A Polymorphism in the TC21 Promoter Associates with an Unfavorable Tamoxifen Treatment Outcome in Breast Cancer

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Abstract
Tamoxifen therapy is a standard in the treatment of estrogen receptor (ER)-positive breast cancer; however, its efficacy varies widely among patients. In addition to interpatient differences in the tamoxifen-metabolizing capacity, there is growing evidence that crosstalk between ER and growth factor signaling contributes to tamoxifen resistance. We focused on TC21, a member of the Ras superfamily, to investigate the influence of the TC21 −582C>T promoter polymorphism on mRNA expression and treatment outcome. Immunohistochemical analyses of breast tumors revealed a higher TC21 expression in ER-negative compared with ER-positive tumors. Expression in ER-positive tumors was higher in carriers of the T allele in an allele dose-dependent manner. Quantitative real-time PCR analyses showed that TC21 mRNA expression is decreased after transfection of ERα in ER-negative breast cancer cells MDA-MB-231, UACC893, and BT-20. In MCF7 ER-positive cells, TC21 expression decreased with 17β-estradiol treatment and increased after treatment with tamoxifen metabolites, 4-OH-tamoxifen, or endoxifen. In patients treated with adjuvant mono tamoxifen, high cytoplasmic TC21 tumor expression or the carriership of the −582T allele conferred increased recurrence rates [n = 45; hazard ratio (HR), 3.06; 95% confidence interval (95% CI), 1.16–8.05; n = 206: HR, 1.79; 95% CI, 1.08–3.00, respectively]. A combined analysis with the data of the known tamoxifen predictor CYP2D6 showed an improvement of outcome prediction compared with CYP2D6 or TC21 genotype status alone (per mutated gene HR, 2.35; 95% CI, 1.34–4.14). Our functional and patient-based results suggest that the TC21 −582C>T polymorphism improves prediction of tamoxifen treatment outcome in breast cancer. [Cancer Res 2008;68(23):9799–808]

Introduction
Recent developments in breast cancer treatment point to tamoxifen as a catalyst for the change to targeted therapy (1). Although 50% to 70% of all estrogen receptor (ER)-positive tumors are responsive to tamoxifen treatment, failure and tumor resistance represent major clinical problems, thus limiting the usefulness of tamoxifen therapy (2–5). Three possible mechanisms of drug resistance are currently under debate: Either the patient can influence the effectiveness of tamoxifen via alterations of drug metabolism called metabolic resistance, or the ER-positive tumor is or can become refractory to treatment called intrinsic or acquired resistance (5). Although tamoxifen itself is a produg, 4-hydroxy tamoxifen and endoxifen have been recognized as the clinically potent metabolites due to their 100 times higher affinity to ER (6, 7) and capability of breast cancer cell growth inhibition (8). To this end, it has become increasingly clear that the hosts cytochrome P450 2D6 (CYP2D6) enzyme activity, which is subject to variation (9) is critical for their formation (6, 10, 11). Detrimental pharmacogenetic effects have been reported in that individuals with a genetically determined impaired metabolizer phenotype had significantly less favorable recurrence-free time (RFT) and disease-free survival (12–14). Although this can be explained by interpatient differences in ER genomic activity, there is growing evidence that also ER nongenomic or membrane-initiated steroid signaling activities and crosstalk with growth factor signal transduction pathways may contribute to tamoxifen resistance. Activation of ER outside the nucleus leads to the activation of surface tyrosine kinase receptors (e.g., IGF-IR, epidermal growth factor receptor, and HER2) as well as interaction with cellular kinases and adaptor molecules [e.g., c-Src, Shc, p85α regulatory subunit of phosphatidylinositol-3-OH kinase (PI3K)], which in turn lead to the activation of mitogen-activated protein kinase (MAPK) and AKT pathways known to orchestrate cell proliferation and survival (15–17). These signaling pathways in turn can activate ER itself or its coactivators and corepressors, thereby increasing the potential of genomic/nuclear ER activity (5, 18). The role of the nongenomic steroid signaling calls for strategies for the identification of relevant factors of this intricate crosstalk that may add to the understanding of disease outcome under tamoxifen.

