Increased expression of biological markers as potential therapeutic targets in Saudi women with triple-negative breast cancer

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ABSTRACT

Aims and background. Triple-negative breast cancer (TNBC) is an aggressive subtype of breast cancer that lacks the expression of hormone receptors and human epidermal growth factor receptor 2 (HER2). Although TNBC represents only 15% of all types of breast cancer, it accounts for a large number of metastatic cases and deaths. Because of the high metastatic rate and both local and systemic recurrence associated with TNBC, extensive research efforts are actively looking for target therapies to effectively treat this aggressive disease. Accordingly, this study has been initiated to investigate the differential expression of biological markers in TNBC and non-TNBC Saudi women that might be utilized as potential targeted therapy and/or predict the sensitivity to currently available therapeutic regimens.

Methods and study design. Two hundred formalin-fixed, paraffin-embedded (FFPE) breast cancer tissues were selected and divided into 3 groups: benign breast tissues (20), TNBC tissues (80) and non-TNBC tissues (100). Expression of mRNA in FFPE tissues was analyzed using real-time polymerase chain reaction (RT-PCR) for the following genes: poly (ADP-ribose) polymerase 1 (PARP-1), topoisomerase 2A (TOPO-2A), vascular endothelial growth factor (VEGF), C-MYC, basic fibroblast growth factor (bFGF), matrix metalloproteinases (MMP-2 and MMP-9), human epidermal growth factor 1 (HER1) and multidrug resistance (MDR) genes.

Results. In the TNBC group, expression of PARP-1, TOPO-2A, HER1, C-MYC, VEGF, bFGF and MMP-2 showed a highly significant increase compared to the non-TNBC group.

Conclusions. The results of this study suggest that (1) TNBC patients will benefit more from TOPO-2A inhibitors as well as antiangiogenic and antimetastatic therapies; (2) inhibition of these target genes is emerging as one of the most exciting and promising targeted therapeutic strategies to treat TNBC in which the intended targets are DNA repair, tumor angiogenesis and metastasis.

Introduction

Breast cancer is a heterogeneous disease that encompasses distinct subtypes with remarkable differences in both biological characteristics and clinical behavior[1]. Triple-negative breast cancer (TNBC) is a subtype of breast cancer that lacks the expression of estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2). This uncommon subtype accounts for approximately 15% of all breast cancer cases and has a poor prognosis despite responding to conventional chemotherapy regimens[2]. Moreover, it has been reported that TNBC is associated with high rates of recurrence, metastasis and death[1]. The incidence, clinico-

pathological characteristics and prognostic factors of TNBC in Saudi women have been investigated recently[3]. The authors reported that the incidence of TNBC in Saudi Arabia is 12%, which is similar to that described in the literature, and that it tends to behave aggressively and is more likely to develop brain metastasis than other breast cancer types[3]. To date, there has been no specific, well-defined treatment protocol for TNBC[4]. Accordingly, chemotherapy and biological targeted therapy optimization needs to be evaluated in TNBC.

The most widely used predictive markers in breast cancer are ER/PR and HER2, whose overexpression allows to tailor antihormonal therapy and therapy with the monoclonal antibody trastuzumab, respectively[5-11]. TNBC is a very aggressive subtype of breast cancer with a very poor prognosis and high recurrence rates[12-14]. Since ER/PR and HER2 are not expressed in TNBC, therapies that decrease estrogen synthesis or block ER or HER2 including aromatase inhibitors, estrogen receptor blockers and trastuzumab are ineffective in its treatment[15,16]. Moreover, characterization of other surface receptors and other biological targets in TNBC as well as novel therapies capable of treating advanced TNBC are lacking. In the currently available literature we have been unable to find any studies investigating the expression of biological markers in TNBC affecting Saudi women and its relation to different treatment interventions. Therefore, the current study was initiated to investigate the expression of some biological markers which play an important role in DNA repair, topological states of DNA during transcription, cellular proliferation, angiogenesis and tumor growth and metastasis including poly (ADP-ribose) polymerase 1 (PARP-1), topoisomerase 2A (TOPO-2A), vascular endothelial growth factor (VEGF), C-MYC, basic fibroblast growth factor (bFGF), matrix metalloproteinases (MMP-2 and MMP-9), human epidermal growth factor 1 (HER1) and multidrug resistance (MDR) genes. Also, these biological molecules are currently used in many tumors as therapeutic targets for newly developed biological therapies such as PARP inhibitors, TOPO-2A inhibitors, monoclonal antibodies, tyrosine kinase inhibitors (TKIs), and inhibitors of angiogenesis and metastasis. Accordingly, investigating the clinical predictive value of the differential expression of these biological markers in Saudi women with TNBC and non-TNBC is of major importance and such expression might be utilized in targeted therapy, which is the trend in clinical practice these days.

