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学位論文

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Candidate biomarkers predictive of anthracycline and taxane efficacy against breast cancer

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Short title: Predictive biomarkers for chemotherapy in breast cancer

Key words: breast cancer, predictive biomarker, anthracycline, taxane, beta-tubulin, topoisomerase-II alpha, tissue inhibitor of metalloprotease-1

SUMMARY

Breast cancer exhibits heterogeneous genetic, biological, and clinical characteristics. Specifically, four breast cancer subtypes have been classified according to biological features, and these vary with respect to clinical behaviors. Therefore, clinically available biomarkers are needed to predict treatment efficacy in each subtype to avoid the administration of ineffective treatments. In this study, to determine whether the candidate molecules would correlate with clinical responses to administered chemotherapeutic agents, the expression statuses of these molecules , including topoisomerase-II alpha (TOP2A), beta-tubulin (B-tub), and tissue inhibitor of metalloprotease-1 (TIMP-1), were immunohistochemically evaluated in 70 breast cancers from 68 patients (mean age, 60 years; range: 32-76 years) with advanced or metastatic breast cancers treated with chemotherapeutic agents (e.g., anthracycline and taxane). Anthracycline and taxane were administered to 82% and 62% of the patients, respectively and the response rates to these agents were 70.5% and 67.2%, respectively. Responses of tumors to the agents did not significantly differ among luminal A /B, luminal-HER2, HER2, and triple negative (TN) cancer subgroups. Overall, 25.1%±29.7%, 8.32%±10.1%, 16.37%±17.5% of cancer cells were positive for B-tub, TOP2A, and TIMP-1 expression, respectively. However, positive molecule expression of the evaluated molecules did not differ between patients who did and did not exhibit clinical responses to treatment. When we compared target molecule expression in tumors from responders and non-responders according to HR and HER2 statuses, the proportion of TOP2A-positive cancer cells was significantly higher among anthracycline responders than among non-responders in HR-negative cancer (15.4%±17.5% vs. 2.0%±2.4%, $p = 0.048$), whereas TOP2A and TIMP-1 expression statuses did not differ in HR-positive

cancer. When patients were stratified according to B-tub, TOP2A, or TIMP-1 expression statuses (B-tub≥10% vs. <10%, TOP2A≥5% vs. 5% ,TIMP-1≤20% vs. TIMP>20%), the proportion of patients with ≥10% B-tub-positive cancer cells was significantly higher in taxane responders than in non-responders (72.4% vs. 37.5%, p=0.016). Although anthracycline responders had a higher proportion of patients with either ≥5% TOP2A-positive cancer cells or ≤20% TIMP-1-positive cancer cells compared to non-responders, these differences were only borderline significant (86.7% vs. 61.5%, p = 0.066). In conclusion, immunohistochemical TOP2A, TIMP-1, and B-tub expression analyses can be completed relatively quickly (approximately -2days) and are expected to be useful as biomarkers predictive of breast cancer responses to chemotherapy.

INTRODUCTION

Breast cancers are known to be heterogeneous with respect to pathological features, hormone receptor and HER2 statuses, and clinical behaviors. Breast cancers can be classified into subtypes according to hormone receptor and HER2 expression statuses, and each subtype is associated with different proliferative capacities, responses to anti-cancer agents, and patient prognoses (1, 2). For example, breast cancers can be classified into four subtypes, luminal A or B, luminal-HER2, HER2, and triple negative cancer, each of which is associated with different prognoses and responses to treatment. However, the extent to which biomarkers, such as HER2 expression, are involved in clinical outcomes remains unclear. In addition to hormone receptor and HER2 expression, tumor size, nodal status, tumor grade, and proliferative ability have been reported as prognostic factors in patients with breast cancer (3). However, little is known about the clinical behaviors associated with each factor. Therefore, a novel strategy is needed to identify biomarkers in each subtype that would allow predictions of treatment efficacy and thus improve the survival of patients with breast cancer.