Among the key components of growth factor signaling are the Ras proteins for which an involvement in tamoxifen resistance has been suggested (19, 20). TC21 also known as R-Ras2 is a member of the Ras superfamily of GTP-binding proteins, which are major regulators of signaling pathways involved in cell division, migration, adhesion, differentiation, and apoptosis (21–24). Analogous to the classic H-, N-, and K-Ras proteins, TC21 is the only Ras member mutated in human cancers (25, 26). Its role in tumorigenesis and cell growth regulation has become evident from the following in vitro observations: GTPase deficient TC21 mutants showed transforming activities in NIH3T3 mouse fibroblasts and...
other cells (26–28), injection of TC21-transformed fibroblasts into nude mouse resulted in the formation of highly aggressive tumors (27, 28), and overexpression of wild-type TC21 caused transformation of MCF10a human breast epithelial cells (29). Although TC21 mutations are infrequent in human breast cancer cell lines and tumors (25), TC21 protein overexpression has been observed rather frequently in breast cancer cell lines (29). The TC21 oncogenic signals are mediated via the PI3K/Akt, nuclear factor-κB (NF-κB), and Ral signaling pathway (30–33), whereas a role in the activation of the extracellular signal-regulated kinase/MAPK cascade is less clear (32, 34–36).

The National Center for Biotechnology Information single nucleotide polymorphism (SNP) database holds numerous entries of genetic variations at Ras genes of which the TC21 (RBAS2) –582C>T promoter polymorphism (rs11023197) is listed with a minor allele frequency of 34% in Europeans. Due to the high prevalence and a possible functionality, we considered it important to follow-up this polymorphism. We performed functional analyses to elucidate the effect of the TC21 –582C>T polymorphism on protein expression and conducted patient-based association studies in breast cancer case collections treated with and without tamoxifen. Our data provide evidence for the pharmacogenetic relevance of a Ras protein in the endocrine treatment of breast cancer.

Materials and Methods

Cells, culture conditions, and transient transfections. MDA-MB-231, UACC893, BT-20 (all ER negative), and MCF7 (ER positive) breast cancer cells were cultured in DMEM supplemented with 10% FCS. ER-negative cells were transfected with 0, 5, 25, 50, 100, or 200 ng of ERα expression plasmid pcMV5ERα using the Effectene transfection reagent (Qiagen) according to manufacturer’s recommendations. Empty pcMV5 plasmid was used to fill up to a total amount of 200 ng of DNA per well. MCF7 cells were treated with 1 mmol/L 17β-estradiol, 50 mmol/L 4-OH-tamoxifen, or 50 mmol/L endoxifen for 24 h. In the case of estradiol treatment experiments, MCF7 cells were switched to medium supplemented with 5% charcoal-stripped serum for 48 h before treatment.

TC21 mRNA transcript quantification. RNA was isolated from cells using the Total RNA Isolation Reagent (ABgene) 24 h after transfection or treatment and reverse transcribed using the Promega M-MLV Reverse Transcriptase (Promega) according to manufacturer’s recommendations. Relative quantitative real-time PCR was performed by SybrGreen chemistry (Eurogentec) using the Applied Biosystems 7500 Real-time PCR System (Applied Biosystems). Gene expression was quantified in triplicates, and each experiment was replicated at least twice. The relative mRNA levels were calculated using the ∆ΔCt method, with the β-glucuronidase (GUS) as a normalizer. Primers were as follows: GUS (Fw: 5’-GCTCATTGT-GAAATTTCGGC-3’, Rv: 5’-ATCCCTTTTTATTCCCCAGC-3’); TC21 (Fw: 5’-AAGTACCCGCTGTCTGGTT-3’, Rv: 5’-GGTTGATCATATAATCGGTACA-3’); and ESR1 (Fw: 5’-CGGGCTCTGAAAATGCTAGC-3’, Rv: 5’-AAGATTC-CTCCTCCCTTCGG-3’). Statistical significance was determined using unpaired t test with Welch correction by GraphPad Prism version 3.03 software (GraphPad Software, Inc.).

Immunoblotting. Cells were lysed in 150 μL 1× Laemmli buffer [50 mmol/L Tris (pH 6.8), 100 mmol/L DTT, 2% SDS, 10% glycerol, 0.1% Bromphenolblue] 48 h after transfection or treatment. Lysates were separated by SDS-PAGE (15% polyacrylamide gel) and transferred to nitrocellulose membranes. Membranes were blocked with 5% skim milk/ TBST for 1 h at room temperature (RT). Immunostaining was done using antibodies specific for TC21 [sc-885 (V-20); Santa Cruz Biotechnology, 1:1,000], ERE [sc-9002 (F-10); Santa Cruz Biotechnology, 1:500], or β-Actin [A-5411 (AC-15); Sigma; 1:5,000] in 1% skim milk/TBST at 4 °C overnight, followed by anti-rabbit- or anti-mouse horseradish peroxidase antibodies (1:10,000; 1 h at RT). Immunoreactive proteins were detected by chemiluminescence using the SuperSignal Western blot kit (Perbio Science) and quantified with AIDA software (Raytest). Protein band intensities were normalized with β-Actin and statistical significance from three independent experiments was determined using unpaired t test with Welch correction by GraphPad Prism version 3.03 software (GraphPad Software).

