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PRECLINICAL STUDY

Impact of Topoisomerase IIα, PTEN, ABCC1/MRP1, and KI67 on triple-negative breast cancer patients treated with neoadjuvant chemotherapy

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Abstract

Purpose Triple-negative breast cancer (TNBC) patients with residual disease following neoadjuvant chemotherapy (NAC) harbor higher risk of relapse, and eventual demise compared to those who achieve pathologic complete response. Therefore, in this study, we assessed a panel of molecules involved in key pathways of drug resistance and tumor progression before and after NAC in TNBC patients, in order to clarify the underlying mechanisms.

Methods We studied 148 TNBC Japanese patients treated with anthracycline/taxane-based NAC. KI67, Topoisomerase IIα (TopoIIα), PTEN, p53, Bcl2, vimentin, ABCG2/BCRP1, ABCB1/MDR1, and ABCC1/MRP1 were immunolocalized in surgical pathology materials before and after NAC.

Results The status of vimentin and increasing labeling index (LI) of TopoIIα and KI67 in biopsy specimens were significantly associated with those who responded to NAC treatment. The abundance of p53 ($p=0.003$), ABCC1/MRP1 ($p=0.033$), ABCB1/MDR1 ($p=0.022$), and a loss of PTEN ($p<0.0001$) in surgery specimens following treatment were associated with pathologic parameters. TopoIIα, PTEN, and ABCC1/MRP1 status predicted pathologic response. In addition, the status of PTEN, ABCC1/MRP1, ABCB1/MDR1, Bcl2, and vimentin in surgical specimens was also significantly associated with adverse clinicopathological factors in surgery specimens, suggesting that these alterations could be responsible for tumor relapse in TNBC patients.

Conclusion KI67, TopoIIα, PTEN, and ABCC1/MRP1 status could predict treatment response and/or eventual clinical outcomes. These results could also provide an insight into the mechanisms of drug resistance and relapse of TNBC patients receiving NAC.

Keywords Triple-negative breast cancer · Neoadjuvant chemotherapy · Mechanistic markers · Drug resistance · Residual disease · Outcomes

Abbreviations

ABCB1/MDR1	Multidrug resistance 1 encoded by the gene *ABCB1*
ABCC1/MRP1	Multidrug resistance protein 1 encoded by the gene *ABCC1*
ABCG2/BCRP1	Breast cancer resistance protein encoded by the gene *ABCG2*
Bcl2	B-cell lymphoma 2
CI	Confidence interval
CR	Complete response
DFS	Disease-free survival
LI	Labeling index
NAC	Neoadjuvant chemotherapy
OR	Odd ratio

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OS	Overall survival
p53	Tumor protein 53
pCR	Pathologic complete response
PD	Progressive disease
PR	Partial response
PTEN	Phosphatase and tensin homolog
RD	Residual disease
SD	Stable disease
TNBC	Triple-negative breast cancer
TNM	Tumor, node, metastasis
TopoIIα	Topoisomerase IIα

Introduction

In addition to its impact on the surgical management, the use of neoadjuvant chemotherapy (NAC) provides useful information regarding the tumors' phenotype and responsiveness to chemotherapy. While a plethora of chemotherapeutic agents have been extensively studied in triple-negative breast cancer patients (TNBC), both anthracyclines and taxanes are the chemotherapy of choice in the great majority of cases [1–3]. TNBC patients respond better to NAC, evidenced by higher pathologic complete response (pCR) rates compared to other subtypes [4, 5]. However, an acquired or de novo resistance to these chemotherapeutic agents could frequently occur [6, 7]. Therefore, one of the challenges in the management of TNBC patients is to overcome its potential resistance.

Several studies have sought to define biomarkers that predict pathologic complete response and clinical outcome in TNBC patients, mostly by employing omics technologies [8–14]. At present, these markers have not universally been adopted in clinical practice [15–19], but their identification provides insights into the existence of multiple molecular alterations, which could be responsible of the inherent or acquired drug resistance detected in TNBC [8, 10, 12, 13, 15, 19, 20–27]. While in isolation a number of pathways have been suggested to be involved in resistance, but an equally important consideration is the possibility of synergism between multiple pathways [15, 16]. These intersecting pathways potentially play an important role in the development of therapeutic resistance. Thus it is important to identify the key molecules and interactions involved with regards to chemotherapeutic response/resistance and eventual clinical outcome of these patients.

Therefore, in this study, we immunolocalized some of the main markers identified by the omics-based studies above. These are KI67 [28–31], Topoisomerase IIα [32, 33], PTEN [12, 34], p53 [28, 35], Bcl2 [28, 36], vimentin [37, 38], ABCG2/BCRP1, ABCB1/MDR1, and ABCC1/MRP1 [39–41]. These proteins could be related to drug resistance mechanisms involved in tumor progression. In addition, several retrospective studies reported the potential prognostic and therapeutic predictive role of these markers in breast cancer but few assessed them in TNBC patients, and the results yield controversial conclusions. Therefore, we first aimed to identify the molecular profiles associated with pCR in the biopsy specimens. Second, we sought to identify the impact of chemotherapeutic treatment on the expression of these proteins. Finally, we aimed to identify which pathways either in isolation or combination, were associated with therapeutic resistance and eventual clinical outcome of patients with residual disease.

Materials and methods

Patient cohort and treatment

The study included 148 TNBC patients who presented with primary disease between 2007 and 2015. The median follow-up was 38 months. Patients underwent neoadjuvant chemotherapy at four different hospitals in Japan (Tohoku University Hospital, Kousai Hospital, Nahanishi Clinic, Sagara Hospital) and the NAC regimen was administered for a median of 4 cycles each before surgery (Table S1). Following NAC treatment, 39.2% (58/148) underwent partial and 60.8% (90/148) total mastectomy and/or axillary lymph node dissection. The clinicopathological characteristics are summarized in Table 1.

The pathological complete response (pCR) was defined as complete disappearance of invasive carcinoma cells in both breast and axillary lymph nodes after NAC. Tumor response was defined according to the Response Evaluation Criteria in Solid Tumors (RECIST 2000) [42] into complete response (CR), partial response (PR), stable disease (SD), and progressive disease (PD). Disease-free survival (DFS) was defined as the length of time from the date of diagnosis to the earliest occurrence of relapse, and overall survival (OS) as the time from diagnosis to the date of death due to any cause in this study. The protocol of the study was approved by local Institutional Review Boards (Kousai Hospital: 2069; Sagara Hospital:14–12; Nahanishi: NNCEC2013004) and the Medical Ethics Committee of Tohoku University Hospital (2014-1-751).

Immunohistochemistry protocol

A total of 126 core-needle biopsies, 104 surgical, and 82 matched paired specimens were available in this study. The details of immunohistochemistry (IHC) of KI67, Topoisomerase IIα (TopoIIα), PTEN, p53, Bcl2, vimentin, ABCG2/BCRP1, ABCB1/MDR1, and ABCC1/MRP1 are summarized in Table S2a, following manufacturer instructions with slight optimization of the conditions. Briefly,

Table 1 Clinicopathological characteristics of the patients

Variables	Absolute	Proportion (%)
Age		
< 60	99	66.9
≥ 60	49	33.1
Breast and ovarian cancer history status		
Presence	25	17.1
Absence	121	82.9
Missing	2	1.4
Clinical tumor size cT		
T1	11	7.4
T2	93	62.8
T3	26	17.6
T4	18	12.2
Clinical lymph node status cN		
N0	28	18.9
N1	79	53.4
N2	23	15.5
N3	18	12.2
Clinical TNM stage		
I	1	0.7
IA	3	2.0
IB	1	0.7
IIA	26	17.6
IIB	50	33.8
IIIA	40	27.0
IIIB	15	10.1
IIIC	7	4.7
IV	5	3.4
Histological grade HG		
I	5	4.8
II	28	26.9
III	63	60.6
Missing	8	7.7
Nuclear grade NG		
I	5	4.8
II	26	25.0
III	68	65.4
Missing	5	4.8
Lymphovascular invasion grade Ly		
Ly0	48	46.2
Ly1	28	26.9
Ly2	15	14.4
Ly3	8	7.7
Missing	5	4.8
Tumor size Ts		
0	56	53.8
≥ 20 mm	46	44.2
Missing	2	1.9
Axillary lymph node positivity *n*		
No	38	38.8
Yes	60	61.2
Missing	6	5.8
Clinical treatment response		
Complete response CR	30	20.3
Partial response PR	79	53.4
Stable disease SD	22	14.9
Progressive disease PD	17	11.5

3 µm sections of 10% formalin-fixed and paraffin-embedded tissue were passed through xylene followed by hydration in graded ethanol. Antigen retrieval was applied for all the markers except ABCG2/BCRP1 and ABCB1/MDR1. Sections were then incubated for 30 min at room temperature with the blocking solution commensurate with the relevant primary antibody and detection kit (Histofine SAB-PO kit; Nichirei, Tokyo, Japan). Slides were then incubated overnight at 4 °C with the primary antibodies. Endogenous peroxidase activity was blocked using absolute methanol and 0.3% H_2O_2 (30 min at room temperature). The slides were then incubated with the relevant secondary antibody for 30 min at room temperature, visualized using liquid DAB and substrate chromogen system (Dako) and subsequently counterstained with hematoxylin. All primary antibodies are monoclonal and have been widely used for immunohistochemistry. The specificity of the antibodies to the antigen has been validated previously using immunoblotting by the manufacturer. External positive and negative controls were included. Representative findings are illustrated in Fig. S1.