Materials and methods

The present study is based on 200 Saudi women living in the province of Riyadh, Kingdom of Saudi Arabia, with primary invasive breast cancer who had undergone surgery between January 2009 and January 2011 (retrospective analysis). Two hundred archival formalin-fixed paraffin-embedded (FFPE) breast carcinoma tissues were collected from the Pathology Department, College of Medicine, King Saud University (135 cases) and the Pathology Department, Al-Shemissy Hospital (65 cases), Riyadh, Saudi Arabia. The inclusion criterion was archived primary breast tumor (T stage 1-3 invasive ductal carcinoma of NOS type) assessed by immunohistochemistry for expression of ER, PR and HER2 at the time of diagnosis. Whole tumor sections were examined to carefully review the histological characteristics of each tissue specimen. The protocol of this study was approved by the Ethical Committee of the College of Medicine, King Saud University.

Study design

A total of 200 FFPE samples were classified into 3 separate groups. Group 1 included 20 benign breast tissues and served as control. Group 2 (non-TNBC) included 100 FPE samples from patients with non-TNBC. Group 3 (TNBC) included 80 samples from patients with TNBC. The demographic and clinical characteristics of the patients and tumors were obtained from the patients' pathology reports and medical records and are summarized in Table 1.

Tissue microdissection for gene expression study

All FFPE samples (200 blocks) were sectioned at 8 μm thickness using a Leica microtome (Manual Rotary Microtome RM2235) at the Pathology Department, College of Medicine, King Saud University. Tissue sections were floated in a DEPC-treated water bath then picked up on clean glass slides and allowed to air dry at 4 °C, after which they were stored at -20 °C until used. Immediately before microdissection, tissue sections were stained with hematoxylin-eosin and examined under a light microscope (Nikon Eclipse E600) without cover-slipping to define and exclude nontumor tissues from each sample. Selected areas of tumor tissues were removed from the slides using a scalpel and placed directly into sterile 2 mL Eppendorf tubes for total RNA extraction.

Quantification of mRNA expression by real-time polymerase chain reaction (RT-PCR)

Total RNA was extracted after microdissection using a RecoverAll Total Nucleic Acid Isolation Kit (Ambion cat #: AM1975) and WaxFree RNA kit (TrimGen Cat #: WR-50) according to the manufacturer's instructions. The quantity and integrity of extracted RNA were characterized using a NanoDrop 8000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA) and expression automated electrophoresis station (BioRad). The isolated RNA had an A 260/280 ratio of 1.9-2.1. First-strand cDNA was synthesized from 1 g of total RNA by reverse transcription with a SuperScript first-strand synthesis system kit (Invitrogen, Carlsbad, CA, USA), according to

Table 1 - **Demographic and clinical characteristics of Saudi women with breast cancer involved in the study**

Characteristics	Breast cancer patients	
	Non-triple negative (n = 100)	Triple negative (n = 80)
Age group		
<40 years	22 (22%)	36 (45%)
40-49 years	46 (46%)	28 (35%)
50 + years	32 (32%)	16 (20%)
Mean age ± SEM (range)	48 ± 1.12 (24-91)	42.5 ± 1.2 (28-70)
Tumor grade		
1	6	9
2	29	36
3	65	35
Tumor size (cm)		
Less than 2	21	21
More than 2	38	50
Unknown	41	9
Lymph node status		
Positive	33	36
Negative	20	37
Unknown	47	7
ER, PR and HER2/neu status		
ER, PR and HER2/neu negative	0	80
ER, PR and HER2/neu positive	53	0
ER, PR negative and HER2/neu positive	19	0
ER, PR positive and HER2/neu negative	15	0
ER, HER2/neu positive and PR negative	13	0

SEM, standard error of mean.

the manufacturer's instructions. The levels of gene expression were measured using the Taqman probe PCR technique. TaqMan Master Mix (Applied Biosystems, USA) with ROX dye was used to measure the gene expression by means of the $2^{-\Delta\Delta Ct}$ method. We used the GAPDH gene as the endogenous control and benign breast tissues as reference samples. The PCR assay was optimized by varying the PCR conditions such as the concentration of cDNA, MgSO4, amplification cycle number, probe and primer concentration and annealing temperature. The PCR reaction mixture (25μL volume) contained a final concentration of 100 ng cDNA and 300 nM of each forward and reverse primer and probe [AUTHORS: Change OK?]. The primers and probes listed in the table hereunder were designed by the Primer Express software, v. 3 (Applied Biosystems) and synthesized by the Biolegio Company. Target genes were amplified in 96-well plates (Applied Biosystems). No template control was used as negative control. The cycling program included 1 cycle of 50 °C for 2 minutes then 95 °C for 10 minutes followed by 40 cycles of denaturation at 94 °C for 30 seconds followed by annealing/extension at 60 °C for 1 minute. The results are represented as fold expression.

