), progesterone receptors (PR) and human epidermal growth factor receptor (HER2). They occur in approximately 20% to 25% of breast cancer patients, and are associated with unfavorable prognosis. TNBC patients do not benefit from molecularly targeted therapy, such as endocrine therapy or trastuzumab, because they lack the appropriate targets for these drugs[(4,5)].

Neoadjuvant chemotherapy (NAC) for primary breast cancer enhances the operability of patients with advanced tumors previously considered inoperable, as well as makes breast-conserving surgery more feasible especially in cases with large tumor size. In addition, it is well established that patients who show a complete response (CR) to NAC can have a better prognosis than those who do not[(6,5)], so the response to NAC can provide valuable information regarding patient prognosis. These advantages of NAC have led to its widespread use for a growing number of breast cancer patients[(7)]. Because adverse effects of various degrees of severity are seen in virtually all patients, it seems to be important to develop predictive factors for the response to NAC to avoid the unnecessary use for patients who are unlikely to derive benefits from such therapy.

Glutathione S-transferase P1 (GSTP1) is a metabolic enzyme that is crucial for the detoxification of toxic substances and anticancer therapy by conjugating them with glutathione. Also, it was reported to inhibit the chemotherapy-induced apoptosis by its direct interaction with the C-terminal of JNK. GSTP1 expression in tumor cells can thus be expected to be associated with resistance to chemotherapy[8]. Although the standard neoadjuvant chemotherapeutic regimen currently consists of a sequential taxane and anthracycline based regimen, the predictive value of GSTP1 expression for this standard regimen in not fully investigated[9].

Topoisomerase II α (TOP2α) is a key enzyme in DNA metabolism, playing a central role in DNA replication. Its primary function is to alter DNA topology from its storage (supercoiled) form to a more exposed (partially uncoiled) form by inducing single strand DNA breaks, and simultaneously passing another intact double helix through the gap, allowing selected regions of DNA to untangle and thus engage in transcription, replication, or repair processes[10]. It is one of the intracellular targets for anthracycline-based therapy. Those drugs create a cleavable complex including the drug, TOP2α and DNA strand, leading to double strand DNA breaks and apoptosis of proliferating tumor cells[11,12].

Aldehyde dehydrogenases (ALDHs) are a family of enzymes involved in the detoxification of a wide variety of aldehydes. ALDH1A mainly functions as a retinoic acid enzyme, catalyzing the conversion of vitamin A (retinol) to retinoic acid. Also, it participates in alcohol metabolism. It was found that the high expression of ALDH1A in tumor cells may provide a route for the tumor to resist chemotherapy, particularly cyclophosphamide. There have been multiple studies linking ALDH1A positivity to clinical outcome and breast cancer phenotypes[13, 14].

Aim of the work:

The aim of this retrospective study was to investigate the ability of GSTP1, TOP2α and ALDH1A expression to predict response to neoadjuvant chemotherapy (AC- Taxol) in triple negative breast cancer, as well as to assess their association with clinicopathological features of the disease.

2. Materials & Methods:

Patients and tissue selection:

This retrospective study included 30 cases of triple negative breast cancer whom were admitted to Clinical Oncology and Nuclear Medicine Department, Zagazig University between 2009 and 2013. Archival formalin-fixed paraffin-embedded core biopsies of these patients were collected from Pathology Department of the same institute. Patients received chemotherapy protocol (AC–Taxol) 4 cycles of doxorubicin *(*Adriamycin*),* and cyclophosphamide 4 cycles of paclitaxel *(*Taxol*)*. Clinical data and follow up information were collected retrospectively from the archives of Clinical Oncology & Nuclear Medicine Department. Histopathologic characteristics, ER, PR and HER2 status were confirmed by blinded review of the original pathology slides and patients' medical files.

The study complied with the guidelines of the local ethics committee.

Evaluation of response to chemotherapy:

Clinical response to AC- TAXOL was evaluated using Ultrasonography to breast which was performed three times: before NAC, after AC and after paclitaxel. Tumor size was determined as tumor length x width (cm^2). A clinical complete remission (CR) was defined as the disappearance of all palpable tumor deposits. Clinical response was scored as partial remission (PR) if the reduction in tumor volume exceeded 50%. Tumor reduction <50% or an increase in volume up to 25% was scored as stable disease (SD). An increase of > 25% was designated as progressive disease (PD)[15].

Immunohistochemistry:

Immunohistochemical staining was carried out using the streptavidin–biotin immunoperoxidase technique. Sections of 3–5 mm thickness were cut from formalin-fixed, paraffin-embedded blocks, mounted on positively charged slides, deparaffinized in xylene, and rehydrated in graded alcohol. Sections were boiled in citrate buffer (pH 6.0) for 20 min and then washed in PBS (pH 7.3). Thereafter, blocking of endogenous peroxidase activity with 6% H2O2 in methanol was carried out. The slides were then incubated overnight with monoclonal antibodies: GSTP1 (1:4000 dilution, clone 3F2C2, catalog #Ab47709, Abcam, Cambridge, UK), TOP2α (ready to use, clone Ki-S1, catalog #MS-1819-R7; Labvision, Fermont, USA) and ALDH1A (dilution 1/100, clone EP1933Y, Abcam, Cambridge, MA, UK). Incubation with a secondary antibody and product visualization were performed with diaminobenzidine substrate (Labvision Corporation, Fermont, USA) as the chromogen. The slides were finally counterstained with Mayer's hematoxylin and washed with distilled water and PBS.