Immunohistochemistry scoring system

For the assessment, sufficient number of cells (> 500 cells) was required. The immunoreactivity was graded in a quantitative or semi-quantities fashion. The details of the scoring system are summarized in Table S2b.

Statistical analysis

Statistical analyses were performed using SPSS statistical program for Windows (version 21.0). The Chi-square test or one-way ANOVA and the logistic regression were performed to evaluate the association of the markers and the clinical parameters with the treatment response in univariate and multivariate analysis, respectively. Pearson or Spearman-rank correlations were used to assess the correlations between the markers. Differences in expression between the matched pairs before and after NAC were assessed using paired samples *t* test, Wilcoxon signed-rank test, or McNemar's test where appropriate. Survival curves were generated using Kaplan–Meier method with the significance evaluated

using the log-rank test. The multivariate Cox model was used to develop a predictive model for risk of relapse or survival. In order to appreciate the intrinsic changes within the markers status, we deliberately did not use the cut-off values for the markers in all the statistical analyses, but we used the receiver operating characteristic curve (ROC curve) analysis to calculate the optimal cut-off values on the original tumor with the survival as referral values, to calculate the frequency of positivity of the markers (PTEN 6, p53 2, ABCG2/BCRP1 and ABCB1/MDR1 4, ABCC1/MRP1 5) and for Kaplan–Meier survival curves for KI67 (53%) and TopoIIα (40%). Unsupervised hierarchical clustering was applied using Ward's method with Squared Euclidean distance measure and *K* means, the number of clusters was determined from the dendrogram. All *P* values given are two-sided, with both univariate and multivariate analysis results given.

Results

Pathological response to NAC and survival analysis

21.6% (32/148) of the patients achieved pathologic complete response (pCR) while the remainder (78.4%-116/148) had residual disease (RD) following NAC. A significant association was detected between pCR and clinical response ($p < 0.0001$), as 25 patients with CR and 7 with PR achieved pCR after surgery. Among the patients with residual disease 40% (46/115) relapsed and 37% (43/116) developed distant metastasis, whereas one with IIIa TNM stage who had previously achieved pCR developed distant metastasis at the regional lymph node. Patients achieving pCR had a significantly more favorable disease-free survival (DFS $p < 0.0001$) and overall survival (OS $p = 0.001$) than those with RD (Fig. 2a, b). In addition, those with CR or PR had significantly better clinical outcomes (DFS $p < 0.0001$, OS $p < 0.0001$) compared to those with SD or PD (Fig. 1c–f).

Clinical factors predictive of treatment response

Pathologic complete response achievement was associated with smaller tumor size ($p = 0.011$) and TNM stage ($p = 0.016$), and a trend with age (below 60 years) ($p = 0.062$) (Table 2). When performing a multivariate analysis, clinical tumor size was the only variable that remained associated with pCR (OR 0.272, 95% CI [0.105–0.739], $p = 0.010$), while the trend in age remained (OR 0.394, 95% CI [0.143–1.083], $p = 0.071$). Clinical treatment response correlated with clinical tumor size ($p = 0.003$) and TNM stage ($p < 0.0001$).

Markers predictive of treatment response

In order to discriminate between patients more prone to achieve pCR from those with residual disease, we further assessed the markers in the biopsy samples. Results demonstrated that patients who achieved pCR had more abundant vimentin ($p < 0.0001$; OR 5.08, CI 95% [1.651–15.645], $p = 0.005$) and a higher KI67 LI ($p = 0.009$; OR 1.038, CI 95% [1.005–1.073], $p = 0.023$), as demonstrated in the univariate and the multivariate analyses, respectively. Those with CR or PR had higher rates of TopoIIα ($p = 0.013$) and KI67 ($p = 0.014$) (Fig. 2). KI67 and TopoIIα double positivity, when using the ROC curve, predicted pCR achievement ($p = 0.051$) and clinical treatment response ($p = 0.048$).

Correlation among the markers examined

We analyzed the correlations among individual markers in both biopsy and surgery specimens. High KI67 LI was negatively correlated with ABCG2/BCRP1 ($p_{biopsy} = 0.018$) and ABCB1/MDR1 ($p_{biopsy} = 0.014$, $p_{surgery} = 0.017$). ABCG2/BCRP1 was correlated with ABCB1/MDR1 ($p_{biopsy} < 0.0001$, $p_{surgery} = 0.019$), and with ABCC1/MRP1 ($p_{biopsy} = 0.001$, $p_{surgery} = 0.013$). Bcl2 was positively correlated with ABCG2/BCRP1 ($p_{biopsy} = 0.03$) and ABCC1/MRP1 ($p_{biopsy} < 0.0001$, $p_{surgery} = 0.006$), and with vimentin ($p_{biopsy} = 0.097$, $p_{surgery} = 0.007$). Vimentin and ABCC1/MRP1 were positively correlated among each other ($p_{biopsy} = 0.004$, $p_{surgery} = 0.046$). An association between TopoIIα with ABCG2/BCRP1 ($p_{biopsy} = 0.001$, $p_{surgery} = 0.003$) and ABCB1/MDR1 ($p_{biopsy} = 0.008$) was demonstrated. Following the treatment, the loss of PTEN ($p_{surgery} = 0.003$) and Bcl2 ($p_{surgery} = 0.028$) was associated with decreased TopoIIα.

Association of the markers examined with the pathological parameters

In order to study the potential impact of the markers on the histopathology of the tumor, we compared their status with the pathological parameters following NAC treatment. High KI67 LI ($p < 0.0001$; $p < 0.0001$) was significantly associated with high nuclear and histological grades, respectively. Low PTEN and TopoIIα was significantly associated with increased tumor size ($p = 0.001$, $p < 0.0001$) and lymphovascular invasion grade ($p = 0.001$, $p = 0.015$), respectively. The metastasis in the axillary lymph nodes was positively correlated with Bcl2 ($p < 0.0001$) and vimentin ($p = 0.03$), and distant metastasis with low TopoIIα ($p = 0.044$) and high ABCC1/MRP1 ($p = 0.036$). The changes detected in the matched pairs were also associated with the pathological characteristics of the tumor. An increased p53 ($p = 0.006$) and ABCB1/