Gene name	Forward primer	Reverse primer	Probe
GAPDH	CCACTCCTCCACCTTTGAC	ACCCTGTTGCTGTAGCCA	FAM-TTGCCCTCAACGACCACTTTGTC-TAMRA
PARP-1	CAAGAGCGATGCCTATTACTG	AGGTAAGAGATTTCTCGGAATTC	FAM-TATGGTCAAGACACAGACACCCAACCGG-TAMRA
TOPO-2A	AGTCGCTTTCAGGGTTCTTGAG	TTTCATTTACAGGCTGCAATGG	FAM-CCCTTCACGACCGTCACCATGGA-TAMRA
HER1	TCCGTGAGTTGATCATCGAATT	GCATTCTTTCATCCCCCTGAA	FAM-CCCGAGACCCCCAGCGCTACC-TAMRA
C-MYC	ACCACCAGCAGCGACTCTGA	TCCAGCAGAAGGTGATCCAGACT	FAM-ACCTTTTGCCAGGAGCCTGCCTCT-TAMRA
VEGF	CGCTTACTCTCACCTGCTTCTG	ATGGGCTGCTTCTTCCAACA	FAM-AGACCACTGGCAGATGTCCCGGC-TAMRA
bFGF	GACTCACTATGGCCAGAAAGCA	GCCAATGGTCAAGGGAACAT	FAM-TCTTGTTTCTCCCCCTGCCAGTCTCTTC-TAMRA
MMP-2	CGCTCAGATCCGTGGTGAG	TGTCACGTGGCGTCACAGT	FAM-TTCTTCTTCAAGGACCGTTCATTTGGC-TAMRA
MMP-9	CCTGGAGACCTGAGAACCAAT	GCCACCCGAGTGTAACCATAG	FAM-ACAGGCAGCTGGCAGAGGAATACCTGTAC-TAMRA
BCRP	TGTCACAAGGAAACACCAATGG	ATGAAAACTTAACACAGCTCCTTCAG	FAM-CCCCGCGACAGCTTCCAATGAC-TAMRA
LRP	CAGCTGGCCATCGAGATCA	TCCAGTCTCTGAGCCTCATGC	FAM-CAACTCCCAGGAAGCGGCGGC-TAMRA
MRP1	TTGGCGCATTCCTTCTTCC	CAGTGACCTCTGGTCCTTAAA	FAM-CGGAACAAGTCGTGCCTGTTTTGGTAAAGA-TAMRA
MDR1	CATGGTGACATCTTCACGACC	ATCATCGGTGTGGTGAGTCAG	FAM-CCTGTATTGTTTGCCACCACGATAGCTGAAAAC-TAMRA

Statistical analysis

Differences between obtained values (mean ± standard error [SEM]) were analyzed by 1-way analysis of variance (ANOVA) followed by the Tukey-Kramer multiple comparison test. A *P* value of 0.05 or less was taken as a criterion for a statistically significant difference.

Results

The demographic and clinical characteristics of TNBC and non-TNBC patients are shown in Table 1. In non-TNBC patients, the mean age at diagnosis was 48 years (SEM 1.12; range 24-91 years). A total of 22 cases were less than 40 years of age, 46 cases were aged 40-50 years and 32 cases were over 50. All non-TNBC cases had invasive ductal carcinoma, with 65 cases having grade 3, 29 grade 2 and 6 grade 1 tumors. Moreover, more than 50% of non-TNBC cases (53) were positive for ER, PR and HER2/neu, 19 cases were ER and PR negative and HER2/neu positive, 15 cases were ER and PR positive and HER2/neu negative, and 13 cases were ER and HER2/neu positive and PR negative. Regarding tumor size in the non-TNBC group, 21 patients had tumors measuring less than 2 cm, 28 had tumors of more than 2 cm, and in 41 patients the tumors were of unknown size. In addition, 33 cases were lymph node positive, 20 cases were lymph node negative, and in 47 cases the lymph node status was unknown. In TNBC patients, the mean age at diagnosis was 42.5 years (SEM 1.2; range 28-70 years). Thirty-six patients were less than 40 years of age, 28 patients were aged 40-50 years, and 16 patients were over 50. Regarding the histological categories, all TNBC cases had invasive ductal carcinoma, with 35 cases having grade 3 tumors, 36 grade 2 tumors, and 9 grade 1 tumors. Regarding tumor size, in the TNBC group the tumors of 21 patients were less than 2 cm, 50 were more than 2 cm, and 9 tumors were of unknown size. In addition, 36 cases were lymph node positive, 37 cases were lymph node negative, and in 7 cases the lymph node status was unknown. Moreover, all TNBC cases (80) were negative for ER, PR and HER2/neu. These selected characteristics of the patients showed that there was no significant difference between TNBC and non-TNBC cases in terms of mean age, histological categories and histological grade of the tumors.