To prevent antigen degradation, sections were stored at 4°C before immunohistochemical analysis. Normal prostate, tonsils and liver were used as positive control to confirm the specificity of staining with GSTP1, TOP2α and ALDH1A respectively. Negative controls were made with primary antibody replaced by PBS. Positive and negative control slides were included within each batch of slides.

Interpretation of immunohistochemistry:

Immunostaining of GSTP1 was evident in the cytoplasm and focally in the nucleus of neoplastic cells. It was classified as positive when 10% or more of tumor cells showed cytoplasmic staining[16].

Positive TOP2α expression was assigned when 10% or more of cells showed brown nuclear staining[17].

ALDH1A immunoreactions was considered positive when 10% or more of tumor cells showed cytoplasmic staining[14].

Statistical analysis

Continuous variables were expressed as the mean ± SD for normally distributed data & median (range) for non-normally distributed data, and the categorical variables were expressed as a number (percentage). Continuous variables were checked for normality by using Kolmogorov-Smirnov test. Data found to be normally distributed were analyzed using the Independent Student-t test for two groups & One way analysis of variance (ANOVA) for three groups. Data found to be non-normally distributed were analyzed using the Mann-Whitney U test for two groups & Kruskal-Wallis H test for three groups. Percent of categorical variables were compared using the Pearson's Chi-square (χ^2) test. Association between staining of GSTP1, TOP2A and ALDH1A were done by McNemar (χ^2) test for paired data with exact correction was done if number of discordant pairs was fewer than 20, while strength of relationship between these nominal data were determined by computing Contingency Coefficient (C). Pearson's correlation coefficient was calculated to assess the relationship between GSTP1, TOP2A and ALDH1A scores except for non-normally distributed parameters, Spearman's rank correlation coefficient was calculated, (+) sign was indicator for direct relationship & (-) sign was indicator for inverse relationship, also values near to 1 was indicator for strong relationship & values near 0 was indicator for weak relationship. Receiver Operating Characteristic (ROC) curves were obtained to calculate the cutoff point for GSTP1, TOP2A and ALDH1A scores & combinations of the three scores to reach the best compromise in the prediction of response. The cutoff point with maximum sensitivity and specificity (validity) is used as the recommended cutoff point and also Area Under Curve (AUC) was calculated. To determine whether GSTP1, TOP2A and ALDH1A staining were predictive for overall response to neoadjuvant chemotherapy, we used uni- and multivariate regression with forward entering of covariates. All tests were two sided, *p*-value <0.05 was considered significant. All statistics were performed using SPSS 22.0 for windows (SPSS Inc., Chicago, IL, USA) & MedCalc 13 for windows (MedCalc Software bvba).

3. Results:

Clinicopathological characteristics:

A total of 30 archival tissues from patients with triple negative breast cancer were included in this study. All cases were diagnosed as infiltrating duct carcinoma (Clinicopathological features of cases are shown in table 1).

Table (1): Clinicopathological features & response to NAC in studied TNBC patients.

	Number	*Percentage (%)*		*Number*	*Percentage (%)*
Age (year)			**GSTP1 staining**		
Mean ± SD	50.43 ± 7.34		*Median (Range)*	25 (0 – 90)	
≤ 50 year	14	46.7 %	*Negative*	14	46.7 %
> 50 year	16	53.3 %	*Positive*	16	53.3 %
Grade			**TOP2α staining**		
Grade II	18	60 %	*Median (Range)*	30 (0 – 90)	
Grade III	12	40 %	*Negative*	11	36.7 %
			Positive	19	63.3 %
Tumor stage (pT)			**ALDH1A staining**		
T2	9	30 %	*Median (Range)*	9 (0 – 85)	
T3	14	46.7 %	*Negative*	16	53.3 %
T4	7	23.3 %	*Positive*	14	46.7 %
Lymph node (pN)			**Response to NAC**		
N1	4	13.3 %	OAR	(19)	(63.3 %)
N2	17	56.7 %	*CR*	9	30 %
N3	9	30 %	*PR*	10	33.3 %
Distant Metastasis (M)			*NR*	(11)	(36.7 %)
M0	30	100 %	*SD*	8	26.7 %
M1	0	0 %	*PD*	3	10 %

Continuous variables were expressed as the mean ± SD for normally distributed data & median (range) for non-normally distributed data; categorical variables were expressed as a number (percentage).

GSTP1 protein expression showed significant direct association with tumor grade, stage and lymph node metastasis (p= 0.039, < 0.001, 0.001 respectively), however a highly significant inverse relationship was observed with response to NAC (p= < 0.001) (table 2, Fig. 1B, 2B).

The association of GSTP1, TOP2α and ALDH1A expression with clinicopathological features & response to NAC in studied TNBC patients:

TOP2α expression level was inversely associated with advanced tumor stage, nodal metastasis, but not with tumor grade (p= < 0.001, 0.028, 0.116 respectively). Conversely, higher TOP2α score was significantly associated with better response to NAC (p= < 0.001) (table 2, Fig. 1C, 2C).

ALDH1A expression levels were directly associated with advanced tumor stage, lymph node metastasis, but not with tumor grade (p= < 0.001, 0.014, 0.552 respectively). Higher ALDH1A score was inversely related to response to NAC (p = 0.001) (table 2, Fig. 1D, 2D).

