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Thymosin beta 15A *(TMSB15A)* is a predictor of chemotherapy response in triple-negative breast cancer

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BACKGROUND: Biomarkers predictive of pathological complete response (pCR) to neoadjuvant chemotherapy (NACT) of breast cancer are urgently needed.

METHODS: Using a training/validation approach for detection of predictive biomarkers in HER2-negative breast cancer, pre-therapeutic core biopsies from four independent cohorts were investigated: Gene array data were analysed in fresh frozen samples of two cohorts ($n = 86$ and $n = 55$). Quantitative reverse transcription polymerase chain reaction (qRT–PCR) was performed in formalin-fixed, paraffin-embedded (FFPE) samples from two neoadjuvant phase III trials (GeparTrio, $n = 212$, and GeparQuattro, $n = 383$).

RESULTS: A strong predictive capacity of thymosin beta 15 *(TMSB15A)* gene expression was evident in both fresh frozen cohorts ($P < 0.0001$; $P < 0.0042$). In the GeparTrio FFPE training cohort, a significant linear correlation between *TMSB15A* expression and pCR was apparent in triple-negative breast cancer (TNBC) ($n = 61$, $P = 0.040$). A cutoff point was then defined that divided TNBC into a low and a high expression group (pCR rate 16.0% *vs* 47.2%). Both linear correlation of *TMSB15A* mRNA levels ($P = 0.017$) and the pre-defined cutoff point were validated in 134 TNBC from GeparQuattro (pCR rate 36.8% *vs* 17.0%, $P = 0.020$). No significant predictive capacity was observed in luminal carcinomas from GeparTrio and GeparQuattro.

CONCLUSION: In TNBC, *TMSB15A* gene expression analysis might help to select patients with a high chance for pCR after NACT.

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Neoadjuvant chemotherapy (NACT), initially an approach for locally advanced, inoperable tumours, has become an established treatment option for operable breast cancer (Goldhirsch *et al*, 2007; Kaufmann *et al*, 2012). Response to chemotherapy is evaluated histologically in the surgical specimen after NACT, and pathological complete response (pCR) is defined as the complete absence of invasive tumour cells in breast and axillary lymph nodes. Pathological complete response is also a surrogate marker for long-term survival for triple-negative breast cancer (TNBC), which lack the expression of hormone receptors (HR) and HER2 (Carey *et al*, 2007; Liedtke *et al*, 2008). Similarly, in HER2-positive carcinomas treated by trastuzumab-containing NACT, pCR indicates a favourable prognosis (Untch *et al*, 2011). The situation is less clear for HR-positive luminal cancers that have an intrinsic good prognosis (von Minckwitz *et al*, 2011a). With current chemotherapeutic approaches, approximately 25% of unselected patients achieve a pCR (von Minckwitz *et al*, 2011b). The strong dependence of pCR from molecular tumour type has been elucidated in the past years. Thus, pCR rates are only in the range of 9% in HR-positive (luminal) carcinomas, whereas TNBC achieve much higher rates (35–40%) (von Minckwitz *et al*, 2011a). Therefore, even in selected breast cancer patient subgroups, a considerable proportion of patients does not respond and is in danger of a dismal outcome. New biomarkers for response prediction are needed that help to stratify patients according to their risk profile for an optimal therapy.

In this project, we identified the predictive capacity of *TMSB15A* gene expression by DNA microarray analysis of fresh-frozen pre-therapeutic breast cancer biopsies of two independent patient cohorts that have been described in previous publications (Modlich *et al*, 2005; Rody *et al*, 2007). *TMSB15A* encodes thymosin beta 15 (TMSB15), a member of the beta thymosin

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family of regulatory proteins (Chen *et al*, 2005). It was the gene with the highest fold change in pCR cases as compared with non-pCR cases in both cohorts and lowest significance level after correction for multiple testing. As *TMSB15A* did not belong to a group of genes from biological motives already known to be associated with pCR (such as for example, proliferation or immune response, Denkert *et al*, 2010; Fasching *et al*, 2011), we decided to systematically validate this individual biomarker in FFPE core biopsies from two additional independent patient cohorts with HER2-negative carcinomas from the neoadjuvant phase III clinical trials GeparTrio (von Minckwitz *et al*, 2005, 2008a,b) and GeparQuattro (von Minckwitz *et al*, 2010). In our methodology, we strictly adhered to the REporting recommendations for tumour MARKer prognostic studies (REMARK) (McShane *et al*, 2006), and used the recently described 'prospective–retrospective' study design (Simon *et al*, 2009).

MATERIALS AND METHODS

Gene expression data sets

From GeparTrio as well as from an independent population-based group of patients treated with neoadjuvant EC, 55 and 86 pre-therapeutic core biopsies were snap frozen and used for Affymetrix (U133A) gene array analysis (Affymetrix, Santa Clara, CA, USA) after isolation of RNA with Qiagen RNeasy (Qiagen, Hilden, Germany). Data were processed and normalised using Affymetrix Microarray Suite 5.0 software and expression values were log2 transformed. Receiver operating characteristic (ROC) analysis and logistic regression was performed for *TMSB15A* (probe set 205347_s_at). Details about the cohorts and generation of the Affymetrix data sets have been previously described (Modlich *et al*, 2005; Rody *et al*, 2007).