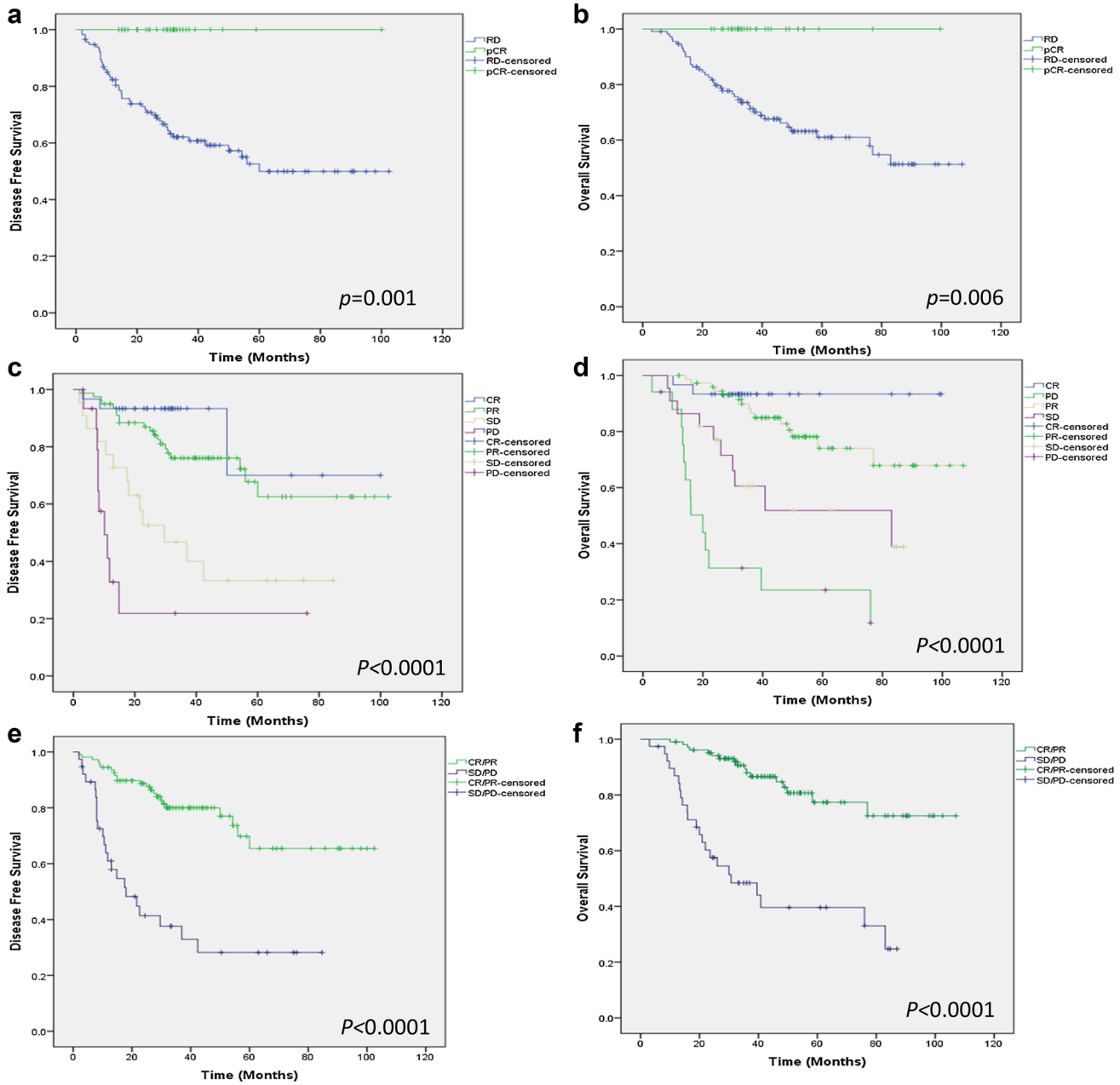

Fig. 1 Kaplan–Meier curves showing differences in disease-free survival and overall survival in relation to clinical and pathologic response after neoadjuvant chemotherapy in patients with triple-negative breast cancer. Kaplan–Meier overall survival curves as stratified in patients having achieved pathologic complete response (pCR, $N=32$) to those with residual disease (RD, $N=116$) (**a, b**), or clinical response into complete response (CR, $N=30$), partial response (PR, $N=79$), stable disease (SD, $N=22$), progressive disease (PD, $N=17$) (**c, d**) or stratified as CR/PR ($N=109$) with SD/PD ($N=39$) (**e, f**). The odd ratio (OR) generated with Cox regression is as follows: **a** DFS OR 0.185, 95% IC [0.044–0.775]; **b** OS OR 0.22, 95% IC [0.05–0.891]; **c** DFS OR 3.07, 95% IC [1.97–4.79]; **d** OS OR 3.36, 95% IC [2.16–5.24]. **e** DFS OR 6.76, 95% IC [3.37–13.57]; **f** OS OR 7.46, 95% IC [3.46–16.08]

MDR1 ($p=0.035$) status was significantly associated with increased number of axillary lymph nodes metastasis, and PTEN loss with higher lymphovascular invasion grade ($p=0.014$) and trend toward significance with tumor size ($p=0.085$).

Association of the markers and the clinicopathological parameters with eventual clinical outcomes of the patients

The patients with a low PTEN expression in the surgery

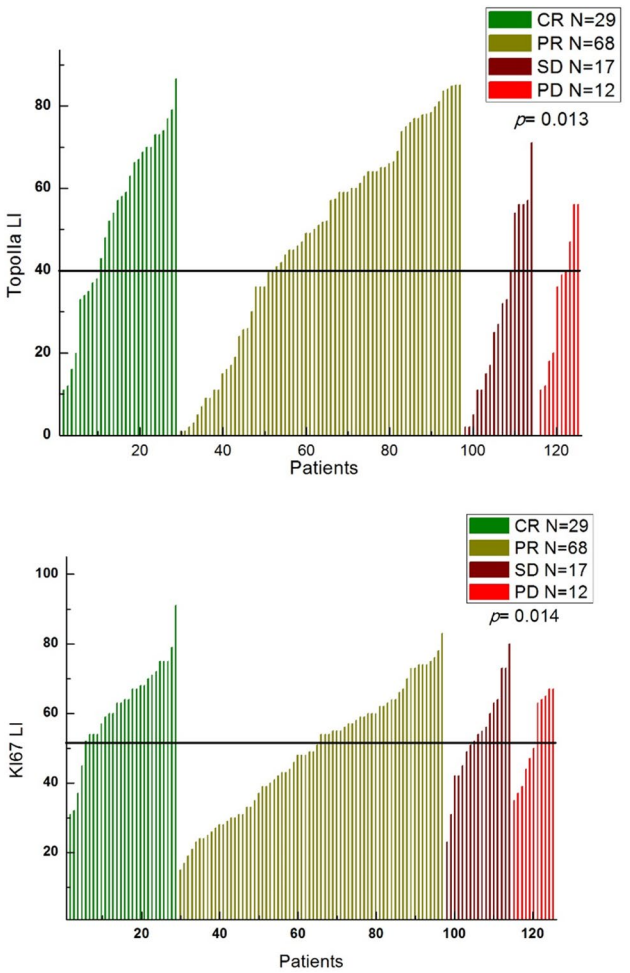

Fig. 2 Bar graphs of the distribution of Topoisomerase IIα and KI67 LI according to patients in response to neoadjuvant chemotherapy. Each bar represents the value of Topoisomerase IIα and KI67 LI for each patient with the corresponding clinical response to the neoadjuvant chemotherapy, (*CR* complete response, *PR* partial response, *SD* stable disease, *PD* progressive disease). The solid horizontal line represents the cut-off value of Topoisomerase IIα (40%) and KI67 (53%) determined by the ROC curve. The proportion of patients with a positive status of the marker using the cut-off who achieved a response to the treatment was as follows: Topoisomerase IIα CR: 19, PR: 47, SD: 6, PD: 5; and KI67 CR: 21, PR: 32, SD: 10, PD: 6. The *p* values are calculated using one-way ANOVA test and are two-sided. Total $N = 148$ patients

specimens ($p = 0.038$; HR 0.867, CI 95% [0.747–1.006], $p = 0.061$) and abundant ABCC1/MRP1 ($p = 0.055$; HR 1.207, CI 95% [1.001–1.456], $p = 0.049$) tended to harbor decreased DFS. In addition, patients with high KI67 LI in their tumor ($p = 0.090$; HR 2.217, CI 95% [0.972–5.059], $p = 0.059$) exhibited a shortened OS (Fig. 3). The positive status of ABCB1/MDR1 ($p = 0.003$) and vimentin ($p = 0.084$) was associated with decreased DFS but demonstrated in the univariate analysis only. PTEN status in the biopsies predicted adverse OS ($p = 0.010$; HR 1.345, CI 95% [1.077–1.68], $p = 0.009$).

Small tumor size ($p < 0.0001$, $p = 0.001$), low lymphovascular invasion grade ($p = 0.017$, $p < 0.0001$), no metastasis in the axillary lymph nodes ($p < 0.0001$, $p = 0.006$), and distant metastasis ($p < 0.0001$, $p < 0.0001$) predicted good DFS and OS of patients with residual disease, respectively (Fig. S2).

Expression analysis of the markers

When using the cut-offs, we could determine the status of each marker in both biopsy and surgery specimens (Fig. S3). Figure 4 represents the distribution of the expression of the markers before and after NAC. KI67 was exclusively detected in the nuclei, whereas all the other markers were detected in both nuclei and cytoplasm (TopoIIα, Bcl2, p53, PTEN, vimentin), and in the cytoplasm and membrane for the multidrug resistance proteins. Tumor infiltrating lymphocytes were relatively positive for KI67, Bcl2, ABCB1/MDR1, TopoIIα, vimentin, and PTEN. The epithelial cells were relatively positive for all the markers except for vimentin. A positive TopoIIα, vimentin, ABCB1/MDR1, and PTEN immunoreactivity was detected in stromal fibroblasts (Fig. S1).

Changes in the status of the markers following NAC in the residual disease

The comparison of markers in the matched pair specimens of patients with residual disease made it possible to study the impact of chemotherapy in these pathways. The finding remained consistent regardless using the cut-offs or not, respectively. Tumors in the surgical specimens had significantly higher status of p53 ($p = 0.013$; $p = 0.003$), ABCC1/MRP1 ($p = 0.059$; $p = 0.033$), ABCB1/MDR1 ($p = 0.026$; $p = 0.022$), and TopoIIα ($p < 0.0001$) compared with that of the biopsies. PTEN ($p < 0.0001$) and Bcl2 ($p < 0.0001$) status were lower in the surgical specimens compared to their matched biopsy. KI67 ($p = 0.256$; $p = 0.166$), vimentin ($p = 0.571$), and ABCG2/BCRP1 ($p = 0.152$; $p = 0.437$) did not exhibit a significant change following NAC (Fig. 4). Figure 5 represents the profile of changes of the markers. An increased expression ranging from negative to more than 50% of positive tumor cells was detected in 32.93% of patients for p53 and 53.66% for ABCC1/MRP1. For ABCB1/MDR1, 24.39% had increased positive tumor cells ranging from 6% to over 50%. TopoIIα increased with an average of 37% in 84.15% of the patients. 56.09% had an average of 50% loss of PTEN and 35% with decreased Bcl2.

