TOB1 Is Regulated by EGF-Dependent HER2 and EGFR Signaling, Is Highly Phosphorylated, and Indicates Poor Prognosis in Node-Negative Breast Cancer

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Abstract
Clinical and animal studies have shown that coexpression of the receptor tyrosine kinases HER2 and epidermal growth factor factor (EGF) receptor (EGFR) indicates a highly metastatic phenotype of breast cancer. In a cellular model of this phenotype using differential gene expression analysis, we identified TOB1 to be up-regulated depending on EGF stimulation and transduction through phosphorylation of HER2 tyrosine 1248. mRNA expression analysis of breast cancers from a cohort of node-negative patients showed significantly shortened distant metastasis-free survival for patients with high TOB1 expression. In subsequent tissue microarray studies of 725 clinical samples, high HER2 and EGF protein levels were significantly correlated with TOB1 expression in breast cancer, whereas EGFR and EGF levels correlated with TOB1 phosphorylation. We did not observe a correlation between TOB1 expression and cyclin D1, which was previously suggested to mediate the antiproliferative effect of unphosphorylated TOB1. A positive correlation of TOB1 phosphorylation status with proliferation marker Ki67 suggests that elevated TOB1 phosphorylation might abrogate the antiproliferative effect of TOB1 in breast cancer. This suggests a new regulatory role for TOB1 in cancer progression with particular significance in HER2- and/or EGFR-positive breast cancers. [Cancer Res 2009;69(12):5049–56]

Introduction
Coexpression of epidermal growth factor (EGF) receptor (EGFR) and HER2 was shown to be associated with shorter survival and shorter time to distant metastasis in breast cancer patients and in animal models than either risk factor alone (1–5). Tumors that simultaneously express EGFR and HER2 are generally characterized by higher tumor grade, early lymph node involvement (6, 7), and an increased rate of cytostatic and tamoxifen resistance (3, 8–10). The important role of EGFR and HER2 in metastasis (11–13) is based on their broad roles in reorganization of the cytoskeleton, cell motility (14, 15), cell adhesion (16–18), and regulation of protease activity (19, 20).

Studies carried out by our group showed that the induction of EGF-induced cell migration was based on site-specific tyrosine 1248 phosphorylation of the HER2 receptor via EGFR, which led to a shift in the timing of PLC-γ1 activation and phosphatidylinositol signaling, resulting in coordinated baseline transient calcium oscillations (21). This may act as the rate-limiting step in enabling single tumor cells to start migration along a growth factor concentration gradient. Consequently, the identification of molecules, which modulate key signaling pathways in metastasis downstream of HER2-Y1248 phosphorylation in these motile cells, was the primary objective of this study. We were further interested in identifying genes involved in other intracellular signaling pathways determining poor clinical outcome induced by EGFR/HER2 heterodimers by investigating phenotypically well-characterized cell lines using whole genome expression analysis.

Therefore, we performed suppression subtractive hybridization assays (SSH) on EGFR-positive MDA-MB-468 cells overexpressing HER2 (MDA-HER2) and compared them with control MDA-MB-468 cells mock transfected with the empty vector (MDA-control) and the YF-mutant of the HER2 expression vector lacking the Y1248 phosphorylation site (MDA-PM). Gene products identified by SSH were validated by real-time reverse transcription-PCR (RT-PCR) and Western blot on a panel of these MDA-MB-468 cells. TOB1 mRNA expression analysis was then done on a cohort of 160 node-negative patients with full clinical records and long-term follow-up to assess the relevance of the HER2-Y1248–related gene expression of TOB1. Lastly, protein expression of TOB1 and phosphorylated TOB1 (pTOB1) was analyzed in tumor tissue from ~700 patients by immunohistochemistry using tissue microarrays (TMA) for the assessment of TOB1 activity.

Materials and Methods

Suppression Subtractive Hybridization of MDA-MB-468 Cells Depending on HER2 Expression
SSH-PCR is a method for comparative gene expression analysis. The procedure leads to a pool of PCR products of differentially expressed genes that are cloned into plasmids for sequencing and identification purposes. Thereby SSH leads to a list of differentially expressed genes with no expression values. The advantage of SSH is the detection of differential expression of low-abundance genes and the high sensitivity for low levels of difference. Furthermore, SSH-PCR allows to identify unknown transcripts and noncoding RNAs (22). For the determination of expression levels and validation purposes, quantitative real-time RT-PCR was carried out.