Anthracycline and taxane are used clinically as standard chemotherapeutic agents in both adjuvant and metastatic settings. Topoisomerase-II alpha (TOP2A) and beta-tubulin (B-tub) are known as target molecules of anthracycline and taxane, respectively. To our knowledge, few reports have described a close relationship between the levels of target molecule expression in tumors and clinical responses to the respective chemotherapeutic agents (4-8). Moreover, suitable methodologies to examine the expression of these molecules in tumors have not been established. The glycoprotein tissue inhibitor of metalloprotease-1 (TIMP-1) is known to inhibit matrix metalloprotease activity and is reportedly associated with prognosis and anti-cancer

agent efficacy in patients with breast cancer. For example, patients with high tumor TIMP-1 expression levels are more likely to experience early cancer relapse and have a poor prognosis (9-13). Regarding chemotherapy responsiveness, patients with TIMP-1-negative tumors have been reported to respond favorably to epirubicin (8, 14), whereas those with high TIPM-1 expression levels exhibited resistance to paclitaxel-based regimens (6). In addition, TIMP-1 expression has been associated with the clinical efficacy of classical chemotherapeutic regimens, including cyclophosphamide/methotrexate/5-fluorouracil (CMF) or epirubicin + CMF (7). Notably, some reports have described the use of combined TIPM-1 and HER2 or TIMP-1 and TOP2A profiling to predict responses to anthracycline-based chemotherapy (4, 5, 15-18). However, these reports did not demonstrate the same results because of the differences in demographic features of patients studied, regimens implemented, and administration settings.

In this study, we examine the intratumoral expression of TOP2A, B-tub and TIMP-1 immunohistochemically in breast cancers to determine whether the expression statuses of these molecules could predict the clinical efficacy of anthracycline- or taxane-based chemotherapeutic regimens.

METHODS

Patients

We examined 70 breast cancer tissues from 68 patients who had locally advanced, metastatic, or recurrent breast cancer and had received chemotherapy at Takamatsu Red Cross Hospital, Kagawa University Hospital, or Ito Breast Surgical Clinic from June 2006 to April 2014. All patients had been diagnosed with breast cancer via histological examination of specimens from

the primary or metastatic lesions. Patients were administered either anthracycline- or taxane-based regimens. For patients with HER2-overexpressing cancers, trastuzumab was administered in combination with chemotherapeutic agents.

Evaluation of therapeutic efficacy

Tumor responses were assessed via physical examination, computed tomography (CT), or magnetic resonance imaging (MRI), according to the Response Evaluation Criteria in Solid Tumors (RECIST) every 2-3 months during treatment. Complete response (CR) was defined as the lack of evidence of disease; partial response (PR) was defined as a reduction in the sum of target lesion diameters by ≧30%, and progressive disease (PD) was defined as an increase in the sum of target lesion diameters by ≧20% or the presence of a new lesion. A clinical response that did not meet any of the above definition was classified as stable disease (SD). Objective response (OR) was defined as CR and PR; disease control (DC) was defined as CR, PR and SD; and clinical benefit (CB) was defined as CR, PR and SD during observation period of ≧6 months.

Immunohistochemical study

Tumor tissues obtained by core needle biopsy were fixed in 10% formalin, embedded in paraffin, and sliced in 10-μm-thick section. The sections were mounted on slides and dehydrated in ethanol followed by incubation in 2% normal goat serum to block non-specific binding for 20 min and a phosphate-buffered saline (PBS) wash. Sections were subsequently incubated overnight at 4°C with monoclonal antibodies against TOP2 (1.00E+02, Abcam, Tokyo, Japan) at a concentration of 10 μg/ml, or B-tub (ab52623, Abnova, Taipei, Taiwan) at a

1:50 dilution, or with an anti-TIMP-1 polyclonal antibody (Abcam) at a 1:250 dilution. The sections were then washed in PBS and incubated with biotinylated anti-mouse or rabbit immunoglobulins (1:500 dilution) for 2 h, followed by incubation with an horse radish peroxidase-conjugated avidin solution (Vector Laboratories, Inc., Burlingame, CA, USA) for 1.5 h at room temperature. Finally, the sections were treated with 0.1 mg/ml 3.3'-diaminobenzidine (Wako Pure Chemical Industries, Ltd., Osaka, Japan) for 10 min at room temperature to induce the colorimetric reaction, washed in tap water, and counterstained with 0.1% hematoxylin solution (19).