Study population An overview on the complete study setup is given in the consort diagram (Figure 1). As an FFPE training cohort, samples from the prospective neoadjuvant phase III GeparTrio study (NCT00544765) or the GeparTrio pilot study performed by the German Breast Group, Neu-Isenburg, Germany were used. The details of study setup and treatments have been published before (von Minckwitz *et al*, 2005, 2008a, b). For mRNA analysis, only patients who had received six cycles of TAC were included. HER2-positive patients had not received trastuzumab in GeparTrio, as this was not the standard of care during the study period; these patients were excluded from the present project. The final study population consisted of 212 patients with HER2-negative tumours, treated with 6× TAC, and with available biomaterial. In all, 16 FFPE samples from GeparTrio were paired to fresh frozen samples used in the Affymetrix array analysis. As a validation cohort, pre-therapeutic core biopsies were used from 383 patients enroled in the prospective, neoadjuvant phase III GeparQuattro trial that compared EC-Doc *vs* EC-DocX *vs* EC-Doc-X (NCT00288002) (von Minckwitz *et al*, 2010). HER2-positive patients were excluded. Baseline clinico-pathological data as well as data on HER2 and HR status were extracted from the study databases. In GeparTrio, centrally evaluated data on HER2 status (based on immunohistochemistry (IHC) and silver-enhanced *in situ* hybridisation according to ASCO/CAP guidelines (Wolff *et al*, 2007)) were used, as HER2 determination was not yet fully established at the time the study was conducted. Grading and histology were also centrally determined; local data on HR expression were used (central evaluation: Institute of Pathology, Charité Berlin). Definition of pCR was complete absence of invasive tumour cells in the breast and lymph nodes as assessed at the time of surgery by the local pathologist (ypT0/Tis; ypN0). The baseline demographic and clinical characteristics of the patients with tissue available for this translational research project are shown in Table 1. The clinical trials were conducted in compliance with the Helsinki Declaration. The protocol was reviewed and approved by all responsible local ethics committees. The leading ethics committee was located at the Johann Wolfgang Goethe University, Frankfurt, Germany (Approval Number: 80/99). Consent of patient, pathologist and investigator to supply tumour material of biopsy and surgery for central pathological evaluation and examination of predictive factors was available.

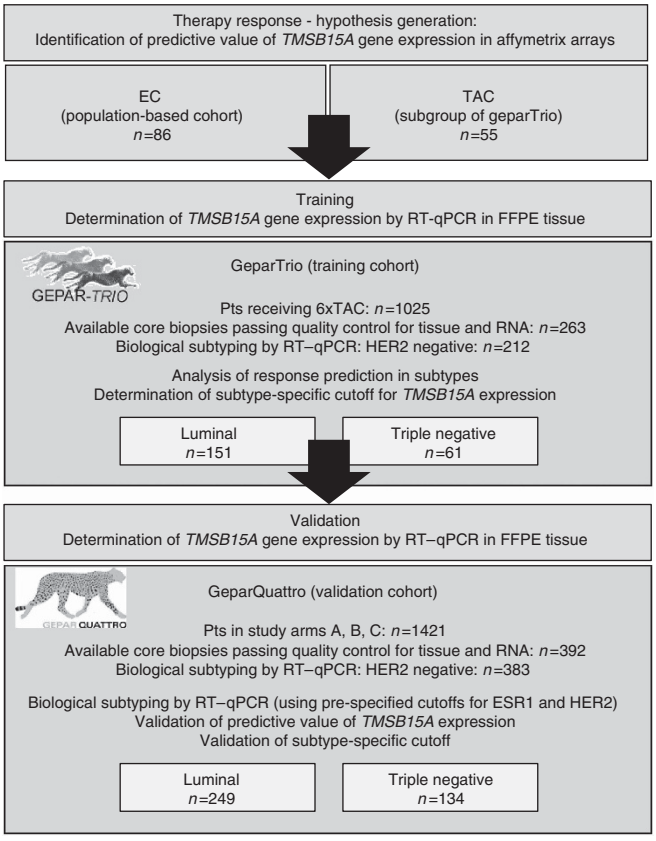

Figure 1 Consort statement: *TMSB15A* was a predictive factor in fresh frozen samples from a population-based cohort treated with EC ($n=86$), as well as in a subset of GeparTrio treated with TAC (fresh frozen tissue, $n=55$). Gene expression analysis was transferred to a qRT–PCR platform using 212 FFPE samples from GeparTrio. Biological tumour type was established by qRT–PCR of *ESR1* and *HER2* using pre-defined cutoff points, and logistic regression analysis for determination of the predictive value of *TMSB15A* gene expression was performed in luminal ($n=151$) and TNBC ($n=61$) separately. In the GeparTrio training cohort, a subtype-specific cutoff point for *TMSB15A* gene expression was determined additionally. The findings from GeparTrio were validated in 383 FFPE samples from GeparQuattro, comprising 249 luminal and 134 TNBC.

Biomarker assays for determination of TMSB15 mRNA levels

RNA was isolated from FFPE tissue sections of core biopsies using a fully automated isolation method of total RNA based on silica-coated magnetic beads in combination with a liquid-handling robot, and RNA was then used in quantitative reverse transcription polymerase chain reaction (qRT–PCR) as described earlier (Bohmann *et al*, 2009; Müller *et al*, 2011). Expression of *TMSB15A*, *ESR1*, and *HER2* as well as the normalisation genes *RPL37A*, *CALM2*, and *OAZ1* was assessed in triplicate. Sequences of primers and probes are listed in Table 2. Biological tumour types were defined based on gene expression data of *ESR1* and *HER2* as follows: *ESR1*+/*HER2*−: luminal; *ESR1*+ or −/*HER2*+: HER2

Table 1 Association of *TMSB15A* mRNA levels with baseline clinico-pathological parameters

	n	Median *TMSB15A* gene expression, ΔCT (s.d.)	*P* (Mann–Whitney)
GeparTrio			
Biological tumour type			<0.0001
Luminal	151	9.14 (2.18)	
Triple-negative	61	12.19 (3.18)	
Age			
<50 years	98	9.71 (2.82)	0.006
⩾50 years	114	9.21 (2.64)	
Tumour histology			
Ductal/other	189	9.47 (2.79)	0.606
Lobular	17	9.66 (2.82)	
Missing	6		
Tumour grade			
G1–2	160	9.38 (2.62)	<0.0001
G3	45	11.36 (2.96)	
Missing	7		
cT			
cT1–2	144	9.47 (2.89)	0.791
cT3–4	62	9.47 (2.51)	
Missing	6		
cN			
cN0	102	9.31 (2.84)	0.31
cN1–3	101	9.55 (2.78)	
Missing	9		
GeparQuattro			
Biological tumour type			<0.0001
Luminal	249	9.58 (2.21)	
Triple-negative	134	12.59 (2.29)	
Age			
<50 years	176	10.35 (2.53)	0.081
⩾50 years	207	10.25 (2.78)	
Tumour histology			
Ductal/other	326	10.40 (2.66)	0.009
Lobular	57	9.58 (2.59)	
Tumour grade			
G1–2	248	9.84 (2.47)	<0.0001
G3	125	11.99 (2.42)	
Missing	10		
cT			
cT1–2	264	11.44 (2.72)	0.119
cT3–4	119	10.09 (2.56)	
cN			
cN0	187	10.41 (2.80)	0.268
cN1–3	196	10.21 (2.55)	

Abbreviations: cN = clinical node stage; cT = clinical tumour stage.

positive; *ESR1 − /HER2 − :* TNBC. The *ESR1* and *HER2* mRNA cutoff values of 16 and 19.5 ΔCT were pre-defined based on two previous studies (Bohmann *et al*, 2009; Pentheroudakis *et al*, 2009). All biomarker analyses were performed blinded to patient outcome.