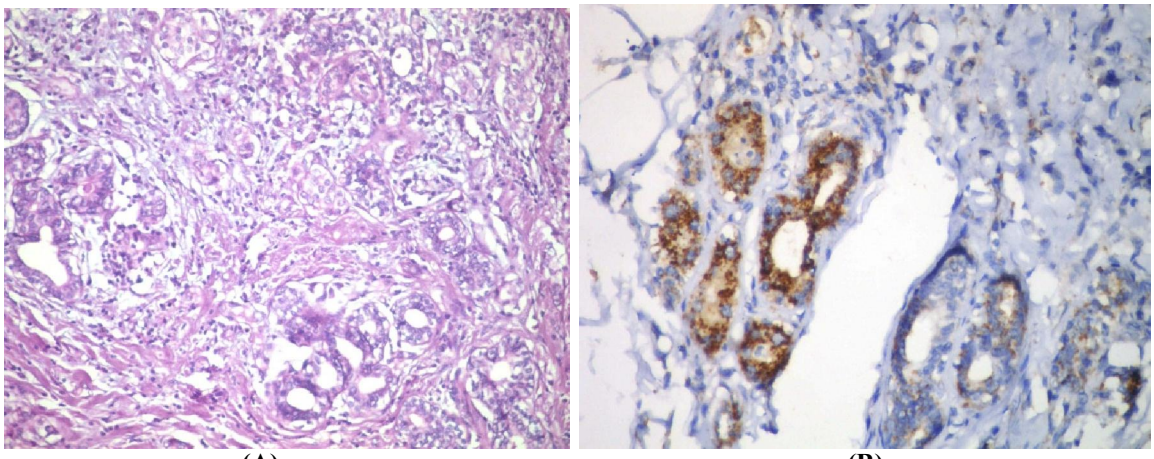
Table (2): GSTP1, TOP2α & ALDH1A scores distribution according to patients' clinicopathological features & response to NAC in studied TNBC patients.

Characteristics	*No*	GSTP1 Median (Range)		p§	TOP2α Median (Range)		p§	ALDH1A Median (Range)		p§
Age (years)										
≤ 50 years	14	42.5	(0 – 90)	0.235	16	(0 – 90)	0.327	40	(0 – 85)	0.297
> 50 years	16	14.5	(0 – 75)		40	(0 – 90)		8.5	(0 – 80)	
Grade										
Grade II	18	8	(0 – 70)	0.039	37.5	(0 – 90)	0.116	12	(0 – 80)	0.552
Grade III	12	50	(0 – 90)		10	(0 – 85)		8.5	(0 – 85)	
Tumor stage (pT)										
T2	9	0	(0 – 60)	<0.001	75	(9 – 90)	<0.001	3	(0 – 57)	<0.001
T3	14	32.5	(5 – 55)		32.5	(0 – 70)		9	(5 – 80)	
T4	7	75	(9 – 90)		0	(0 – 15)		70	(8 – 85)	
Lymph node (pN)										
N1	4	1.5	(0 – 3)	0.001	72.5	(30 – 80)	0.028	0	(0 – 8)	0.014
N2	17	9	(0 – 70)		35	(0 – 90)		15	(0 – 80)	
N3	9	65	(9 – 90)		5	(0 – 55)		45	(6 – 85)	
Response to NAC										
OAR	19	5	(0 – 45)	<0.001	50	(0 – 90)	<0.001	6	(0 – 85)	0.001
NR	11	65	(8 – 90)		5	(0 – 20)		57	(8 – 85)	
Response to NAC										
CR	9	0	(0 – 20)	<0.001	75	(8 – 90)	<0.001	3	(0 – 15)	0.001
PR	10	19.5	(3 – 45)		37.5	(0 – 55)		14	(5 – 85)	
SD	8	57.5	(8 – 75)		5	(0 – 20)		51	(9 – 80)	
PD	3	90	(85 – 90)		0	(0 – 12)		70	(8 – 85)	

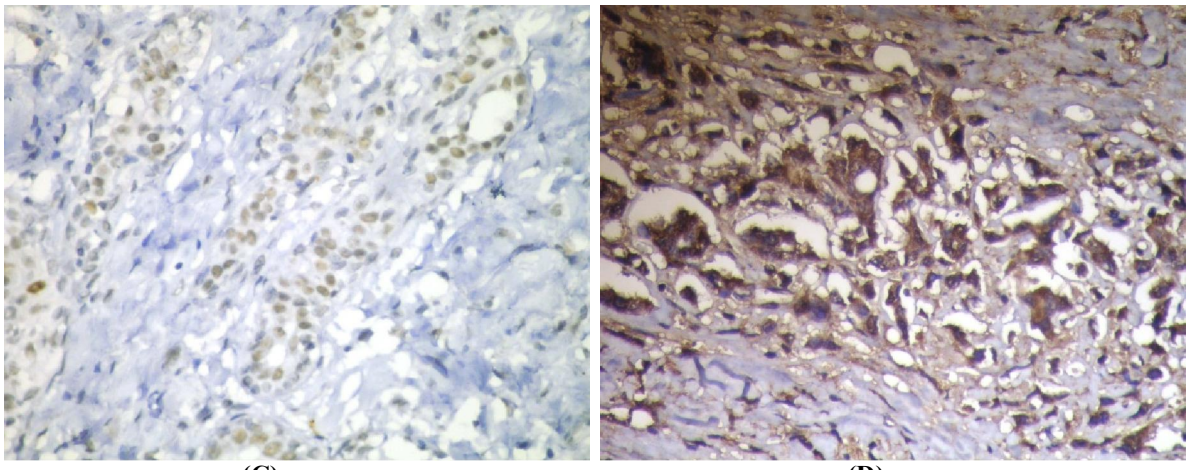
Continuous variables were expressed as the median (range); § *Mann Whitney U test* for two groups, *Kruskal-Walls H test* for three groups . p< 0.05 is significant.

