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Gene Expression Profiles of Breast Cancer Obtained from Core Cut Biopsies Before Neoadjuvant Docetaxel, Adriamycin, and Cyclophoshamide Chemotherapy Correlate with Routine Prognostic Markers and Could Be Used to Identify Predictive Signatures

Genexpressionsanalyse an Stanzbiopsien des Mammakarzinoms vor neoadjuvanter Chemotherapie mit Docetaxel, Adriamycin und Cyclophosphamid korreliert mit prognostischen Routinemarkern und ist zur Identifikation prädiktiver Gensignaturen geeignet

Abstract

Background: Neoadjuvant administration of chemotherapy provides a unique opportunity to monitor response to treatment in breast cancer and assesses response exactly. Global gene expression profiling by microarrays has been used as a valuable tool for the identification of prognostic and predictive marker genes. Even though this technology is now wide spread and relatively standardized, there are only few data available which compare established parameters with expression values to determine reliability of this method. Therefore we analyzed gene expression data of pretreatment biopsies of breast cancer patients and compared them with the results of the immunohistochemical receptor expression for ER/ PR and Her-2, as well as FISH testing for HER-2 amplification. We analyzed the change of expression of these markers before and after neoadjuvant chemotherapy. Furthermore we evaluated the predictive significance of prognostic gene signatures as described by Sorlie, van't Veer and Ahr for response to neoadjuvant chemotherapy. **Methods:** Pretherapeutic core biopsies were obtained from 70 patients undergoing neoadjuvant TAC chemotherapy within the GEPARTRIO-trial. Samples were characterized according to standard pathology including ER, PR and HER2 IHC and amount of cancer cells. Only biopsies with more than 80% tumor cells were considered for further examination. RNA was isolated and expression profiling performed using Affymetrix Hg U133 Arrays (22 500 genes). GeneData's Expressionist software was used for bioinformatic analyses. **Results:** More than two thirds of the biopsies yielded sufficient amounts (> 5 µg) of RNA for expression profiling and

Zusammenfassung

Hintergrund: Die neoadjuvante Chemotherapie des Mammakarzinoms bietet die einzigartige Möglichkeit, das Ansprechen auf die Therapie zu beobachten und exakt zu monitoren. Globale Genexpressionsanalysen durch DNA-Chiptechnologie werden als wertvolles Hilfsmittel zur Identifizierung prognostischer und prädiktiver Gensignaturen eingesetzt. Obwohl diese Technik inzwischen weit verbreitet und standardisiert ist, sind nur wenige Daten verfügbar, die gut etablierte Marker mit den jeweiligen Expressionswerten vergleichen, um die Zuverlässigkeit dieser Methode besser einschätzen zu können. Daher haben wir Genexpressionsdaten prätherapeutischer Stanzbiopsien von Mammakarzinompatientinnen analysiert und mit den Ergebnissen der immunhistochemischen Rezeptorexpression von ER/PR und Her-2, sowie der FISH-Analyse bezüglich einer Her-2-Amplifikation verglichen. Weiterhin wurde die Expressionsänderung dieser Marker vor und nach neoadjuvanter Chemotherapie untersucht. Etablierte prognostische Gensignaturen, wie sie bereits von Sorlie, van't Veer und Ahr beschrieben wurden, sind des Weiteren auf ihre prädiktive Wertigkeit hin untersucht worden. **Methoden:** Prätherapeutische Stanzbiopsien wurden von 70 Patientinnen erhalten, die sich einer neoadjuvanten TAC-Chemotherapie im Rahmen der GEPARTRIO-Studie unterzogen. Die Proben wurden im Rahmen der Routinepathologie hinsichtlich der immunhistochemischen Expression von ER, PR und Her-2 sowie des Tumorzellanteils untersucht. Es wurden für die weitere Analyse nur Stanzbiopsien mit einem Tumorzellgehalt von mehr als 80% verwendet. Nach RNA-Isolation wurde ein Genexpressions-