Cell culture Wild-type Cal-51 (DSMZ, Braunschweig, Germany) were cultured in RPMI supplemented with 10% fetal calf serum (FCS), and MCF-7 cells (DSMZ) were cultured in 5% FCS in the absence of phenol red. Paclitaxel (Sigma-Aldrich Chemie Gmbh, Munich, Germany)-resistant clones were generated by increasing drug concentration from 1.56 nM to 6.25 nM and repeated limiting dilution subcloning. Affymetrix arrays were performed as described above. Wild-type Cal-51 cells were transiently transfected with *TMSB15A* siRNA (100 ng, Sigma-Aldrich Chemie GmbH). Decrease of *TMSB15A* mRNA levels was determined by qRT–PCR. Relative gene expression was calculated from duplicate samples using the comparative CT method. Cytotoxicity assays for cell survival were performed as described previously (Stege *et al*, 2004). At 24 h after siRNA transfection, doxorubicin or paclitaxel were added in increasing concentrations. Cells were incubated with cytotoxic drugs for 72 h. Subsequently, cell viability was determined by sulforhodamine B assay. The absorbance difference of control cells without drug was set to be 100%. Each experiment was performed in triplicate at least.

Statistical evaluation Statistical analysis was performed using SPSS Statistics 19 (IBM Corporation, Somers, NY, USA), GraphPad PRISM 5.01 (GraphPad software, La Jolla, CA, USA), JMP 7.0.2 (SAS, Cary, NC, USA), and the statistical language R (www.r-project.org). In logistic regression analyses, significance of the correlation with pCR was assessed by the Wald test. The cutoff point for *TMSB15A* gene expression was determined using an R-based software designed by our group, and which is available in the internet (http://molpath.charite.de/cutoff/): for cutoff determination, this software correlates the dichotomised biomarker with a binary outcome variable using logistic regression. The optimal cutoff is defined as the point with the most significant split (Fisher's exact test). The details about this method will be the subject of a separate publication (manuscript submitted). All tests were two-sided, *P*-values <0.05 were considered as significant.

RESULTS

Gene expression analysis

To identify biomarkers predictive of a pCR, gene expression array data from previously published studies were analysed and searched for genes with significant expression differences in pCR and non-pCR cases. *TMSB15A* was the gene with the highest fold change in pCR cases as compared with non-pCR cases in both cohorts. In comparison with patients who had not achieved a pCR, we found a 5.3-fold ($P=0.0002$) and a 3.8-fold ($P=0.01$) higher *TMSB15A* isoform expression in those patients who had achieved a pCR following EC (in the population-based cohort) and TAC NACT (in the GeparTrio cohort), respectively (Figure 2A). Receiver operating characterisitic (ROC) analysis revealed predictive power of *TMSB15A* gene isoform expression in both the EC as well as the TAC cohort (auc = 0.89, $P<0.0001$, and auc = 0.82, $P<0.0042$, respectively; Figure 2B and C). After combination of both cohorts, subgroup analysis was performed with molecular tumour types. A significant predictive value for *TMSB15A* gene expression in TNBC (auc = 0.730, $P=0.037$), as well as in HER2-positive carcinomas (auc = 0.821, $P=0.012$), and in the luminal subgroup (auc = 0.918, $P=0.046$), although there were only two events (pCRs) within the luminal subgroup. As *TMSB15A* did not belong to a group of genes from biological motives already known to be associated with pCR (such as for example, proliferation or immune response), we decided to validate this individual biomarker in FFPE cohorts and to examine it functionally using siRNA technology.

TMSB15A gene expression in FFPE tissue of GeparTrio *TMSB15A* gene expression was subsequently investigated in routinely used FFPE samples from the GeparTrio trial using qRT–PCR. HER2-positive tumours had not been treated with neoadjuvant

Table 2 Sequences of PCR primers and probes

Gene	FAM/TAMRA probe	Forward primer	Reverse primer	Amplicon length (bp)
TMSB15A	CAAGCTTTCAGGCTATCTTCTAGTCAA	CCGCGAACAGCCTTTCAC	CGACAAGTCTGGCTTATCACTCA	77
ESR1	ATGCCCTTTTGCCGATGCA	GCCAAATTGTGTTTGATGGATTAA	GACAAAACCGAGTCACATCAGTAATAG	73
HER2	ACCAGGACCCACCAGAGCGGG	CCAGCCTTCGACAACCTCTATT	TGCCGTAGGTGTCCCTTTG	87
OAZ1	TGCTTCCACAAGAACCGCGAGGA	CGAGCCGACCATGTCTTCAT	AAGCCCAAAAAGCTGAAGGTT	83
CALM2	TCGCGTCTCGGAAACCGGTAGC	GAGCGAGCTGAGTGGTTGTG	AGTCAGTTGGTCAGCCATGCT	72
RPL37A	TGGCTGGCGGTGCCTGGA	CCGCGAACAGCCTTTCAC	CGACAAGTCTGGCTTATCACTCA	77

Sequences are given in 5′ to 3′ orientation.