MDA-MB-468 cells overexpressing HER2 (MDA-HER2) and the respective G418 resistant control cells were generated and cultured as described by Roetger and colleagues (23).
Twenty-four hours before harvesting of the cells, standard medium was replaced with medium containing 0.1% FCS. For RNA isolation, the cells were cooled on ice, medium was removed, and RNA was isolated after cell lysis in the culture flask with the RNeasy Midi Kit (Qiagen) according to the manufacturer's manual. Residual genomic DNA was removed by a DNase digestion step.

For comparative gene expression analysis, 2 μg of RNA from MDA-HER2 and MDA-control were reverse transcribed with Moloney murine leukemia virus reverse transcriptase (Clontech), and SSH was done with the PCR-Select cDNA Subtraction Kit (Clontech) in forward and reverse direction according to the manufacturer's recommendations.

This experiment was repeated with RNA from the same cell lines, which were additionally stimulated with 100 ng EGF/mL medium, which was added 1 h before harvesting or as indicated in the relevant experiment.

Creation of a Subtracted cDNA Library

Freshly prepared 10 cycle secondary SSH-PCR reactions (0.2 μL) resulting from the forward and the reverse subtraction were cloned into the pCR 2.1 vector after an additional extension step (8 min, 72°C) using the Original T/A Cloning Kit (Invitrogen) and transformed to E. coli TOP 10 F’ according to the manufacturer's instructions. Bacterial suspension was plated on Luria Broth agar plates containing ampicillin, isopropyl-β-D-thiogalactopyranoside, and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside for selection and identification of clones carrying plasmids containing inserts.

Sequencing of Subtracted cDNA Library

Positive clones resulting from each of the subtractions were picked and cultured in 1.5 mL TB-Medium in DeepWell-plates at 37°C overnight. Plasmids were isolated using the Wizard Magnesium Plasmid Purification System (Promega). Sequencing was done using a M13 forward promoter (−21) primer (5’-TGTTAGCGACGCGCATG-3’) and BigDye 3.0 Sequencing Mix (Applied Biosystems) before analysis by capillary electrophoresis on an ABI 3700 Genetic Analyzer (Applied Biosystems). The sequences of the inserts of differentially expressed genes were identified using National Center for Biotechnology Information (NCBI) Blast search (blastn; ref. 24).

Relative Expression Quantification in Cultured Cells by Real-time RT-PCR

Expression of putatively differentially expressed genes was quantified by SYBR Green real-time RT-PCR in cell lines originally used for SSH analysis. Additionally, expression was analyzed in the cell line MDA-PM to identify genes that are induced by signals transduced by phosphorylation of tyrosine 1248 of the HER2 receptor. Twenty-four hours before harvesting of the cells, standard medium was replaced with medium containing 0.1% FCS before EGF was added at 100 ng/mL and the cells incubated for the time indicated for each experiment. DNase-treated total RNA was reverse transcribed using Multiscribe Reverse Transcriptase and random hexamers (Applied Biosystems) according to the manufacturer's instructions. Quantitative RT-PCR analyses were done on an ABI 7900 Sequence Detection System (Applied Biosystems) using the Quantitect SYBR Green PCR kit (Qiagen) and qPCR MasterMix Plus (Eurogentec). Individual expression values were normalized to 18S rRNA (Pre-Developed TaqMan Assay Reagents Control Kit; Applied Biosystems). Primer design was done with the "Primer Express 2.0" software (Applied Biosystems) according to the manufacturer's grid in the DNA primer design optimization for genomic sequences, at least one primer of each pair was designed spanning two exons. Specificity of amplification was further checked by melting curve analysis with the SDS 2.1 software (Applied Biosystems). Primer oligonucleotides were purchased from Operon, except prespecified TOB2 primers, which were purchased from SuperArray. PCR conditions were initial denaturation for 10 min at 95°C, followed by 50 cycles consisting of 15 s at 95°C and 1 min at 60°C. Expression ratios between two samples were calculated from differences in threshold cycles at which an exponential increase in reporter fluorescence could first be detected (Ct values). Results of triplicates were averaged. For primer sequences, see Supplemental Data A1.

