Original Article

Low Expression of NQO1 Predicts Pathological Complete Response to Neoadjuvant Chemotherapy in Breast Cancer Patients Treated with TAC Regimen

(breast cancer / neoadjuvant therapy / predictive biomarkers)

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Abstract. The aim of this study was to evaluate pre-operative tumour expression of NAD(P)H:quinone oxidoreductase 1 (NQO1) along with other biological markers as potential predictors of pathological complete response (pCR) to neoadjuvant docetaxel, doxorubicin, and cyclophosphamide-containing (TAC) chemotherapy in patients with primary breast cancer. Sixty-one patients who received neoadjuvant chemotherapy (NCT) with TAC regimen were enrolled in this prospective study. The pre- and post-NCT expression of oestrogen receptor (ER), progesterone receptor (PR), epidermal growth factor receptor 1 and 2 (EGFR and HER2), NQO1, Ki-67 proliferation index, multidrug resistance protein 1 (MDR1), p53 and BCL2 were evaluated by immunohistochemistry. The pCR was reached in 14 patients (23 % of the study group). Multivariate analysis demonstrated that patients with ER-, PR-, NQO1-negative, and Ki-67-positive tumours had a significantly higher chance to achieve pCR. Within the biological subtypes, the highest pCR rate (50 %) was seen in triple-negative (i.e. ER-, PR-, HER2-) tumours. Post-operative evaluation showed that in comparison to pre-operative tissue samples, NQO1 expression was significantly increased, while Ki-67 and HER2 decreased, in the residual tissue after NCT. In conclusion, the present data suggests that NQO1 expression may be a novel diagnostic biomarker for the prediction of positive response to NCT in patients with breast cancer.

Introduction

Neoadjuvant chemotherapy (NCT) has become a standard of care in large and locally advanced breast cancer (BC), as it commonly switches the tumour from inoperable into operable status, or increases the chance for breast-conserving surgery. Moreover, 10 % to 30 % of patients respond to NCT by complete histological disappearance of the tumour, referred to as pathological complete response (pCR) (Caudle and Hunt, 2011). Numerous studies have demonstrated that this subset of patients has a distinctly better chance for longer disease-free and overall survival (Fisher et al., 1998; Kuerer et al., 1999; Ogston et al., 2003; Rastogi et al., 2008). Hence, pCR became routinely used as a standard surrogate marker of favourable response to NCT. However, BC is a heterogeneous group of tumour types with high inter-individual variability in NCT outcomes (Liu et al., 2010). This fact has led to an intensive search for tools allowing better classification of the disease, and consequent individualization of therapy (Leong and Zhuang, 2011).

Currently, only a small number of single biomarkers have been selected as useful tools in the management of

BC. Those which demonstrate significant prognostic value for overall survival include the oestrogen receptor (ER), the progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2) (Weigel and Dowsett, 2010). The hormonal receptors have also strong predictive power for pCR achievement after NCT. While pCR in ER-negative tumours is seen in 21 % to 33 % of patients, ER-positive tumours have only a 7 % to 8 % rate (Colleoni et al., 2004; Ring et al., 2004). The relationship between HER2 and pCR remains less clear as variable data, i.e. both absence and presence of predictive value of HER2 in patients treated by NCT, have been presented (Andre et al., 2008; Iwase et al., 2011; Li et al., 2011b). Similarly conflicting results are known for numerous other molecules, such as proliferation marker Ki-67 (Stuart-Harris et al., 2008), tumour suppressor p53 (Linjawi et al., 2004), apoptosis regulator BCL2, or multidrug resistance transporter MDR1 (Surowiak et al., 2005). Moreover, recent data suggest that the NAD(P)H:quinone oxidoreductase 1 (NQO1) enzyme may play an important role in the pathology of BC since homozygous mutation, which disables NQO1 activity, predicts worse survival in BC patients (Fagerholm et al., 2008; Hubackova et al., 2009; Yuan et al., 2011; Hubackova et al., 2012). On the other hand, the predictive value of NQO1 protein expression itself has not yet been described.