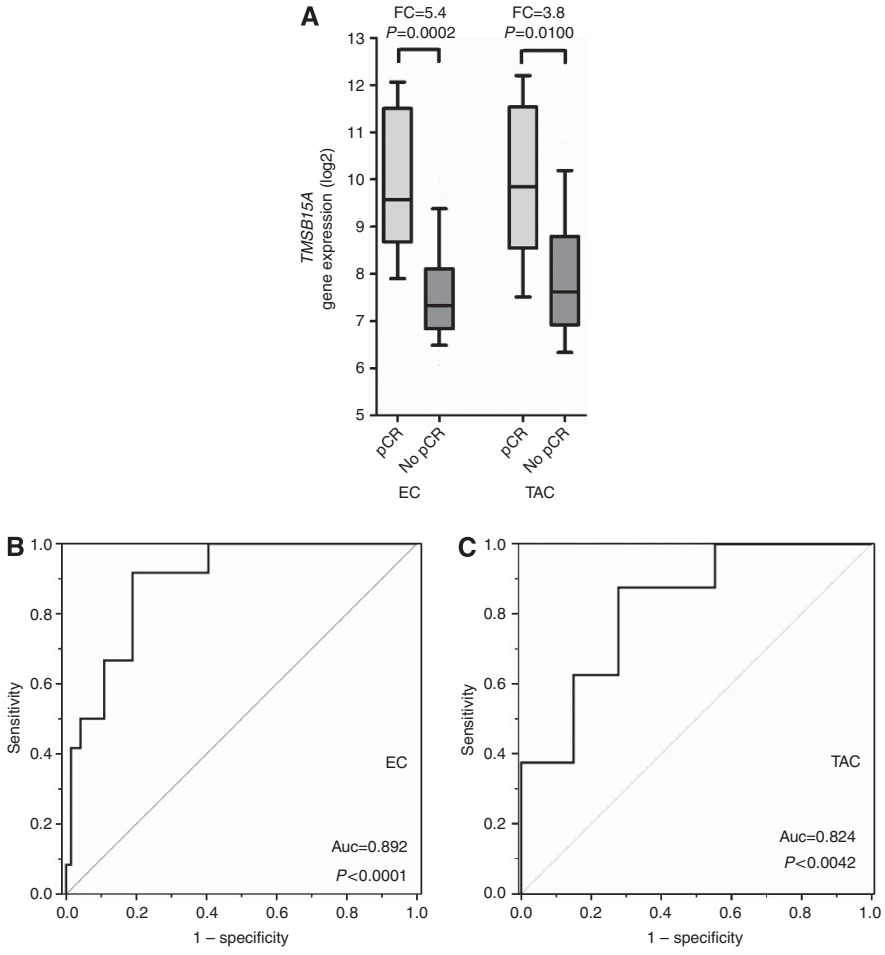

Figure 2 Distributions of *TMSB15A* gene expression in dependence of pCR in the EC cohort and the TAC cohort bold lines: medians; whiskers, 10–90th percentile; FC = fold change, *P*, Mann–Whitney test. (**B, C**) Reciever operator characteristic (ROC) curves for *TMSB15A* gene expression in the EC and TAC cohort. Abbreviation: Auc = area under the curve.

trastuzumab in GeparTrio, therefore only HER2-negative cases were included in the subsequent analyses. *TMSB15A* mRNA levels ranged from 3.32 to 16.44 ΔCT, with a median of 9.55 ΔCT. In TNBC, *TMSB15A* was strongly overexpressed as compared with luminal tumours (Figure 3A): median *TMSB15A* expression was 12.19 ΔCT in TNBC and 9.14 ΔCT in luminal carcinomas ($P<0.0001$, Table 1). A strong association was further seen with poor tumour differentiation (G3, $P<0.0001$); a weak association with younger age was evident, too ($P=0.006$, Table 1). Because of the different expression levels in TNBC and luminal carcinomas, further statistical investigations were performed separately in each biological subtype. An overview of clinico-pathological characteristics of the luminal and TNBC subgroup is given in Table 3; significant differences were seen for tumour grading, and pCR rate ($P<0.0001$ in each case). Logistic regression revealed that *TMSB15A* gene expression was a positive predictor of therapy response in TNBC. A doubling of the mRNA amount represented a 1.25-fold increase of the odds ratio (OR) for pCR (95% CI = 1.01–1.55, $P=0.040$). A ΔCT of 11.7 was identified as the optimal cutoff point (Figure 3B) splitting the cohort into a low expression group ($n=25$) with a pCR rate of 16.0% and a high expression group ($n=36$) with an almost three-fold higher pCR rate of 47.2% (OR 4.70, 95% CI = 1.34–16.45, $P=0.016$, Table 4, Figure 3C). This cutoff point had a sensitivity of 81.0% (95% CI = 60–92.3%), and specificity of 52.5% (95% CI = 37.5–67.1%) for response prediction (Figure 3B). Logistic regression with continuous *TMSB15A* mRNA data in luminal tumours did not show a significant predictive effect: OR = 1.35 (95% CI = 0.96–1.89, $P=0.082$). When molecular tumour types were defined by IHC, results were quite similar: In TNBC (ER − /PR − /HER2 − , $n=43$) *TMAB15A* gene expression

