Advances in Brief

Combined Vascular Endothelial Growth Factor and *TP53* Status Predicts Poor Response to Tamoxifen Therapy in Estrogen Receptor-positive Advanced Breast Cancer[1]

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Abstract

Purpose: **In recent studies, we showed that *TP53* gene mutation or high levels of cytosolic vascular endothelial growth factor (VEGF) in estrogen receptor (ER)-α-positive primary breast tumors predict a poor disease outcome for patients treated with first-line tamoxifen for advanced disease. Mutant TP53 may up-regulate VEGF, whereas, on the other hand, wild-type TP53 may decrease VEGF production.**

Experimental Design: **In the present study, we aimed to assess the combined predictive value of *TP53* gene mutation and VEGF status of 160 advanced breast cancer patients with ER-positive tumors who were treated with tamoxifen (median follow-up from start of tamoxifen treatment, 64 months). To assess *TP53* gene mutation status, the entire open reading frame was sequenced; for VEGF status, an ELISA was used.**

Results: **In univariate analysis, both *TP53* gene mutation (28% of the tumors) and a VEGF level above the median value were significantly associated with a short progression-free survival, post-relapse overall survival, and a poor rate of response to tamoxifen. In Cox multivariate regression analysis including the traditional predictive factors, the addition of *TP53* gene mutation and VEGF status, alone or in combination, significantly predicted a poor efficacy of tamoxifen treatment. When the two factors were combined, a significantly decreased odds ratio was seen for the rate of response (odds ratio, 0.27). Similarly, an increased hazard ratio (HR) was seen for progression-free survival (HR, 2.32) and post-relapse overall survival (HR, 1.68) in the group with mutant *TP53* and high VEGF compared with the group with both risk factors absent.**

Conclusions: **Combined *TP53* gene mutation status and high VEGF levels of ER-positive primary breast tumors independently predict a poor course of the disease of patients with advanced breast cancer treated with tamoxifen. These patients, having unfavorable tumor characteristics, might benefit more from other types of (individualized) treatment protocols.**

Introduction

Although tamoxifen is the endocrine treatment of choice for all stages of breast cancer (1), approximately one-half of the patients with ER[3]-α-positive breast tumors either do not respond or rapidly develop resistance to tamoxifen treatment. Because response rates in patients with ER-α-negative primary tumors are low, resistance to this nonsteroidal antiestrogen is a major clinical problem. A large number of cell biological factors, other than ER, were shown to be related with a favorable or poor type of response to endocrine treatment (for review, see Ref. 2). The estrogen-regulated PgR or pS2 gene expression has, for example, been associated with a favorable response. Conversely, other tumor cell biological factors, tyrosine kinases such as epidermal growth factor receptor (3) or its family member HER2 (4), the tumor suppressor gene *TP53* (5), urokinase-type plasminogen activator (6), human kallikrein 10 (7), and VEGF (vascular permeability factor; Ref. 8), were associated with a poor response of metastatic breast cancer to tamoxifen.

VEGF is a potent angiogenic factor, and the common oncogenes and tumor suppressor genes associated with neoplastic cell transformation also play an integral part in activating the angiogenic switch. Alterations in these genes, including *RAS*, *SRC*, *HER2*, and *TP53*, have been characterized as inducers of VEGF expression (9). Mutant TP53 is reported to up-regulate VEGF, whereas wild-type TP53 may decrease VEGF production and increase levels of the angiogenesis inhibitors (10).

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[3] The abbreviations used are: ER, estrogen receptor; VEGF, vascular endothelial growth factor; PFS, progression-free survival; PR-OS, post-relapse overall survival; OR, odds ratio; HR, hazard ratio; CI, confidence interval; PgR, progesterone receptor; CR, complete remission; PR, partial remission; SDis, stable disease; DFI, disease-free interval; d.f., degree(s) of freedom.

Interestingly, Pal *et al.* (11) recently showed a direct effect of TP53 on VEGF, in that TP53 makes a complex with Sp1 and thereby inhibits Sp1-mediated VEGF transcriptional activation in breast cancer cell lines.

In view of the facts that (*a*) TP53 regulates VEGF production, (*b*) *TP53* and VEGF each predict a poor efficacy of tamoxifen treatment in ER-positive primary breast cancer (5, 8), and (*c*) the combined VEGF with TP53 status resulted in better survival prediction in primary breast cancer (12), we hypothesized that combining these biological factors may give additional information on tamoxifen response in patients with advanced breast cancer.