This is the first study to investigate the predictive potential of NQO1 protein expression measurement in BC for the effects of NCT. The aim of the presented research was to correlate the rate of pCR with the expression of the established as well as some novel promising biomarkers in BC patients treated in a neoadjuvant setting with doxorubicin- and taxane-based TAC regimen, one of the current standards (Aebi et al., 2010). Also, the levels of expression of the biomarkers before and after NCT were compared.

Material and Methods

Study population

Patients with newly diagnosed BC, treated at the Department of Oncology, University Hospital in Hradec Králové, were enrolled for the participation in the study. Eligibility criteria were age above 30, locally advanced tumour, and indication for NCT by TAC regimen. The initial diagnostic workup included a complete history, biochemical, haematological and clinical examination, bilateral mammography, and breast ultrasound examination with quantification of the main tumour diameter. A diagnosis of BC was done on the basis of core needle biopsy of primary tumour. Presence of metastases was evaluated by chest X-ray and liver ultrasonography.

From January 2009 until April 2011, 61 patients were enrolled to the study. All patients gave written informed consent, and the study was performed in accordance with the Helsinki Declaration, under supervision of the University Hospital Ethical Committee at the Depart-

ment of Oncology, University Hospital in Hradec Králové. All patients received treatment with six cycles of TAC regimen – i.e. docetaxel 75 mg/m^2, doxorubicin 50 mg/m^2, cyclophophamide 500 mg/m^2, on day 1, every 3 weeks. Patients with HER2 receptor positivity were treated with NCT add-on trastuzumab. Within 21 days after completion of the last chemotherapy cycle and after overall assessment of response, patients underwent surgery and post-operative treatment according to standard recommendations. Post-operative irradiation was delivered to the chest wall, internal mammary lymph nodes, and supraclavicular/axillary lymph nodes.

Definition of response to neoadjuvant therapy

The clinical response to NCT was evaluated after each cycle of chemotherapy and prior to definitive surgery 21 days after the last cycle of NCT. The examination focused on determination of the main tumour diameter, axillary node status and presence of metastases. Tumour diameter was measured by ultrasonography. Pathological response at surgery was based on the criteria presented by Chevallier et al. (1993). Pathological complete response (pCR) was defined as absence of any tumour cells (either invasive or *in situ* component) in the breast, and axillary tissue removed at surgery. The histological tumour type was defined according to the WHO definitions. All pathological examinations including immunohistochemistry were performed without information on the clinical details by a single pathologist, and reviewed independently by another pathologist at the Department of Pathology, University Hospital in Hradec Králové.

Immunohistochemical evaluation

All antigens were detected in tissue sections of the core-cut-biopsies obtained at the time of primary diagnosis before NCT, and in the residual tumour tissue (in case of no pCR) obtained during surgery for the tumour excision after completion of NCT. Tumour samples were initially fixed in 10% neutral buffered formalin and embedded in paraffin. Consequently, 4-μm-thick sections were used for immunohistochemistry. Antibodies, dilutions and pre-treatment procedures used are summarized in Table 1. The heat-induced epitope retrieval was used in all methods. The tissue sections were incubated for 40 min in a water bath at a temperature of 96 °C (for pH 9 pre-treatment), or for 10 min in Histoprocessor (for pH 6 pre-treatment). The antibodies were incubated at room temperature for 30 min and the reaction was visualized by EnVision peroxidase kit (DAKO, Glostrup, Denmark). The staining was evaluated and quantified by light microscopy. Kits EGFR pharmDx and HercepTest (both DAKO) were used for detection of EGFR and HER2, respectively.