Correlation between GSTP1, TOP2α and ALDH1A proteins:

Both GSTP1 and ALDH1A expression levels were inversely correlated to TOP2 α (r = -0.733, p = < 0.001& r = -0.720, p = <0.001 respectively), however they were directly correlated to each other (r = +0.626, P = <0.001) (table 3).

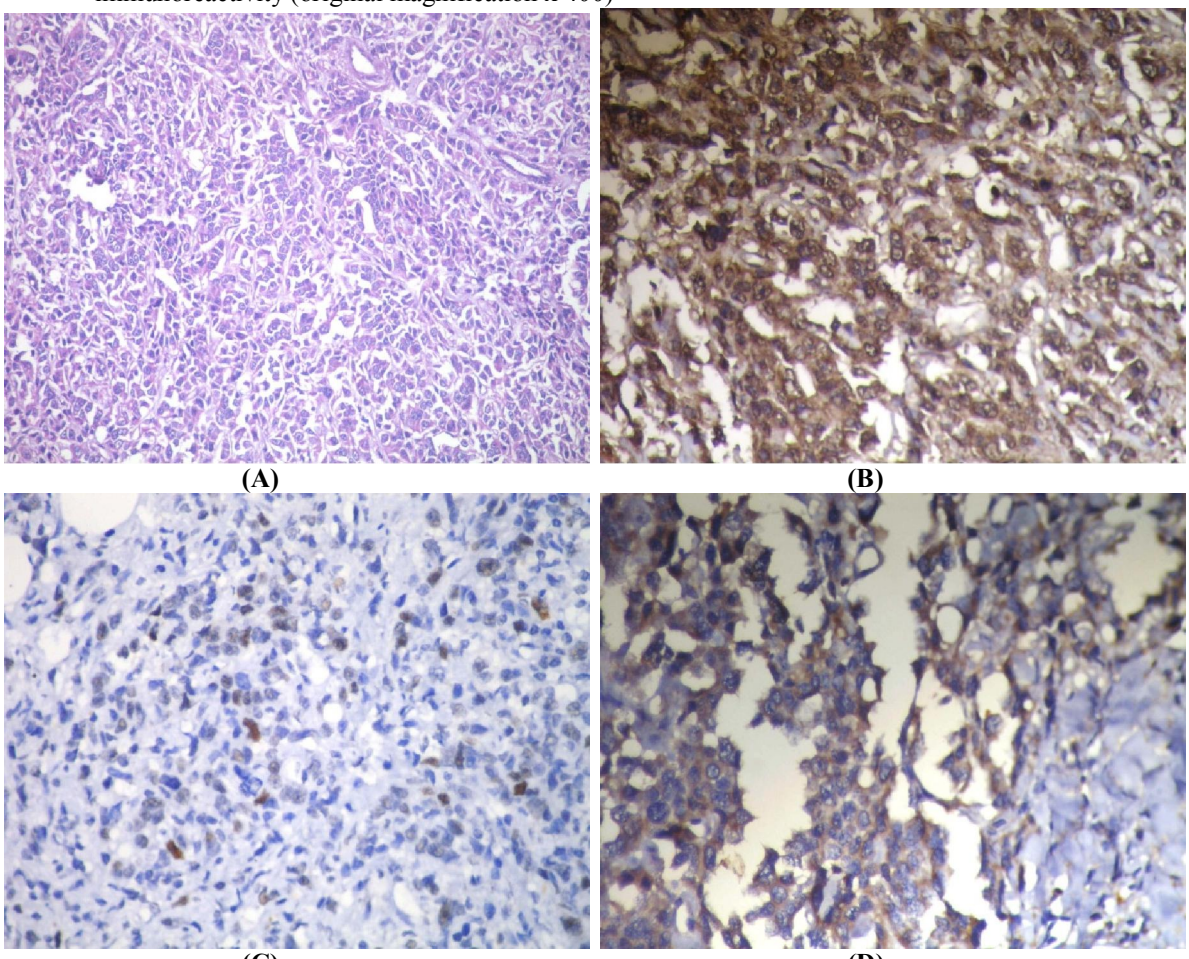


(A) **(B)**



(C) **(D)**

Fig. 1: A case of triple negative breast cancer showing: (A) malignant ductal epithelial cells arranged in groups with tubular formation; (B) cytoplasmic GSTP1 immunoreactivity (original magnification x 400); (C) diffuse nuclear TOP2α immunoreactivity (original magnification x 400); (D) cytoplasmic ALDH1A immunoreactivity (original magnification x 400)



(A) **(B)**

(C) **(D)**

Fig. 2: A case of triple negative breast cancer showing: (A) cords and sheets of malignant ductal epithelial cells; (B) diffuse cytoplasmic GSTP1 & scattered nuclear immunoreactivity (original magnification x 400); (C) focal nuclear TOP2α immunoreactivity (original magnification x 400); (D) cytoplasmic ALDH1A immunoreactivity (original magnification x 400)

Table (3): Association & correleation between GSTP1, TOP2α and ALDH1A staining.