Statistical analysis

The Mann-Whitney U-test or standard chi-square test was used as appropriate to compare the two groups. For each variable, a 95% confidence interval (CI) was calculated for the median value using the method of Brookmeyer and Crowley (20). Significance was defined a $P < 0.05$, and all P-values were two-sided. The SPSS statistical software package (SPSS Inc. Tokyo, Japan) was used for all calculations.

RESULTS

Clinicopathologic characteristics of the patients

The median age of the patients was 60 (32 - 76) years. The 70 evaluated tumors represented 37 locally advanced, 17 metastatic, and 16 recurrent breast cancers (Table 1); of these, 42 (60%) were hormone-sensitive and 17 (24.3%) overexpressing HER2. Invasive ductal carcinoma accounted for 89.5% of the tumors, and 50%, 8.6%, 25.7%, and 15.7% of the tumors were classified as luminal A/B, luminal-HER2, triple negative and HER2, respectively. In addition,

62% and 81.7% of the patients received anthracycline- and taxane-based chemotherapy, respectively. Anthracycline was administered as the first, second, and third or later line of chemotherapy in 34 (47.9%), 15 (21.1%) and one patient (1.4%), respectively (data not shown), whereas taxane was administered as the first, second, and third or later line in 32 (45.1%), 27 (38%) and 7 patients (9.9%), respectively.

Treatment efficacy

Overall, 85.5% of all patients exhibited responses to either anthracycline- or taxane-based chemotherapy (Table 2); the agent-specific response rates were 70.5% for anthracycline, 67.2% for taxane, and 88.9% for trastuzumab. The response rates to either anthracycline or taxane according to the subtypes were 58.6% for luminal A/B, 83.3% for luminal-HER2, 45.5% for triple negative, and 75.0% for HER2 tumors (Table 3). To compare the efficacy of administered agents among subtypes, response rates to anthracycline and taxane were 74.1% and 60.9%, respectively in luminal A/B; 100% and 66.7%, respectively in luminal-HER2; 50.0% and 42.1%, respectively in triple negative; 83.3% and 83.3%, respectively in HER2 groups (Table 3). Patients with HER2-overexpressing tumors had a fairly high response rates to trastuzumab-containing regimens

Candidate molecule expression in the tumor

The expression of candidate molecules, including B-tub, TOP2A, and TIMP-1, was evaluated in primary or metastatic tumors from all enrolled patients; 25.6% ± 29.8%, 8.5% ± 10.1%, and 16.6% ± 17.7% of tumors were positive for these molecules, respectively (Table 4). As both the hormone receptor and HER2 status are known as predictors of chemotherapeutic efficacy, for instance, patients with hormone receptor-negative or HER2 overexpressing cancers

have been reported to exhibit good responses to anthracycline or taxane. We compared tumor responses to chemotherapy and candidate molecule expression statuses in tumors according to HR or HER2 status. The response rates to taxane did not differ significantly between patients with HR-positive and HR-negative tumors (75.8% vs. 58.3%, p = 0.166, Table 5), and taxane responders and non-responders did not differ with respect to proportion of B-tub-positive cancer cells (24.0% ± 29.0% vs. 25.4% ± 27.4%, p = 0.841). Similarly, the rates of responses to anthracycline did not differ between patients with HR-positive and -negative tumors (76.9% vs. 61.1%, p = 0.264). Furthermore, patients with HR-positive and –negative tumors did not differ with respect to TOP2A or TIMP-1 expression (TOP2A, 7.1% ± 9.6% vs. 10.5% ± 14.0%, p = 0.307; TIMP-1, 14.6% ± 22.5% versus 19.5% ± 28.1%, p = 0.44). Patients with and without HER2-overexpressing tumors did not differ in terms of responses to taxane or anthracycline (taxane, 87.5% and 65.3%, respectively, p = 0.215; anthracycline, 50.5% and 67.7%, respectively, p = 0.703, Table 6) or the tumor expression of B-tub (32.2% ± 32.7% vs. 23.9% ± 26.6%, p = 0.606) or TOP2A (19.0% ± 21.5% vs. 6.9% ± 9.3%, p = 0.325). However, compared to patients with HER2-normal tumors, those with HER2-overexpressing tumors had a significantly lower proportion of TIMP-1-positive cancer cells (8.8% ± 9.3% vs. 20.4% ± 25.6%, p = 0.031).