Table 2 Association of the clinical parameters with treatment response by univariate analysis

Variables	pCR		RD		χ^2 p value
	N	(%)	*N*	(%)	
Age					
< 60	26	17.6	74	50.0	3.48 (0.062)
≥ 60	6	4.1	42	28.4	
Breast and ovarian cancer history status					
Presence	7	4.8	18	12.3	0.652 (0.419)
Absence	25	17.1	96	65.8	
Clinical tumor size cT					
T1	5	3.4	6	4.1	
T2	24	16.2	69	46.6	11.17 (0.011)
T3	3	2.0	23	15.5	
T4	0	0	18	12.2	
Clinical Lymph node status cN					
NO	9	6.1	19	12.8	
Ni	14	9.5	65	43.9	3.18
N2	4	2.7	19	12.8	(0.364)
N3	5	3.4	13	8.8	
Clinical TNM stage					
I	0	0	1	0.7	
IA	3	2.0	0	0	
IB	0	0	1	0.7	
IIA	6	4.1	20	13.5	
IIB	11	7.4	39	26.4	18.86 (0.016)
IIIA	9	6.1	31	20.9	
IIIB	0	0	15	10.1	
IIIC	3	2.0	4	2.7	
IV	0	0	5	3.4	

This table lists the associations between patients' age and tumor characteristics (clinical tumor size, lymph node status, TNM stage) with treatment response in patients with pathologic complete response (pCR) and residual disease (RD). Chi-square (χ^2) test was used for the analysis, Chi-square (χ^2) and p values are represented

Hierarchical clustering analysis of the changes and their association with prognosis

In order to capture the profile of the changes following treatment across the multiple markers, we ran hierarchical clustering analysis, to evaluate whether multiple changes were associated with prognostic outcomes of patients.

We therefore dichotomized the markers' status into "0" for either positive or negative expression, and "1" for an increase or decrease of the expression detected in the matched pairs (gain or loss). Thereafter, when we conducted an unsupervised hierarchical clustering analysis associated with K means clustering, three main grouping inherent in our cohort were detected (Fig. S4). The number of patients per cluster (N) and the corresponding number of changes within each marker (M) per group were as follows: Group 1, $N=38$, $M=3$; Group 2, $N=18$, $M=5$; Group 3, $N=19$, $M=7$.

Patients with adverse clinical outcomes corresponded to those harboring higher rate of change involving potentially KI67, TopoIIα, PTEN, p53, vimentin, ABCB1/MDR1, and ABCC1/MRP1 (Fig. S4d). Results demonstrated that the three cluster-groups were prognostically significant as associated with DFS ($p=0.049$) and OS ($p=0.035$) of the patients (Fig. S4a). The group clusters also tended to be associated with tumor size ($p<0.0001$), TNM stage ($p=0.078$), and histological grade ($p=0.094$).

Discussion

This study aimed to characterize the expression of the markers that could explain the responsiveness of TNBC patients to NAC, and identify biological pathways associated with drug resistance, loco-regional recurrence, and outcomes. This is important as the molecular

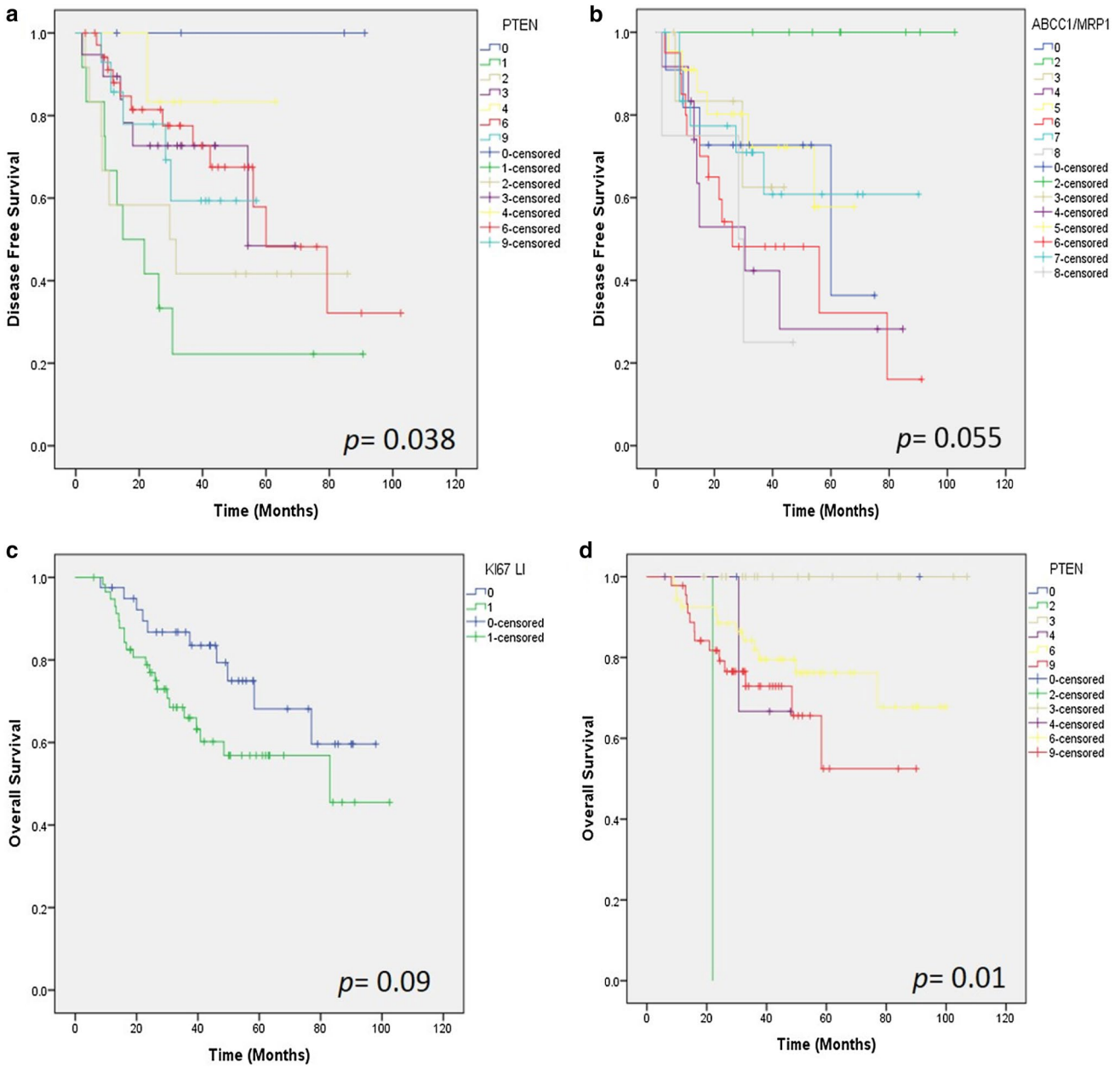

Fig. 3 Kaplan–Meier curves showing the association of the markers with disease-free survival and overall survival. Association of the markers expression post-neoadjuvant chemotherapy with disease-free survival PTEN (**a**), ABCC1/MRP1 (**b**), and KI67 with overall survival [cut-off value 53% determined by ROC curve (**c**)]. **d** Shows the association of the expression of PTEN in the biopsy specimens with overall survival. The *P* values are calculated using log-rank test and are two-sided

characterisation of drug-resistant cancer cells in residual disease of TNBC tissue and the mechanisms that allow TNBC tumors to survive to NAC have remained elusive. Thereby, we demonstrated that ABCC1/MRP1, PTEN, Topoisomerase IIα, vimentin, and KI67 are all involved in the response to chemotherapy and/or clinical outcome of patients, which could also support the development of effective interventions for TNBC patients with residual disease.

The association of the overexpression of the drug efflux pumps with the aggressive nature of multidrug-resistant tumors is well known [7, 43, 44]. The correlation between upregulation of ABCC1/MRP1 and ABCB1/MDR1 and clinical outcomes in breast carcinoma cells in response to treatment [43–45], but not in TNBC was reported. In this study, we did firstly demonstrate the associations between ABCC1/MRP1 and NAC therapeutic resistance in TNBC, suggesting its involvement in the progression