In the present study, we aimed to assess whether the combined VEGF and TP53 status may be predictive for the type of response to tamoxifen therapy, PFS, and PR-OS in a series of ER-positive patients with advanced breast cancer.

Materials and Methods

Patients and Treatment. Our study design was approved by the medical ethical committee of the Erasmus MC (Rotterdam, the Netherlands). To evaluate the predictive value of combined *TP53* gene mutation and cytosolic VEGF status in relation to tamoxifen treatment in patients with advanced breast cancer, the 160 ER-positive tumors (≥10 fmol/mg protein) overlapping from earlier studies on the predictive value of *TP53* gene mutation (5) and VEGF protein levels (8) were used. The HER2 status is not known. All of the patients received tamoxifen (40 mg daily) as first-line treatment after diagnosis of advanced disease (median age, 61 years; range, 33–82 years). At the start of tamoxifen treatment, 125 (78%) patients were postmenopausal, and 35 (22%) were premenopausal. None of the patients had received neoadjuvant therapy or were exposed to hormonal treatment at an earlier stage. Eleven patients (7%) were diagnosed with metastatic disease (M_1) at time of primary surgery (breast-conserving lumpectomy, 51 patients; modified mastectomy, 109 patients). Twenty-one patients (13%) received systemic adjuvant chemotherapy [cyclophosphamide, methotrexate, and 5-fluorouracil (CMF), 14 patients; cyclophosphamide, Adriamycin, and 5-fluorouracil (CAF), 7 patients]. The median follow-up of patients alive is 108 months (range, 66–167 months) from primary surgery, and 64 months (range, 21–100 months) from the onset of tamoxifen treatment. During follow-up, 140 patients (88%) have died with a median survival time of 24 months. Tumor progression occurred in 157 (98%) of the patients. After tumor progression on first-line tamoxifen treatment, 118 patients were treated with one or more additional endocrine agents (mainly high-dose progestins), whereas, thus far, 100 patients were subsequently treated with one or more regimens of chemotherapy (mainly CMF or CAF) after the occurrence of hormonal resistance. Criteria for PFS, PR-OS, and type of response were described previously (8). As explained, CR and PR (CR, 13 patients; PR, 18 patients) and SDis for more than 6 months (SDis, 64 patients) were combined for overall response (8).

Assays of *TP53* Gene Mutation and VEGF. Results from the cDNA-based sequencing of the entire open reading frame (exons 2 through 11) of the *TP53* gene were taken from Berns *et al.* (5). The results of the measurements of the cytosolic levels of VEGF isoforms 121 and 165, ER, and PgR were taken from Foekens *et al.* (8).

Statistics. The strength of the associations of VEGF with ER and PgR were tested with Spearman rank correlation (r_s). The associations of VEGF (used as a continuous variable) with other variables (used as grouping variables) were tested with the nonparametric Wilcoxon rank-sum test or the Kruskal-Wallis test. The associations of *TP53* gene mutation with other variables were tested with the Pearson's χ^2 test, whereas the Wilcoxon rank-sum test was used to test the association between PgR and gene mutation. In uni- and multivariate analysis, the relation with response to therapy was examined with logistic regression analysis, and the ORs were calculated. Cox univariate and multivariate regression analysis was used in the analysis of PFS and PR-OS, and HRs were calculated. The associated likelihood ratio test was used to test for differences between multivariate models with variables included and excluded. ORs and HRs are presented with their 95% CIs. Survival curves were generated using the method of Kaplan and Meier, and the log-rank test for trend was used to examine survival data. For all tests, all available data during the follow-up period were included. All computations were performed with the STATA statistical package, release 7.0 (STATA Corp., College Station, TX). All *P*s are two-sided.

Results

VEGF Levels, *TP53* Gene Status, and Associations. The entire open reading frame of the *TP53* gene of 160 female breast tumors was sequenced. Mutations were detected in 44 (28%) of the tumors, of which 33 were missense mutations. The remaining 11 mutations (referred to as nonmissense mutations) included 3 in-frame deletions/insertions and 8 nonsense or frameshift mutations, both leading to a premature stop codon. Therefore, one-fourth of the mutations would be false negatives when immunohistochemical analyses were used to assess the TP53 status.