Electrophoretic mobility shift assays. DNA-protein interaction was investigated by electrophoretic mobility shift assay (EMSA). The nuclear extract from MCF7 and MDA134 breast cancer cell lines was prepared as described in Schreiber and colleagues (37). Double stranded oligonucleotides used were TC21 –582C:T 5’-ggCTATTCTTCGCACGCCGTC-3’, TC21 –582T: 5’-ggCTATTCTTTCGACCGGCTC-3’, consensus ERE: 5’-gattGACAAATGCGTCATACGTACGTAACG-3’, and consensus E2F: 5’-ggTGGAATTTTCGCCCGTATG-3’. Experiments were performed as described in Rokavec and colleagues (38).

Immunohistochemistry. A tissue array containing 73 breast tumor samples (45 ER positive and 28 ER negative) was used to analyze the TC21 protein expression. Clinical characteristics of patients are given in Table 1 (expression collection). Immunostaining was done using 2 μg/mL of specific primary anti-TC21 rabbit polyclonal antibody (Santa Cruz Biotechnology) as described elsewhere (39). Stained tissues were evaluated by an expert pathologist and a scientist without knowledge of other sample characteristics including TC21 genotype. A cytoplasmic staining score was calculated from the percentage of stained cells (0–100%) and staining intensity (0–3) using the Remmnele method (40). The cytoplasmic staining of tumor tissues with scores 0 to 3 was considered negative/weakly positive, and with scores 4 to 8 strongly positive. Nuclear staining was scored from 0 (no staining) to 3 (strong staining). The differences in mean expression with respect to the genotype were calculated using one-way ANOVA. The correlations between TC21 expression and genotype status were calculated using nonparametric Spearman’s rank test. Statistical tests were done using GraphPad Prism 3.03 software (GraphPad Software, Inc.).

Patients, DNA extraction, and genotyping. Formalin-fixed, paraffin-embedded tumor specimens of 486 patients diagnosed with primary invasive breast cancer between 1986 and 2000 were obtained from the archival database at the Robert Bosch Hospital Breast Center, Stuttgart, Germany. This patient collection has been recently described by Schroth and colleagues (12) who reported on adjuvant tamoxifen breast cancer treatment outcome relative to the patients CYP2D6 and CYP2D19 genotypes. The adjuvant mono-tamoxifen treatment group (mTAM) included 206 ER-positive cases. A group of 280 cases without tamoxifen treatment that received either adjuvant chemotherapy or had no drug therapy served as control (noTAM group). Clinical characteristics of mTAM and noTAM patient groups are given in Table 1 (genotyping collection). A subset of this patient collection (n = 73) was used to set up a tissue array for immunohistochemical analyses referred to as expression collection (Table 1). The use of archival patient materials was approved by the local ethics committee of the University Tübingen. After histologic inspection, DNA was extracted from normal breast tissue using standard procedures. For genotyping, we used matrix-assisted laser desorption/ionization time-of-flight mass spectrometry described by Jaremko and colleagues (41).

Statistical analyses of treatment outcome associations. We tested for an association between TC21 expression or TC21 genetic variants and RFT or overall survival (OS). RFT was defined as the time from surgery to the occurrence of a breast event, i.e., local or distant recurrence, or contralateral breast cancer and OS was defined as the time from surgery to death from any cause. Patients who were alive and were without a breast event were censored at the date of the last follow-up inquiry. Time-to-relapse and survival data were analyzed by calculating Kaplan-Meier distributions. For combined CYP2D6/TC21 analysis, we used the genotype-derived CYP2D6-metabolizer status that categorizes patient's metabolic capacity with respect to the formation of active tamoxifen metabolites 4-OH tamoxifen and endoxifen taken from Schroth and colleagues (12). Metabolizer phenotypes are as follows: poor metabolizer (PM), intermediate metabolizers (IM), heterozygous extensive metabolizers (hetEM), and extensive metabolizer (EM). CYP2D6 PM, IM, and hetEM were combined into the variable CYP2D6 decreased. Statistical significance of a relationship between outcome and polymorphism was assessed by log-rank test. Cox regression analyses showed that tumor size and nodal status were
with 200 ng control vector (pCMV5) or with 200 ng ERα pCMV5ER cells BT-20, UACC893, and MDA-MB-231 transiently transfected endogenous UACC893, C/C; BT-20, C/C; MCF7, C/C. On RNA level, we compared TC21 showing the expression of ERα mRNA expression in ER-negative breast cancer cells BT-20, UACC893, and MDA-MB-231 transiently transfected with 200 ng control vector (pCMV5) or with 200 ng ERα pCMV5ER expression vector. TC21 mRNA expression was down-regulated in cells expressing ERα (in BT20 to 60%; P = 0.042, in UACC893 to 67%; P = 0.0021, and in MDA-MB-231 to 73%; P = 0.21; Fig. 1A). The control reverse transcription-PCR (RT-PCR) gel images show the expression of ERα after transfection with ERα expression