We compared the mRNA expression of PARP-1, TOPO-2A, HER1 and C-MYC in TNBC and non-TNBC patients (Figure 1). In TNBC patients, PARP-1 expression was significantly increased (10-fold and 2.5-fold compared with control and non-TNBC cases, respectively). Similarly, the expression of TOPO-2A, HER1 and C-MYC in TNBC cases was significantly increased (2.6-fold, 2.7-fold and 1.7-fold, respectively, compared with non-TNBC cases). Increased expression of these genes was observed in the non-TNBC group compared with the control group. The expression of these target genes and its relationship with tumor grade are summarized in Table 2.
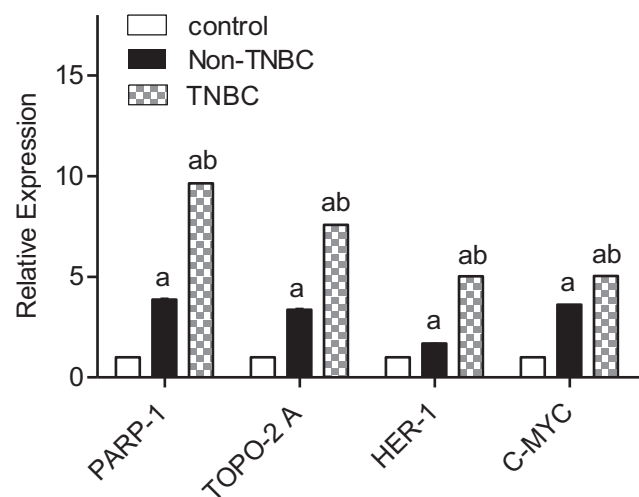

Figure 1 - Differential expression of poly (ADP-ribose) polymerase 1 (PARP-1), topoisomerase 2A (TOPO-2A), human epidermal growth factor 1 (HER1) and C-MYC genes in Saudi women with triple-negative breast cancer (TNBC) and non-triple negative breast cancer (non-TNBC). Data are presented as mean ± SEM (n = 20, 100 and 80 for control, non-TNBC and TNBC groups, respectively). a and b indicate significant change with respect to the control and non-TNBC groups, respectively, at *P* <0.05 using ANOVA followed by Tukey-Kramer as a post-ANOVA test.

To investigate the potential role of antiangiogenic and antimetastatic therapy in TNBC, we analyzed the mRNA expression of VEGF, bFGF, MMP-2 and MMP-9 (Figure 2). Our results showed a differential significant increase in the expression of these target genes in TNBC cases compared with non-TNBC cases. The expression of angiogenic and metastatic factors and its relationship with tumor grade are summarized in Table 3.

To predict the differential sensitivity of TNBC and non-TNBC cases to the available chemotherapeutic regimens and different therapeutic interventions, we monitored the mRNA expression of multidrug resistance genes including breast cancer resistance protein (BCRP), lung resistance protein (LRP), multidrug resistance-associated protein 1 (MRP1) and multidrug resistance gene 1 (MDR1) (Figure 3). No significant changes in mRNA expression of BCRP, LRP and MDR1 among TNBC and non-TNBC cases were observed. Only the expression of MRP1 showed a significant increase in TNBC compared with non-TNBC cases. On the other hand, the expression of multidrug resistance genes was increased in both TNBC and non-TNBC cases compared with controls. The expression of multidrug resistance genes and its relationship with tumor grade are summarized in Table 4.