Expression of candidate molecules in responders and non-responders to chemotherapy

A comparison of candidate molecule expression in patients who did and did not respond to chemotherapy revealed no significant differences (B-tub, 27.3% ± 28.4% vs. 24.0% ± 27.6%, p = 0.349; TOP2A, 6.6% ± 9.2% vs. 14.9% ± 20.9%, p = 0.481; TIMP-1, 15.2% ± 24.0% vs. 6.4% ± 11.7%, p = 0.444, Table 4). For further analysis, patients were divided into subgroups

according to HR (positive vs. negative) or HER2 status (overexpressing vs. normal), and the proportions of cancer cells positive for the candidate molecules were compared between responders and non-responders to taxane or anthracycline within these subgroups. Among both HR subgroups, the proportions of B-tub-positive cancer cells did not differ between taxane responders and non-responders (HR-positive group, 24.5% ± 28.2% vs. 27.5% ± 32.8%, p = 0.82; HR-negative group, 22.4% ± 26.1% vs. 17.3% ± 22.7%, p = 0.594, Table 7). Similarly, the proportions of B-tub-positive cancer cells did not differ between taxane responders and non-responders in the HER-normal group (23.9% ± 26.9% vs. 23.8% ± 27.8%, p = 0.989); however, patients with HER2-overexpressing tumors were administered taxane in combination with trastuzumab, and therefore, data regarding the efficacy of taxane alone were unavailable.

Further comparison of patients in the HR-positive subgroup found that the proportions of TOP2A- or TIMP-1-positive cancer cells did not differ between anthracycline responders and non-responders (TOP2A, 6.4% ± 8.4% vs. 9.4% ± 15.4%, p = 0.718; TIMP-1, 13.4% ± 18.5% vs. 16.7% ± 21.9%, p = 0.765, Table 8). However, among HR-negative patients, although TIMP-1 expression did not differ between anthracycline responders and non-responders (24.6% ± 34.5% vs. 13.2% ± 17.8%, p = 0.407), TOP2A positivity was significantly more frequent among the responders than among the non-responders (15.4% ± 17.5% vs. 2.0% ± 2.4%, p = 0.048).

Finally, the responders and non-responders were compared with respect to the proportions of patients in whom B-tub-, TOP2A, or TIMP-1 positive cells accounted for ≥10%, ≥5% or ≤ 20% of all tumors cells, respectively. Although the proportions of patients with ≥5% TOP2A or ≤20% TIMP-1 positive cells did not differ between responders and non-responders (TOP2A,

46.4% vs. 18.2%, p = 0.123; TIMP-1, 80.0% vs. 67.8%, p = 0.365, Table 9), responders included a significantly higher proportions of patients with ≥10% B-tub positivity as compared with non-responders (72.4% vs. 37.5%, p = 0.023). Taken together, although the proportion of patients with either ≥5% TOP2A or ≤20% TIMP-1 positivity was higher among responders than among non-responders, this difference was not significant (86.7% vs. 61.5%, p = 0.066).

DISCUSSION

The biological and genetic heterogeneity associated with breast cancer have been reported to influence variations in tumor progression, responses to treatment, and patient prognosis. Since breast cancer demonstrates heterogeneity biologically as well as genetically. Recent systems have allowed the classification of breast cancers into several subtypes, according to hormone sensitivity, HER2 status, and proliferation ability, in attempt to gain a better understanding of clinical behaviors. Because each breast cancer subtype exhibits particular clinical behaviors (21), specific treatment strategies must be developed for each.