### Results

**TC21 expression is regulated by ERα.** First, we identified the TC21 –582T genotypes of the cell lines: MDA-MB-231, T/T; UACC893, C/C; BT-20, C/C; MCF7, C/C. On RNA level, we compared endogenous TC21 mRNA expression in ER-negative breast cancer cells BT-20, UACC893, and MDA-MB-231 transiently transfected with 200 ng control vector (pCMV5) or with 200 ng ERα pCMV5ER expression vector. TC21 mRNA expression was down-regulated in cells expressing ERα (in BT20 to 60%; P = 0.042, in UACC893 to 67%; P = 0.0021, and in MDA-MB-231 to 73%; P = 0.21; Fig. 1A). The control reverse transcription-PCR (RT-PCR) gel images show the expression of ERα after transfection with ERα expression vector. Next, we investigated the effect of estrogen and tamoxifen metabolites on TC21 expression. Compared with nontreated ER-positive MCF7 cells, the expression of TC21 was reduced by 23% when the cells were treated with 1 nmol/L 17β-estradiol (P = 0.16; Fig. 1B). In contrast, TC21 expression was 2.3- and 2.4-fold higher when treated with 50 nmol/L 4-OH-tamoxifen (P = 0.019) or endoxifen (P = 0.003), respectively (Fig. 1C) for 24 hours.

Subsequently, we analyzed the regulation of TC21 expression on protein level. In BT-20 and UACC893 cells (TC21 –582C/C genotype), the TC21 protein levels were down-regulated to 20% and 15%, respectively, after transfection of 50 ng pCMVErα with no further reduction with increasing amounts of pCMVErα (Fig. 2A). However, in MDA-MB-231 cells (–582T/T), the TC21 protein levels were less strongly down-regulated to 40% only with higher amounts of pCMVErα (Fig. 2A). TC21 protein quantification revealed significant differences in the capacity of TC21 down-regulation by ERα between MDA-MB-231 and BT-20 or UACC893 cells (P = 0.0083 and 0.0075, respectively; Fig. 2A). There was no difference between the two C/C genotype cell lines BT-20 and UACC893 (P = 0.22). After treatment of estrogen-deprived MCF7 cells with 1 nmol/L 17β-estradiol for 48 hours, the TC21 protein levels decreased (Fig. 2B). When MCF7 cells were treated with 50 nmol/L 4-OH-tamoxifen or endoxifen for 48 hours, the protein levels of TC21 increased (Fig. 2C). Of note, the normalized TC21 expression in all nontransfected ER-negative cells was ~3 to 4 times higher compared with nontreated MCF7 cell line. We also observed a strong reduction of ERα expression after treatment with 17β-estradiol (Fig. 2B), which may explain the weak nonsignificant reduction of TC21 expression observed in Figs. 1B and 2B.

The **TC21 –582T allele is associated with elevated TC21 protein expression in ER-positive breast tumors.** We analyzed the TC21 expression in tumor tissues from 73 breast cancer patients by immunohistochemistry (Fig. 3C and D). Of these specimens, 45 tumors were previously diagnosed ER positive and 28 ER negative. TC21 protein was observed as cytoplasmic and/or nuclear staining. The cytoplasmic TC21 expression was higher in ER-negative compared with ER-positive tumors; however, the difference did not reach statistical significance (P = 0.09). With respect to nuclear TC21 expression, there was no difference. In ER-positive tumors, the cytoplasmic TC21 expression was significantly higher in carriers of the heterozygote –582C/T genotype and even higher in carriers of the homozygous minor –582T/T genotype, suggesting an allele dose-dependant effect (C/C versus C/T, P < 0.05; C/T versus T/T, P < 0.01; C/C versus T/T, P < 0.001; Fig. 3A). The correlation between protein expression and genotypes was significant for percentage of stained cells (r = 0.52; P = 0.0006), staining intensity (r = 0.46; P = 0.003), and expression score (r = 0.55; P = 0.0003). There was no correlation between cytoplasmic TC21 expression and –582 genotypes in ER-negative