Discussion

Individualized therapy based on the expression of molecular biology markers of tumors and patients is an

Table 2 - **Differential expression of PARP-1, TOPO-2A, HER1 and C-MYC genes and its relation to tumor grade in Saudi women with breast cancer**

Tumor grade	Non-TNBC Fold expression					TNBC Fold expression				
	Number	PARP-1	TOPO-2A	HER1	C-MYC	Number	PARP-1	TOPO-2A	HER1	C-MYC
1	6	3.46±0.19	3.30±0.41	2.0±0.63	3.26±0.53	9	7.68 ±0.92[b]	7.72±0.46[b]	6.12±0.50[b]	4.6±0.82[b]
2	29	3.47±0.13	3.41±0.23	1.67±0.09	3.27±0.23	36	10.05±0.39[bc]	7.35±0.45[b]	4.77±0.29[b]	5.38±0.34[b]
3	65	4.08±0.14[d]	3.34±0.14	1.66±0.05	3.81±0.16	35	9.73±0.34[bc]	7.79±0.51[b]	5.19±0.29[b]	4.81±0.33[b]
Total	100	3.87±0.10	3.36±0.11	1.68±0.05	3.62±0.13	80	9.64±0.26[b]	7.58±0.31[b]	5.03±0.18[b]	5.04±0.23[b]

Data are presented as mean ± SEM. [b]Indicates a significant change with respect to non-TNBC; [c]indicates a significant change with respect to grade 1; [d]indicates a significant change with respect to grade 2, at $P < 0.05$ using ANOVA followed by Tukey-Kramer as a post-ANOVA test. SEM, standard error of mean; TNBC, triple-negative breast cancer; PARP-1, poly (ADP-ribose) polymerase 1; TOPO-2A, topoisomerase 2A; HER1, human epidermal growth factor 1
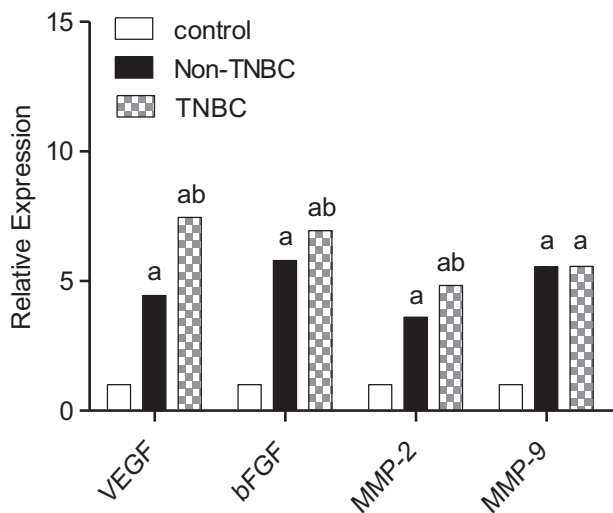
Figure 2 - Differential expression of vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF) and matrix metalloproteinases (MMP-2 and MMP-9) genes in Saudi women with triple-negative breast cancer (TNBC) and non-triple negative breast cancer (non-TNBC). Data are presented as mean ± SEM (n = 20, 100 and 80 for control, non-TNBC and TNBC groups, respectively). a and b indicate significant change with respect to control and non-TNBC groups, respectively, at $P < 0.05$ using ANOVA followed by Tukey-Kramer as a post-ANOVA test.

important strategy in the treatment of different types of human cancer. Although some promising agents are being developed for the treatment of TNBC, no targeted treatment is available in routine clinical practice today. Accordingly, the development of targeted agents based on the expression of biological markers to be used as combined or monotherapy is urgently needed for patients with TNBC. The current study was undertaken to investigate the clinical predictive value of the differential expression of biological markers in Saudi women with TNBC and non-TNBC, which might be utilized in targeted therapy, the trend in clinical practice these days.

PARP-1, a nuclear enzyme, plays an important role in the repair of single-strand DNA breaks via the base excision repair pathway and represents an important novel target in cancer therapy[17]. The data presented in this study have demonstrated a differential increase in the mRNA expression of the PARP-1 gene in TNBC compared with non-TNBC cases (Figure 1). PARP-1 expression also showed a positive correlation with tumor grade in both TNBC and non-TNBC cases. Our results are consistent with the data presented by Ossovskaya *et al*. in 2010[18], which demonstrated more than 2-fold up-

Table 3 - Differential expression of VEGF, bFGF, MMP-2 and MMP-9 genes and its relation to tumor grade in Saudi women with breast cancer