Western Blot Analysis

Twenty-four hours before harvesting of the cells, standard medium was replaced with medium containing 0.1% FCS before EGF was added at 100 ng/mL, and the cells were incubated for the time indicated for each experiment. Cell lysates were prepared using radiolimunoprecipitation assay buffer [10 mmol/L Tris pH 7.2, 150 mmol/L NaCl, 1% Triton X-100, 0.1% sodium deoxycholate, 5 mmol/L EDTA, 0.1% SDS, complete protease inhibitor cocktail (Roche, Mannheim, Germany)], 1 mmol/L Na3VO4, 1 mmol/L NaF]. Twenty micrograms of protein were fractionated by SDS-PAGE, transferred to an Immobilon-PQ membrane (Millipore), and analyzed by immunodetection. Antibodies were used α-phospho-TOB1 (1:750), α-TOB1 (1:1;500; IBL), and α-tubulin (Santa Cruz). Blots were blocked with 3% bovine serum albumin (BSA)/5% milk powder (for TOB1) and 5% BSA (for pTOB1) and detected with horseradish peroxidase–conjugated secondary antibodies and ECL+ reagents (Amersham Biosciences).

TMAs and Immunohistochemistry

A TMA composed of tissues from 725 invasive breast cancer cases was constructed according to standard protocols using a dedicated instrument (Beecher Instruments; refs. 25, 26). The cores with a diameter of 0.6 mm each were placed in a distance of 0.2 mm. Two cores per specimen from each breast cancer case were taken for the composition of the TMA. For the localization of representative tumor areas, H&E-stained sections were prepared from each original tumor block. The tumor series comprised all stages and grades (G1, 110; G2, 370; G3, 245; T1, 180; T2, 257; T3, 193; T4, 95; N0, 456; N>0, 269).

The immunohistochemical analysis of TMAs has been previously done for c-erbB2, EGFR, cyclin D1, p53, and Ki67 and described in detail elsewhere (27). Antibodies for TOB1 (clone 4B1) and pTOB1 (both IBL) were used at dilutions of 1:25 and 1:5, respectively. The antibody for EGF (Acris) was used at 1:20. Pretreatment conditions are listed in Supplemental Data A2. In brief, the LSAB/AP method was used for all immunohistochemical staining (DAKO; ref. 27).

The stained tissues were evaluated by two pathologists, with a score from 0 to 3 (0 corresponding to negative staining and 3 corresponding to strongest staining). Discrepant cases were discussed and decided on consensus.

Real-time RT-PCR from RNA from Breast Cancer Tissue

Sample collection. A fresh frozen breast tumor sample set accrued from 1986 to 1998 at the University Medical Center Muenster served as the source of samples for the prognostic evaluation of TOB1 expression. The inclusion criteria for the primary study included T1 or T2 tumors, lymph node negative, follow-up for 5 y. One hundred eighty-six patients had known recurrence status at last follow-up. A subset of 160 patients (of 186) was evaluable for clinical follow-up that has been classified for estrogen receptor (ER), progesterone receptor (PgR), HER2, and therapy.

Sixty-six percent of the tumors were ER+, 15.6% of the tumors were high-/moderately positive for EGFR, and 15% of the tumors were positive for HER2 with Dako-Score 2 and 3. Treatment regimens were applied as follows: Forty percent of the patients received tamoxifen therapy, which was combined in 22% of these cases with radiation or chemotherapy. Approximately 57% received no therapy; 13% received chemotherapy, mostly cyclophosphamide, methotrexate, 5-fluorouracil and Epirubicin, cyclophosphamide; 5% radiation alone; and 3% gonadotropin-releasing hormone analogues.

We chose distant metastasis-free survival (DMFS) as the primary end point because it is closely linked to cancer-related death and cancer cell motility.

Sample processing. Total RNA was isolated from 50 to 100 mg of frozen breast cancer tissue using RNeasy Mini kit (Qiagen). To ensure free of DNA contamination, an on-column DNase I treatment was done. The amplifiable RNA was determined by measuring the expression level of a housekeeping gene (EEF1A1).

Gene expression profiling. One-step RT-PCR with SYBR Green was used for gene expression profiling, and the PCR components were described previously (28). PCR primers were designed to amplify all known splice variants. Amplification plates in 384-well format with 15-μL reactions
in duplicates were done using Applied Biosystems Prism 7900HT Sequence Detection System. The cycle profile consists of 50°C 2 min, 95°C 1 min, 60°C 30 min, followed by 45 cycles of 95°C 15 s and 60°C 30 s ending with dissociation analysis to ensure target specific amplification. Serially diluted run-off transcripts were included in each 384-well plate to monitor instrument-to-instrument variation.

Together with three normalization genes, the expression levels of mRNAs in 160 RNA samples were profiled. The expression level of each gene was normalized with the average of expression levels of three housekeeping genes (\textit{NUP214}, \textit{PPIG}, and \textit{SLU7}). The normalized expression levels of mRNAs were used for statistical analyses.