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high quality data were obtained for 50 samples. Unsupervised clustering broadly revealed a correlation with hormone receptor status. When ER-α, PR and HER2 as analyzed by immunohistochemistry were compared to the corresponding mRNA data from gene chips more than 90% concordance was observed. We could observe a switch of receptor expression for ER, PR or HER-2 from positive to negative and vice versa in 16/35 cases (45.7%) and 5/22 cases (22.7%) respectively. The prognostic marker sets of Sorlie, van't Veer and Ahr could not discriminate responders from non-responders in our patient group. **Conclusions:** Our results demonstrate that reliable expression profiles can be achieved by using limited amounts of tissue obtained during neoadjuvant chemotherapy. Microarray data capture conventional prognostic markers but might contain additional informative gene sets correlated with treatment outcome. Prognostic marker sets are not suitable to predict tumor response in the neoadjuvant setting, suggesting the necessity of class prediction methods to identify marker sets predictive for the type of therapy used.

profil unter Verwendung des Affymetrix Hg U133 arrays (22 500 Gene) erstellt. Die bioinformatische Analyse erfolgte mit der GeneData Expressionist Software. **Ergebnisse:** Von mehr als zwei Drittel aller Stanzbiopsien konnten ausreichende RNA-Mengen (> 5 µg) zur Genexpressionsanalyse gewonnen werden, so dass von 50 Proben qualitativ hochwertige Daten zu erhalten waren. Ein unsupervised clustering erbrachte eine hohe Korrelation mit dem Hormonrezeptorstatus. Die immunhistochemische Expression von ER, PR und Her-2 wurde mit den korrespondierenden mRNA-Daten der Chipanalyse verglichen und zeigte eine Konkordanz der Expression in mehr als 90% der Fälle. Eine Veränderung der Rezeptorexpression von ER, PR oder Her-2 von positiv nach negativ und umgekehrt wurde in 16/35 Fällen (45,7%) bzw. 5/22 Fällen (22,7%) beobachtet. Die prognostischen Markersets von Sorlie, van't Veer und Ahr waren innerhalb unseres Patientenkollektivs nicht in der Lage, responder von non-respondern zu unterscheiden. **Schlussfolgerung:** Unsere Ergebnisse zeigen, dass zuverlässige Genexpressionsdaten aus limitiertem Tumorgewebe erhältlich sind. Die Genexpressionsdaten spiegeln zum Einen die konventionellen Prognosefaktoren zuverlässig wieder und liefern darüber hinaus zusätzliche informative Gensets, die mit dem Therapieansprechen korrelieren. Prognostische Markersets sind allerdings nicht geeignet, das Tumoransprechen im Rahmen der neoadjuvanten Chemotherapie vorherzusagen, so dass dies die Notwendigkeit unterstreicht, mittels „class prediction"-Methoden prädiktive Markersets für die eingesetzte spezifische Therapie zu identifizieren.

Key words
Neoadjuvant chemotherapy · breast cancer · gene expression profiling · estrogen receptor · progesterone receptor · Her-2

Schlüsselwörter
Neoadjuvante Chemotherapie · Brustkrebs · Genexpressionsprofil · Östrogenrezeptor · Progesteronrezeptor · Her-2