The relationships of *TP53* gene mutation and the level of VEGF with patient and tumor characteristics and with each other are listed in Table 1. The median level of VEGF determined in all 160 cytosols was 0.22 ng/mg protein (range, 0–27 ng/mg protein). Consistent with the reported up-regulation of VEGF by mutant TP53 and down-regulation by wild-type TP53, in cytosols prepared from 44 tumors with a *TP53* gene mutation, the median VEGF level (0.39 ng/mg protein) was twice as high ($P = 0.04$) compared with the 116 cytosols prepared from tumors without a *TP53* gene mutation (0.19 ng/mg protein). Of the 44 tumors with a *TP53* gene mutation, higher cytosolic levels of VEGF were found in the 11 tumors with a nonmissense mutation (median, 0.43 ng/mg protein) compared with the 33 tumors with a missense mutation (0.30 ng/mg protein). This difference was not statistically significant ($P = 0.21$). In the present series of 160 tumors, the prevalence of *TP53* gene mutation and the levels of VEGF, in accordance with their reported association with a poor prognosis in primary breast cancer, are highest in tumors of patients with a short DFI (Table 1). There were no significant relationships between the prevalence of *TP53* gene mutation or the level of VEGF and the levels of ER (data not shown) or PgR within these ER-positive tumors

Table 1 Relationships of *TP53* gene status and VEGF with patient and tumor characteristics

Characteristic	Frequency	Mutated *TP53* gene		VEGF	
		No. (%)	*P*	Median value (quartiles)[a]	*P*
All patients	160	44 (28)		0.22 (0, 0.75)	
Menopausal status[b]					
Premenopausal	55	12 (22)		0.18 (0, 0.43)	
Postmenopausal	105	32 (30)	0.24[c]	0.23 (0.02, 0.79)	0.15[d]
Dominant site of relapse[e]					
Soft tissue	20	9 (45)		0.12 (0, 0.52)	
Bone	81	17 (21)		0.22 (0.02, 0.56)	
Viscera	59	18 (31)	0.08[c]	0.28 (0, 0.81)	0.54[f]
DFI					
≤1 yr	45	18 (40)		0.48 (0.18, 2.34)	
>1 yr	115	26 (23)	0.03[c]	0.17 (0, 0.45)	<0.0001[d]
PgR status[g]					
Negative	24	8 (33)		0.34 (0.10, 0.48)	
Positive	135	36 (27)	0.13[d]	0.20 (0, 0.78)	0.48[h]
TP53 gene mutation					
No	116			0.19 (0, 0.54)	
Yes	44			0.39 (0.10, 1.45)	0.04[d]

[a] All of the values are in ng/mg protein (25th and 75th percentiles).
[b] At time of primary surgery.
[c] *P* for Pearson's χ^2 test.
[d] *P* for Wilcoxon's rank-sum test.
[e] In case of multiple sites, the site with the worst prognosis was considered dominant.
[f] *P* for Kruskal-Wallis test.
[g] Cutoff point used: 10 fmol/mg protein. PgR value is missing for one patient.
[h] *P* for Spearman rank correlation.

(Table 1). In an exploratory analysis, the factors were combined and related to the dominant site of relapse. Those tumors with no mutation and low ($n = 63$) or high ($n = 53$) levels of VEGF, respectively, revealed bone (51% or 60%, respectively) and viscera (38% or 32%, respectively) as dominant sites of relapse. In tumors with a *TP53* gene mutation but with low levels of VEGF ($n = 17$), bone was the dominant site of relapse (47%), followed by soft tissue (29%) and viscera (24%). In contrast, for those tumors with high levels of VEGF and with a TP53 mutation ($n = 27$), viscera (52%) was the main site of relapse, followed by bone (33%) and soft tissue (15%).

Univariate and Multivariate Analysis for PFS and PR-OS. In Cox univariate regression analysis, *TP53* gene mutation status was associated with a poor PFS (HR, 1.53; 95% CI, 1.08–2.19; $P = 0.02$) and PR-OS (HR, 1.56; 95% CI, 1.08–2.26; $P = 0.02$). Similarly, a high level of VEGF (equal to or above the median value of 0.22 ng/mg protein) was associated with a short PFS (HR, 1.55; 95% CI, 1.12–2.13; $P = 0.007$) and PR-OS (HR, 1.63; 95% CI, 1.16–2.29; $P = 0.005$). To assess whether there was an independent relationship of these factors with PFS or PR-OS, Cox multivariate regression analysis was performed. After inclusion of the traditional predictive factors age and menopausal status, dominant site of relapse (bone *versus* soft tissue, viscera *versus* soft tissue, DFI of <1 year *versus* >1 year), ER and PgR (both as a continuous variable), and stepwise elimination of the nonsignificant variables, a base model containing dominant site of relapse and DFI was defined (Table 2). The other factors, as well as adjuvant chemotherapy (which was added as an indicator variable), did not significantly add to the multivariate models for PFS and PR-OS in these ER-positive tumors. When added separately or together to the base model for PFS, *TP53* gene mutation and a high VEGF level both independently predicted a poor PFS. The increase in χ^2 ($\Delta\chi^2$) by the addition of *TP53* gene mutation and VEGF status as a combined variable to the base model of PFS was 11.0 (d.f. = 2; $P = 0.004$). Similarly, in analysis for PR-OS, the addition of the combined variable showed a $\Delta\chi^2$ of 8.0 (d.f. = 2; $P = 0.02$).