Tumor grade	Non-TNBC Fold expression					TNBC Fold expression				
	Number	VEGF	bFGF	MMP-2	MMP-9	Number	VEGF	bFGF	MMP-2	MMP-9
1	6	5.04±0.38	5.12±0.34	3.2±0.29	6.25±0.86	9	7.51±0.72[b]	6.9±0.52[b]	5.34±0.40[b]	6.12±0.50
2	29	4.38±0.20	5.38±0.19	3.3±0.20	5.26±0.39	36	7.50±0.46[b]	6.93±0.27[b]	4.66±0.20[b]	5.32±0.28
3	65	4.41±0.14	6.04±0.17	3.78±0.09[d]	5.58±0.23	35	7.40±0.44[b]	7.0±0.23[b]	4.87±0.18[b]	5.68±0.24
Total	100	4.44±0.11	5.79±0.13	3.61±0.07	5.55±0.13	80	7.46±0.29[b]	6.95±0.17[b]	4.83±0.13[b]	5.57±0.17

Data are presented as mean ± SEM. [b]Indicates a significant change with respect to non-TNBC; [d]indicates a significant change with respect to grade 2, at $P < 0.05$ using ANOVA followed by Tukey-Kramer as a post-ANOVA test. SEM, standard error of mean; TNBC, triple-negative breast cancer; VEGF, vascular endothelial growth factor; bFGF, basic fibroblast growth factor; MMP-2 and MMP-9, matrix metalloproteinases
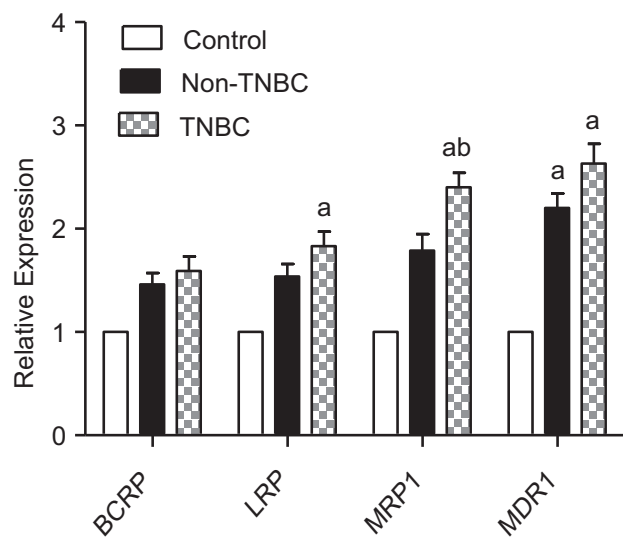
Figure 3 - Differential expression of breast cancer resistance protein (BCRP), lung resistance protein (LRP), multidrug resistance-associated protein 1 (MRP1) and multidrug resistance gene 1 (MDR1) in Saudi women with triple-negative breast cancer (TNBC) and non-triple negative breast cancer (non-TNBC). Data are presented as mean ± SEM (n = 20, 100 and 80 for control, non-TNBC and TNBC groups, respectively). a and b indicate significant change with respect to control and non-TNBC groups, respectively, at *P* <0.05 using ANOVA followed by Tukey-Kramer as a post-ANOVA test.

BRCA2 mutations and cancers harboring defects in other DNA repair proteins[21,22]. Earlier and recent studies confirmed that loss of BRCA-dependent DNA repair mechanisms combined with the PARP inhibitor olaparib (AZD2281) is associated with synthetic lethality and augmented cell death[21,23,24]. Preliminary results of a recent randomized phase II trial of chemotherapy (carboplatin plus gemcitabine) combined with the PARP inhibitor BSI-201 in metastatic TNBC showed significantly improved clinical benefit, progression-free survival and overall survival compared with carboplatin plus gemcitabine alone[25]. On the other hand, data from a phase II study with the PARP inhibitor olaparib reported that PARP inhibitors combined with chemotherapy are more effective in the treatment of patients with sporadic TNBC than PARP inhibitors as monotherapy[26]. However, combined treatment of TNBC patients with olaparib and paclitaxel was associated with grade 2 to 4 neutropenia, which required dose modifications for both olaparib and paclitaxel[27]. More recently, Chuang *et al.*[28] investigated the effects of 4 different PARP inhibitors (AG-014699, AZD-2281, ABT-888 and BSI-201) in 3 genetically distinct TNBC cell lines (MDA-MB-468, MDA-MB-231 and Cal-51) and reported that the PARP inhibitors currently in clinical trials have different antitumor mechanisms beyond PARP inhibition and these PARP-independent mechanisms warrant further investigation. In 2012, Patel *et al.*[29] compared the actions of the PARP inhibitor iniparib with the more extensively characterized PARP inhibitors olaparib and veliparib. The authors reported that iniparib failed to sensitize cells to cisplatin, gemcitabine, or paclitaxel and that its effects are unlikely to reflect PARP inhibition and should not be used to guide decisions about other PARP inhibitors. Accordingly, one can anticipate that PARP-1 might not be considered a good therapeutic target in patients with TNBC.