Introduction

Neoadjuvant or primary systemic therapy (PST) is the standard care for inflammatory and inoperable mammary carcinomas. Large randomized trials comparing PST and adjuvant therapy revealed no difference in clinical outcome. However PST offers several avantages. (i) The rate of breast conservation surgery can be increased by 10–15% depending on the drugs used and (ii) the observed therapeutic effect of PST as response of the tumor to treatment can directly be monitored and excactly assessed by both clinican and patient resulting in an in vivo assay for the chemosensibility of the tumor [1, 2]. In particular the rate of pathological complete remissions (pCR) correlates very well with disease free (DFS) and overall survival (OAS) and thus can be used as a surrogate marker for clinical outcome of the disease [3 – 5]. To date many efforts have been undertaken to detect specific marker genes for predicting tumor response and disease prognosis. Global gene expression profiling by microarrays has been used as a valuable tool for the identification of prognostic marker genes [6 – 9]. For example a 41 gene signature [10] allowed us to identify patients with an unfavorable prognosis [11]. In combination with the setting of primary systemic therapy, this approach can be used to correlate gene expression profiles with response to chemotherapy in order to identify predictive gene signatures guiding the selection of the individual therapy [12 – 14]. In previous work we could show that neoadjuvant chemotherapy with docetaxel (T), adriamycin (A) and cyclophosphamide (C) resulted in a rate of pathologic complete remission of 17.9% in stage II–IV breast cancer disease [15]. Goal of our study presented here was to examine the feasibility of performing gene expression profiling on pretherapeutic material of the neoadjuvant GEPARTRIO trial and evaluate the reliability of the methods.

Material and Methods

Selection of patients

Patients with previously untreated, unilateral or bilateral primary breast cancer were enrolled in the GEPARTRIO trial after informed consent. Inclusion criteria encompassed a measurable tumor lesion by palpation in two dimensions with one diameter of at least 2 cm, age of 18 years or older and a good performance status. Assessment of diagnosis by core cut biopsy under ultrasound guidance was mandatory in all patients after determination of tumor dimensions by clinical examination, ultrasound, bilateral mammography and/or magnetic resonance imaging (MRI). When breast cancer was diagnosed patients were screened for metastatic disease by chest-X-ray, abdominal ultrasound and/or computed tomography (CT) scan and bone scan.

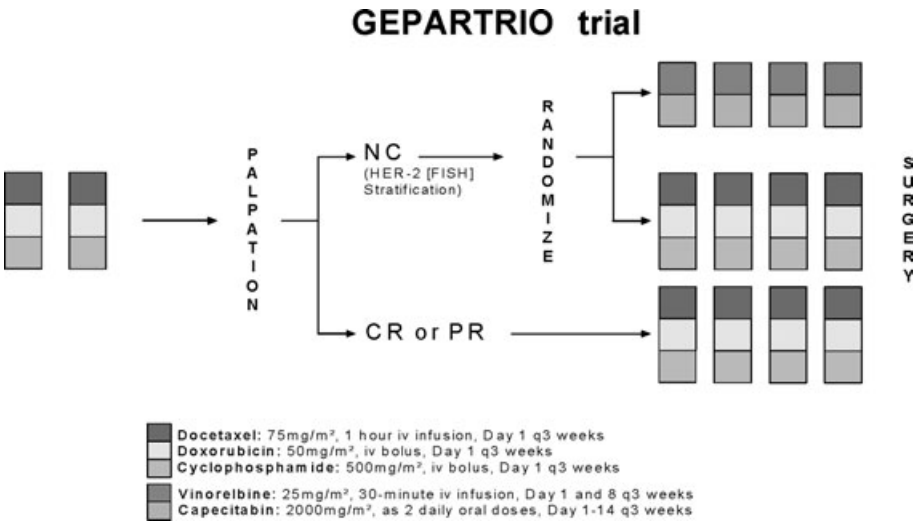

Fig. **1** Study design of the GeparTrio trial.

Study design

Study design of this neoadjuvant TAC trial is depicted in Fig. **1**. Initially all patients received two cycles of TAC (doxorubicin 50 mg/m^2, cyclophosphamide 500 mg/m^2 and docetaxel 75 mg/m^2 all on day 1, every 3 weeks). Tumor response was determined by palpation during the third week of the second cycle. Patients who demonstrated a tumor response defined as a tumor shrinkage of more than 50% were randomized to four or six further cycles of TAC. In case of no tumor response patients received either four further cycles of TAC or four cycles of NX (vinorelbine 25 mg/m^2 day 1 and 8 plus capecitabine 1000 mg/m^2 orally twice/day on days 1–14 every 3 weeks) as a non-crossresistant schedule after randomization.