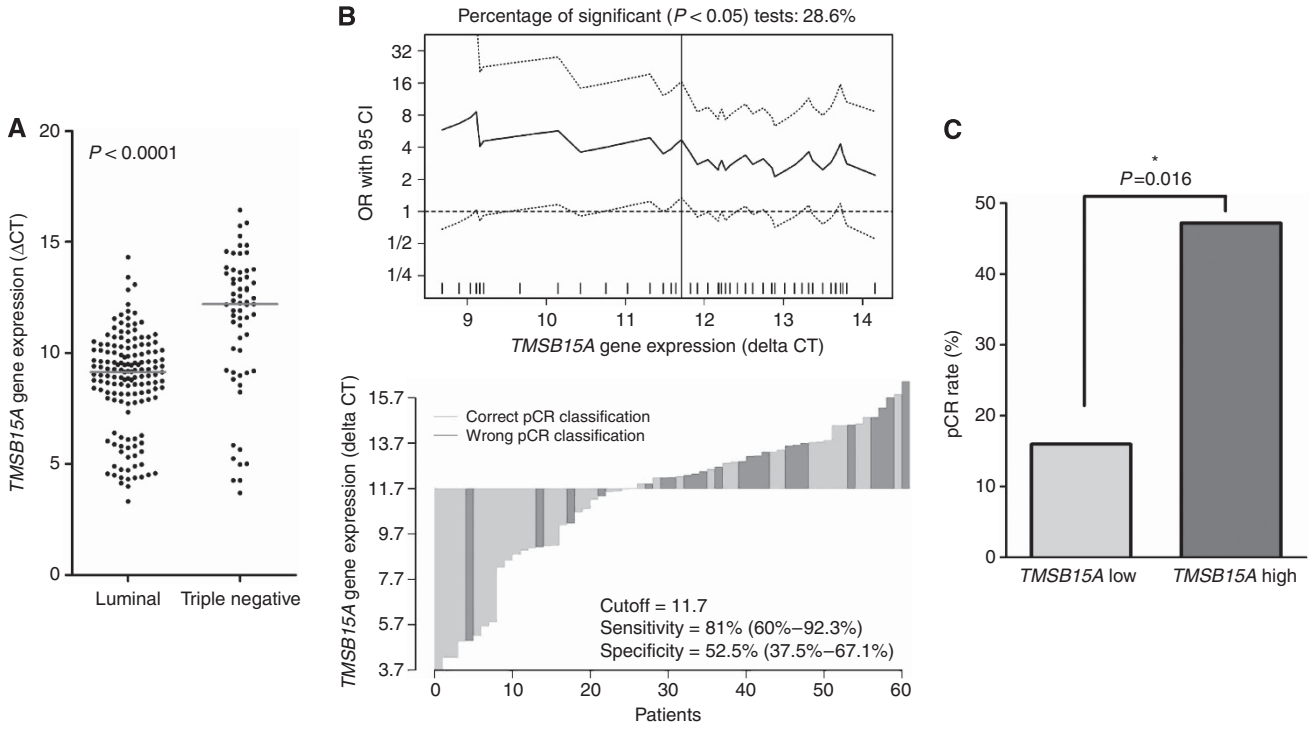

Figure 3 (**A**) Distribution of *TMSB15A* expression in luminal carcinomas and TNBC from GeparTrio. Dots indicate individual tumours. Red lines, medians. *P*, Mann–Whitney test. (**B**) Upper panel: determination of a cutoff point of *TMSB15A* gene expression for TNBC in GeparTrio. All possible cutoff points were considered and the corresponding odds ratios were calculated and plotted. Each data point in the line gives the corresponding OR and the 95% CI (dotted lines) on the y-axis. Vertical line: most significant split. Lower panel: waterfall plot for TNBC in GeparTrio charts each tumour as a vertical bar. Green bars represent cases with correct pCR classification, red bars represent cases with wrong pCR classification. Sensitivity and specificity of the cutoff point are indicated. (**C**) Pathological complete response (pCR) rates in dependence of *TMSB15A* status in TNBC from GeparTrio. For dichotomisation into a *TMSB15A* low and high expression group, the subtype-specific cutoff point was used (11.7 ΔCT). *P*-values were calculated by logistic regression. *Indicates significant values. The colour reproduction of this figure available at the *British Journal of Cancer* online.

(continuous) was a significant predictor of pCR (OR = 1.32 per ΔCT, 95% CI = 1.00–1.74, $P = 0.049$), however not in luminal carcinomas (ER+ or PR+/HER2−, $n = 141$; OR = 1.33 per ΔCT, 95% CI = 0.96–1.83, $P = 0.083$, data not shown).

Validation of the predictive impact of TMSB15A gene expression in GeparQuattro In the validation cohort GeparQuattro, distribution of *TMSB15A* mRNA levels was similar as in GeparTrio (range 3.67–16.67 ΔCT, median 10.29 ΔCT). A strong correlation with triple-negative subtype was seen as well: Median *TMSB15A* expression was 12.59 in TNBC and 9.58 in luminal tumours ($P < 0.0001$, Table 1, Figure 4A). Association with non-lobular histology ($P = 0.009$), and poor differentiation ($P < 0.0001$) were further found (Table 1). As in GeparTrio, luminal and TNBC differed in tumour grading, and pCR rate but also in histology ($P < 0.0001$ in each case, Table 3). The predictive value of *TMSB15A* gene expression found in GeparTrio was validated in TNBC from GeparQuattro: OR was 1.26 (95% CI = 1.04–1.52) per doubling of the mRNA amount (ΔCT, $P = 0.017$). The cutoff point of 11.7 ΔCT, which had been defined in GeparTrio, could be validated as well: pCR rate was 17.0% in TNBC with *TMSB15A* low expression and 36.8% in the high expression group (OR = 2.84, 95% CI = 1.18–6.82, $P = 0.020$, Figure 4C, Table 4). Sensitivity was 80.0% and specificity was 41.5% (Figure 4B). Multivariate logistic regression analysis showed that in TNBC from GeparQuattro, TMSB15A gene expression was the only significant predictive factor, independently of patient age, as well as clinical tumour (cT) and nodal stage (cN, $P = 0.041$). No significant predictive effect was seen in luminal carcinomas (OR = 1.14 per ΔCT, 95% CI = 0.91–1.43, $P = 0.249$). Similarly to GeparTrio, the use of IHC-defined molecular tumour types yielded the same results. In TNBC (ER−/PR−/HER2−, $n = 126$) TMSB15A gene expression was a significant predictive factor (OR = 1.23 per ΔCT, 95% CI = 1.01–1.49, $P = 0.041$), and in luminal carcinomas (ER+ or PR+/HER2-, $n = 257$) it was not (OR = 1.05, 95% CI = 0.85–1.31, $P = 0.652$, data not shown).

Functional evaluation of the relationship between TMSB15A expression and chemosensitivity To investigate whether there is a functional link between *TMSB15A* mRNA levels and response to chemotherapy, exploratory cell culture experiments were conducted. Gene expression arrays that had been performed from the breast cancer cell lines MCF-7 and Cal-51 showed relatively low levels of *TMSB15A* mRNA detected in ER-positive (luminal) MCF-7 (Figure 5A). Triple-negative Cal-51 cells, however, showed high-level *TMSB15A* gene expression, and were therefore chosen for further experiments. Induction of secondary paclitaxel resistance in Cal-51 resulted in a significant decrease of *TMSB15A* gene expression as compared with parental cells (fold change 5.2, $P = 0.004$; Mann–Whitney test, Figure 5A). Parental Cal-51 cells were then used for siRNA experiments. As shown in Figure 5B, transient transfection with *TMSB15A* siRNA successfully reduced gene expression by up to 98%, as determined by qRT–PCR. Transfected cells were subsequently tested for sensitivity to paclitaxel (Figure 5C) and doxorubicin (Figure 5D). However, no difference in sensitivity to both drugs was determined as compared with cells transfected with control siRNA ($P = 0.470$ and $P = 0.206$, respectively).