Table 1. Patient and tumor characteristics for the two breast cancer patient collections used for TC21 genotyping and TC21 protein expression measurements

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Genotype collection</th>
<th>Expression collection</th>
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<tbody>
<tr>
<td></td>
<td>mTAM* (n = 206)</td>
<td>NoTAM* (n = 280)</td>
</tr>
<tr>
<td>Age (y)</td>
<td>Median 68.4</td>
<td>Median 56.1</td>
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<tr>
<td>Follow up (mo)</td>
<td>Range 40.1 to 91.8</td>
<td>Range 28.7 to 88.1</td>
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<tr>
<td>Tumor size</td>
<td>Median 76.6</td>
<td>Median 68.9</td>
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<tr>
<td>≤2 cm</td>
<td>Range 8.1 to 227.2</td>
<td>Range 4.3 to 198.6</td>
</tr>
<tr>
<td>2–5 cm</td>
<td>92 (45.1%)</td>
<td>112 (40.6%)</td>
</tr>
<tr>
<td>&gt;5 cm</td>
<td>94 (46.1%)</td>
<td>128 (46.4%)</td>
</tr>
<tr>
<td>Nodal status</td>
<td>N0 129 (69.4%)</td>
<td>N1 148 (55.6%)</td>
</tr>
<tr>
<td></td>
<td>Range 18 (8.8%)</td>
<td>111 (41.7%)</td>
</tr>
<tr>
<td></td>
<td>N1 52 (28.0%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>N2 5 (2.6%)</td>
<td></td>
</tr>
<tr>
<td>Grading</td>
<td>G1 18 (8.8%)</td>
<td>G2 166 (59.7%)</td>
</tr>
<tr>
<td></td>
<td>17 (6.1%)</td>
<td>1 (1.4%)</td>
</tr>
<tr>
<td>ER status</td>
<td>ER+ 206 (100.0%)</td>
<td>ER 147 (53.8%)</td>
</tr>
<tr>
<td></td>
<td>— —</td>
<td>126 (46.2%)</td>
</tr>
</tbody>
</table>

Abbreviations: mTAM, patients with adjuvant tamoxifen monotherapy; noTAM, patients with other than tamoxifen regimens.

*Differences in numbers refer to unavailable information.

significantly correlated to RFT in the patient collections. Thus, in multivariate Cox regression analyses, these two prognostic factors were used for adjustment and to test for an independent contribution of genetic factors to the outcome variable assuming an additive genetic model. Statistical tests were run using SPSS software version 12.1.
tumors (Fig. 3A). With respect to nuclear TC21 expression, we did not observe any significant correlation with -582 genotypes, neither in ER-negative, nor in ER-positive tumors (data not shown). We furthermore compared cytoplasmic TC21 expression between ER-positive and ER-negative tumors according to patients' C0 genotype. In carriers of the C/C genotype, the TC21 expression was significantly higher in ER-negative compared with ER-positive tumors. No significant differences were observed in carriers of the C/T and T/T genotypes (Fig. 3B).

No specific protein-DNA interactions were observed in EMSA assays. We tested whether the observed variation of the allele-dependent TC21 expression may be explained by direct DNA binding of nuclear factors and performed EMSA assays using nuclear proteins from ER-positive MCF7 and MDA134 cells. Experiments were carried out with different binding buffers and different nonspecific competitors, but no DNA-protein complexes, neither with the C, nor with the T allele oligonucleotide probes were observed (data not shown). Control reactions with ERE consensus oligonucleotide probe showed a specific DNA-bound ERα complex using identical conditions. Furthermore, in silico analyses suggested that E2F proteins may bind to the TC21/C0 region, a reason why we included control reactions with E2F consensus oligonucleotides in which we observed a DNA-bound E2F complex.

