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Comparative value of tumour grade, hormonal receptors, Ki-67, HER-2 and topoisomerase II alpha status as predictive markers in breast cancer patients treated with neoadjuvant anthracycline-based chemotherapy

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Abstract

The aim of this study was to evaluate the predictive value of five different biological factors in breast cancer patients treated with neoadjuvant anthracycline-based chemotherapy: (1) tumour grade scored according to the Elston–Ellis classification, (2) hormonal receptor (HR) status; (3) tumour cell proliferation evaluated by Ki-67 staining, (4) HER-2 and topoisomerase II alpha (TopoIIα) expression evaluated by immunohistochemistry (IHC), (5) *HER-2* and *TopoIIα* amplification evaluated by real-time polymerase chain reaction (PCR). 119 patients with operable breast cancer were treated with six cycles of FEC (100 5-fluorouracil (5-FU) 500 mg/m^2, Epirubicin 100 mg/m^2, Cyclophosphamide 500 mg/m^2). Tumour response was assessed clinically and by computed tomography (CT) scan, then by pathological assessment. The clinical overall response (OR) was 80%, with 19% of complete responders (CR). The radiological OR was 71%, with 16% of CR. A pathological CR was demonstrated in 13% of the patients according to the Sataloff classification. In the multivariate analysis, the absence of HR expression and Ki-67$\geqslant$20% were predictive for a clinical CR. A high tumour grade was predictive for a pathological CR. Overexpression or amplification of *HER2* or *Topollcα* were not predictive of response.

Keywords: Breast cancer; Neoadjuvant chemotherapy; Predictive factors

1. Introduction

As the clinical and pathological responses of breast cancer to neoadjuvant chemotherapy are short-term markers for a long-term outcome [1,2], it is important to identify accurately biological factors that may predict the response to neoadjuvant therapy. These factors should identify patients who will benefit most from treatment, and spare patients from the toxicity of treatment that is known to be ineffective. These factors could also provide mechanistics insights into understanding tumour biology. Recent retrospective studies have suggested a correlation between HER2 overexpression and a benefit from anthracycline therapy [3,4], but a biological mechanism linking HER2 overexpression to anthracycline sensitivity is lacking. Topoisomerase II alpha (*TopoIIα*) is localised close to *HER2* on chromosome 17 q12-q21 and the enzyme is a molecular target for anthracyclines [5]. Because of the physical proximity to *HER2*, *TopoIIα* is frequently co-amplified with *HER2* in breast cancer [6]. This co-amplification could

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provide a plausible explanation for the link between HER2 overexpression and anthracycline response.

In the present study, the role of tumour grade, hormonal receptors (HR), Ki-67, HER-2 and TopoIIα status as predictive markers of response to neoadjuvant anthracycline-based chemotherapy was evaluated in primary tumour specimens from 119 patients with breast carcinomas.

2. Patients and methods

2.1. Patient and tumour characteristics

All patients with operable breast cancer and treated between January 2000 and December 2001 at the Paul Strauss Center with neoadjuvant anthracycline-based chemotherapy were included in this retrospective study. Neo-adjuvant chemotherapy was indicated when the largest tumour diameter was equal to or larger than 20 mm. Patients with a primary inflammatory carcinoma or with a long clinical history of neglected tumour in breast were excluded. A microbiopsy was performed before the chemotherapy treatment to allow a pathological diagnosis and evaluation of biological parameters. All tumours were adenocarcinomas. Patients received six cycles of an anthracycline-based therapy (5-fluorouracil (5-FU) 500 mg/m^2, epirubicin 100 mg/m^2, cyclophosphamide 500 mg/m^2, on day 1 of a 21-day cycle). No patient received tamoxifen as part of her neoadjuvant treatment. Patients were scheduled to undergo surgery 4 weeks after the sixth cycle with tumour excision and axillary node dissection. Table 1 shows the patient and tumour characteristics.

2.2. Assessment response

Assessment of the tumour response was undertaken by bidimensional clinical and computed tomography (CT) measurements both prior to chemotherapy treatment and after six cycles, but before surgery. The clinical baseline and preoperative measurements were obtained with a caliper by the same medical oncologist. Clinical and radiological responses were recorded according to the International Union Against Cancer (UICC) criteria: a complete response (CR) indicating the disappearance of the primary tumour; a partial response (PR) indicating a reduction of $\geqslant 50\%$; stable disease (SD) indicating a reduction of $<50\%$ or increase in size of $<25\%$; progressive disease (PD) indicating an increase in size of $\geqslant 25\%$.