DISCUSSION

In this project, we investigated *TMSB15A* gene expression as a novel biomarker for prediction of response to NACT in HER2-negative

Table 3 Clinico-pathological characteristics of luminal carcinomas and TNBC in GeparTrio and GeparQuattro

	GeparTrio				*GeparQuattro*			
	Total *n* (%)	**Luminal *n* (%)**	**Triple-negative *n* (%)**	***P***	**Total *n* (%)**	**Luminal *n* (%)**	**Triple-negative *n* (%)**	***P***
Total no. of samples	212	151	61		383	249	134	
Age								
<50 years	98 (46.2)	65 (43.0)	33 (54.1%)	0.095[a]	176 (46.0)	108 (43.4)	68 (50.7)	0.123[a]
⩽50 years	114 (53.8)	86 (57.0)	28 (45.9%)		207 (54.0)	141 (56.6)	66 (49.3)	
Tumour histology								
Ductal	184 (89.3)	126 (86.3)	58 (96.7)	0.076[b]	299 (78.1)	180 (72.3)	119 (88.8)	<0.0001[b]
Lobular	17 (8.3)	16 (11.0)	1 (1.7)		57 (14.9)	53 (21.3)	4 (3.0)	
Other	5 (2.4)	4 (2.7)	1 (1.7)		27 (7.0)	16 (6.4)	11 (8.2)	
Missing	6	5	1					
Tumour grade								
G1	21 (10.2)	19 (13.1)	2 (3.3)	<0.0001[b]	9 (2.4)	9 (3.8)	0 (0)	<0.0001[b]
G2	139 (67.8)	109 (75.2)	30 (50.0)		239 (64.1)	188 (78.8)	51 (38.1)	
G3	45 (22.0)	17 (11.7)	28 (46.7)		125 (33.5)	42 (17.6)	83 (61.9)	
Missing	7	6	1		10	10		
cT								
cT1	1 (0.5)	0 (0)	1 (1.7)	0.246[b]	5 (1.3)	1 (0.4)	4 (1.3)	0.051[b]
cT2	138 (69.0)	99 (70.2)	39 (66.1)		259 (67.6)	163 (65.5)	96 (71.6)	
cT3	38 (19.0)	24 (17.0)	14 (23.7)		59 (15.4)	40 (16.1)	19 (14.2)	
cT4	23 (11.5)	18 (12.8)	5 (8.5)		60 (15.7)	45 (18.1)	15 (11.2)	
Missing	12	10	2		10			
cN								
cN0	102 (50.2)	76 (53.5)	26 (42.6)	0.120[a]	187 (48.8)	115 (46.2)	72 (53.7)	0.096[a]
cN1–3	101 (49.8)	66 (45.5)	35 (57.4)		196 (51.2)	134 (53.8)	62 (46.3)	
Missing	9	9						
pCR								
pCR	32 (15.1)	11 (7.3)	21 (34.4)	<0.0001[a]	60 (15.7)	20 (8.0)	40 (29.9)	<0.0001[a]
No pCR	180 (84.9)	140 (92.7)	40 (65.6)		323 (84.3)	229 (92.0)	94 (70.1)	

Abbreviations: cN = clinical node stage; cT = clinical tumour stage; pCR = pathological complete response; TNBC = triple-negative breast cancer. [a]Fisher's exact test. [b]Pearson's chi-square test.

Table 4 Correlation with pCR: logistic regression analysis within TNBC

***TMSB15A* gene expression**	***n***	**Events**	**% pCR**	**OR**	**95% CI**	***P***
GeparTrio						
Low (⩽11.7 ΔCT)	25	4	16.0	1	—	
High (>11.7 ΔCT)	36	17	47.2	4.70	1.34–16.45	0.016
GeparQuattro						
Low (⩽11.7 ΔCT)	47	8	17.0	1	—	
High (>11.7 ΔCT)	87	32	36.8	2.84	1.18–6.82	0.020

Abbreviations: CI = confidence interval; OR, odds ratio; pCR = pathological complete response; TNBC = triple-negative breast cancer.

breast cancer. We identified the clinical validity of *TMSB15A* mRNA for prediction of response by gene expression analysis in two independent cohorts, and systematically validated it in the neoadjuvant clinical phase III GeparTrio and GeparQuattro trials. Taking into account the differing expression levels of *TMSB15A* in luminal tumours and TNBC, we investigated each biological subtype separately. In the GeparTrio training cohort and GeparQuattro validation cohort, *TMSB15A* significantly predicted pCR in TNBC, whereas in luminal carcinomas, no reliable predictive value could be observed. A cutoff point for *TMSB15A* mRNA expression levels that was defined in GeparTrio TNBC could be validated in GeparQuattro as well.

A prominent finding in our project is that *TMSB15A* expression is a marker for triple-negative tumour type as compared with luminal subtype. Based on the strongly differing expression levels in luminal cancers and TNBC evident in GeparTrio, we hypothesised that the utility as a predictive marker could be different within biological subtypes. Indeed, *TMSB15A* mRNA levels were reliably predictive only in TNBC, both on the continuous scale and after dichotomisation using a cutoff point. This cutoff point divided the triple-negative group in a low expression group with a pCR rates of 16.0%/17.0% in GeparTrio/GeparQuattro, which is lower than the pCR rate in unselected patients (von Minckwitz *et al*, 2011b), and a high expression group with a pCR rate of 47.2%/36.8% in GeparTrio/GeparQuattro. TNBC constitute a heterogeneous group of tumours that partially overlaps with the basal-like intrinsic subtype as defined by gene expression profiling. Several subtypes of TNBC have been described to date with the aid of molecular methods, for example the claudin-low or the HER2-enriched subtype (Perou, 2011) as described by gene expression profiling, or the 5 negative/core basal phenotype by IHC (Blows *et al*, 2010). The potential diagnostic or prognostic use is not clear up to now, therefore this subtyping has not yet found its way into the clinics. The current pragmatic definition of triple-negative disease used in the clinic is negativity for ER, PR, and HER2 by IHC/ISH (Badve *et al*, 2011). In our project, we molecularly classified luminal and TNBC similarly, however, determined ER (*ESR1*) and HER2 expression by qRT–PCR, which is not the clinical standard. We nevertheless showed that the predictive value of *TMSB15A* gene expression was also present when TNBC were defined by standard IHC/ISH, which indicates that it is not an artifact due to the use of a novel typing method. The systematic comparison between