regulation of PARP-1 in approximately 70% of primary breast adenocarcinomas including TNBC compared with syngeneic nonmalignant breast tissues. In the current study, the observed progressive increase in PARP-1 expression in TNBC compared with non-TNBC patients suggests that TNBC patients would benefit more from PARP-1 inhibitor therapy than non-TNBC patients. It is well documented that in tumors with BRCA mutations, as frequently reported in TNBC, PARP-1 is the major player in DNA repair processes and that inhibition of PARP-1 in these tumors ultimately leads to cell death[19,20]. Consistent with this hypothesis, preclinical studies reported that inhibitors of PARP-1 can act as monotherapy to selectively kill cancers with BRCA1 or

The TOPO-2A gene is the molecular target of anthracycline-based chemotherapy and therapy with other TOPO-2A inhibitors, and is predictive of the response to these agents[30]. The data presented in this study showed a highly significant increase in TOPO-2A in NTBC cases,

Table 4 - Differential expression of BCRP, LRP, MRP1 and MDR1 and its relationship with tumor grade in Saudi women with breast cancer

Tumor grade	Non-TNBC Fold expression					TNBC Fold expression				
	Number	BCRP	LRP	MRP1	MDR1	Number	BCRP	LRP	MRP1	MDR1
1	6	1.78±0.31	1.79±0.36	1.80±0.95	1.61±0.27	9	2.58±0.63	2.02±0.31	2.35±0.81	3.69±0.89
2	29	1.17±0.21	1.20±0.21	1.86±0.21	2.18±0.18	36	1.57±0.20	1.93±0.24	2.20±0.22	2.55±0.21
3	65	1.39±0.15	1.66±0.16	1.76±0.21	2.44±0.32	35	1.36±0.17c	1.68±0.18	2.63±0.40	2.44±0.31
Total	100	1.46±0.11	1.54±0.12	1.79±0.16	2.20±0.14	80	1.59±0.14	1.83±0.14	2.40±0.22b	2.63±0.19

Data are presented as mean ± SEM. bIndicates a significant change with respect to non-TNBC; cindicates a significant change with respect to grade 1, at *P* <0.05 using ANOVA followed by Tukey-Kramer as a post-ANOVA test. SEM, standard error of mean; TNBC, triple-negative breast cancer; BCRP, breast cancer resistance protein; LRP, lung resistance protein; MRP1, multidrug resistance-associated protein 1; MDR1, multidrug resistance gene 1

suggesting that TNBC would be more sensitive to TOPO-2A inhibitors than non-TNBC. It is well documented that increased sensitivity to TOPO-2A inhibitors is associated with TOPO-2A amplification, while its deletion may be accompanied by resistance to TOPO-2A inhibitor therapy[30]. Consistent with this hypothesis is the finding that decreased levels of TOPO-2A in tumors increased resistance to the TOPO-2A inhibitor doxorubicin[31]. Moreover, amplification of TOPO-2A in HER2-negative breast cancers has been reported[32]. Tan et al.[33] found that decreased TOPO-2A expression in TNBC patients was responsible for the poor response to adjuvant anthracycline-based chemotherapy. Moreover, downregulation of TOPO-2A among TNBC patients might explain the differences in their response to chemotherapy[34,35].

The HER1 gene codes for the epidermal growth factor receptor (EGFR). It belongs to the HER family of transmembrane tyrosine kinase receptors and plays an important role in cell proliferation, migration and protection against apoptosis. In the present study, the observed differential increase in the expression of HER1 mRNA in TNBC cases may point to the possible advantage of anti-HER1 therapies in patients with TNBC over non-TNBC. In 25 TNBC patients, Pintens et al.[36] showed that HER1 overexpression was 52% using immunohistochemistry while HER1 amplification was 16% using fluorescence hybridization. Poor prognosis of breast tumors overexpressing HER1 has been previously reported[30,37]. Although studies have demonstrated increased HER1 expression in breast cancer, clinical studies utilizing HER1 as a therapeutic target in breast cancer are still lacking[38,39]. It is well documented that the HER1 protein is targeted by inhibiting its extracellular ligand-binding domain using monoclonal antibodies and/or its tyrosine kinase activity in the cytoplasmic domain by TKIs. Accordingly, several monoclonal antibodies including cetuximab and panitumumab have been clinically tested in many tumors[1,2,30]. However, several clinical studies reported that targeting HER1 in breast cancer yielded no credible results[38,40,41]. In addition, it has been reported that tumor response to the TKIs erlotinib and gefitinib depends on HER1 protein expression[42]. Recently, the anti-HER1 monoclonal antibody cetuximab has been tested alone and in combination with carboplatin in patients with metastatic TNBC[43]. The response rates were 6% in patients administered cetuximab alone, 16% in patients administered cetuximab plus carboplatin after progression, and 17% in those treated with cetuximab plus carboplatin from the beginning. The authors concluded that the combination of cetuximab plus carboplatin in metastatic TNBC produced responses in fewer than 20% of patients and that cetuximab blocked the expression of the EGFR pathway in only a minority, suggesting that most had different mechanisms for activation of this pathway[43]. Therefore, EGFR cannot be considered a good therapeutic target in TNBC patients.