Methods

Pretherapeutic samples from 70 patients were snap frozen in liquid nitrogen and part of the removed tumor tissue was used for diagnostic purposes. Samples were characterized according to standard pathology including IHC of ER, PR and HER2 and amount of cancer cells as well as FISH analysis of HER2. Only biopsies with more than 80% tumor cells were considered further. RNA was isolated with Qiagen RNeasy reagents and expression profiling performed using Affymetrix Hg U133 Arrays (22 500 genes) according to protocols of the manufacturer. Adequate RNA amounts could be achieved in n = 50 patients according a rate of 71.4%. For bioinformatic analyses the EXPRESSIONIST software from GeneData (Basel, Switzerland) was used. Genelists as used for cluster analysis were mapped to Affymetrix probe sets by utilizing Unigene annotation and genomic sequence information. Classification of response to treatment was characterized (i) as clinical response based primarily on palpation as well as ultrasound imaging and (ii) as pathological response using post surgery data from the pathologist.

Results

We enrolled n = 70 patients in our study who had histologically confirmed invasive breast cancer by core cut biopsy and met the inclusion criteria for neoadjuvant chemotherapy within GEPARTRIO trial. From 55 biopsies of different patients high quality chip data could be obtained for gene expression analysis. Pretreatment biopsies could be examined from 50 patients. RNA isolation of snap frozen tissue with more than 80% tumor cell proportion was verified by capillary gel electrophoresis (Agilent Bioanalyzer 2100). Adequate RNA amounts could be achieved in n = 55 samples according a rate of 78.6%.

Clinical characteristics of the 50 patients with pre-treatment biopsies are given in Table **1**.

Fig. **2** gives the result of a global unsupervised clustering approach. A filter was applied to select for those 3707 genes with highest variance in expression between samples. Then hierarchical clustering was used to group the samples (in rows) based on the expression pattern of those genes. Red branches of the sample tree on the right represent ER positive tumors and blue branches ER negative tumors. As seen in the figure this unsupervised approach broadly revealed a correlation with the hormone receptor status of the tumors. This result is in line with previous observations that the ER status is the major determinant of the expression profile of mammary carcinomas (van't Veer et al. [7]) and can be viewed as a proof of principle for the quality of the data.

After performing DNA microarray analysis we compared the expression levels of estrogen receptor, progesterone receptor and Her-2 in a subcohort of patients with data of routine pathology revealed by immunohistochemistry (IHC) and fluorescence in-situ hybridization (FISH). To assign threshold value for discrimination between positive or negative receptor expression we compared immunohistochemical scores with RNA expression as displayed exemplary for ER in Fig. **3**.

As shown in Table **2**, when the results of ER, PR and HER2 analysis by immunohistochemistry were compared to the corresponding mRNA data from gene chips more than 90% concordance was observed. To evaluate the predictive significance of prognostic gene signatures for response to neoadjuvant chemotherapy we tried to correlate response to chemotherapy and gene signatures described by Sorlie et al., van't Veer et al. and Ahr et al. As depicted in Fig. **4** the hierarchical clustering revealed that marker sets of Sorlie, van't Veer and Ahr could not discrimi-

Table **1** Patient characteristics

age	median 53	range 30–69				
menopausal status	pre 25	post 25				
tumour stage	T2 34	T3 10	T4 6			
lymph node status	positive 22	negative 28				
histological type	duktal 44	lobular 6				
grading	G1 4	G2 34	G3 11	GX 1		
receptor status	ER+ 33	ER– 17	PR+ 24	PR– 26	HER2+* 20	HER2–* 28
study arm	TAC 40	TAC-NX 10				
clinical response	cCR	cPR	cSD	cPD		
OP	pCR 8	residual disease 40	not completed 2			