### Statistical Analysis
DMFS was chosen as the primary end point of this study. DMFS is defined as the interval between the date of definitive breast cancer surgery and diagnosis of first distant metastasis or last date of follow-up (whichever occurred first). Contralateral recurrences and deaths without recurrence were regarded as censoring events, whereas local recurrences were considered as neither events nor censoring events. The definition of DMFS endpoints, its events, and censoring rules were aligned with those adopted by the National Surgical Adjuvant Breast and Bowel Project for the prognostic molecular marker studies (29).

Kaplan-Meier-survival estimates were generated and compared with the log-rank test. Multivariate analysis was done using Cox proportional hazards regression model. Level of significance was \( P < 0.05 \).

### Results
Gene expression analysis revealed up-regulation of genes involved in cellular adhesion, calcium signaling, and cytoskeleton structure and reorganization in \textit{EGFR/HER2–expressing cells}. Sequencing and subsequent database search of gene products amplified in the SSH procedure led to a list of 112 gene products, up-regulated in MDA-MB-468 cells, dependent on \textit{HER2} expression and \textit{EGF} stimulation. For the complete list, see Supplemental Data A.3.

A general analysis of the designated functions of these 112 gene products revealed that those with a known function were mainly involved in cellular adhesion, calcium signaling, and cytoskeleton structure and reorganization. A list of the respective gene products is given in Supplemental Table A.5.

Relative expression quantification by quantitative RT-PCR confirmed accuracy of screening results. To confirm the results obtained by SSH-PCR, we performed real-time RT-PCR. Of the 112 genes putatively differentially expressed according to SSH results between MDA-MB-468 cells differing in \textit{HER2} expression and stimulated by \textit{EGF}, 18 were selected for quantitative expression analysis by real-time RT-PCR in the same cell lines analyzed by SSH. Gene products were chosen depending on their known function and background information data availability. Generally, differential expression indicated by SSH could be confirmed by real-time RT-PCR. Whereas eight examined gene products showed only small expression differences by real-time PCR, which were within the range of SD, about half of the validated gene products were differentially expressed by more than 2-fold in the compared cDNAs, as indicated by SSH. Only 2 of 18 genes detected by SSH were false positives (\textit{HIF1A} and \textit{ZNF165}). For expression differences, see Supplemental Data A.4.

\textit{TOB1} is up-regulated by \textit{EGF} stimulation through phosphorylation of \textit{HER2-Y1248}. To find out which of the \textit{HER2}-dependent gene products identified in this study were in fact stimulated by signal transduction through Y1248, we added MDA-PM cells to the original panel of SSH analyzed cells for real-time RT-PCR analysis.

![Figure 1. Real-time RT-PCR expression analysis of \textit{TOB1} and \textit{TOB2}. Expression rate is given as relative expression with values of MDA-control considered as 100%. Bars, SE. A, relative expression values with and without \textit{EGF} stimulation (stimulation for 1 h). *, \( P < 0.05 \) in two-tailed unpaired Student’s \( t \) test. B, time course of \textit{TOB1} expression after \textit{EGF} stimulation. C, time course of \textit{TOB2} expression after \textit{EGF} stimulation.](image)

![Figure 2. Western blot analysis of expression of \textit{TOB1}, \textit{TOB2}, and \textit{pTOB1} after \textit{EGF} stimulation. Left, MDA-HER2; right, MDA control cells.](image)
The cell line MDA-PM was constructed to express a mutated form of HER2 that could not phosphorylate tyrosine 1248 due to an exchange by phenylalanine. These cells show no transendothelial invasion in vitro due to impaired migration capability and related cytoskeleton reorganization (30).

Furthermore, it was shown by Dittmar and colleagues (21) that EGF stimulation is necessary for migratory activity in MDA-HER2 cells in culture. Therefore, candidate genes for a crucial role in a HER2-dependent invasive and highly motile phenotype, mediated by HER2/EGFR heterodimers and tyrosine 1248 phosphorylation, had to be up-regulated in steady state in MDA-HER2 and must additionally respond to EGF stimulation in MDA-HER2 cells but not in MDA-control cells and MDA-PM cells. Thus, we quantified expression values of candidate genes in MDA-control cells, MDA-HER2, and MDA-PM, with and without EGF stimulation, respectively.