	GSTP1		TOP2α		ALDH1A	
	r	p	r	p	r	p
GSTP1	----		- 0.733	<0.001	+ 0.626	<0.001
TOP2α	- 0.733	<0.001	----		- 0.720	<0.001
ALDH1A	+ 0.626	<0.001	- 0.720	<0.001	----	

r: correleation coefficient; p< 0.05 is significant.

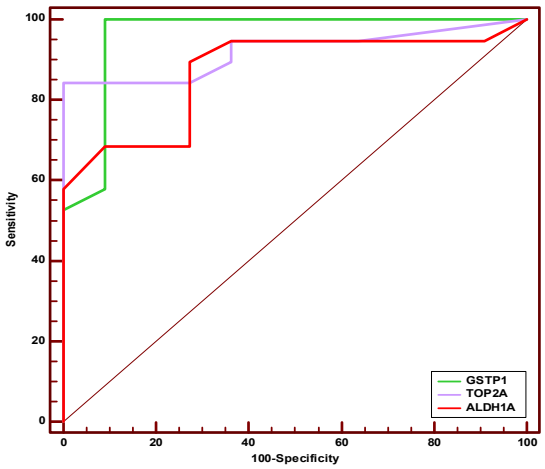
GSTP1, TOP2 α and ALDH1A proteins as predictors for response of TNBC patients to NAC:

Receiver Operating Characteristics (ROC) was calculated to explore the performance and the partial threshold values of GSTP1, TOP2 α and ALDH1A expression to predict response to neoadjuvant chemotherapy (table 4). GSTP1 expression level had 100% sensitivity and 90.9% specificity with AUC of 0.959 in predicating overall response versus no response to NAC, however adding ALDH1A and TOP2 α expression to GSTP1 had 94.7% sensitivity, 100% specificity and AUC of 0.976. (table 4, fig. 3).

Table (4): GSTP1, TOP2α and ALDH1A scores as a predictor for response of TNBC patients to NAC; ROC curve Analysis

IHC	Cut-off values	Sens. % (95% CI)	Spec. % (95% CI)	PPV % (95% CI)	NPV % (95% CI)	Accuracy (95% CI)	AUC (95% CI)
Overall response (OAR) vs No response (NR)							
GSTP1	**≤ 45**	100 % (82.4-100)	90.9 % (58.7-99.8)	95 % (75.1-99.9)	100 % (69.2 – 100)	96.6 % (73.7 – 99.92)	0.959‡ (0.817-0.998)
TOP2α	**> 20**	84.2 % (60.4 – 96.6)	100 % (71.5 – 100)	100 % (79.4 – 100)	78.6 % (49.2 – 95.3)	89.9 % (64.4 – 97.8)	0.921‡ (0.762-0.988)
ALDH1A	**≤ 30**	89.4 % (66.9-98.7)	72.7 % (39-94)	85 % (62.1-96.8)	80 % (44.4 – 97.5)	83.2 % (56.6 – 96.9)	0.871‡ (0.698-0.965)
GSTP1 & TOP2α	**≤ 26**	100 % (82.4 – 100)	90.9 % (58.7 – 99.8)	95 % (75.1 – 99.9)	100 % (69.2 – 100)	96.6 % (73.7 – 99.9)	0.986‡ (0.858-1.000)
GSTP1 & ALDH1A	**≤ 55**	94.7 % (74 – 99.9)	100 % (71.5 – 100)	100 % (81.5 – 100)	91.7 % (61.5 – 99.8)	96.6 % (73 – 99.9)	0.981‡ (0.850-1.000)
ALDH1A & TOP2α	**≤ -3**	84.2 % (60.4 – 96.6)	100 % (71.5 – 100)	100 % (79.4 – 100)	78.6 % (49.2 – 95.3)	89.9 % (64.4 – 97.8)	0.921‡ (0.762-0.988)
GSTP1, TOP2α & ALDH1A	**≤ 46**	94.7 % (74 – 99.9)	100 % (71.5 – 100)	100 % (81.5 – 100)	91.7 % (61.5-99.8)	96.6 % (73 – 99.9)	0.976‡ (0.843-1.000)
Complete response (CR) vs Partial response (PR)							
GSTP1	**≤ 5**	88.8 % (51.8 – 99.7)	80 % (44.4 – 97.5)	80 % (44.4 – 97.5)	88.9 % (51.8 – 99.7)	84.2 % (47.9 – 98.5)	0.917‡ (0.697-0.993)
TOP2α	**> 55**	88.8 % (51.8 – 99.7)	100 % (69.2 – 100)	100 % (63.1 – 100)	90.9 % (58.7 – 99.8)	94.7 % (61 – 99.9)	0.900‡ (0.675-0.989)
ALDH1A	**≤ 5**	88.8 % (51.8 – 99.7)	90 % (55.5 – 99.7)	88.9 % (51.8 – 99.7)	90 % (55.5 – 99.7)	89.4 % (53.7 – 99.7)	0.928‡ (0.712-0.996)
GSTP1 & TOP2α	**≤ - 60**	88.8 % (51.8 – 99.7)	100 % (69.2 – 100)	100 % (63.1 – 100)	90.9 % (58.7 – 99.8)	94.7 % (61 – 99.9)	0.900‡ (0.675-0.989)
GSTP1 & ALDH1A	**≤ 25**	100 % (66.4 – 100)	90 % (55.5 – 99.7)	90 % (55.5 – 99.7)	100 % (66.4 – 100)	94.7 % (60.7 – 99.8)	0.978‡ (0.786-1.000)
ALDH1A & TOP2α	**≤ - 57**	88.8 % (51.8 – 99.7)	100 % (69.2 – 100)	100 % (63.1 – 100)	90.9 % (58.7 – 99.8)	94.7 % (61 – 99.9)	0.922‡ (0.705-0.995)
GSTP1, TOP2α & ALDH1A	**≤ - 57**	88.8 % (51.8 – 99.7)	100 % (69.2 – 100)	100 % (63.1 – 100)	90.9 % (58.7 – 99.8)	94.7 % (61 – 99.9)	0.922‡ (0.705-0.995)
Stable disease (SD) vs progressive disease (PD)							
GSTP1	**≤ 75**	100 % (63.1 – 100)	100 % (29.2 – 100)	100 % (63.1 – 100)	100 % (29.2 – 100)	100 % (53.8 – 100)	1.000‡ (0.715-1.000)
TOP2α	**> 0**	75 % (34.9 – 96.8)	66.6 % (9.4 – 99.2)	85.7 % (42.1 – 99.6)	50 % (6.8 – 93.2)	72.7 % (27.9 – 97.5)	0.667§ (0.334-0.908)
ALDH1A	**≤ 65**	75 % (34.9 – 96.8)	66.6 % (9.4 – 99.2)	85.7 % (42.1 – 99.6)	50 % (6.8 – 93.2)	72.7 % (27.9 – 97.5)	0.604§ (0.281-0.871)
GSTP1 & TOP2α	**≤ 70**	100 % (63.1 – 100)	100 % (29.2 – 100)	100 % (63.1 – 100)	100 % (29.2 – 100)	100 % (53.8 – 100)	1.000§ (0.715-1.000)
GSTP1 & ALDH1A	**≤ 135**	100 % (63.1 – 100)	66.6 % (9.4 – 99.2)	88.9 % (51.8 – 99.7)	100 % (2.5 – 100)	90.9 % (48.4 – 99.8)	0.792§ (0.454-0.968)
ALDH1A & TOP2α	**≤ 80**	100 % (63.1 – 100)	33.3 % (0.8 – 90.6)	80 % (44.4 – 97.5)	100 % (50 – 100)	81.8 % (46.1 – 97.4)	0.625§ (0.298-0.884)
GSTP1, TOP2α & ALDH1A	**≤ 135**	100 % (63.1 – 100)	66.6 % (9.4 – 99.2)	88.9 % (51.8 – 99.7)	100 % (2.5 – 100)	90.9 % (48.4 – 99.8)	0.833§ (0.499-0.982)