Scoring system used for quantification of detected antigens

The expression of evaluated antigens was initially assessed semi-quantitatively by using either the percent-

Table 1. Antibodies used for immunohistochemical evaluation

Marker	Clone	Manufacturer	Dilution	Sample processing
ER	1D5	DAKO, Glostrup, Denmark	1:75	buffer S 2367 (DAKO), pH 9
PR	PgR636	DAKO, Glostrup, Denmark	1:300	buffer S 2367 (DAKO), pH 9
Ki-67	MIB-1	DAKO, Glostrup, Denmark	1:30	buffer S 2367, (DAKO) pH 9
BCL2	124	DAKO, Glostrup, Denmark	1:200	citrate buffer, pH 6
p53	DO-7	DAKO, Glostrup, Denmark	1:150	buffer S 2367 (DAKO), pH 9
NQO1	Polyclonal	Sigma-Aldrich, St. Louis, MO	1:150	citrate buffer, pH 6
MDR1	Polyclonal	Sigma-Aldrich, St. Louis, MO	1:250	citrate buffer, pH 6

age of positive tumour cells (PPTC), and/or by the intensity of immunostaining (none = 0; weak = 1; moderate = 2; strong = 3). The proliferation marker Ki-67 was evaluated quantitatively in the periphery of the tumour as a percentage of positive tumour cells. HER2 expression was scored according to manufacturer's instructions with the currently published ASCO/CAP modification (Wolff et al., 2007) on a scale of 0–3 (0 = no staining, or membrane staining in less than 10 % of the tumour cells; 1 = faint/barely perceptible membrane staining detected in more than 10 % of tumour cells; 2 = weak to moderate staining of the entire membrane observed in more than 30 % of tumour cells; and 3 = strong staining of the entire membrane observed in more than 30 % of tumour cells). For the univariate and multivariate analysis of predictive potential of detected antigens, with the exception of Ki-67, all other antigens were considered as positive if at least 10 % of tumour cells showed positive staining, or the intensity/scaling value was more than 1. Ki-67 was scored positive if more than 20 % of tumour cells were stained (Li et al., 2011b).

Statistical analysis

Predictive factors for pCR were initially screened by univariate analysis with χ^2 test. The factors associated with pCR probability at a significance level of 0.1 or less were consequently processed by a multivariate logistic regression analysis. The Wilcoxon signed-rank test was used to study the correlation between the expression of evaluated biomarkers in the tumour specimens obtained before (core-needle biopsy) and after (excised residual tumour tissue) NCT. Statistical analysis was performed using Graphpad Prism 5.0 (Graphpad Software, Inc., San Diego, CA) and NCSS (Kaysville, UT). A P value of less than 0.05 was considered to be significant.

Results

All patients included in the study completed NCT regimen; complications occurred with the expected spectrum, incidence, and intensity. Most often they comprised mild (24/61) or moderate (i.e. necessitating gabapentine pharmacotherapy) paresthaesias of extremities (6/61), and neutropoenia, which appeared mostly after the third cycle of AC and was grade 3 in 4/61 and
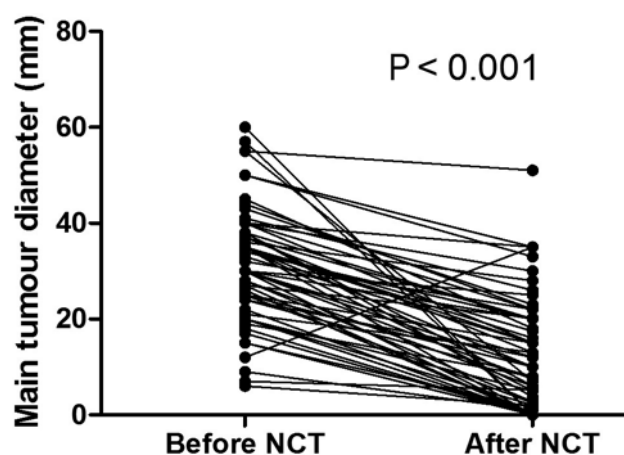

Fig. 1. Clinical response of tumour mass to neoadjuvant chemotherapy. Main tumour diameter was measured by sonography before and after completion of NCT.

grade 4 in 2/61 patients. No patient suffered anthracycline-based cardiac failure during NCT. The tumour response to TAC regimen is presented in Fig. 1. The vast majority of patients showed significant decrease in the main tumour diameter. Only three patients presented with no response and progressive disease. Out of 61 patients, 14 reached pCR during NCT.