* Her-2 status was not available in n = 2

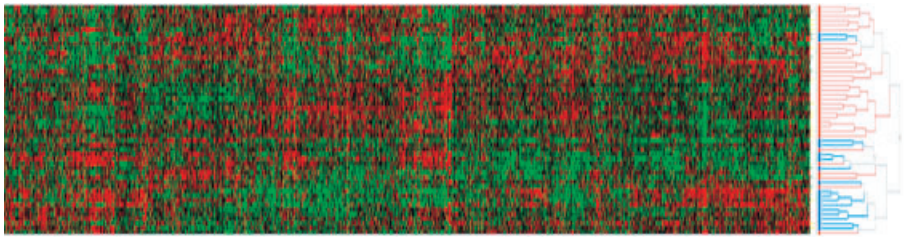

Fig. **2** Unsupervised clustering of tumor samples. Hierarchical clustering was used to group the samples (in rows) based on the expression pattern of genes with highest variance in expression between samples. Red branches of the sample tree on the right represent ER positive tumors and blue branches ER negative tumors.

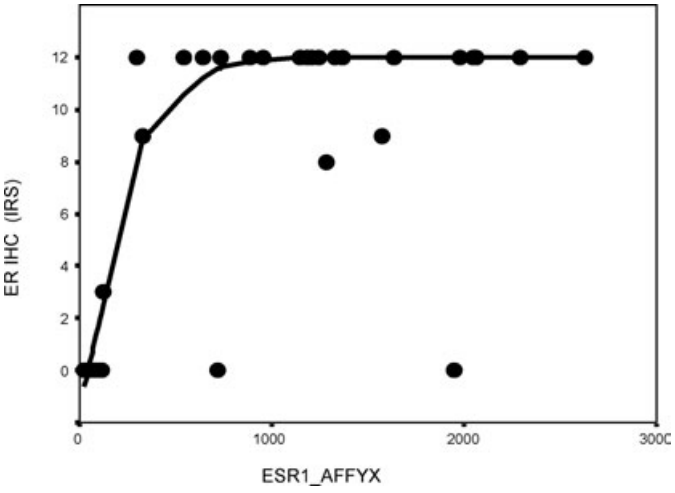

Fig. **3** Comparison of immunohistochemical score for ER with RNA expression on microarray to determine threshold value for discrimination between positive or negative receptor expression.

Table **2** Concordance of microarray and IHC/FISH data from core biopsies in a subcohort of patients

	ER (n = 45)		*PR (n = 41)*		*HER2* (n = 36)*	
microarray cut off median	>120 1142.2	<120 51.4	>7.4 19.7	<5.8 1.8	>500 2565.2	<500 241.3
# of samples	31	14	21	20	12	24
IHC/FISH positive *negative*	29 2	0 14	18 3	1 19	10 2	0 24
concordance (%)	43/45 (95.6)		37/41 (90.2)		34/36 (94.4)	

* HER2 status was obtained primary by FISH analysis (n = 28), if not available IHC results were used (n = 8)

nate responders from non-responders in our patient group suggesting the necessity of class prediction methods to identify marker sets predictive for the type of therapy used.

A switch of hormone receptor status in pretreatment biopsies compared to breast cancer specimens after chemotherapy is a frequently observed phenomenon. In our study immunohistochemical hormone receptor status of both pre- and posttreatment biopsies could be obtained from 35 cases (Fig. **5**). Data regarding HER-2 expression were available for 22 cases. We could observe a switch of receptor expression for ER, PR or HER-2 from positive to negative and vice versa in 16/35 cases (45.7%) and 5/22 cases (22.7%) respectively. Interestingly we found a loss of PR expression in posttreatment biopsies in 12/19 cases (63.2%) whereas a switch from negative to positive was observable in only two cases. A correlation of receptor switch with tumor re-