The pathological CR was evaluated by tumour excision and axillary node resection after six cycles of chemotherapy. Two experienced breast pathologists looked at all samples. Two grading classifications were utilised to evaluate the tumour response after chemotherapy. In the Chevallier classification, the absence of invasive tumour cells, or persistence of *in situ* disease, and negative axillary lymph nodes define a pathological CR [7]. In the Sataloff classification, we have similar criteria for a pathological CR, but also observe the persistence of small clusters of widely dispersed cells in these cases [8].

2.3. Evaluation of pathological factors

Five different pathological factors were determined on the initial microbiopsy and studied as predictive factors in a single laboratory (Centre Paul Strauss): Tumour grade, tumour cell proliferation evaluated by Ki-67 staining [9], HR, HER2 and TopoIIα status (Table 2). All slides were reviewed by the two pathologists without knowledge of the treatment response.

The tumour grade was scored according to the Elston–Ellis classification on a scale of 1–3 [10].

The four other pathological factors were evaluated by immunohistochemistry (IHC). Reagents used for IHC studies are listed in Table 3. 3-μm sections from formalin-fixed, paraffin-embedded tissues were cut and mounted on positively-charged slides. Tissue sections were deparaffinised and rehydrated in graded alcohol. The slides were subjected to heat-induced epitope retrieval by immersing them in 0.01 M boiling citrate buffer (pH 6) in a pressure cooker for 3 min, followed by a 20-min cooling-off period. Slides were then

Table 1
Patient and tumour characteristics

Number of patients	119
Median age (range) (years)	52 (29–71)
Median largest tumour diameter (range) (mm)	45 (20–100)
Clinically-positive axillary nodes	49%
Tumour type (%)	
Invasive ductal	77
Invasive lobular	13
Mucinous	1
Other	9

Table 2
Pathological variables

Tumour grade	
1	32
2	49
3	19
HER2 status	
0-1	60
2	18
3	22
HR status	
Positive	75
Negative	25
Ki-67-median score (range)	19 (1–90)
TopoIIα-median score (range)	9 (1–47)

HR, hormone receptor.

incubated overnight with the monoclonal antibody. The antibody was localised using the Dako detection system according to the manufacturer's instructions. Diaminobenzidine (Sigma Inc.) was used to visualise antibody binding. Negative and positive control slides were included with each assay. Ki-67 staining equal to or more than 20% was considered as high level [9]. Tumours were considered HR-positive if either or both oestrogen (ER) and progesterone receptors (PR) were positive in $\geqslant$10% of tumour cells. HER2 status was scored on a scale of 0–3+ according to the Dako scoring system. A score of 2+ or 3+ was considered as overexpression. TopoIIα status was scored quantitatively and, based on prior studies, staining equal to or more than 10% was considered as overexpression [11].

The number of *HER2* and *TopoIIα* gene copies was also evaluated using a real-time polymerase (PCR) method with the Light Cycler™ system (Roche Molecular Biochemicals). DNA was obtained from 5×18μm sections of formalin-fixed paraffin-embedded blocks using a QIAamp DNA mini-kit (QIAGEN), with a modified version of protocol (WilKam, QiAgenNews). *HER2* amplification was measured with the LightCycler–Her2 DNA Quantification Kit (Cat. No. 3 113 922, Roche, France), according to the supplier's instructions, in which a 112-bp fragment of the *HER2* gene and a 133-bp fragment of the reference gene are simultaneously amplified by the use of specific sets of primers. Both genes are localised on chromosome 17. Specific pairs of hybridisation probes (labelled with fluorescein at the 3′ end for both genes and at the 5′ end with LightCycler-Red 705 for *HER2* and LightCycler-Red 640 for the reference gene) allowed the differential fluorescent detection of the amplified DNA. This was observed after light excitation, due to the FRET (fluorescence energy transfer) between fluorescein and the fluorophors, which occured only when the probes were hybridised on the template DNA and were therefore in close proximity. The amount of measured fluorescence was proportional to the increasing amount of DNA generated during the PCR process). The calculation of the relative amounts of the *HER2* gene was performed by the LightCycler Relative Quantification Software. The final results were expressed as a ratio between the number of *HER2* gene copies and number of reference gene copies. A ratio <2 was assumed to be negative for *HER2* amplification, a ratio $\geqslant 2$ was assumed to be positive for *HER2* amplification.