‡p < 0.05; § p > 0.05; ROC curve: Receiver Operating Characteristic curve; Sens: Sensitivity; Spec: Specificity; PPV: Positive Predictive Value; NPV: Negative Predictive Value; AUC: Area Under Curve; 95%CI: 95% Confidence Interval; *p* < 0.05 is significant.



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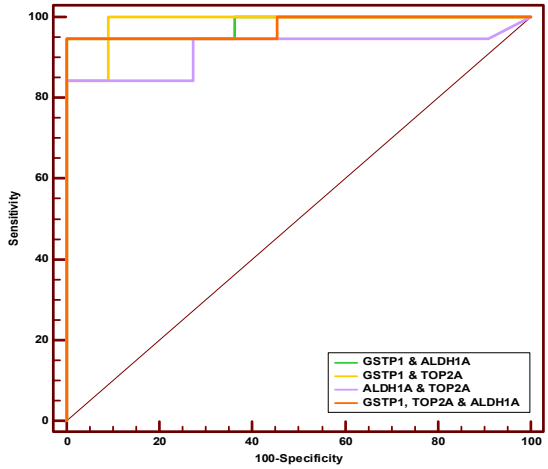

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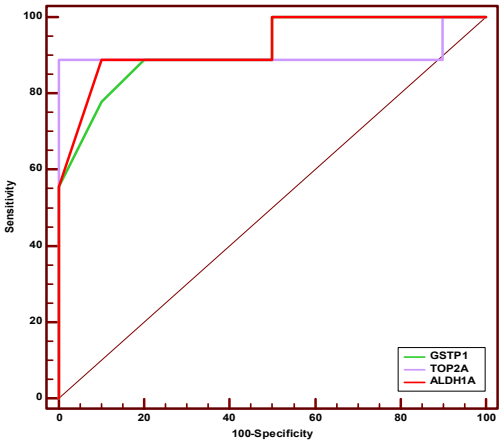

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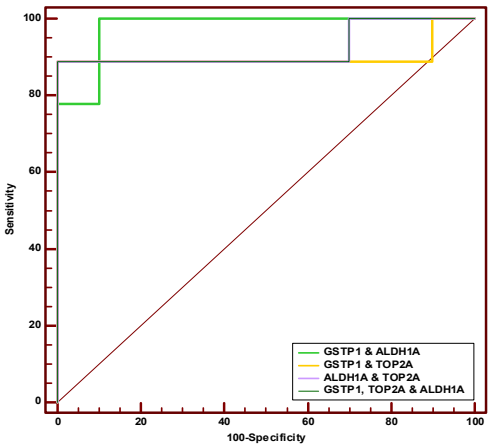