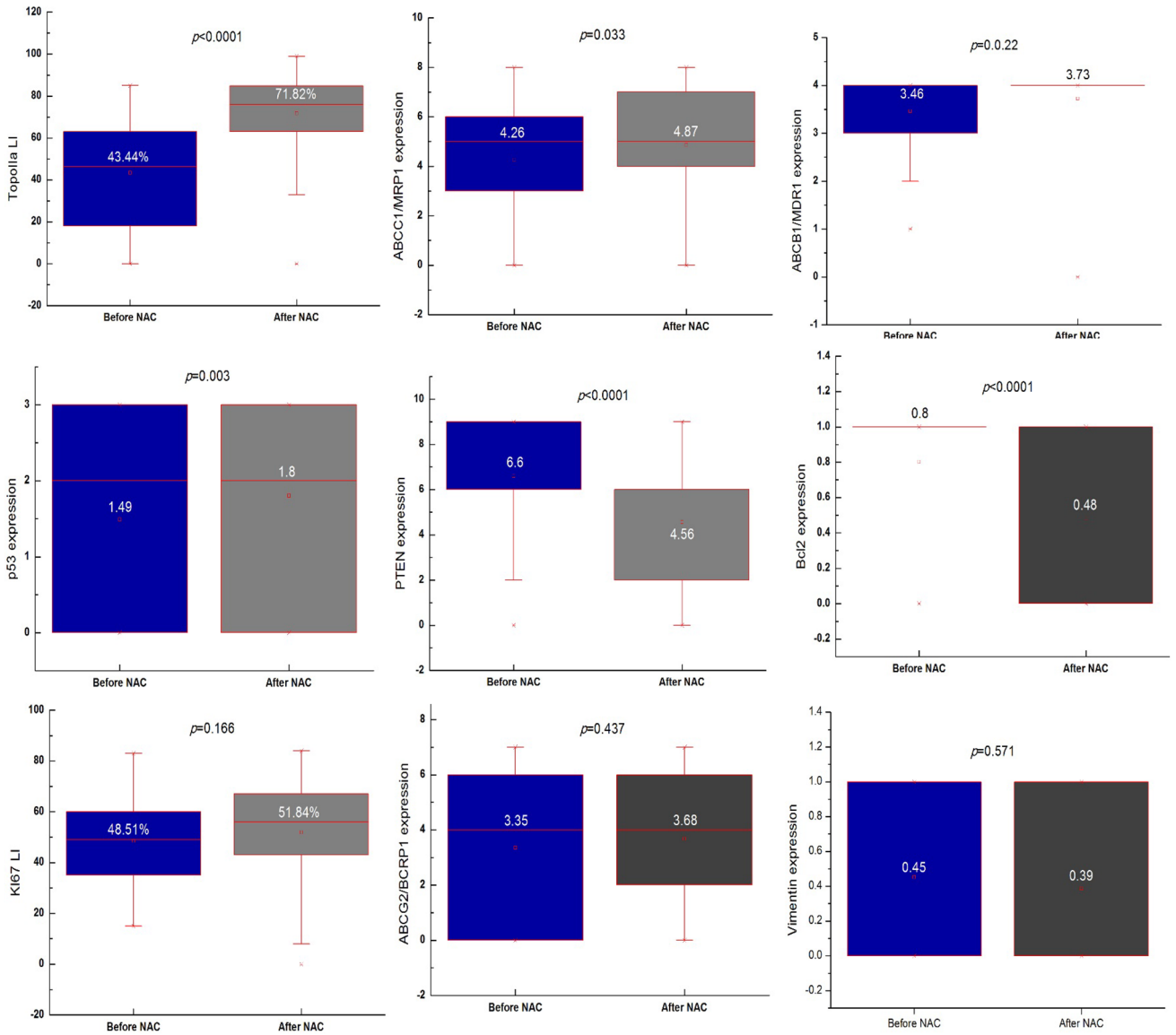

Fig. 4 Expression of the markers before and after neoadjuvant chemotherapy. The expression of Topoisomerase IIα, ABCC1/MRP1, ABCB1/MDR1, and p53 in resected triple-negative breast cancer tissues after neoadjuvant chemotherapy was significantly increased compared with that in biopsy specimens before chemotherapy, whereas PTEN and Bcl2 expression significantly decreased after neoadjuvant chemotherapy. KI67, ABCG2/BCRP1, and vimentin expression did not have a significant change when compared to the biopsy and surgery matched pairs specimens. $N = 82$ patients

of drug-resistant TNBC tumors following NAC. The main multidrug resistance proteins often associated with drug resistance includes ABCC1/MRP1, ABCB1/MDR1, and ABCG2/BCRP1, and share substrates widely used in TNBC NAC regimen [40, 46]. ABCB1/MDR1 in cancer cell lines was reported to lead to resistance to its substrate paclitaxel [47]. In our study, we demonstrated that 67.1% of those with residual disease had more than 70% of tumor cells positive to ABCB1/MDR1 before NAC, and 15% more had increased expression following NAC, associated with lymph nodes metastasis and decreased DFS. We also demonstrated the correlation between the co-expression of ABCB1/MDR1 and ABCC1/MRP1 with ABCG2/BCRP1 suggesting the effective use of agents which are not substrates of these proteins, but rather of ABCG2/BCRP1 as a way to overcome the development of resistance to regimen including ABCC1/MRP1 or ABCB1/MDR1 substrate agents in the NAC setting. Targeting ABCB1/MDR1 and blocking autophagy were also suggested as an effective mean of reducing Epirubicin resistance [48].

We also demonstrated that the status of the anti-apoptotic protein Bcl2 was correlated with vimentin, and that both

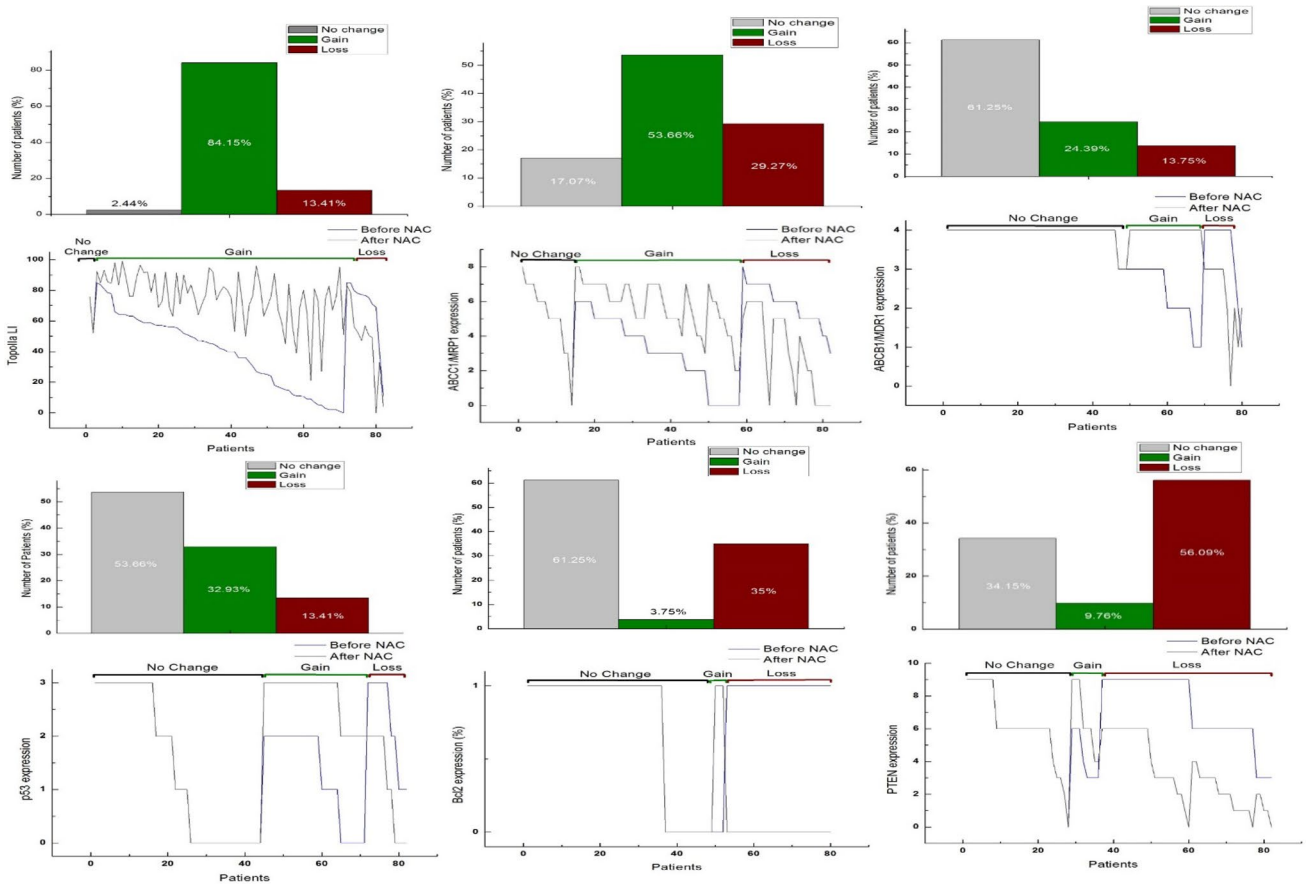

Fig. 5 Molecular profile of the changes in markers expression following neoadjuvant chemotherapy and their frequency among the patients. The histograms represent the proportion of patients according to the molecular changes status: no change (either positive or negative expression), gain (increase in the expression), loss (decrease in the expression) when comparing the expression of the markers in the biopsy and surgery matched pairs specimens. The corresponding fill area graphs highlight the inherent variations within the markers' expression profile that had a significant change following neoadjuvant chemotherapy for the patients with triple-negative breast cancer. $N = 82$

were associated with metastasis in the axillary lymph nodes, but only vimentin was associated with decreased DFS, which is consistent with previous studies demonstrating the association of vimentin with basal-like subgroup in highly proliferative carcinoma cells and poor DFS [37, 38, 49, 50]. Bcl2 has occasionally been proposed as prognostic factor for breast cancer [36, 51–53], but our study suggests only its direct involvement in tumor progression and dissemination.

Of particular interest, we also demonstrated that the resistant carcinoma cells harbored enriched Bcl2 and vimentin and an upregulation of drug efflux pumps. We also demonstrated the correlations between these markers and their association with the promotion of therapeutic resistance. These results above suggested targeting selectively Bcl2 family members [54–56] to sensitize cells harboring this phenotype toward chemotherapy-induced death in the neoadjuvant setting.