In this study, we aimed to determine whether the expression of key molecules that serve as targets of chemotherapeutic agents or are closely involved in tumor sensitivity to these agents, such as B-tub, TOP2A and TIMP-1, could predict clinical responses to treatment in 70 tumors from 68 patients who had primary, advanced, or recurrent breast cancer and were treated with anthracycline or taxane. We selected an immunohistochemical method that could be completed in 2 days because the ability to rapidly predict responses to cheomotherapy is crucial to the development of a novel strategy for predicting treatment efficacy.

In this study, we observed higher than expected overall anthracycline and taxane response rates of 70.5% and 67.2%, respectively (Table 2); these high rates were attributed to the fact that either agents was administered to all patients as either the first or second of chemotherapy (data not shown). Reasons for their high response rates than had been expected were that these two agents were administered in all the patients at early lines such as either first or second line of chemotherapy (data not shown). When we assessed tumor responses to these agents according to the subtype, we noted a low response rate or TN cancer as compared to that for other types of cancer (Table 3). However, the response rate did not significantly differ among the groups (data not shown). Our data suggest that these chemotherapeutic agents exhibited strong activity against breast cancer when used at an early stage of treatment regardless of subtype.

Regarding the tumor expression of the three molecules of interest, chemotherapy responders and non-responders did not differ with respect to the proportions of cancer cells positive for B-tub, TOP2A, and TIMP-1 expression (Table 4). In further analysis of tumor responses or molecule expression according to the HR or HER2 status, we found that although chemotherapeutic responses did not differ according to HR or HER2 status, TOP2A expression was significantly higher among anthracycline responders than among non-responders with HR-negative tumors (Table 8). These data were compatible with the findings of previous reports (22-24). However, TOP2A expression did not different between the anthracycline responders and non-responders in the HR-positive group, and TIMP-1 expression did not differ regardless of HR status. Therefore, another biomarker may indicate sensitivity to anthracycline in HR-positive breast cancers.

Although the proportion of cancer cells expressing TIMP-1 was significantly lower in patients with HER2-overexpressing tumors than in patients with HER2-normal tumors, tumor responses to anthracycline did not differ by HER2 status and were not found to associate with intratumoral TIMP-1 expression (Tables 4 and 6). However, as shown in Table 9, the combined TOP2A and TIMP-1 status, indicating high TOP2A /low TIMP-1 expression, was expected to be useful for predicting tumor responses to anthracycline. Similarly, neither taxane efficacy nor B-tub expression appeared to be affected by the HR or HER2 status in this study (Table 7). However, the overall proportion of patients with a proportion of B-tub positive cells $\geq$10% was significantly higher among taxane responders than among non-responders (Table 9). These data contradicted an earlier report by Huang in which patients expressing high tumor levels of B-tub exhibited resistance to taxane (25). This discrepancy may be attributed to the fact that the earlier study investigated lung cancer responses to taxane in combination with carboplatin.

In conclusion, immunohistochemical analysis of TOP2A, TIMP-1, and B-tub may yield biomarkers predictive of responses to chemotherapy for breast cancer. Overall, these biomarkers are expected to become available for clinical predictions of anthracycline and taxane efficacy. However, a prospective study involving a large number of patients is needed to confirm correlation between tumor responses and molecular expression.

Competing interests

The authors declare that they have no competing interests.

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FIGURE LEGENDS

Table 1. Characteristics of patients and tumors

Table 2. Overall response to anti-cancer agents

Table 3. Response rates to anthracycline and taxane according to tumor subtype

Table 4. Proportions of cancer cells with positive target molecule expression among responders and non-responders

Table 5. Chemotherapeutic efficacy and tumor target molecule expressions according to hormone receptor status

Table 6. Chemotherapeutic efficacy and tumor target molecule expressions according to HER2 status

Table 7. Comparison of the proportions of cancer cells positive for beta-tubulin expression between taxane responders and non-responders among patients stratified according to HR or HER2 status

Table 8. Comparison of the proportions of cancer cells positive for TOP2A and TIMP-1 expression between anthracycline responders and non-responders among patients stratified according to HR or HER2 status