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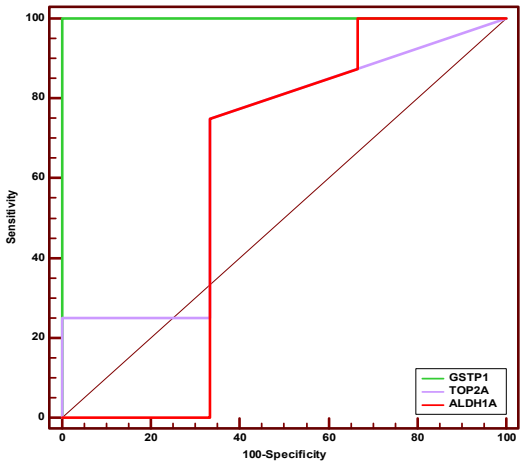
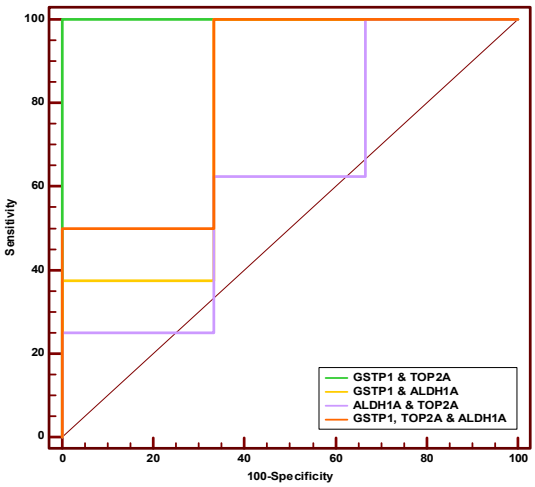


(e)



(f)

Figure (3) ROC curves comparing GSTP1, TOP2α and ALDH1A scores & combinations of the three markers as a predictor for response of triple negative breast cancer to NAC: (a) &(b) OAR vs NS;(c) & (d) CR vs PR; (e) & (f) SD vs PD; GSTP1 & TOP2α = GSTP1 - TOP2 α; GSTP1 & ALDH1A = GSTP1 + ALDH1A; ALDH1A & TOP2α = ALDH1A – TOP2α; GSTP1, TOP2α and ALDH1A = GSTP1 - TOP2α + ALDH1A

Multivariate logistic regression showed that the GSTP1 expression level was the only independent predictive factor for response to neoadjuvant chemotherapy ($p = 0.005$) (table 5).

Table (5): Predictive of value of GSTP1, TOP2α and ALDH1A staining for overall response of TNBC to NAC:

	Univariate analysis				Multivariate analysis			
	β	OR (95%CI)		p	β	OR (95%CI)		p
Age group	1.099	3.000	(0.968 – 9.302)	0.057				
Grade	0.000	1.000	(0.323 – 3.101)	1.000				
T	-1.791	0.167	(0.020 – 1.385)	0.121				
N	-0.693	0.500	(0.125 – 1.999)	0.327				
GSTP1	-0.017	0.983	(0.964 – 1.002)	0.080	-0.1023	0.902	(0.839 – 0.970)	0.005
TOP2α	0.055	1.056	(1.011 – 1.104)	0.014				
ALDH1A	-0.012	0.988	(0.968 – 1.008)	0.230				
Constant					4.4478			

β: regression coefficient; 95%CI: 95% Confidence Interval; OR: Odd Ratio; Overall model fit: chi square=39.429, d.f=1, p<0.001. $p< 0.05$ is significant.

4. Discussion:

In spite of the tremendous efforts done to determine the appropriate chemotherapy for individual breast cancer patients, there is no reliable marker to select the best treatment regimen or to monitor response during therapy. Previous studies have shown that the response to anthracycline-based chemotherapy is the most important predictive factor for outcome, and that patients who are responsive to anthracycline-based combinations derive the most benefit from adding other regimens, especially taxans[18,15]. Theoretically, monitoring biological markers early during the course of NAC would help in making personalized treatment decisions, thus preventing unnecessary toxicity from a nonresponsive regimen.

GSTP1protein is thought to be involved in chemoresistance through the detoxification of chemotherapeutic agents and inhibition of chemotherapy-induced apoptosis. In this study, we investigated the association of GSTP1 protein expression with clinicopathological features of TNBC cases, and found that higher GSTP1 expression levels showed significant direct association with tumor grade, stage and lymph node metastasis (p= 0.039, < 0.001, 0.001 respectively). These findings confirmed those from several previous studies[19,20], suggesting a possibility that GSTP1 protein expression can be useful as a marker for biological aggressiveness of breast cancers. However, **Huang et al.**[21] and **Franco et al.**[22] reported that GSTP1 immunoreactivity was not associated with any clinicopathological features. **Miyake et al.**[16] suggested that GSTP1 promoter hypermethylation, especially in ER-positive tumors, results in diminished detoxification of DNA-damaging estrogen metabolites such as E2-2,3-Q and E2- 3,4-Q and in the development of breast tumors with relatively high histological grade.

Top2α protein is one of the intracellular targets for anthracycline-based chemotherapy. Drugs trap the enzyme in a cleavable complex, thereby inhibiting its function, leading to double strand DNA breaks and cell death. We found that TOP2α expression level was inversely associated with advanced tumor stage, nodal metastasis, but not tumor grade (p=<0.001, 0.028, 0.116 respectively). Similar results were reported by **Romero et al.**[23] **and Mrklic et al.**[17], but others showed that Top2α immunoreactivity was not associated with clinicopathological features of the disease[24,25].