Of the 18 gene products quantified by real-time RT-PCR, TOB1 was the only one to be up-regulated in HER2-overexpressing MDA-MB-468 cells and at the same time being almost nonresponsive to EGF stimulation in parental cells and cells expressing mutated HER2 receptor (Y1248F; Fig. 1A). TOB1 expression showed a strong reaction to EGF stimulation in MDA-HER2 cells compared with MDA-control (3-fold compared with 1.5-fold) and even the basic expression level of TOB1 was 2.5-fold higher in MDA-HER2 compared with MDA-control and MDA-PM. Therefore, TOB1 fulfilled the criteria to be regulated by EGF-stimulated HER2-Y1248 phosphorylation.

TOB1 protein expression and phosphorylation is associated with EGF-dependent erbB signaling and proliferation in breast cancer. To confirm that our RNA expression data have relevance on the protein level and further shed light on the dynamic by which the differential RNA expression translates to the protein level, we performed time course experiments on the mRNA and protein levels. Due to reported cross-reactivity of the TOB1 antibody with TOB2, we also included TOB2 in these studies on the protein and

### Table 1. Statistical correlations of TOB1 and pToB protein levels with each other and a panel of breast cancer relevant markers including HER2, EGF, EGFR, and cyclin D1 as determined by TMA analysis

<table>
<thead>
<tr>
<th>TOB1, n</th>
<th>χ² contingency coefficient</th>
<th>pTOB, n</th>
<th>χ² contingency coefficient</th>
</tr>
</thead>
<tbody>
<tr>
<td>T</td>
<td>575</td>
<td>0.208</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>N</td>
<td>575</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>HER2</td>
<td>523</td>
<td>0.26</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>N/D</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HER2&lt;3</td>
<td>154</td>
<td>0.404</td>
<td>0.0004</td>
</tr>
<tr>
<td>HER2&gt;2</td>
<td>N/D</td>
<td>N/D</td>
<td>N/D</td>
</tr>
<tr>
<td>EGF</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Overall</td>
<td>542</td>
<td>0.236</td>
<td>0.0002</td>
</tr>
<tr>
<td>HER2&lt;3</td>
<td>427</td>
<td>0.243</td>
<td>0.0015</td>
</tr>
<tr>
<td>HER2&gt;2</td>
<td>78</td>
<td>0.366</td>
<td>0.061</td>
</tr>
<tr>
<td>EGF</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Overall</td>
<td>549</td>
<td>0.262</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Cyclin D1</td>
<td>519</td>
<td>0.262</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Ki67</td>
<td>567</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

NOTE: Dashes indicate no statistical significant correlation. N/D indicates that theses correlations were not determined due to very low case numbers in those groups.
RNA levels. These experiments revealed that up-regulation of TOB1 after EGF stimulation lasts for at least 1 h before reaching sub-base-line levels after 4 hours (Figs. 1B and 2). The poststimulation sub-base-line levels of TOB1 coincide with up-regulation of TOB2 and phosphorylation of TOB1 serine 152 and 154. Both up-regulation of TOB2 and phosphorylation of TOB1 are stronger in the MDA-control cells than in MDA-HER2 (Figs. 1C and 2).

TMA analysis (compare Table 1) confirmed that the results that were obtained on the mRNA and protein levels in cell lines have relevance in human breast tumors. TMA analysis on 725 mammary carcinomas indicated that TOB1 protein expression is up-regulated depending on HER2 levels and EGF stimulation. On the TMA, HER2 and EGF protein expression levels correlated with high significance (P < 0.0001 and P = 0.0002, respectively) to TOB1 expression. In contrast, we saw no correlation between TOB1 expression and EGFR. TOB1 phosphorylation status, on the other hand, was independent of HER2 levels, whereas EGFR and particularly EGF expression seemed to be involved in TOB1 phosphorylation. EGF levels were significantly correlated to both TOB1 and pTOB1 levels (P = 0.0001 and P = 0.0001, respectively). However, the χ² contingency coefficient (0.696 for EGF/pTOB1 versus 0.236 for EGF/TOB1) pointed to a remarkably higher grade of correlation between EGF and pTOB1, indicating that EGF stimulation leads to a high proportion of phosphorylated TOB1. Notably, overall TOB1 expression was positively and highly significantly correlated to cyclin D1 status (P < 0.0001), but not with the proliferation marker Ki67. Because, on the other hand, pTOB1 was correlated with both of the latter, our data substantiate a role for phosphorylation of TOB1 in proliferation.

TOB1, but not pTOB1, was significantly correlated to tumor size (P < 0.05). Nodal status seems to be independent of TOB1 levels and shows close to borderline correlation with pTOB1. The percentage of ER-positive cells was correlated neither to TOB1 expression nor to TOB1 phosphorylation in the tumor samples on the TMA. Due to these data and the primary focus of this study, a subgroup analysis related to ER status was not done in the following experiments.