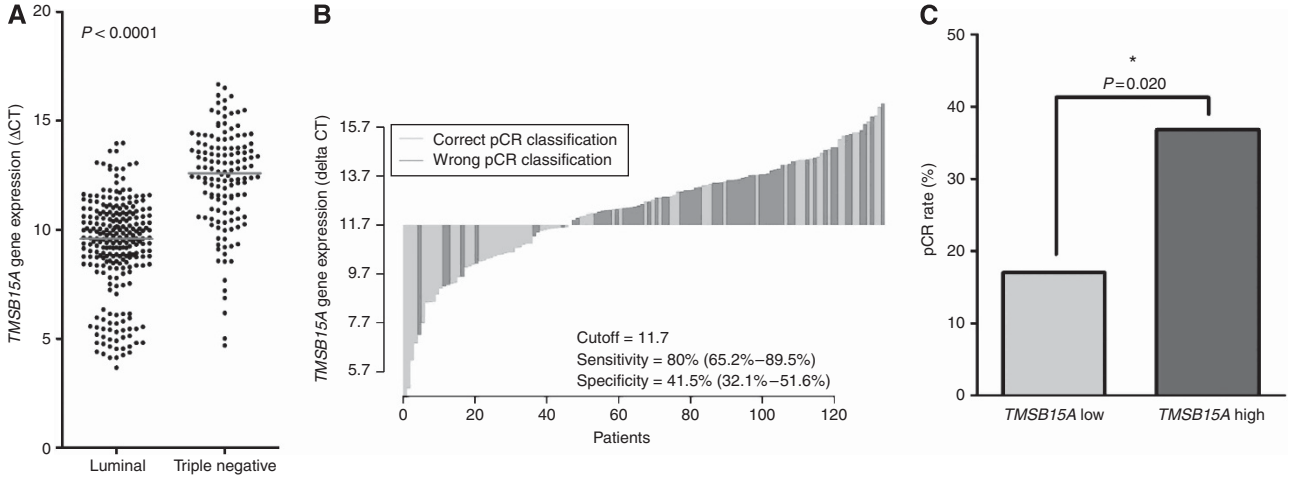

Figure 4 (**A**) Distribution of *TMSB15A* expression in luminal cancers and TNBC from GeparQuattro. Dots indicate individual tumours. Red lines, medians. *P*, Mann–Whitney test. (**B**) Waterfall plot for TNBC in GeparQuattro charts each tumour as a vertical bar. Green bars represent cases with correct pCR classification, red bars represent cases with wrong pCR classification. Sensitivity and specificity of the cutoff point are indicated. (**C**) Pathological complete response (pCR) rates in dependence of *TMSB15A* status in TNBC from GeparQuattro. For dichotomisation into a *TMSB15A* low and high expression group, the cutoff point of 11.7 ΔCT, which had been pre-defined in GeparTrio, was used. *P*-values were calculated by logistic regression. *Indicates significant values. The colour reproduction of this figure available at the *British Journal of Cancer* online.