Relevant clinico-pathological features, together with results of the evaluation of selected antigens (as exemplified in Fig. 2), were analysed by univariate analysis and correlated to pCR (Table 2). We found that the negativity of NQO1 and oestrogen/progesterone receptors and the positivity of Ki-67 and p53 in pre-operative bioptic samples are associated with pCR after NCT. Combined expression analysis of hormone receptors and HER2 (Fig. 3) also demonstrated a higher pCR rate in the triple-negative tumours, while the presence of hormone receptor positivity demonstrated a lower pCR rate irrespective of the HER2 status. Subsequent multivariate regression analysis further confirmed that hormone receptor (P = 0.05 for ER, P = 0.01 for PR), NQO1 negativity (P = 0.0001), and Ki-67 positivity (P = 0.03) are the strongest factors predictive of pCR (Table 3).

Evaluation of expression changes pre- and post-NCT showed that NQO1 is significantly more expressed in the residual tissue after NCT. In contrast, the expression of the proliferation marker Ki-67 and HER2 receptor
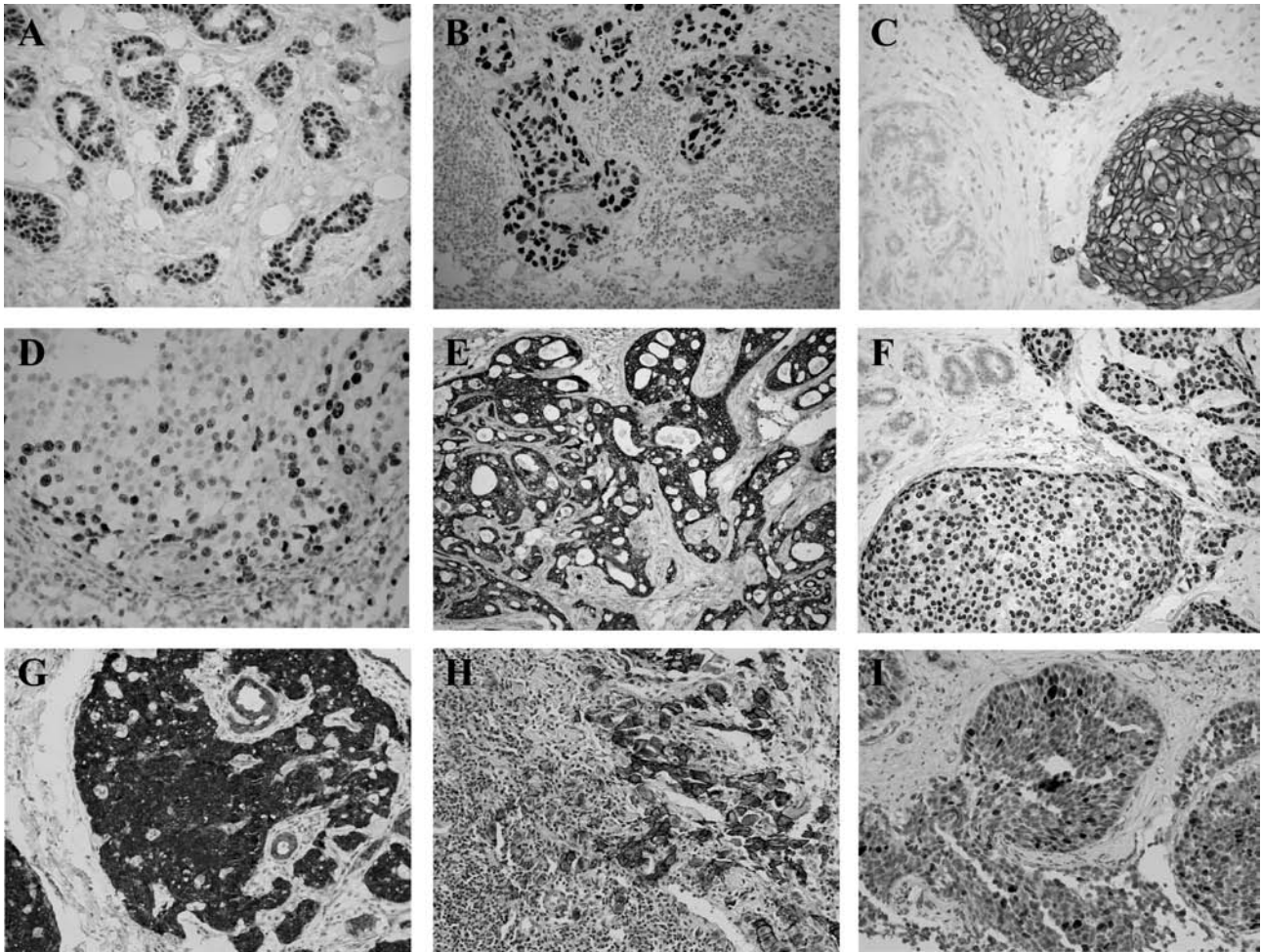
Fig. 2. Immunohistochemistry of selected antigens in biopsy samples – representative pictures demonstrating positive presence of the evaluated antigen. **A.** ER; **B.** PR; **C.** HER2; **D.** Ki-67; **E.** BCL2; **F.** p53; **G.** NQO1; **H.** EGFR; **I.** MDR1.