Overall, less than 1% of the tumors displayed a nuclear localization of TOB1. Representative examples of TOB1 and pTOB1 staining are shown in Fig. 3.

TOB1 mRNA expression has an additional independent influence on prognosis in stage I and II disease. To find out whether TOB1 expression has prognostic relevance, 160 frozen tissue samples from node-negative breast cancer stage I and II patients with known clinical records and long-term follow-up were analyzed. Among them, 66 developed distant metastasis with a mean follow-up time of 27 months. The mean (SD) age of patients was 58 (13) years with 68.7% older than 55 years. Most tumors were of medium (41%) and high grade (34%). Fifteen percent of tumors are unclassified for grade.

TOB1 mRNA levels were determined by quantitative RT-PCR. Significant amounts of TOB1 mRNA were detectable in all tumor tissues whereas the relative level of expression deviated by a factor of 10,000. Survival data analyzed by Kaplan-Meier test revealed a significant association between high expression levels of TOB1 mRNA and shortened 5-year metastasis-free survival (Fig. 4A). This result was confirmed by a univariate Cox analysis and an additional in silico analysis on the data NCBI data set GSE 2034, which was generated for expression profiling (31). Using TOB1 mRNA expression from normalized array values from the Wang data set dichotomized at median in Kaplan-Meier analysis, a significant association of TOB1 with DMFS was revealed (Fig. 4B). Furthermore, we classified the Wang data set for array-based phenotypes according to Sorlie and colleagues (32). We found a significant association of TOB1 mRNA expression and DMFS in the small subgroup of basal-like breast cancers (n = 66, P = 0.014; Fig. 4C), which might support the results obtained by immunohistochemistry on TMA that EGFR and TOB1 expression are not statistically significantly correlated.

To evaluate an independent prognostic value of TOB1 in relationship to established parameters, we performed multivariate survival analysis using Cox proportional hazard model on both our stage I/II cohort and the Wang data set, which is also a pure node-negative.
cohort of patients. As shown in Table 2, TOB1 mRNA expression turned out significantly in multivariate analysis in both stage I/II cohorts (\(P = 0.026\) and \(P = 0.012\), respectively). TOB1 contributes independent prognostic value in the Cox model besides the highly significant progesterone receptor mRNA in both cohorts. Noteworthy, TOB1 has a statistical significant effect in the model besides the effect of EGFR mRNA expression. In contrary, HER2 mRNA levels did not reach significance in the model in either of the cohorts.

### Discussion

Breast cancers simultaneously expressing EGFR and HER2 are associated with an early onset of metastasis and early death (3, 33–35). Data from our group and others suggest that coexpression of EGFR and HER2 leads to a dominant lateral signaling from EGFR via HER2 (30, 36–39). To discover which genes are involved in this highly aggressive phenotype of breast cancer, we performed comparative gene expression studies by SSH comparing the global gene expression in breast cancer cases with long-term follow-up and full clinical records. Our data showed a significantly shorter distant metastasis-free survival for breast cancer patients with high TOB1 expression levels. This holds also true in a data set obtained from the NCBI data bank provided by Wang and colleagues (31), composed of expression data from stage I/II patients. Furthermore, we performed multivariate survival analysis using Cox proportional hazard model on both our stage I/II cohort and the Wang data set. The Wang data set was chosen because it represents a pure node-negative cohort of patients (35).

Of the 18 candidates that were quantified by real-time RT-PCR, only TOB1 fulfilled these conditions (Fig. 1A). TOB1 has been identified as a substrate of mitogen-activated protein kinase (MAPK)–dependent phosphorylation and interacts directly with HER2. It has been shown to regulate proliferation by affecting cyclin D1 promoter activity (41–47). Thereby, TOB1 has been thought of as a tumor suppressor (48, 49). In contrast, there have been no reports in the literature suggesting a role in migration and metastasis for any kind of cell type. To look for relevance of TOB1 mRNA expression in breast cancer and an association with clinical outcome, we performed expression analysis on a patient cohort of 160 node-negative breast cancer cases with long-term follow-up and full clinical records. Our data showed a significantly shorter distant metastasis-free survival for breast cancer patients with high TOB1 expression levels. This holds also true in a data set obtained from the NCBI data bank provided by Wang and colleagues (31), composed of expression data from stage I/II patients. Furthermore, we performed multivariate survival analysis using Cox proportional hazard model on both our stage I/II cohort and the Wang data set. The Wang data set was chosen because it represents a pure node-negative cohort of patients. TOB1 showed up with independent prognostic value in the model besides the highly significant progesterone receptor mRNA in both cohorts, and which might indicate independence from ER function.