For the first time, we demonstrated that PTEN protein status in pre-NAC specimens was an independent predictor of adverse outcomes. Similarly, the association of high intratumoral PTEN gene expression with poor prognosis in patients with locally advanced breast cancers harboring wild-type *TP53* was reported, exhibiting a weak correlation between *PTEN* gene expression and PTEN protein expression [57]. We did not detect any correlation between PTEN and p53, but patients who moved from high to lower levels of PTEN in response to NAC tended to have adverse clinical outcomes, as 50% of the them had decreased PTEN status following NAC, associated with high lymphovascular invasion grade, increased tumor size, and shorter DFS and OS. Considering the pivotal roles of PTEN in cell growth, proliferation, and survival [58], results of this study suggest that a disruption of its functions, originating from either direct *PTEN* gene mutation/deletion or caused by perturbation of its post-translational regulation [59, 60], could be involved in the acquired resistance following treatment and subsequent adverse clinical outcomes [61–64]. Therefore, agents inhibiting downstream signaling pathways of PTEN,

in the adjuvant setting, are still of great benefit for those who did not respond to NAC [65, 66]. Nevertheless, one should expect the potential resistance to those agents [67]. Therefore, further investigations are required to clarify alternative targets [59].

We also demonstrated that PTEN deletion was associated with decreased TopoIIα. A potential mechanistic link between these molecules is the regulation of TopoIIα by deubiquitylases [68–70]. The deubiquitylases OTUD3 interacts with de-polyubiquitylates and stabilizes PTEN, which stabilizes also TopoIIα. The concomitant reduction of both was reported to lead to decreased TopoIIα, dysfunction of the decatenation checkpoint, compromising genomic stability and this fosters breast cancer progression [58, 68–70].

While previous genomics findings highlighted the molecular markers frequently altered in the residual tumor of TNBC patients [12, 15, 70, 71], our study brings a novel approach of identifying altered pathways by examining protein expression indicative of the state of these pathways. Therefore, here we demonstrated that patients with adverse clinical outcome were more prone to have alterations in key proteins involved in cell growth and cell proliferation, drug bioavailability, and epithelial–mesenchymal transition. Therefore, the changes observed in these different cellular pathways confirm the usefulness of targeting multiple pathways in TNBC [70–72].

Finally, consistent with previous studies [32, 73–79], our results demonstrated that a high TopoIIα and KI67 LI increased the sensitivity of TNBC patients to anthracycline-based chemotherapy. It was also associated with tumor size and lymphovascular invasion. This suggests a significant potential of TopoIIα as a prognostic and predictive factor for NAC treatment anthracycline-based in TNBC patients alongside KI67 [61, 62].

This study has some limitations. First, we could not calculate residual cancer burden due to the retrospective nature of the study. Nevertheless, we could demonstrate that tumor size, the presence of metastasis in the lymph nodes, and distant metastasis could predict early DFS and OS for those with residual disease. Second, the diversity in NAC regimen due to the multi-center nature of the study may increase the generalizability of our findings, but it may also represent a limitation. To address this, when we restricted the analysis exclusively to the treatment arm including FEC-Taxane ($N = 115$, data not shown), similar results were obtained. Third, the imbalance in the size of the cohort within the subgroups, regarding the clinical response, makes it difficult to detect the main interaction effects within each subgroup. Fourth, the absence of a molecular characterization of the TNBC subtypes within our cohort has made it difficult to associate the markers with the different TNBC molecular subtypes and therefore to their differential response to treatment and prognoses.

Conclusion

We demonstrated that KI67, TopoIIα, PTEN, and ABCC1/MRP1 expression predicted pathologic outcomes, and were associated with clinicopathological parameters. We also demonstrated that multiple alterations of the markers controlling cell proliferation, cell cycle, and drug efflux were responsible for the adverse clinical outcome of TNBC patients with residual disease following NAC.

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References

1. Liedtke C, Mazouni C, Hess KR, André F, Tordai A, Mejia JA, Symmans WF, Gonzalez-Angulo AM, Hennessy B, Green M, Cristofanilli M, Hortobagyi GN, Pusztai L (2008) Response to neoadjuvant therapy and long-term survival in patients with triple-negative breast cancer. J Clin Oncol 26(8):1275–1281
2. Wu K, Yang Q, Liu Y, Wu A, Yang Z (2014) Meta-analysis on the association between pathologic complete response and triple-negative breast cancer after neoadjuvant chemotherapy. World J Surg Oncol 12:95. https://doi.org/10.1186/1477-7819-12-95
3. Martin M, Romero A, Cheang MCU, Lopez Garcıa-Asenjo JU, Garcıa-Saenz JA, Oliva B, Roman JM, He X, Casado A, de la Torre J, Furio J, Puente J, Caldes T, Vidart JA, Lopez-Tarruella S, Diaz-Rubio E, Perou CM (2011) Genomic predictors of response to doxorubicin versus docetaxel in primary breast cancer. Breast Cancer Res Treat 128:127–136
4. Wahba HA, El-Hadaad HA (2015) Current approaches in treatment of triple-negative breast cancer. Cancer Biol Med 12(2):106–116
5. Székely B, Silber AL, Pusztai L (2017) New therapeutic strategies for triple-negative breast cancer. Oncology 31(2):130–137
6. Zhang LH, Yang AJ, Wang M, Liu W, Wang CY, Xie XF, Chen X, Dong JF, Li M (2016) Enhanced autophagy reveals vulnerability of P-gp mediated epirubicin resistance in triple negative breast cancer cells. Apoptosis 21(4):473–488
7. Yamada A, Ishikawa T, Ota I, Kimura M, Shimizu D, Tanabe M, Chishima T, Sasaki T, Ichikawa Y, Morita S, Yoshiura K, Takabe K, Endo I (2013) High expression of ATP-binding cassette transporter ABCC11 in breast tumors is associated with aggressive subtypes and low disease-free survival. Breast Cancer Res Treat 137(3):773–782
8. Xu H, Eirew P, Mullaly SC, Aparicio S (2014) The omics of triple-negative breast cancers. Clin Chem 60(1):122–133
9. Florea AM, Busselberg D (2013) Breast cancer and possible mechanisms of therapy resistance. J Local Glob Health Sci. https://doi.org/10.5339/jlghs
10. O'Reilly EA, Gubbins L, Sharma S, Tully R, Ho Zhing Guang M, Weiner-Gorzel W, McCaffrey J, Harrison M, Furlong F, Kell M, McCanna A (2015) The fate of chemoresistance in triple negative breast cancer (TNBC). BBA Clin 3:257–275
11. Bouchalova K, Cizkova M, Cwiertka K, Trojanec R, Hajduch M (2009) Triple negative breast cancer-current status