TopoIIα amplification was measured using the Master SYBR green I dye kit (Roche Molecular Biochemicals) with primers: TOP2A-3F 5′-CAGCGTGTTGAGCCTGAATG-3′ and TOP2A-3R 5′-TTGCAGGACCACCCAGTACC-3′. An optimal concentration of 4 mM of $MgCl_2$ was used.

The reaction mixture was preheated at 95 °C for 10 min, followed by *five* cycles at 98° for 10 s, 62 °C for 10 s, 72 °C for 10 s and 35 cycles at 95° for 10 s, 62 °C for 10 s, 72 °C for 10 s. As a reference gene, beta-globin was measured using the LightCycler-control Kit DNA (Cat No. 2 158 833 Roche Molecular Biochemicals). All analyses were performed in at least two independent experiments. For each experimental sample, the amount of target and reference were determined from the standard curve included in the LightCycler-control Kit DNA. Then the target amount was divided by the reference amount to obtain a normalised *TopoIIα* value. A ratio $\geqslant 2$ was assumed to be positive for *TopoIIα* amplification.

Table 3
Reagents used for the IHC studies

	Clone	Dilution	Manufacturer
Tumour cell proliferation	Ki-67/MiB1	1/50	Dako
ER	6F11	1/50	Dako
PR	PGR 312	1/200	Dako
HER2	CB 11	1/50	Novocastra
TopoIIα	3F6	1/50	Novocastra

IHC, immunohistochemistry; ER, oestrogen receptor; PR, progesterone receptor.

2.4. Statistical analysis

To study the agreement between pathological CR and the clinical and radiological CR, Cohen's kappa coefficient was used [12]. If there is complete agreement, kappa is equal to 1. Kappa values close to or less than 0 show poor agreement (no better than by chance).

The effect of the different biological factors on the response rate to neoadjuvant chemotherapy was assessed by means of the Chi-square test or Fisher's exact test in comparisons with small numbers. $P<0.05$ was considered to indicate statistical significance, and all resulting P were two-tailed. To identify variables independently related to the response, a multivariate analysis using a logistic regression model was performed. All variables with a P value less than 0.20 in the univariate analysis were included in a stepwise forward logistic regression model, allowing for interaction. P values are presented for the final model. 'Goodness of fit' was assessed by the Hosmer–Lemeshow test.

The statistical analysis was performed using BMDP (BMDP Statistical Software, Inc., Los Angeles, CA, USA).

3. Results

119 patients with operable breast cancer were treated with neoadjuvant anthracycline-based chemotherapy at the Paul Strauss Cancer Center between January 2000 and December 2001 (Table 1). All breast cancers were stage IC–IIIA invasive adenocarcinomas.

3.1. Tumour response to neoadjuvant treatment

As assessed by the physical examination, 61% of the patients had a PR and 19% had a CR, allowing a clinical overall response (OR) of 80%. There was no PD. Following CT measurement, the OR was 71% (16% CR and 55% PR). No PD was observed in the radiological measurements.

After the six cycles of neoadjuvant chemotherapy, lumpectomy was achieved in 72% of the patients. The median number of excised axillary lymph nodes was 11. According to the Chevallier classification, the pathological CR rate was 9%. According to the Sataloff classification, this pathological CR rate was 13%.

We studied the agreement of clinical and radiological CR with the pathological CR. The kappa coefficient between clinical CR and pathological CR was 0.42 ($P = 1 \times 10^{-6}$). The kappa coefficient between radiological CR and pathological CR was 0.28 ($P = 6 \times 10^{-3}$). With a higher kappa value, clinical response seemed to be superior to radiological response in predicting the pathological CR in the present study. However, the difference was not statistically significant.

3.2. Predictive value of tumour grade, HR. Ki-67, HER2 and TopoIIα evaluated by IHC

99 patients were assessable for these five biological variables. These variables were not evaluated in 20 patients because of a lack of tumour samples. In the univariate analysis (Table 4), high tumour grade, high tumour cell proliferation (Ki-67 $\geqslant$ 20%) and absence of HR expression were predictive of a clinical CR ($P = 0.004$, 0.002, 0.003, respectively). These three biological factors were also predictive of a pathological CR according to the Sataloff classification, ($P =$ 0.0001, 0.01, 0.008, respectively). In our series, the pathological CR rate (Sataloff) was 42% for grade 3 tumours compared with 10% for grade 2 tumours and 0% for grade 1 tumours. The pathological CR rate (Sataloff) was 27% for negative HR tumours, compared with 8% for positive HR tumours. The pathological CR rate (Sataloff) was 23% for highly proliferative tumours, compared with 4% for tumours with a low cell proliferation. Overexpression of HER2 and TopoIIα (with a cut-off at 10%) was not predictive of response to anthracycline-based chemotherapy. The pathological CR rate (Sataloff) was 15% for tumours overexpressing HER2 compared with 8% for tumours without overexpressed HER2. The pathological CR rate (Sataloff) was 14% for tumours overexpressing TopoIIα compared with 10% for tumours without overexpressed TopoIIα.