### Table 2. Univariate and multivariate Cox regression analysis for DMFS in the validation set of 160 stage I/II breast cancer patients (Data set Helms) and the data set provided by Wang and colleagues (31) in the NCBI data base GSE2034 (Data set Wang)

<table>
<thead>
<tr>
<th>Covariate</th>
<th>Data set Helms (n = 160)</th>
<th>Data set Wang (n = 255)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HR (95% CI)</td>
<td>HR (95% CI)</td>
<td></td>
</tr>
<tr>
<td>Age</td>
<td>1.13 (0.62–1.98)</td>
<td>n.a.</td>
</tr>
<tr>
<td>Clinical tumor size (T)</td>
<td>2.65 (1.19–3.32)</td>
<td>n.a.</td>
</tr>
<tr>
<td>Tumor grade (G)</td>
<td>0.99 (0.59–1.03)</td>
<td>n.a.</td>
</tr>
<tr>
<td>ER status</td>
<td>1.19 (0.69–2.04)</td>
<td>1.03 (0.63–1.68)</td>
</tr>
<tr>
<td>PgR Status</td>
<td>0.53 (0.32–0.88)</td>
<td>0.96 (0.60–1.55)</td>
</tr>
<tr>
<td>PGF mRNA</td>
<td>n.a.</td>
<td>n.a.</td>
</tr>
<tr>
<td>TOB1 mRNA</td>
<td>1.81 (1.1–2.98)</td>
<td>1.84 (1.22–2.76)</td>
</tr>
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</table>

NOTE: All parameters were used as binary variables in the analysis. Thereby, dichotomization of parameters was done as follows: age, <50 versus ≥50 y; clinical tumor size (T), T1 versus T2; ER status and PgR status (immunohistochemical), negative versus positive; ER mRNA (probe set 205225_at), <2,500 versus ≥2,500 [cutoff value determined in correlation to ER (immunohistochemical) measurement]; PgR mRNA, EGFR mRNA, HER2 mRNA, TOB1 mRNA, all < median versus ≥ median; x, not considered; n.a., not available from database GSE2034.

Abbreviations: HR, hazard ratio; 95% CI, 95% confidence interval.
Noteworthy, TOB1 has a statistical significant effect in the model besides the effect of EGFR mRNA expression, which fits well with the finding obtained by immunohistochemistry on our TMA that EGFR and TOB1 are not correlated significantly. It has to be mentioned that HER2 did not reach any significance in the statistical analysis, which might be due to the low numbers of high HER2 expressing tumors.

Western blot and real-time RT-PCR analyses in cultured cells showed that the up-regulation of TOB1 after EGF stimulation lasts less than 4 hours before it is phosphorylated in a way that is not positively affected by HER2 overexpression (Figs. 1B and 2). Conversely, the homologue TOB2, which increases expression during cell cycle progression, we see between TOB1 and cyclin D1 expression, which points to the finding obtained by immunohistochemistry on our TMA that EGFR and TOB1 are not correlated significantly.

Table 2. Univariate and multivariate Cox regression analysis for DMFS in the validation set of 160 stage I/II breast cancer patients (Data set Helms) and the data set provided by Wang and colleagues (31) in the NCBI database GSE2034 (Data set Wang) (Cont’d)