Breast cancer treatment outcome with adjuvant tamoxifen is related to TC21 expression in breast tumors. High cytoplasmic TC21 expression was observed in 17 tumors (44%) of 39 cases treated with adjuvant tamoxifen. These patients had increased recurrence rates when compared with patients with low or no TC21 expression (22 patients; $P = 0.0053$; Fig. 4A). Adjusted analysis for the two prognostic factors tumor size and nodal status showed a significant nonfavorable RFT [hazard ratio (HR), 3.06;
95% confidence interval (95% CI), 1.16–8.05; P = 0.023] for patients with high expression of TC21. No such association was observed in patients not treated with adjuvant tamoxifen (Fig. 4B). In neither group, we observed a significant association between TC21 expression and OS.

Breast cancer treatment outcome after adjuvant tamoxifen is related to patients’ TC21 –582 genotypes. In the total of 486 breast cancer patients, genotype frequencies were 38.9% for C/C, 45.9% for C/T, and 15.2% for T/T; they were in Hardy-Weinberg equilibrium and matched those reported for populations of European descent. Genotype frequencies did not differ between treatment groups. Patient and tumor characteristics are shown in Table 1. In the mTAM group, the minor TC21 –582T allele was associated with a higher frequency of relapse (odds ratio, 1.87; P = 0.018). Kaplan-Meier estimates showed a significant allele-dose-dependent unfavorable RFT for carriers of the T allele compared with carriers of the major C/C genotype (P = 0.022; Fig. 4C). Furthermore, adjusted analysis for the two prognostic factors tumor size and nodal status showed a significant nonfavorable RFT (per allele HR, 1.79; 95% CI, 1.08–3.00; P = 0.025) for patients carrying the minor T allele. No associations between genotypes and treatment outcome were observed in ER-positive patients not treated with tamoxifen (n = 141; Fig. 4D). This was also true for ER-negative patients. In neither group, we observed a significant association between genotypes and OS.

Analyses of the combined TC21 and CYP2D6 genotypes in patients treated with adjuvant tamoxifen monotherapy. We combined the TC21 genotypes with CYP2D6 genotype/phenotype data previously reported for this patient collection by Schroth and colleagues (12) for an extended analysis of joint effects on treatment outcome. Kaplan-Meier estimates showed a significant unfavorable RFT for carriers of mutated genotypes at both genes. Patients with a TC21 –582 C/T or T/T genotype who also were carriers for CYP2D6 genotype predisposing to decreased enzyme function (IM, PM, and hetEM) had a significantly unfavorable RFT when compared with patients with wild-type genotypes in both genes. Patients with mutations in either one gene had less favorable RFT when compared with patients being wild-type at both genes but more favorable RFT when compared with patients with both genes mutated. The RFT was similar between patients with wild-type TC21 and mutated CYP2D6 and patients with mutated TC21 and wild-type (EM) CYP2D6. Altogether, the distribution of Kaplan Meier curves suggest an allele dose-dependent effect of the combined TC21/CYP2D6 associated RFT (log-rank P = 0.0022; Fig. 5). The combined analyses revealed that patients that were wild-type at both genes (26%) had a relapse-free probability of >93% at 5 years. In contrast, patients with both genes

**Figure 2.** TC21 protein expression is regulated by ERα in an allele-dependent manner. A, BT-20, UACC893, and MDA-MB-231 cells (TC21 –582 genotypes: C/C, C/C, and T/T, respectively) were transiently transfected with increasing amounts of the pCMV5ERα expression vector. TC21 protein levels were quantified, normalized with β-Actin, and expressed as a percentage of the nontransfected cells based on three experiments. TC21 expression was down-regulated at significantly lower amounts of ERα in BT-20 or UACC893 cells, compared with MDA-MB-231 cells (P = 0.0083 and 0.0075, respectively). There was no significant difference between BT-20 and UACC893 cells (P = 0.22). B, treatment of MCF7 cells with estradiol led to a decrease in TC21 protein levels. MCF7 cells were estrogen deprived and treated with 1 nmol/L 17β-estradiol or vehicle (ethanol) for 48 h. C, treatment of MCF7 cells with tamoxifen metabolites increased TC21 protein levels. MCF7 cells were treated with 50 nmol/L 4-OH-tamoxifen or 50 nmol/L endoxifen for 48 h. Lysates were immunoblotted as indicated.

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mutated (22%) had a relapse-free probability of only 70% at 5 years. In Cox-regression analyses, we combined the intermediate groups of patients with either mutated gene to obtain the following three patient groups: TC21_wt/CYP2D6_EM, TC21_wt/CYP2D6_decreased plus TC21_mut/CYP2D6_EM, and TC21_mut/CYP2D6_decreased. By adjusting for the two prognostic factors, tumor size and nodal status, a significant nonfavorable RFT for patients with one or two genes mutated was observed (per mutated gene HR,
A comparison of the distribution of TC21 and CYP2D6 genotypes in our patient collection showed that they segregated independently (P = 0.59). We did not observe a significant association between combined genotypes and OS.