followed an opposite trend with the decrease of its expression after NCT (Table 4).

Discussion

In the present study of patients with breast carcinoma treated with TAC neoadjuvant regimen, we have detected predictive potential of low ER, PR, and NQO1, and high Ki-67 expression in the initial pre-operative core-needle biopsy samples for the achievement of pCR. Moreover, comparison of biomarker expression, quantified in pre-operative biopsy samples with expression in tumour samples excised during surgery, showed an increase of NQO1 and decrease of Ki-67 and HER 2 expression after the NCT.

We have used pCR as a commonly accepted surrogate marker of positive outcome of the BC therapy after NCT (Caudle and Hunt, 2011). The overall pCR rate observed in our study was 23 %. This corresponds with the results of a recent large-scale GeparTrio study using identical TAC regimen, where the incidence of pCR was 20.5 % (Huober et al., 2010). However, the occurrence of pCR reported in different studies, and/or after different protocols, is highly variable and the values presented fall usually within the interval of 7 % to 33 % (Weigel

and Dowsett, 2010). There are two potential reasons for such a high variation. Firstly, one of them can be the variability of the definition of pCR and different classification schemes of pathological response used by different authors. The second is the variation of tumour phenotype in the individual reported cohorts. The highest pCR rates are usually seen in the narrowly defined subsets of patients characterized on the basis of molecular diagnostics of selected gene products, and treated with targeted therapy (Buzdar et al., 2007). Variations in the detected markers confirm the fact that BC is a highly heterogeneous disease and each potential subtype differs in terms of gene expression and molecular features (Goldhirsch et al., 2009). This further emphasises the requirement for the research of reliable biomarkers.

The most commonly used molecular marker already included in the routine diagnostic procedure in all BC samples is the measurement of ER and PR expression. Both receptors have demonstrated not only the ability to predict pCR after neoadjuvant regimens, but namely good prognostic value for overall survival (Weigel and Dowsett, 2010). Interestingly, hormone receptor positivity contrasts with therapeutic outcomes in BC; while in case of pCR, low expression of ER means a higher chance to reach complete response after NCT, the pro-