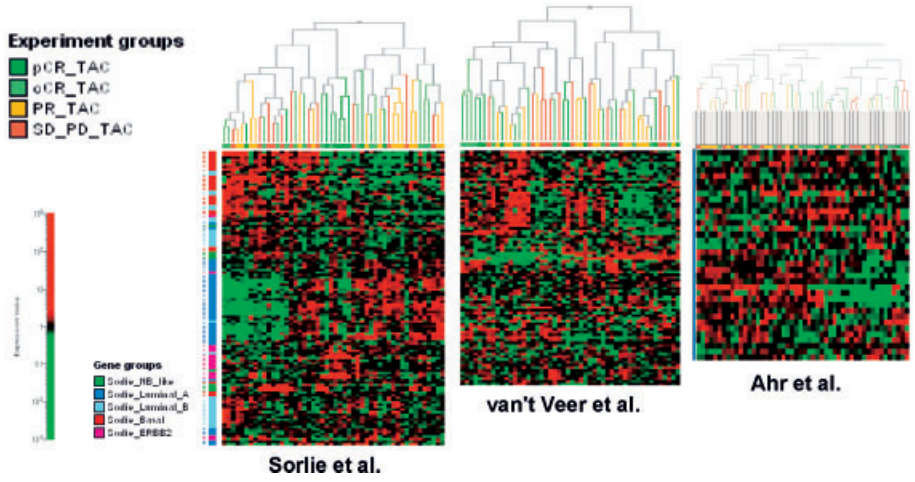

Fig. **4** Correlation of prognostic gene signatures and tumor response described by Sorlie et al., van't Veer et al. and Ahr et al.

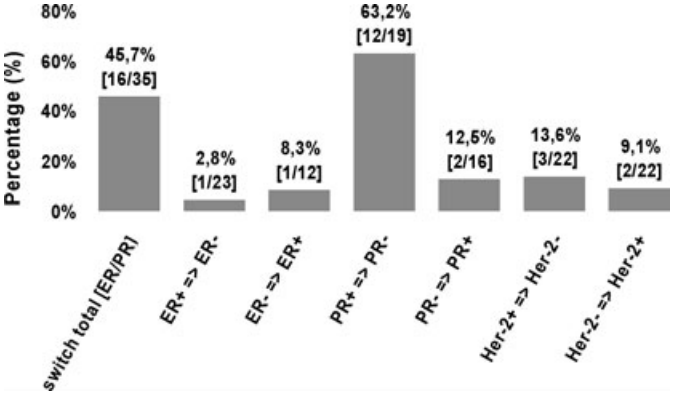

Fig. **5** Changes of ER-, PR- and Her-2-expression of pre-treatment core cut biopsies compared with posttreatment tumor tissue.

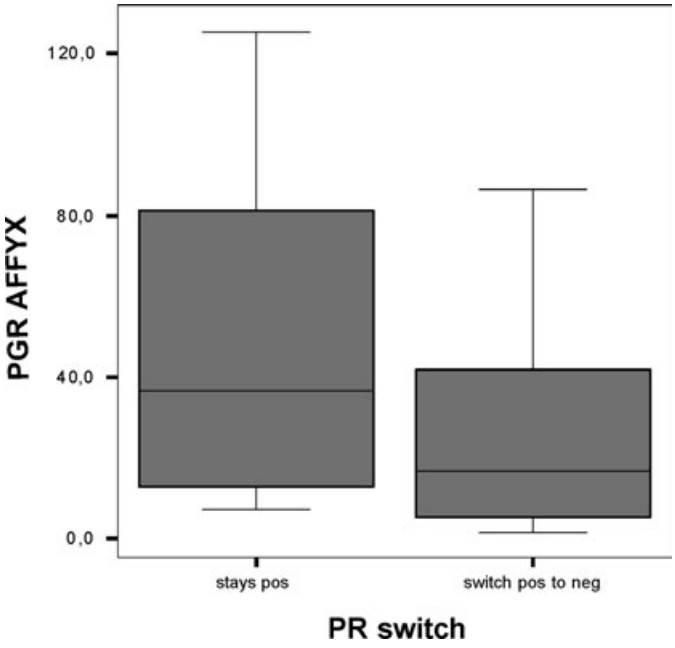

Fig. **6** Comparison of gene expression data with immunohistochemical PR expression in tumor specimens with or without receptor switch.

sponse could not be determined. One might speculate that an initial low receptor expression might be causal for the loss of expression. However, as shown in Fig. **6** comparison of gene expression data with immunohistochemical analysis in tumor specimens with or without receptor switch revealed no significant difference, demonstrating that an initial low receptor expression might not be causal for the loss of receptor expression.