Discussion

This study focused on TC21, a member of the Ras protein family, which may participate in membrane receptor signaling mediated nongenomic ER action and, hence, potentially contributes to tamoxifen resistance. We performed functional analyses of TC21 expression as well as patient-based investigations to provide evidence that TC21 may be involved in ER/growth factor crosstalk and influences disease outcome under tamoxifen.

Microarray analyses of breast tumors showed a consistent association between TC21 mRNA levels and ER status in that TC21 expression was higher in ER-negative compared with ER-positive tumors (ONCOMINE-CANCER PROFILING DATABASE). Our own data showed that also on the protein level the TC21 expression is higher in ER-negative compared with ER-positive tumors and cell lines. These findings, together with our quantitative RT-PCR and Western blot data, indicate that TC21 expression is repressed by ER. Moreover, our observation of the −582 genotype based interpatient variation of TC21 expression in ER-positive breast tumors suggests that this repression is allele dependent. TC21 expression was significantly higher in carriers of the minor −582T allele and the increase of expression followed an allele dose–dependent manner. In a comparison of TC21 expression between ER-positive and ER-negative tumors according to patients −582 genotype, we observed a significant difference only in patients with the C/C genotype. In ER-negative cell lines carrying the C/C

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genotype, TC21 protein levels were strongly decreased at low amounts of ERs, whereas in a T/T cell line only, a modest decrease was observed even in the presence of high amounts of ERs. Altogether, these data suggest that ERs predominantly repress the C allele but not the T allele.

The observed allelic difference in expression may have its origin in variable binding affinities of ERs to C and T alleles. Yet, our in silico search did not identify sequences reminiscent of estrogen responsive elements (ERE) within the critical region, and on the experimental level, specific EMSA experiments did not reveal any ER binding even when using various nuclear extracts and experimental conditions. It is well-known that in addition to direct binding of ER to specific EREs, ER may transactivate target genes by interacting with other transcriptional factors such as Sp1, activator protein, and NF-kB. We performed EMSA experiments with nuclear extracts of cell lines in which we observed ER-mediated changes in TC21 mRNA expression, pointing to the presence of proteins relevant to these changes. However, in the light of EMSA being an in vitro method, there is a possibility that we might have not been able to reproduce the in vivo conditions under which the regulatory mechanisms of the TC21 promoter may involve ER-dependent transcriptional regulation. An alternative explanation would be that the TC21 −528C>T is not the causative variant but rather is in linkage disequilibrium with another functional polymorphism in the upstream promoter region. We therefore examined linkage disequilibrium patterns across the chromosomal region surrounding the −582C>T polymorphism and performed in silico analyses to study transcription factor binding sites that could be altered by SNPs linked with the −582C>T (Supplementary Table S1). We did not observe any EREs in these SNP regions, but there are Sp1 and Oct-1 binding sites, which could be indirectly regulated by ER. Future functional and association studies therefore should address the biological relevance of these linked SNPs.

Based on our hypothesis of TC21 being involved in ER/growth factor crosstalk, we further asked the question whether TC21 protein expression and in particular the TC21 −582C>T polymorphism hold the potential to improve breast cancer prognosis and prediction of disease outcome under tamoxifen. In the adjuvant tamoxifen patient group in which tamoxifen was the sole anticancer drug treatment, patients with strong cytoplasmic TC21 expression in their tumors had significantly less favorable RF rates when compared with patients with no or weak TC21 expression. No such differences were observed among patients without tamoxifen treatment. Our observation of TC21 expression being a predictor of tamoxifen response is in line with data from a microarray gene expression study by Ma and colleagues (42), which is accessible at the Gene Expression Omnibus (accession number GSE1378). This study provided expression and clinical data of 60 ER-positive breast cancer patients treated with adjuvant tamoxifen. Upon calculation of Kaplan-Meier distributions, we observed a significant nonfavorable RF probability for patients with high TC21 mRNA expression (log2 Cy5/Cy3 ratio, >−1.0) compared with patients with low TC21 expression (log2 Cy5/Cy3 ratio, <−1.0).

Stratification of our adjuvant mono-tamoxifen-treated patient collection by the TC21 −582 genotype showed a significant less favorable RF probability for carriers of the minor T allele. Again, these differences were not observed in patients without tamoxifen treatment. We conclude that the TC21 is a useful classifier of tamoxifen treatment outcome, a notion that is in agreement with the current literature (16, 43) on ER and growth factor signaling.