Table 2. Univariate analysis of factors predictive of pCR

Characteristic	pCR (No.)		Odds ratio	95% CI	P
	Yes No.	No No.			
Age			2.62	0.76–8.93	0.12
< 50	7	13			
≥ 50	7	34			
T status			2.81	0.94–14.19	0.2
T1/T2	12	32			
T3/T4	2	15			
Nodal status			0.64	0.16–2.66	0.54
Negative	3	14			
Positive	11	33			
Grade			2.81	0.56–14.19	0.2
Grade I/II	12	32			
Grade III	2	15			
ER status			3.49	1.0–12.1	0.04
Negative	9	16			
Positive	5	31			
PR status			12.46	1.51–1.03	0.005
Negative	13	24			
Positive	1	23			
HER 2			1.88	0.55–6.40	0.31
Negative	9	23			
Positive	5	24			
Ki-67			0.095	0.011–0.790	0.01
Negative	1	21			
Positive	13	26			
NQO1			17.08	4.04–72.25	0.001
Negative	10	6			
Positive	4	41			
BCL2			1.47	0.44–4.80	0.52
Negative	7	19			
Positive	7	28			
p53			0.25	0.05–1.23	0.04
Negative	2	19			
Positive	12	28			
EGFR			0.34	0.1–1.0	0.08
Negative	7	35			
Positive	7	12			
MDR1			0.71	0.12–4.10	0.70
Negative	12	42			
Positive	2	5			

longation of overall survival is commonly related to positive ER receptor status. The reason for such discrepancy may be due to the lower aggressiveness of ER-positive tumours with generally slower proliferation, and also due to the use of effective hormonal treatment with ER antagonists or aromatase inhibitors (Takei et al., 2011). Our data of lower ER/PR expression in preoperative samples of patients with pCR are in agreement with this concept.

Another biomarker commonly used in the diagnostics of BC is HER2 (Leong and Zhuang, 2011). As a receptor associated with intracellular signaling cascade activating BC cell proliferation, high expression of this protein on the membrane of BC cells indicates higher proliferation potential. The predictive role of HER2 was
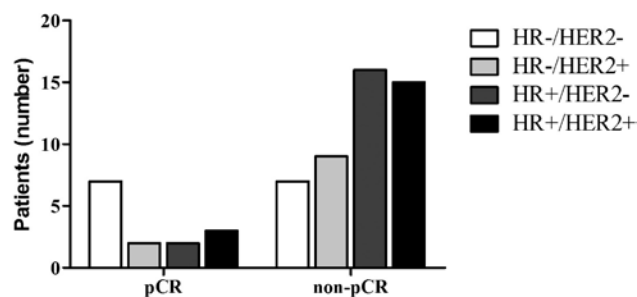

Fig. 3. Association between pCR and hormone receptor/ /HER2 phenotype.

Table 3. Multivariate logistic regression analysis of factors predictive of pCR

Factor	Odds ratio	95% CI	P value
ER	0.29	0.08–1.00	0.05
PR	0.08	0.01–0.66	0.01
NQO1	0.06	0.01–0.25	0.0001
EGFR	2.92	0.85–10.04	0.09
Ki-67	10.50	1.27–86.93	0.03
p53	4.07	0.82–20.29	0.09

indeed described in some studies (Andre et al., 2008). On the other hand, other studies, including ours, have failed to find such association (Gonzalez-Angulo et al., 2004; Li et al., 2011a). Recent works ascribed this discrepancy to the necessity of assessing the HER2 status in association with the ER/PR (HR) status of the tumour (Darb-Esfahani et al., 2009). Indeed, significantly higher pCR rate is observed in HR+ tumours also positive for HER2 (luminal B type) than in tumours with HR+ but HER2-negative status (luminal A type), even without usage of trastuzumab (Darb-Esfahani et al., 2009; Huober et al., 2010). In our study we have seen a similar trend, though not statistically significant, perhaps due to the limited number of patients. However, we observed the highest pCR rate in the group of triple-negative tumours (i.e. HR-/HER2-), which is in agreement with all other reports. Patients with triple-negative BC who achieved pCR also have a good prognosis, while the subset of triple-negative cases who do not reach pCR represent highly aggressive tumours with a poor prognosis (Carey et al., 2007, 2010).