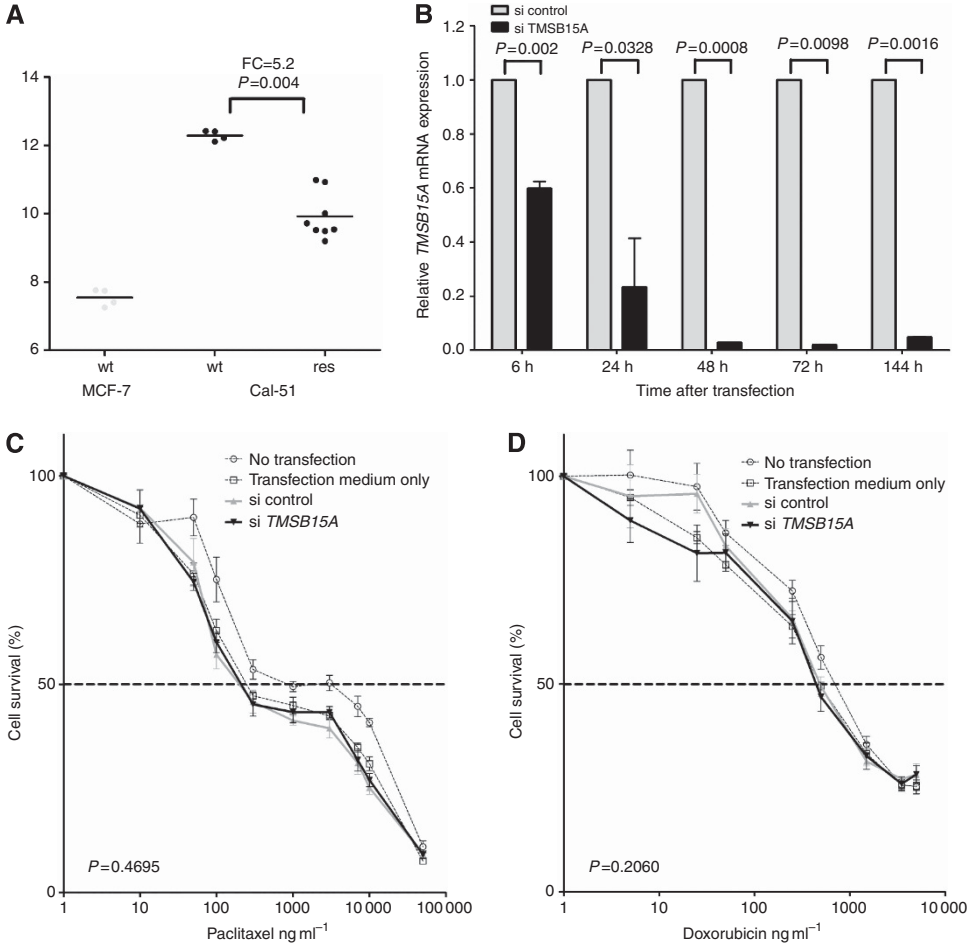

Figure 5 (**A**) *TMSB15A* mRNA levels (Affymetrix HG U133A microarray; probe set 205347_s_at) in MCF-7 and Cal-51 breast cancer cell lines. For clones of wild type (wt), cells were measured (dots), four clones of paclitaxel-resistant (res) Cal-51 were measured twice (dots). Expression levels in wt and res Cal-51 were compared by Mann–Whitney test. Bold lines, mean; FC = fold change. (**B**) *TMSB15A* mRNA expression in parental Cal-51 cells transfected with siRNA against *TMSB15A* (si *TMSB15A*) relative to cells transfected with control siRNA (si control). *P*, columnar *t*-test. (**C**) Dose–response curves for wild-type Cal-51 treated with increasing concentrations of paclitaxel (left panel) and doxorubicin (right panel) 24 h after siRNA transfection. Comparison of si control (bold grey line) with si *TMSB15A* treated cells (bold black line) were made by paired *t*-test. Dashed line, IC50.

qRT–PCR and IHC/ISH for determination of tumour types is described in a recent publication of our group, which shows that molecular tumour types defined by qRT–PCR reveal a quite similar prognostic and predictive impact as those defined by IHC/ISH (Denkert *et al*, 2012).

The heterogeneity of TNBC is also reflected by the fact that, although in comparison to luminal carcinomas, response rates to CTX are significantly higher and are in the range of 40% for modern NACT (von Minckwitz *et al*, 2011b), a significant number of patients with TNBC do not achieve a pCR. Large retrospective studies have shown that pCR is a robust surrogate marker for long-term survival in patients with TNBC treated with NACT (Carey *et al*, 2007; Liedtke *et al*, 2008) Thus, patients with pCR can expect a quite favourable long-term outcome, but in patients without pCR, triple-negative disease follows an aggressive course with early metastasis and death from disease (Hudis and Gianni, 2011). Biomarkers that predict pCR in the triple-negative subgroup are therefore helpful to select those patients for whom NACT is the optimal treatment option. The cutoff point of 11.7 ΔCT detected those patients with a sensitivity of 81%/80% in GeparTrio/GeparQuattro and might therefore be an aid in the decision for NACT in combination with clinical parameters such as age (Huober *et al*, 2010), to which *TMSB15A* adds independent predictive information. On the other hand, patients with TNBC who are less likely to achieve a pCR and therefore are in danger of a dismal outcome, could be identified, too. For those patients unfortunately, no standard specific treatment exists today. However, much effort is made to develop and test experimental therapeutics for TNBC (Hudis and Gianni, 2011). In this regard, *TMSB15A* gene expression analysis might help to select patients with a lower chance for pCR for clinical trials investigating novel therapies in this poor prognosis subgroup.

The function of TMSB15, the protein encoded by *TMSB15A*, in TNBC or the mechanisms by which it is upregulated has not been investigated to date. TMSB15 belongs to the beta thymosin family of highly conserved 5-kDa proteins with three members in humans, TMSB4, TMSB10, and TMSB15 (Chen *et al*, 2005). All beta thymosins bind and sequester (monomeric) G-actin and remove it from the dynamic assembly/deassembly process, which constantly takes place during essential cellular functions, such as mitosis, migration, intracellular transport, and phagocytosis (Mannherz and Hannappel, 2009). Beta thymosins further have extracellular functions due to their ability to interact with various other proteins and are thereby involved in the regulation of immune response, wound healing, and angiogenesis (Chen *et al*, 2005). TMSB15 is the least well studied family member, however, there is increasing evidence for an essential role in tumour progression. It has been shown to be upregulated in cancer cell lines of various origin and be implicated in tumour cell migration and proliferation (Bao *et al*, 1996; Abdulrahman *et al*, 2007; Banyard *et al*, 2009). In breast cancer, *TMSB15A* might be upregulated to increase cell motility and transformation, which would explain that it is found in higher levels in aggressive, triple-negative and poorly differentiated carcinomas. Our cell culture data show an upregulation of *TMSB15A* in triple-negative Cal-51 cells as compared with luminal MCF-7 cell and therefore, reflect the situation found *in vivo*. These results further indicated that decreased *TMSB15A* expression seems to be a side-effect of chemoresistance, rather than a causal factor for chemoresistance, as no increase of sensitivity neither to anthracycline nor to taxane could be determined by *TMSB15A* knockdown with siRNA.

In luminal carcinomas, we did not observe a significant predictive effect of *TMSB15A* gene expression. Luminal tumour type by itself is a negative predictor for pCR, with a response rate of approximately 9% with current regimes (von Minckwitz *et al*, 2011). Although the identification of those few patients who benefit from NACT would be desirable, the detection of predictive biomarkers is hampered by the low number of events.

Some limitations of our study need to be mentioned. Sample size was limited by dealing with very small amounts of tissue (core biopsies) that have already been used for diagnostic purposes. Therefore, we did not reach two-thirds of the original study population as postulated by Simon for category IB evidence (Simon *et al*, 2009). Further, in our study cohorts, standard anthracyline/taxane-based NACT was applied, whereas the treatment protocols for triple-negative disease are changing. Especially platinum agents are increasingly applied (Hudis and Gianni, 2011). Therefore, we plan to investigate *TMSB15A* gene expression prospectively in patient cohorts treated with different agents to see whether it is a general maker for response to cytotoxic therapy.

In conclusion, we identified and validated *TMSB15A* mRNA levels as a predictive marker for response to NACT in TNBC. *TMSB15A* expression analysis might be helpful for the identification of patients who would benefit from anthracycline/taxane-based NACT. For patients with *TMSB15A*-negative TNBC, who have a smaller chance for therapy response, novel treatment options, and experimental therapeutics should be considered.

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