We also studied TopoIIα as a quantitative variable, using the Mann-Whitney non-parametric test. Increased TopoIIα expression was related to a clinical and radiological CR ($P = 0.01$ and 0.05, respectively). Median TopoIIα expression level was higher when a pathological CR (Sataloff) was achieved than when it was not, but the difference was not statistically significant ($P = 0.07$).

In multivariate analysis, using a logistic regression model (Table 5), Ki-67 and HR status were identified as independent variables in predicting a clinical complete response ('goodness of fit': $P = 0.54$). Tumour grade was identified as the only independent variable to predict a pathological CR according to the Sataloff classification ('goodness of fit': $P = 0.98$). In this model, 64% of patients with a negative HR tumour and a high tumour cell proliferation had a clinical CR, and 42% of patients with a grade 3 tumour had a pathological CR.

3.3. Predictive value of HER2 and TopoIIα evaluated by real-time PCR

Evaluation of the amplification of *HER2* and *TopoIIα* by real-time PCR was performed on 99 tumour samples and results were obtained in 88 and 82 tumour samples, respectively. Amplification of *HER2* gene was demonstrated in 88% of HER2 3+ tumours, 25% of HER2 2+ tumours and 5% of HER2 0-1+ tumours. Amplification of the TopoIIα gene was demonstrated in 24% of tumours with a high TopoIIα expression and in 26% of tumours with a low TopoIIα expression. *HER2* status and *TopoIIα* status, assessed by quantitative PCR, were not related to either the clinical or radiological tumour response or to the pathological response (Table 6).

Table 4
Univariate analysis: *P* values

	Clinical response		Radiological response		Pathological CR	
	OR	CR	OR	CR	Chevallier	Sataloff
Tumour grade	0.13	0.004	0.05	0.57	0.008	0.0001
Ki-67	0.64	0.002	0.08	0.40	0.02	0.01
HR	0.70	0.003	0.51	0.42	0.08	0.008
HER2	0.83	0.06	0.72	0.45	0.40	0.38
TopoIIα	0.18	0.07	0.10	0.06	0.57	0.56

OR, overall response; CR, complete response.

Table 5
Multivariate analysis: *P* values

	Tumour grade	HR	Ki-67
Clinical CR	0.44	0.009	0.003
Pathological CR (Sataloff)	0.0001	0.32	0.49

CR, complete response.

Table 6
HER2 and *TopoIIα* amplification, univariate analysis: *P* values

	Clinical CR	Radiological CR	Pathological CR (Sataloff)
HER2	0.67	0.61	0.99
TopoIIα	0.75	0.22	0.87

CR, complete response.

4. Discussion

In the present study, 119 patients with stage IC to IIIA invasive adenocarcinomas were treated with six cycles of an anthracycline-based therapy. The clinical OR was 80%, with 19% of CR. The radiological OR was 71%, with 16% of CR. No PD was observed during the chemotherapy. After surgery, a pathological CR was demonstrated in 9% of the patients according to the Chevallier classification and in 13% of the patients according to the Sataloff classification. With a higher kappa value, clinical response seemed to be superior to the radiological response evaluated by CT to predict the pathological CR. In univariate analysis, high tumour grade, Ki-67 $\geqslant$ 20%, and absence of HR expression were predictive factors for a clinical CR and pathological CR according to the Sataloff classification. Increased TopoIIα expression studied as a quantitative variable was related to a clinical CR ($P = 0.01$). In multivariate analysis, absence of HR expression and Ki-67 $\geqslant$ 20% were predictive for a clinical CR, high tumour grade was predictive for a pathological CR according to the Sataloff classification. HER2 expression was not predictive of response. Amplification of *HER2* or *TopoIIα* was not predictive of the response to anthracycline-based chemotherapy.