ALDH1A is an important enzyme, in cancer stem cell differentiation, that regulates the conversion of retinoic acid to oxidizing retinol. Breast cancer stem cells (CSCs) are associated with a biologically aggressive phenotype, also they express high levels of ATP-binding cassette transporters and thus are thought to be resistant to various chemotherapeutic agents effluxed by ATP-binding cassette transporters. CSCs have been shown to be resistant to paclitaxel, doxorubicin, 5-flurouracil and platinum[26,27].

In our study, cases with higher ALDH1A expression levels were directly associated with advanced tumor stage, lymph node metastasis, but not with tumor grade (p=<0.001, 0.014, 0.552 respectively). In the study of **Khoury et al.**[14] and the meta-analysis of **Liu et al.**[28], ALDH1A immunoreactivity was found to be significantly associated with higher tumor grade, advanced stage as well as nodal metastasis, but not with patient age. Conversely, other studies reported no association with any clinicopathological feature of the tumor[27,29]. The different cut off values of TOP2α and ALDH1A expression, difference in tumor stages, grades of selected patients and different antibodies used in studies, all contribute to the controversy among studies.

An interesting finding of the present study is that both GSTP1 and ALDH1A expression levels were inversely correlated to TOP2 α (r = -0.733, p = <0.001& r = -0.720, p = <0.001 respectively), however they were directly correlated to each other (r = +0.626, P = <0.001). The triple negative basal-like subtype of breast cancer was suggested to have stem

cell-like phenotype with higher levels of GSTP1 protein expression[16, 30].

Although using the Mann-Whitney U test & Kruskal-Wallis H test for analysis of relationship of GSTP1, Top2α and ALDH1A proteins expression to the response to NAC proved highly significant relationship (p = <0.001, <0.001 &0.001 respectively), but multivariate logistic regression showed that GSTP1 expression was the only independent predictive factor for response to NAC (p = 0.005), indicating that GSTP1 plays a significant role in suppression of anti tumor activity of NAC. Also the ROC curve showed that GSTP1 expression had 100% sensitivity and 90.9% specificity with AUC of 0.959 in predicating overall response versus no response to NAC. Adding ALDH1A and TOP2 α expression to GSTP1 had 94.7% sensitivity, 100% specificity and AUC of 0.976. These findings taken together lead us to consider that GSTP1 protein expression may serve as an independent marker for prediction of response to NAC, however combining TOP2 α and ALDH1A with GSTP1 seems to increase its predicatively.

GSTP1 expression was found to be associated with poor response to chemotherapeutic agents. In vitro studies confirmed these findings[20,21,31]. In mammary carcinoma cell line, the development of resistance to the chemotherapeutic agent doxorubicin was followed by increase in GSTP1 gene expression[32]. This is consistent with data showing that GST overexpression protects against anthracyclin-induced cell death, highlighting that detoxification of anthracyclins is mediated through the GSTP system[31]. Because GSTP1 is a major phase II detoxification enzyme, it is possible that tumor cells lacking GSTP1 are more vulnerable to the cytotoxic effect of certain chemotherapeutic agents, due to decrease in cellular defense mechanisms in response to oxidative stress[15].

In most studies, Top2α gene amplification, with subsequent protein expression, has been detected in a setting of co-amplification, and protein expression, of HER2 gene, but rarely in HER2 non-amplified patients, this may be explained by the fact that Top2α gene is in close proximity to HER2 gene at 17q21-22[24,33,34]. Some studies reported that Top2α amplification, but not the protein expression, predicted benefit from chemotherapy only in HER2- positive tumors[35,36]. While others found no association[27,33,37,38]. High Top2α expression in TNBCs was proved to be associated with better response to anthracycline-based chemotherapy[17,39]. The discrepancy among researchers regarding the predictive value of Top2α expression might be, in part, because of different proportions of breast cancer subtypes between study populations, **Romero et al.**[23] demonstrated that Top2α expression is substantially different across the known molecular subtypes. Also the various methods of assessment, usually semiquantitative, and the small number and diversity of patients studied may contribute to this discrepancy.

Tanei et al.[27] concluded that ALDH1A-positive cells have a role in resistance to anthracycline-based therapy. This is consistent with previous studies reporting that ALDH1A expression is associated with decrease overall survival and recurrence free survival of patients as a consequence of treatment failure[14,29]**.** It was speculated that the reason for this resistance was that these tumors contain a higher proportion of cancer stem cells, also ALDH1A functions as an enzyme that inactivates cyclophosphamides[14].

Conclusion:

In conclusion, due to lack of expression of ER, PR, and HER2 receptors in TNBC, specific targeted therapies are not effective, and chemotherapy is currently the only modality of available systemic therapy. GSTP1 protein expression has the potential to serve as an independent marker for identifying patients with triple negative breast cancer that are unlikely to benefit from neoadjuvant chemotherapy (AC-Taxol), however combining TOP2 α and ALDH1A with GSTP1 seems to increase its predicatively. So a panel of these markers might be of value in detecting patients who would not respond to AC-Taxol, making them candidates for other chemotherapeutic regimens. Further studies including large number of cases and different molecular subtypes of breast cancer would be required to confirm these findings.

Conflicts of interests: Non.

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