Table 9. Comparison of target molecule expression statuses between responders and non-responders

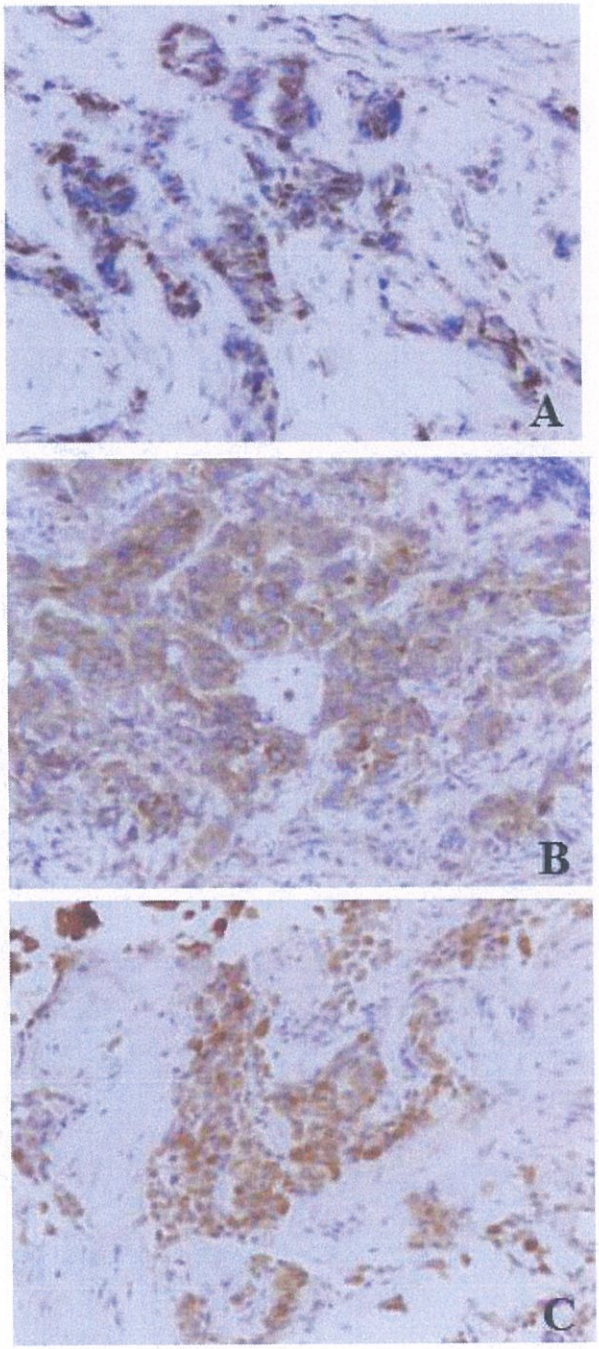
A
B
C

Table 1. Patients and tumor characteristics

No. patients	68
No. breasts	70
median age	60 (32-76)
HR-positive (%)	42 (60)
HER2-positive (%)	17 (24.3)
% invasive ductal carcinoma	89.5
Cancer status (%)	
locally advanced	37 (52.9)
metastatic	17 (24.3)
recurrent	16 (22.9)
Subtype (%)	
Luminal A/B	35 (50)
Luminal HER2	6 (8.6)
triple negative	18 (25.7)
HER2	11 (15.7)
% Administered agents	
anthracycline	62.0
taxane	81.7
trastuzumab	11.3
others	19.7

Table 2. Overall response to anti-cancer agents

Agent	Response rate (%)
All	85.5
Anthracyclin	70.5
Taxane	67.2
Trastuzumab	88.9
Others	36.5

Table 3. Response rates to anthracycline and taxane according to tumor subtypes

	All (%)	Anthracycline	*p-value*	Taxane	*p-value*	Others	*p-value*
Luminal-A/B	58.6	74.1		60.9		21.4	
Luminal-HER2	83.3	100	*N.T.**	66.7	*N.T.*	75	*N.T.*
Triple negative	45.5	50.0	*0.12*	42.1	*0.089*	50	*0.79*
HER2	75.0	83.3	*0.704*	83.3	*0.634*	33.3	*0.90*