and prospective targeted treatment based on HER1 (EGFR), TOP2A and C-MYC gene assessment. Biomed Pap Med Fac Univ Palacky Olomouc Czech Repub 153(1):13–17
12. Balko JM, Giltnane JM, Wang K, Schwarz LJ (2014) Molecular profiling of the residual disease of triple-negative breast cancers after neoadjuvant chemotherapy identifies actionable therapeutic targets. Cancer Discov 4(2):232–245
13. Lonning PE, Knappskog S (2013) Mapping genetic alterations causing chemoresistance in cancer: identifying the roads by tracking the drivers. Oncogene 32(46):5315–5330
14. McShane LM, Altman DG, Sauerbrei W, Taube SE, Gion M, Clark GM (2005) Reporting recommendations for tumour MARKer prognostic studies (REMARK). Br J Cancer 93(4):387–391
15. Davis SL, Eckhardt SG, Tentler JJ, Diamond JR (2014) Triple-negative breast cancer: bridging the gap from cancer genomics to predictive biomarkers. Ther Adv Med Oncol 6(3):88–100
16. Lehmann BD, Pietenpol JA (2014) Identification and use of biomarkers in treatment strategies for triple negative breast cancer subtypes. J Pathol 232(2):142–150
17. Guestini F, McNamara KM, Ishida T, Sasano H (2016) Triple negative breast cancer chemosensitivity and chemoresistance: current advances in biomarkers identification. Exp Opin Ther Targets 20(6):705–720
18. Yadav BS, Chanana P, Jhamb S (2015) Biomarkers in triple negative breast cancer: a review. World J Clin Oncol 6(6):252–263
19. Fleisher B, Clarke C, Ait-Oudhia S (2016) Current advances in biomarkers for targeted therapy in triple-negative breast cancer. Breast Cancer 8:183–197
20. Longley DB, Johnston PG (2005) Molecular mechanisms of drug resistance. J Pathol 205(2):275–292
21. Rivera E, Gomez H (2010) Chemotherapy resistance in metastatic breast cancer: the evolving role of ixabepilone. Breast Cancer Res 12(2):S2
22. Ossovskaya V, Wang Y, Budoff A, Xu Q, Lituev A, Potapova O, Vansant G, Monforte J, Daraselia N (2011) Exploring molecular pathways of triple-negative breast cancer. Genes Cancer 2(9):870–879
23. Lips EH, Michaut M, Hoogstraat M, Mulder L, Besselink NJM, Koudijs MJ, Cuppen E, Voest EE, Bernards R, Nederlof PM, Wesseling J, Rodenhuis S, Wessels LFA (2015) Next generation sequencing of triple negative breast cancer to find predictors for chemotherapy response. Breast Cancer Res 17(1):134
24. Chen Y-H, Hancock BA, Solzak JP, Brinza D, Scafe C, Miller KD, Radovich M (2017) Next-generation sequencing of circulating tumor DNA to predict recurrence in triple-negative breast cancer patients with residual disease after neoadjuvant chemotherapy. NPJ Breast Cancer 3(1):24
25. Santuario-Facio SK, Cardona-Huerta S, Perez-Paramo YX, Trevino V, Hernandez-Cabrera F, Rojas-Martinez A, Uscanga-Perales G, Martinez-Rodriguez JL, Martinez-Jacobo L, Padilla-Rivas G et al (2017) A new gene expression signature for triple-negative breast cancer using frozen fresh tissue before neoadjuvant chemotherapy. Mol Med 23:101–111
26. Wein L, Loi S (2017) Mechanisms of resistance of chemotherapy in early-stage triple negative breast cancer (TNBC). Breast 34(Suppl 1):S27–S30
27. Bareche Y, Venet D, Ignatiadis M, Aftimos P, Piccart M, Rothe F, Sotiriou C (2018) Unravelling triple-negative breast cancer molecular heterogeneity using an integrative multiomic analysis. Ann Oncol 29:895–902
28. Kim T, Han W, Kim MK, Lee JW, Kim J, Ahn SK, Lee H-B, Moon H-G, Lee K-H, Kim T-Y et al (2015) Predictive significance of p53, Ki-67, and Bcl-2 expression for pathologic complete response after neoadjuvant chemotherapy for triple-negative breast cancer. J Breast Cancer 18(1):16–21
29. Wang W, Wu J, Zhang P, Fei X, Zong Y, Chen X, Huang O, He J-R, Chen W, Li Y et al (2016) Prognostic and predictive value of Ki-67 in triple-negative breast cancer. Oncotarget 7(21):31079–31087
30. Elnemr GM, El-Rashidy AH, Osman AH, Issa LF, Abbas OA, Al-Zahrani AS, El-Seman SM, Mohammed AA, Hassan AA (2016) Response of triple negative breast cancer to neoadjuvant chemotherapy: correlation between Ki-67 expression and pathological response. Asian Pac J Cancer Prev 17(2):807–813
31. Nakashoji A, Matsui A, Nagayama A, Iwata Y, Sasahara M, Murata Y (2017) Clinical predictors of pathological complete response to neoadjuvant chemotherapy in triple-negative breast cancer. Oncol Lett 14(4):4135–4141
32. Nogi H, Uchida KEN, Kamio M, Kato K, Toriumi Y, Akiba T, Morikawa T, Suzuki M, Kobayashi T, Takeyama H (2016) Triple-negative breast cancer exhibits a favorable response to neoadjuvant chemotherapy independent of the expression of Topoisomerase IIα. Mol Clin Oncol 4(3):383–389
33. Li XY, Mu L, Feng J (2016) Topoisomerase IIα and BRCA1 expression as predictive factors for anthracycline-based adjuvant chemotherapy response and prognosis in triple-negative breast cancers. Int J Clin Exp Pathol 9(9):9249–9258
34. Ignatiadis M, Singhal SK, Desmedt C, Haibe-Kains B, Criscitiello C, Andre F, Loi S, Piccart M, Michiels S, Sotiriou C (2012) Gene modules and response to neoadjuvant chemotherapy in breast cancer subtypes: a pooled analysis. J Clin Oncol 30(16):1996–2004
35. Duffy MJ, Synnott NC, Crown J (2018) Mutant p53 in breast cancer: potential as a therapeutic target and biomarker. Breast Cancer Res Treat 170:213. https://doi.org/10.1007/s10549-018-4753-7
36. Abdel-Fatah TM, Perry C, Dickinson P, Ball G, Moseley P, Madhusudan S, Ellis IO, Chan SY (2013) Bcl2 is an independent prognostic marker of triple negative breast cancer (TNBC) and predicts response to anthracycline combination (ATC) chemotherapy (CT) in adjuvant and neoadjuvant settings. Ann Oncol 24(11):2801–2807
37. Karihtala P, Auvinen P, Kauppila S, Haapasaari KM, Jukkola-Vuorinen A, Soini Y (2013) Vimentin, zeb1 and Sip1 are up-regulated in triple-negative and basal-like breast cancers: association with an aggressive tumour phenotype. Breast Cancer Res Treat 138(1):81–90
38. Yamashita N, Tokunaga E, Kitao H, Hisamatsu Y, Taketani K, Akiyoshi S, Okada S, Aishima S, Morita M, Maehara Y (2013) Vimentin as a poor prognostic factor for triple-negative breast cancer. J Cancer Res Clin Oncol 139(5):739–746
39. Wang L, Jiang Z, Sui M, Shen J, Xu C, Fan W (2009) The potential biomarkers in predicting pathologic response of breast cancer to three different chemotherapy regimens: a case control study. BMC Cancer 9:226–226
40. Kovalev AA, Tsvetaeva DA, Grudinskaja TV (2013) Role of ABC-cassette transporters (MDR1, MRP1, BCRP) in the development of primary and acquired multiple drug resistance in patients with early and metastatic breast cancer. Exp Oncol 35(4):287–290
41. Delou JMdA, Vignal GM, Índio-do-Brasil V, Accioly MTdS, da Silva TSL, Piranda DN, Sobral-Leite M, de Carvalho MA, Capella MAM, Vianna-Jorge R (2017) Loss of constitutive ABCB1 expression in breast cancer associated with worse prognosis. Breast Cancer 9:415–428
42. Eisenhauera EA, Therasseb P, Bogaertsc J, Schwartzd LH, Sargente D, Fordf R, Danceyg J, Arbuckh S, Gwytheri S, Mooneyg M, Rubinsteing L, Shankarg L, Doddg L, Kaplanj R, Lacombec D, Verweijk J (2009) New response evaluation criteria in solid tumours: revised RECIST guideline (version 1.1). Eur J Cancer 45:228–247
43. Leslie EM, Deeley RG, Cole SP (2005) Multidrug resistance proteins: role of P-glycoprotein, MRP1, MRP2, and BCRP (ABCG2) in tissue defense. Toxicol Appl Pharmacol 204(3):216–237