Based on our observation that the presence of tamoxifen metabolites increases TC21 expression, we suggest that a tamoxifen-mediated increase of TC21 levels may contribute to an increase of growth factor signaling, thus promoting the adaptive type of tamoxifen resistance. To this end, the crosstalk between ER and growth factor signaling associated with resistance to tamoxifen is contributed by ER nuclear and ER membrane-initiated steroid signaling (17). Of note, the latter can be activated by both estrogen and selective ER modulators such as tamoxifen (44, 45). Accordingly, the survival of breast cancer cells even in the presence of tamoxifen (46) has been attributed to the bidirectional crosstalk between ER and growth factor receptor-initiated signaling cascades, e.g., PI3K/Akt and MAPK. This self-stimulatory cycle-intensifying ER activity may be contributed by TC21 at the level of Ras signaling, which may be driven by ER-dependent TC21 regulation on the level of ER target gene expression.

In the light of our recent findings of genetically determined impaired CYP2D6 activity being a predictor for nonfavorable outcome, we performed TC21/CYP2D6 genotyping. Carriers of risk alleles at both genes (TC21/mut/CYP2D6 decreased) had increased recurrence rates when compared with patients with one (TC21/wt/CYP2D6 decreased or TC21/mut/CYP2D6/EM) or no (TC21/wt/CYP2D6/EM) risk allele in an allele dose-dependent manner. The number of patients included in Kaplan-Meier analyses differs from those of the entire collection due to failure of genotyping.
tamoxifen treatment outcome of breast cancer (12, 47), we
extended the analysis of RF probability as a function of combined
TC21 and CYP2D6 genotypes. Importantly, the individual TC21
and CYP2D6 analyses were performed in the same patient
collection and both polymorphisms segregated independently;
therefore, the assessment of combined effects was reasonable.
At 5-year follow-ups, patients with major genotypes at both loci
(TC21^wt/2D6^EM) had an RF probability of 93% and this
remained at high level even on long term. Of note, the combined
data show a 12% to 14% improved RF probability compared
with CYP2D6EM or TC21^wt status alone. In contrast, the 5-year RF
probability was only 70% in patients with mutated genotypes in
either genes (TC21^mut/2D6^decreased) and worsened over time in
that at 10 years, every other patient had experienced recurrence.
Patients with a mutated genotype in either TC21 or CYP2D6 had
less favorable RFI when compared with patients with both regular
genotypes but more favorable RFI when compared with patients with both genes mutated. This pattern suggested a compound
TC21-CYP2D6 gene dose-dependent relationship.
Interestingly, RFI probability curves were similar, independent
of whether TC21 or CYP2D6 was mutated. Accordingly, a
nonmutated TC21 was capable to partially overcome the disadvantage of an impaired CYP2D6 genotype. In contrast, a
mutated TC21 was able to partially override the favorable effect
of a CYP2D6EM genotype toward a less favorable effect on RFI.
Strikingly, the effect of TC21 on RF rates was most pronounced in patients with CYP2D6-decreased genotype, suggesting an additive
effect of TC21 that modulates a patient's constitutional resistance
based on CYP2D6 genotypes. For a judgment of the combined
pharmacogenetic relevance of both genes in tamoxifen resistance,
it is important to recall that although genotypes have been
established at the constitutional level, their functional conse-
quences in case of CYP2D6 affect the hosts' tamoxifen metabolic
capacity and, in case of TC21, the tumor bound ER growth factor
crosstalk. Our findings therefore support the notion that
tamoxifen resistance may depend on the level of both, host
genetic, and tumor-adaptive elements (5). We speculate that
complementary TC21 and CYP2D6 analyses will identify patients
likely to benefit from tamoxifen and those in need for alternative
treatment with a higher accuracy due to the different underlying
biological principles.

In summary, our TC21 functional and patient-based pharmaco-
genetic findings are congruent and in line with current views on
tamoxifen resistance. TC21 may therefore be regarded as a novel
candidate for the testing of its value in the prediction of tamoxifen
treatment outcome in larger studies and prospective clinical trials.
To the best of our knowledge, this study for the first time draws
attention to the relevance of a Ras protein in the context of
tamoxifen pharmacogenomics of breast cancer.

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A Polymorphism in the TC21 Promoter Associates with an Unfavorable Tamoxifen Treatment Outcome in Breast Cancer

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