Measurement of other biomarkers in primary BC samples shows contradictory results. There is no consensus regarding the predictive value of these markers, either for pCR or for overall survival. Most investigated have been markers of proliferation (e.g. Ki-67), basal cell type (CK5/6), or markers associated with regulation of apoptosis – p53 and BCL2 (Malamou-Mitsi et al., 2006; Hornychova et al., 2008; Weigel and Dowsett, 2010; Li et al., 2011a). We also evaluated key molecules involved in these pathways. However, we did not detect any significant relationship to pCR achievement for any of them, with the exception of Ki-67. High levels of this antigen demonstrated that tumours with high prolifera-

Table 4. Changes in the expression of selected antigens between tumour samples obtained before and after NCT

	Before NCT		After NCT		P value
	Median	25–75% Percentile	Median	25–75% Percentile	
ER	80.0	0.0–90.0	70.0	0.0–90.0	0.09
PR	10.0	0.0–90.0	0.0	0.0–77.5	0.71
HER 2	2.0	1.0–2.0	0.0	0.0–1.0	< 0.0001
Ki-67	25.0	10.0–60.0	22.5	10.0–50.0	0.03
NQO1	80.0	60.0–90.0	100.0	67.5–100.0	0.0035
BCL2	50.0	0.0–97.5	35.0	0.0–87.5	0.50
p53	10.0	3.5–40.0	10.0	3.0–30.0	0.55
EGFR	0.0	0.0–7.5	0.0	0.0–0.0	0.83
MDR1	0.5	0.0–5.0	0.0	0.0–8.75	0.24

tion fraction have a better chance to respond to NCT by pCR. This may seem to be a logical consequence of the administration of cytostatics, targeted on rapidly dividing cells. Such results are in agreement with previous reports (Stuart-Harris et al., 2008; Darb-Esfahani et al., 2009) and comply with the concept that significant proliferation is seen particularly in ER-negative tumours.

The most interesting finding of the present study was the predictive potential of low NQO1 expression in the tumour tissue for the pCR occurrence in TAC-treated patients. NQO1 is an almost ubiquitous cellular component, which under different pathophysiological situations provides protection of the cells against oxidative damage by reduction of quinolone substrates (Dinkova-Kostova and Talalay, 2010). NQO1 activation also induces pro-apoptotic p53 and interferes with the anti-apoptotic NF-κB pathway. Thus, depending on the micro-environmental factors and the cell type, the effects of NQO1 on the cell proliferation and survival may vary (Jamshidi et al., 2011). In breast cancer, the alteration of NQO1 expression in subjects with mutant alleles in the genome is known to increase susceptibility of mammary gland for tumour development, perhaps due to alteration of cellular defence mechanisms against noxious stimuli. Moreover, the presence of variant alleles in the genome of BC patients also indicates poor response to adjuvant chemotherapy (Jamieson et al., 2011). In the only study examining the relationship between the protein expression of NQO1 directly in BC and outcomes of chemotherapy, Jamshidi et al. (2011) reported that negative NQO1 was associated with ER negativity but not with other tumour characteristics. This complies with our data where both ER- and NQO1-negative status corresponded with better outcome. On the other hand, the authors were unable to find a significant relationship between NQO1 expression and any other clinico-pathological parameters, such as patient survival or outcome after adjuvant anthracycline treatment. We assume that this contradiction to our results is the consequence of the pre-operative nature of our study with pCR as the principal outcome, which could not reflect any contribution of surgical stress, inflammation, wound healing, or other factors influencing results in

adjuvant studies. The anticipated mechanism why the NQO1-negative BC patients respond better to NCT in our study may be higher susceptibility of these cancer cells to injury induced by cytostatics, but detailed cellular study to confirm this is currently unavailable. Nevertheless, increased NQO1 expression in patients with incomplete response, i.e. at least partial resistance to the applied NCT, may indicate selection of NQO1-positive cells and supports this hypothesis.

In conclusion, we have identified low NQO1 protein expression as a new potential diagnostic biomarker for the prediction of positive response to pre-operative NCT with TAC-containing regimen by induction of pCR. In agreement with the majority of previous reports, we have also seen similar predictive potential of low ER/PR, and high Ki-67 expression. Our results may offer improvement of current diagnostic procedures to further refine rational therapy individualization in breast cancer patients.

Acknowledgment

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