44. Kim B, Fatayer H, Hanby AM, Horgan K, Perry SL, Valleley EM, Verghese ET, Williams BJ, Thorne JL, Hughes TA (2013) Neoadjuvant chemotherapy induces expression levels of breast cancer resistance protein that predict disease-free survival in breast cancer. PLoS ONE 8(5):e62766
45. Collina F, Di Bonito M, Li Bergolis V, De Laurentiis M, Vitagliano C, Cerrone M, Nuzzo F, Cantile M, Botti G (2015) Prognostic value of cancer stem cells markers in triple-negative breast cancer. Biomed Res Int 2015:158682
46. Chen Z-S, Tiwari AK (2011) Multidrug resistance proteins (MRPs/ABCCs) in cancer chemotherapy and genetic diseases. FEBS J 278(18):3226–3245
47. Kathawala RJ, Gupta P, Ashby CR, Chen ZS (2015) The modulation of ABC transporter-mediated multidrug resistance in cancer: a review of the past decade. Drug Resist Updates 18:1–17
48. Zhang L-h, Yang A-j, Wang M, Liu W, Wang C-y, Xie X-f, Chen X, Dong J-f, Li M (2016) Enhanced autophagy reveals vulnerability of P-gp mediated epirubicin resistance in triple negative breast cancer cells. Apoptosis 21(4):473–488
49. Lehmann BD, Jovanovic B, Chen X, Estrada MV, Johnson KN, Shyr Y, Moses HL, Sanders ME, Pietenpol JA (2016) Refinement of triple-negative breast cancer molecular subtypes: implications for neoadjuvant chemotherapy selection. PLoS ONE 11(6):e0157368
50. Jang MH, Kim HJ, Kim EJ, Chung YR, Park SY (2015) Expression of epithelial-mesenchymal transition-related markers in triple-negative breast cancer: ZEB1 as a potential biomarker for poor clinical outcome. Hum Pathol 46(9):1267–1274
51. Tawfik K, Kimler BF, Davis MK, Fan F, Tawfik O (2012) Prognostic significance of Bcl-2 in invasive mammary carcinomas: a comparative clinicopathologic study between "triple-negative" and non-"triple-negative" tumors. Hum Pathol 43(1):23–30
52. Hwang KT, Woo JW, Shin HC, Kim HS, Ahn SK, Moon HG, Han W, Park IA, Noh DY (2012) Prognostic influence of BCL2 expression in breast cancer. Int J Cancer 131(7):E1109–E1119
53. Vargas-Roig LM, Cuello-Carrión FD, Fernández-Escobar N, Daguerre P, Leuzzi M, Ibarra J, Gago FE, Nadin SB, Ciocca DR (2008) Prognostic value of Bcl-2 in breast cancer patients treated with neoadjuvant anthracycline based chemotherapy. Mol Oncol 2(1):102–111
54. Chen W, Dong J, Haiech J, Kilhoffer MC, Zeniou M (2016) Cancer stem cell quiescence and plasticity as major challenges in cancer therapy. Stem Cells Int 2016. https://doi.org/10.1155/2016/1740936
55. Guestini F, McNamara KM, Sasano H (2017) The use of chemosensitizers to enhance the response to conventional therapy in triple-negative breast cancer patients. Breast Cancer Manag 6(4):127–131
56. Hu Y, Yague E, Zhao J, Wang L, Bai J, Yang Q, Pan T, Zhao H, Liu J, Zhang J (2018) Sabutoclax, pan-active BCL-2 protein family antagonist, overcomes drug resistance and eliminates cancer stem cells in breast cancer. Cancer Lett 423:47–59
57. Yndestad S, Austreid E, Knappskog S, Chrisanthar R, Lilleng PK, Lonning PE, Eikesdal HP (2017) High PTEN gene expression is a negative prognostic marker in human primary breast cancers with preserved p53 function. Breast Cancer Res Treat 163(1):177–190
58. Brandmaier A, Hou SQ, Shen WH (2017) Cell cycle control by PTEN. J Mol Biol 429(15):2265–2277
59. Wang X, Jiang X (2008) Post-translational regulation of PTEN. Oncogene 27(41):5454–5463
60. Bermúdez Brito M, Goulielmaki E, Papakonstanti EA (2015) Focus on PTEN regulation. Front Oncol 5:166
61. Iqbal J, Thike AA, Cheok PY, Tse GM, Tan PH (2012) Insulin growth factor receptor-1 expression and loss of PTEN protein predict early recurrence in triple-negative breast cancer. Histopathology 61(4):652–659
62. Inanc M, Ozkan M, Karaca H, Berk V, Bozkurt O, Duran AO, Ozaslan E, Akgun H, Tekelioglu F, Elmali F (2014) Cytokeratin 5/6, c-Met expressions, and PTEN loss prognostic indicators in triple-negative breast cancer. Med Oncol 31(1):801
63. Beg S, Siraj AK, Prabhakaran S, Jehan Z, Ajarim D, Al-Dayel F, Tulbah A, Al-Kuraya KS (2015) Loss of PTEN expression is associated with aggressive behavior and poor prognosis in Middle Eastern triple-negative breast cancer. Breast Cancer Res Treat 151(3):541–553
64. Li S, Shen Y, Wang M, Yang J, Lv M, Li P, Chen Z, Yang J (2017) Loss of PTEN expression in breast cancer: association with clinicopathological characteristics and prognosis. Oncotarget 8(19):32043–32054
65. Fouque A, Jean M, Weghe PV, Legembre P (2016) Review of PI3K/mTOR inhibitors entering clinical trials to treat triple negative breast cancers. Recent Pat Anticancer Drug Discov 11(3):283–296
66. Costa RLB, Han HS, Gradishar WJ (2018) Targeting the PI3K/AKT/mTOR pathway in triple-negative breast cancer: a review. Breast Cancer Res Treat. https://doi.org/10.1007/s10549-018-4697-y
67. Massihnia D, Galvano A, Fanale D, Perez A, Castiglia M, Incorvaia L, Listi A, Rizzo S, Cicero G, Bazan V, Castorina S, Russo A (2016) Triple negative breast cancer: shedding light onto the role of PI3K/Akt/mTOR pathway. Oncotarget 7(37):60712–60722
68. Kang X, Song C, Du X, Zhang C, Liu Y, Liang L, He J, Lamb K, Shen WH, Yin Y (2015) PTEN stabilizes TOP2A and regulates the DNA decatenation. Sci Rep 5:17873
69. Yuan L, Lv Y, Li H, Gao H, Song S, Zhang Y, Xing G, Kong X, Wang L, Li Y, Zhou T, Gao D, Xiao ZX, Yin Y, Wei W, He F, Zhang L (2015) Deubiquitylase OTUD3 regulates PTEN stability and suppresses tumorigenesis. Nat Cell Biol 17:1169
70. Fielding AB, Concannon M, Darling S, Rusilowicz-Jones EV, Sacco JJ, Prior IA, Clague MJ, Urbé S, Coulson JM (2018) The deubiquitylase USP15 regulates Topoisomerase II alpha to maintain genome integrity. Oncogene. https://doi.org/10.1038/s41388-017-0092-0
71. Millis SZ, Gatalica Z, Winkler J, Vranic S, Kimbrough J, Reddy S, O'Shaughnessy JA (2015) Predictive biomarker profiling of > 6000 breast cancer patients shows heterogeneity in TNBC, with treatment implications. Clin Breast Cancer 15(6):473–481
72. Zeichner SB, Terawaki H, Gogineni K (2016) A review of systemic treatment in metastatic triple-negative breast cancer. Breast Cancer 10:25–36
73. Keam B, Im SA, Lee KH, Han SW, Oh DY, Kim JH, Lee SH, Han W, Kim DW, Kim TY, Park IA, Noh DY, Heo DS, Bang YJ (2011) Ki-67 can be used for further classification of triple negative breast cancer into two subtypes with different response and prognosis. Breast Cancer Res 13(2):R22
74. Li XR, Liu M, Zhang YJ, Wang JD, Zheng YQ, Li J, Ma B, Song X (2011) CK5/6, EGFR, Ki-67, cyclin D1, and nm23-H1 protein expressions as predictors of pathological complete response to neoadjuvant chemotherapy in triple-negative breast cancer patients. Med Oncol 28(Suppl 1):S129–S134
75. Tan QX, Qin QH, Yang WP, Mo QG, Wei CY (2014) Prognostic value of Ki67 expression in HR-negative breast cancer before and after neoadjuvant chemotherapy. Int J Clin Exp Pathol 7(10):6862–6870
76. Tian M, Zhong Y, Zhou F, Xie C, Zhou Y, Liao Z (2015) Effect of neoadjuvant chemotherapy in patients with triple-negative breast cancer: a meta-analysis. Oncol Lett 9(6):2825–2832
77. Pan Y, Yuan Y, Liu G, Wei Y (2017) P53 and Ki-67 as prognostic markers in triple-negative breast cancer patients. PLoS ONE 12(2):e0172324
78. Wang J, Xu B, Yuan P, Zhang P, Li Q, Ma F, Fan Y (2012) TOP2A amplification in breast cancer is a predictive marker of

anthracycline-based neoadjuvant chemotherapy efficacy. Breast Cancer Res Treat 135(2):531–537
79. Bravaccini S, Rocca A, Bronte G (2018) Is Ki67 still a powerful ally in predicting the clinical benefit of anthracyclines for the adjuvant treatment of early breast cancer? Breast Cancer Res Treat 168:767–768
80. Rossi L, Laas E, Mallon P, Vincent-Salomon A, Guinebretiere JM, Lerebours F, Rouzier R, Pierga JY, Reyal F (2015) Prognostic impact of discrepant Ki67 and mitotic index on hormone receptor-positive, HER2-negative breast carcinoma. Br J Cancer 113:996
81. Mueller RE, Parkes RK, Andrulis I, O'Malley FP (2004) Amplification of the TOP2A gene does not predict high levels of Topoisomerase II alpha protein in human breast tumor samples. Genes Chromosom Cancer 39:288–297
82. Khan F, Esnakula A, Ricks-Santi LJ, Zafar R, Kanaan Y, Naab T (2018) Loss of PTEN in high grade advanced stage triple negative breast ductal cancers in African American women. Pathology 214(5):673–678
83. Dillon LM, Miller TW (2014) Therapeutic targeting of cancers with loss of PTEN function. Curr Drug Targets 15(1):65–79
84. Darb-Esfahani S, Denkert C, Stenzinger A, Salat C, Sinn B, Schem C, Endris V, Klare P, Schmitt W, Blohmer J-U et al (2016) Role of TP53 mutations in triple negative and HER2-positive breast cancer treated with neoadjuvant anthracycline/taxane-based chemotherapy. Oncotarget 7(42):67686–67698
85. Yamashita H, Toyama T, Nishio M, Ando Y, Hamaguchi M, Zhang Z, Kobayashi S, Fujii Y, Iwase H (2006) p53 protein accumulation predicts resistance to endocrine therapy and decreased post-relapse survival in metastatic breast cancer. Breast Cancer Res 8(4):R48
86. Varga Z, Cassoly E, Li Q, Oehlschlegel C, Tapia C, Lehr HA, Klingbiel D, Thürlimann B, Ruhstaller T (2015) Standardization for Ki-67 assessment in moderately differentiated breast cancer. A retrospective analysis of the SAKK 28/12 study. PLoS ONE 10(4):e0123435
87. Biesaga B, Niemiec J, Ziobro M (2014) BCL-2, Topoisomerase IIα, microvessel density and prognosis of early advanced breast cancer patients after adjuvant anthracycline-based chemotherapy. J Cancer Res Clin Oncol 140:2009–2019
88. Gonzalez-Angulo AM, Ferrer-Lozano J, Stemke-Hale K, Sahin A, Liu S, Barrera JA, Burgues O, Lluch AM, Chen H, Hortobagyi GN, Mills GB, Meric-Bernstam F (2011) PI3K pathway mutations and PTEN levels in primary and metastatic breast cancer. Mol Cancer Ther 10(6):1093–1101
89. Britton KM, Eyre R, Harvey IJ, Stemke-Hale K, Browell D, Lennard TWJ, Meeson AP (2012) Breast cancer, side population cells and ABCG2 expression. Cancer Lett. https://doi.org/10.1016/j.canlet.2012.1003.1041
90. Soini Y, Jarvinen K, Kaarteenaho Wiik R, Kinnula V (2001) The expression of P-glycoprotein and multidrug resistance proteins 1 and 2 (MRP1 and MRP2) in human malignant mesothelioma. Ann Oncol 12:1239–1245