For ER we found only one switch of receptor expression from positive to negative (clinical tumor response: cPR) and from negative to positive (clinical tumor response: cPD) respectively. A loss of HER-2 expression in posttreatment biopsies was seen in 3 cases and overexpression despite initial absence of HER-2 expression in 2 cases. Also a correlation with tumor response could not be seen.

Discussion

Gene expression profiling of breast cancer treated by neoadjuvant chemotherapy is an interesting tool for developing predictive as well as prognostic markers. Even though this technology is now wide spread and relatively standardized, there are only few data available which compare established parameters with expression values to determine reliability of this method. Therefore we analyzed gene expression data of pretreatment biopsies of breast cancer patients and compared them with the results of the immunohistochemical receptor expression for ER/PR and FISH testing for HER-2 amplification. Our results demonstrate that reliable expression profiles can be achieved by using limited amounts of tissue obtained during neoadjuvant chemotherapy. Correlation of routine parameters as ER, PR and HER-2 revealed by IHC/FISH and expression levels in microarray analysis demonstrates concordance of more than 90% suggesting that expression data are valid and can be used for further analysis. Unsupervised clustering of gene expression revealed a strong discrimination due to estrogen receptor status as previously described by several authors, which strongly emphasizes the usefulness of our data. Our results demonstrate that reliable expression profiles can be achieved by using limited amounts of tissue obtained during neoadjuvant chemotherapy. Microarray data capture conventional prognostic markers but might contain additional informative gene sets correlated with treatment outcome. However, prognostic marker sets are not suitable to predict tumor response in the neoadjuvant setting, suggesting the necessity of class prediction methods to identify marker sets predictive for

the type of therapy used. In 648 patients of the GEPARTRIO trial the HER-2/neu status centrally determined by FISH did not predict for response to TAC chemotherapy [16].

In 63.2 % of all cases with a core cut biopsy positive for PR we could detect a loss of the receptors expression in posttreatment tumor tissue. Mostly a loss of PR expression was seen after neoadjuvant chemotherapy. The underlying causes for this observation and its biological impact still remain unclear. Gene expression data suggest that a weak receptor expression before treatment start is not responsible for receptor loss. Our data are consistent with results published by Taucher et al., who found a shift in 51.7 % from pretreatment positivity of PR to negative expression after neoadjuvant chemotherapy ($p = 0.0005$) [17]. The authors also found a decrease of ER status to loss of ER expression during chemotherapy in 14 % ($p = 0.02$). However, Burcombe et al. found a change in hormone receptor classification in only 10 of 118 tumors after neoadjuvant chemotherapy (three ER, seven PR) [18]. HER-2 status changed in nine of 118 patients (five 2+ tumors were scored 3+, four 3+ tumors shifted to 2+ at surgery). In terms of Ki-67 the authors found a decrease of expression before and after chemotherapy from 24.9 to 18.1 % ($p = 0.002$). Higher Ki-67 proliferation indices were associated with PR negative tumors (median 28.3 %, PR positive 22.9 %, $p = 0.042$). Our results suggest that initial PR positive tumors with high Ki-67 proliferation index will have a reduced proliferation and a loss of PR expression during chemotherapy (data not shown). Therefore PR seems to be a marker for proliferative potential of tumors.

A change of hormone receptor status after neoadjuvant chemotherapy could have implications for the subsequent endocrine therapy. Particularly a loss of PR with remaining ER expression could be an indication for upfront antiestrogen therapy with an aromatase inhibitor for five years, instead of tamoxifen or switching to an aromatase inhibitor after two or five years of tamoxifen. Furthermore it still remains unclear if HER-2 overexpression in posttreatment tumors could be an indication for a subsequent therapy with trastuzumab. Overall clinical trials are needed investigating if pre- or posttreatment receptor status should be used for further treatment decisions.

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