<table>
<thead>
<tr>
<th>Analysis with TOB1 mRNA</th>
<th>Analysis without TOB1 mRNA</th>
<th>Analysis with TOB1 mRNA</th>
<th>Analysis without TOB1 mRNA</th>
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<tr>
<td>Data set Helms (n = 160)</td>
<td>Data set Wang (n = 255)</td>
<td>Data set Helms (n = 160)</td>
<td>Data set Wang (n = 255)</td>
</tr>
<tr>
<td>HR (95% CI)</td>
<td>P</td>
<td>HR (95% CI)</td>
<td>P</td>
</tr>
<tr>
<td>1.93 (0.79–4.72)</td>
<td>0.15</td>
<td>2.07 (0.85–5.05)</td>
<td>0.11</td>
</tr>
<tr>
<td>2.24 (1.19–4.15)</td>
<td>0.015</td>
<td>2.69 (1.29–5.61)</td>
<td>0.0084</td>
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<tr>
<td>1.02 (0.47–2.32)</td>
<td>1.0</td>
<td>1.01 (0.52–1.88)</td>
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</tr>
<tr>
<td>2.94 (1.24–6.98)</td>
<td>0.014</td>
<td>3.89 (1.64–9.22)</td>
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</tr>
<tr>
<td>0.32 (0.16–0.68)</td>
<td>0.0028</td>
<td>0.32 (0.15–0.67)</td>
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<td>n.a.</td>
<td>n.a.</td>
<td>n.a.</td>
<td>n.a.</td>
</tr>
<tr>
<td>6.21 (2.85–13.55)</td>
<td>0.0000045</td>
<td>6.21 (2.77–13.94)</td>
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</tr>
<tr>
<td>1.34 (0.64–2.81)</td>
<td>0.43</td>
<td>1.69 (0.82–3.48)</td>
<td>0.16</td>
</tr>
<tr>
<td>2.45 (1.11–5.41)</td>
<td>0.026</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>2.00 (1.29–3.11)</td>
<td>0.0019</td>
<td>x</td>
<td>x</td>
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We sought to corroborate the relevance of our expression data showing an up-regulation of TOB1 expression in a cohort of 725 breast cancer patients. The protein expression data from TMAs clearly confirmed high TOB1 protein levels in tumors that express high levels of HER2 and EGF. Conversely, EGFR expression alone was not associated with regulation of TOB1 expression. However, EGFR probably plays an important role in the regulation of TOB1 activity through extracellular signal-regulated kinase-1/2-mediated phosphorylation of serine residues (42, 44). On the other hand, pTOB1 levels did not correlate with HER2 expression in TMA analysis. This finding is supported by our Western blot analysis (Fig. 2) and implicates that TOB1 phosphorylation seems to be largely independent of HER2 levels, although high expression levels of TOB1 (which are linked to HER2 levels) correlated with a high phosphorylation level in the TMAs. These observations point toward an EGFR and EGF determined erbB signaling as a mechanism of regulating TOB1 phosphorylation via MAPK, whereas HER2 levels are involved in regulation of TOB1 expression levels. These findings imply that tumors with coexpression of HER2 and EGFR might have high levels of TOB1 with a high degree of phosphorylation.

Because TOB1 has been described for its antiproliferative properties, we looked at tumor cell proliferation by assessing Ki67 expression levels. Our data substantiated the reported antiproliferative effect of TOB1, which is abrogated on phosphorylation (42, 44). We did not see any correlation of total TOB1 levels and Ki67 whereas pTOB shows a highly significant correlation with Ki67. This is in accordance with the findings of Suzuki and colleagues (44), who showed that TOB1 phosphorylation functions as a MAPK-dependent switch that can trigger cell cycle progression.

The antiproliferative activity of TOB1 has been attributed to the suppression of cyclin D1 promoter activity (47). However, our data did not corroborate a direct relationship between TOB1 expression and decreased cyclin D1 protein levels in human breast cancer tissues. In contrast, we found that TOB1 protein expression levels were positively correlated with cyclin D1 expression in a cohort of more than 700 cases. We also observed a correlation between pTOB1 and both TOB1 and cyclin D1, which may imply that the antiproliferative activity of TOB1 is frequently overridden in breast cancer cells by TOB1 phosphorylation. Furthermore, we observed a nuclear localization of TOB1, which was shown to be important for its antiproliferative activity (50), in less than 1% of the cases (data not shown). This is in concordance with the correlation we see between TOB1 and cyclin D1 expression, which points to an abrogated antiproliferative TOB1 activity in the majority of examined tumors. These findings are supported by earlier studies that reported an increase of cytoplasmic TOB1 after transformation of cells with HER2 and concomitant suppression of antiproliferative activity despite high levels of TOB1 (41, 50). This is in agreement with our observations that TOB1 expression is significantly correlated with tumor size.

It is also of further interest to study the role of TOB1 in other breast cancer subgroups. In this study, we did not see an association of ER or PgR expression with TOB1 or pTOB1 on the protein level. Further studies might be able to shed light on TOB1 in clinically better-defined subgroups according to protein expression and treatment with tamoxifen, Herceptin, and Taxol.

Additional functional studies are needed to examine if TOB1 is a determinant of poor prognosis in HER2-positive breast cancers or simply an indicator of this phenotype. It seems of particular interest in this context to determine if TOB1 activity has relevance in...
the treatment of HER2- and EGFR-positive breast tumors with clinically approved or future therapies targeting erbB signaling.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

References
TOB1 Is Regulated by EGF-Dependent HER2 and EGFR Signaling, Is Highly Phosphorylated, and Indicates Poor Prognosis in Node-Negative Breast Cancer

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