C-MYC is an important transcription factor which regulates the expression of many genes involved in cell proliferation and tumorigenesis[44,45]. Our results showed higher levels of C-MYC mRNA expression in TNBC than non-TNBC cases. This could explain the aggressiveness of TNBC and the poor prognosis of TNBC patients. An earlier study reported that C-MYC is highly expressed in breast cancer[46]. In estrogen-dependent breast cancer, estrogen is the major player in regulating C-MYC expression, whereas in estrogen-independent breast cancer, constitutive expression of C-MYC is usually high[30]. It has been reported that the response of breast cancer to chemotherapy is affected by the C-MYC protein, probably through DNA damage response regulation[47-49]. Therefore, the role played by the C-MYC protein in BRCA1-mutant breast cancer makes it an important target in TNBC.

Tumor angiogenesis plays crucial role in cancer cell survival, tumor growth and development of distant metastasis. The formation of new blood vessels within the tumor mass is essential for providing an adequate oxygen and nutrient supply to the tumor and for initiating metastatic spread[50]. Tumor angiogenesis is enhanced by different angiogenic factors including bFGF, VEGF and TGF-, which is secreted by cancer cells to stimulate proliferation of endothelial cells through paracrine mechanisms[51-54]. The data presented in this study showed a differential increase in the expression of angiogenic factors in TNBC versus non-TNBC. Accordingly, targeting angiogenesis in TNBC patients may improve the therapeutic outcome and reduce recurrence and metastasis. Enhanced expression of VEGF has been observed in human cancers including breast cancer[55]. Increased progression-free survival in breast cancer patients who received the monoclonal antibody bevacizumab in combination with paclitaxel or cisplatinum has been reported[30,56]. Moreover, combined therapy with the TKIs sunitinib or sorafenib and capecitabine increased progression-free survival compared to capecitabine given as monotherapy[57,58].

It is well known that the matrix metalloproteinases MMP-2 and MMP-9 play an important role in tumor metastasis[12,13,59]. The observed increase in MMP-2 in TNBC patients may explain the early metastasis often occurring in these patients. Agents which target these endopeptidases should be considered in the treatment of TNBC. Although it has been reported that MMPs are expressed in many tumors[60-63], studies on the clinical predictive value of MMP-2 and MMP-9 in breast cancer are still limited[64-67].

Multidrug resistance in breast cancer and other malignancies represents a major obstacle to successful treatment and causes therapeutic failure. The multidrug resistance phenomenon is associated with decreased cellular uptake and retention of substrate chemotherapy drugs due to overexpression of the ATP-binding cassette (ABC) proteins including BCRP, LRP, MRP1 and

MDR1[68,69]. The data presented in the current study showed increased mRNA expression of LRP, MRP1 and MDR1 in TNBC cases compared with control benign breast tissues. Although MDR1 showed a similar increase in the expression pattern in both TNBC and non-TNBC, only the MRP1 gene showed a significant differential increase in TNBC compared with non-TNBC. It is well documented that the decreased response of breast tumors to chemotherapy is secondary to increased MDR1 expression[70]. Burger *et al.*[71] demonstrated that the efficacy of cancer chemotherapy in patients with breast tumors is inversely related to the expression of MDR genes.

In conclusion, the results of this study suggest that (1) TNBC patients will benefit more from TOPO-2A inhibitors as well as antiangiogenic and antimetastatic therapies than their non-TNBC counterparts; (2) inhibition of these target genes is emerging as one of the most exciting and promising targeted therapeutic strategies to treat TNBC, in which the intended targets are DNA repair, tumor angiogenesis and metastasis. It is worth mentioning that the correlation between the mRNA expression levels of the genes investigated in the current study and the levels of the proteins encoded by these genes has not yet been verified.

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