The superiority of a physical examination compared with radiology to predict pathological findings on the primary breast tumour, although not formally demonstrated in our study, possibly due to a lack of statistical power, has been reported in previous studies. Herrada and colleagues [13] evaluated 100 patients to correlate physical examination, sonographic and mammographic measurements of breast tumours with pathological findings after neoadjuvant chemotherapy. Physical examination provided the best correlation with pathology findings on the primary tumour, and sonography showed the best correlation for the lymph nodes. Lluch and colleagues [14] evaluated 60 patients to study the role of physical examination and CT in defining the tumour response after neoadjuvant chemotherapy. Physical examination provided the best assessment of the primary breast tumour, whereas CT was the best non-invasive method to detect axillary node involvement.

Several studies have already assessed the predictive value of tumour grade, HR status, and tumour cell proliferation in predicting the response to neoadjuvant chemotherapy. High-grade tumours generally show a significantly higher percentage of responders compared with low-grade tumours [15,16]. Negative HR status [17,18] and a high tumour cell proliferation [19,20] are also correlated with chemosensitivity. In the present study, 64% of patients with a negative HR tumour and a high tumour cell proliferation had a clinical CR, compared with 3% for tumours with the opposite characteristics. A pathological CR was demonstrated in 42% of patients with grade 3 tumours compared with 0% for those with grade 1 tumours.

Amplification and overexpression of HER2 are strongly linked in breast cancer [21,22]. The overall concordance of IHC with the CB11 antibody with quantitative PCR was also 88% in a prospective study on 254 breast cancers [23]. Our data support the hypothesis that TopoIIα could be amplified simultaneously with HER2, because they are located close to one another on chromosome 17. In the present study, 90% (18/20) of tumours with *TopoIIα* amplification also had a *HER2* amplification. There was no relationship between *TopoIIα* amplification and expression in our study. The fact that *TopoIIα* is a cell-cycle-regulated protein might explain these results [24]. TopoIIα expression can be considered as a marker of cell proliferation [25]. In the present study, Ki-67 and TopoIIα expressions were strongly correlated ($P = 4 \times 10^{-9}$, Spearman rank correlation coefficient = 0.62).

Most data on HER2 expression and anthracycline sensitivity have been derived from adjuvant clinical trials. The addition or dose increase of doxorubicin in the adjuvant treatment significantly improved the outcome of patients with HER-2+ tumours, but not of patients with HER-2− tumours in three retrospective studies [26–28]. Data in the neoadjuvant setting are conflicting. Some studies have failed to show any predictive value for HER2 expression [17,29,30]. A recent study from the MD Anderson Cancer centre did not found any correlation between the clinical and pathological response to neoadjuvant anthracycline-based chemotherapy and HER2 expression in 97 patients [31]. Similarly, HER2 status did not predict response to neoadjuvant anthracycline-based chemotherapy in 97 patients in a Netherlands Cancer Institute study [32]. In contrast, Willsher and colleagues found a correlation between HER2 expression and resistance to mitoxantrone-based chemotherapy [33]. Geisler and colleagues demonstrated that HER2 overexpression predicted resistance to a low-dose anthracycline chemotherapy

[34]. We recently showed in the neoadjuvant setting that low-dose anthracycline and HER-2 overexpression predicted a poor OR, low- or high-dose anthracycline and the absence of HER-2 overexpression predicted an intermediate OR, and high-dose anthracycline and HER-2 overexpression predicted a high OR [35]. In the present study, the high dose of anthracycline (epirubicin 100 mg/m^2) used for all patients could explain the absence of a predictive value of HER2 overexpression or amplification.

Two studies have evaluated the predictive value of *HER2* and *TopoIIα* amplification, in 35 and 67 patients, respectively, treated with an anthracycline-based chemotherapy (doxorubicin 50 mg/m^2) [36,37]. In the first study, *HER2* and *TopoIIα* amplification evaluated by *in situ* hybridisation were significantly associated with an increased response rate [36]. In the second study, response to chemotherapy was increased in cases with a coamplification of *HER2* and TopoIIα evaluated by a chromogenic *in situ* hybridisation [37].

In conclusion, HER2 or TopoIIα were not predictive markers of response to neoadjuvant anthracycline-based chemotherapy in breast cancer patients in the present study. Patients with a negative HR status, high grade breast tumours with a high tumour cell proliferation (Ki-67 $\geqslant$ 20%) were much more likely to respond to neoadjuvant anthracycline-based chemotherapy than patients with the opposite characteristics. Nevertheless, the predictive value of these markers for an individual patient is limited. Microarray technology should allow, in the future, the individual adjustment of treatment and selection of patients who would benefit most from neoadjuvant chemotherapy [38].

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