* not tested

Table 4. Proportions of cancer cells with positive target molecule expressions among responders and non-responders

target molecule (agent)	all patients	responders	non-responders	*p-value*
beta-tubulin (taxane)	25.1%±29.7%	27.3%±27.6%	24.0%±27.6%	*0.349*
TOP2A (anthracycline)	8.32%±10.09%	6.6%±9.2%	14.9%±20.9%	*0.481*
TIMP-1 (anthracycline)	16.37%±17.48%	15.2%±24.0%	6.4%±11.7%	*0.444*

Table 5. Chemotherapeutic efficacy and tumor target molecule expressions according to hormone receptor status

	HR*(+)	HR(-)	*p-value*
response to taxane (%)	75.8	58.3	*0.166*
% B-tub (+) cells	24.0 ± 29.0	25.4 ± 27.4	*0.841*
response to anthracycline (%)	76.9	61.1	*0.264*
% TOP2A (+) cells	7.1 ± 9.6	10.5 ± 14.0	*0.307*
% TIMP-1 (+) cells	14.6 ± 22.5	19.5 ± 28.1	*0.44*

* hormone receptor

Table 6. Chemotherapeutic efficacy and tumor target molecule expressions according to HER2 status

	HER2 (+)	HER2 (-)	*p-value*
response to taxane (%)	87.5	65.3	*0.215*
% B-tub (+) cells	32.2 ± 32.7	23.9 ± 26.6	*0.606*
response to anthracycline (%)	50.5	67.7	*0.703*
% TOP2A (+) cells	19.0 ± 21.5	6.9 ± 9.3	*0.325*
% TIMP-1 (+) cells	808 ± 9.3	20.4 ± 25.6	***0.031***

Table 7. Comparison of the proportions of cancer cells positive for B-tub expression between taxane responders and non-responders among patients stratified according to HR or HER2 status

Patients population	responders	non-responders	*p-value*
Pts with HR*(+) tumor	24.5 ± 28.2 (%)	27.5 ± 32.8	*0.82*
Pts with HR(-)tumor	22.4 ± 26.1	17.3 ± 22.7	*0.594*
Pts with HER2(+) tumor	N.T.[#]	N.T.	
Pts with HER2(-)tumor	23.9 ± 26.9	23.8 ± 27.8	*0.989*

*hormone receptor

[#]not tested

Table 8. Comparison of the proportions of cancer cells positive for TOP2A and TIMP-1 expression between anthracycline responders and non-responders among patients stratified according to HR or HER2 status

Patients population		responders	non-responders	*p-value*
Pts with HR(+) tumor	% TOP2A(+) cells	6.4 ± 8.4	9.4 ± 15.4	*0.718*
	% TIMP-1(+) cells	13.4 ± 18.5	16.7 ± 21.9	*0.765*
Pts with HR(-) tumor	% TOP2A(+) cells	15.4 ± 17.5	2.0 ± 2.4	***0.048***
	% TIMP-1(+) cells	24.6 ± 34.5	13.2 ± 17.8	*0.407*
Pts with HER2(+) tumor	% TOP2A(+) cells	N.T.	N.T.	
	% TIMP-1(+) cells	N.T.	N.T.	
Pts with HER2(-)tumor	% TOP2A(+) cells	6.1 ± 8.5	5.4 ± 11.7	*0.854*
	% TIMP-1(+) cells	25.7 ± 29.4	16.3 ± 21.4	*0.315*

Table 9. Proportions of patients with target molecule expressions between responders and non-responders

	responders	non-responders	*p-value*
B-tub(+) ≥ 10%	72.4	37.5	***0.023***
TOP2A(+) ≥ 5%	46.4	18.2	*0.125*
TIMP-1(+) < 20%	80.0	67.8	*0.365*
TOP2A(+) ≥ 5% / TIMP-1(+) < 20%	86.7	61.5	***0.066***