In Kaplan-Meier analysis as a function of combined *TP53* gene mutation and VEGF status, patients with a nonmutated *TP53* gene and a low VEGF level in their primary tumor showed the longest PFS (Fig. 1*A*) and PR-OS (Fig. 1*B*), with a median time to progression and death of 10 and 35 months, respectively. Patients with tumors containing one of two of the unfavorable factors showed an intermediate PFS (median, 8 months; HR, 1.46; 95% CI, 1.03–2.08; $P = 0.03$) and PR-OS (median, 23 months; HR, 1.69; 95%, 1.16–2.45; $P = 0.006$), and those with both a *TP53* gene mutation and a high VEGF level showed the shortest PFS (median, 5 months; HR, 2.17; 95% CI, 1.37–3.44; $P = 0.001$) and PR-OS (median, 19 months; HR, 2.13; 95% CI, 1.31–3.46; $P = 0.002$).

Response to Tamoxifen Treatment. Of the 160 patients, 95 (59%) responded (13 CR, 18 PR, and 64 SDis) with a median duration of response of 14 months (range, 3–71 months). In tumors with a *TP53* gene mutation or a high level of VEGF, the overall response rates were 45% (OR, 0.46; 95% CI, 0.23–0.92; $P = 0.03$) for tumors with a mutated *TP53* gene and 51% (OR, 0.51; 95% CI, 0.27–0.96; $P = 0.04$) for those with high levels of VEGF. Consistent with the results observed in the analyses of PFS and PR-OS, in multivariate logistic regression analysis for response, the combined variable of *TP53* gene mutation and VEGF status independently ($\Delta\chi^2 = 6.3$; d.f. = 2;

Table 2 Multivariate analysis for PFS and PR-OS after start of first-line tamoxifen treatment in patients with advanced breast cancer[a]

	PFS			PR-OS		
	P	HR	(95% CI)	*P*	HR	(95% CI)
Base model						
Dominant site of relapse[b]						
Bone *vs.* soft tissue	0.07	1.61	(0.96–2.69)	0.57	1.18	(0.67–2.07)
Viscera *vs.* soft tissue	0.03	1.81	(1.06–3.08)	0.03	1.90	(1.07–3.37)
DFI						
>1 yr *vs.* ≤1 yr	0.001	0.55	(0.39–0.79)	<0.001	0.43	(0.29–0.62)
Additions to base model						
+*TP53* gene mutation (yes *vs.* no)[c]	0.01	1.65	(1.14–2.38)	0.09	1.42	(0.96–2.10)
+VEGF status (high *vs.* low)[c,d]	0.02	1.48	(1.06–2.05)	0.04	1.45	(1.02–2.04)
+*TP53* gene mutation/VEGF status[d,e]						
No *TP53* mutation + VEGF-low		1			1	
TP53 mutation or VEGF-high	0.04	1.45	(1.02–2.07)	0.01	1.66	(1.14–2.42)
TP53 mutation + VEGF-high	0.01	2.32	(1.40–3.83)	0.05	1.68	(1.00–2.82)

[a] All multivariate models included 160 patients.
[b] In case of multiple sites, the site with the worst prognosis was considered dominant.
[c] Added separately to the base model.
[d] Low, below the median value of 0.22 ng/mg protein; high, ≥0.22 ng/mg protein.
[e] Added as a combined variable to the base model.

$P = 0.04$) added to the model containing dominant site of relapse and DFI. Compared with tumors with both a wild-type *TP53* gene and a low level of VEGF (response rate, 71%; OR, 1), those without a *TP53* gene mutation and a high level of VEGF (response rate, 57%; OR, 0.64; 95% CI, 0.28–1.46; $P = 0.29$) or with a *TP53* gene mutation and a low level of VEGF (response rate, 53%; OR, 0.29; 95% CI, 0.08–1.03; $P = 0.06$) showed an intermediate response. However, when both factors are unfavorable (response rate, 41%; OR, 0.27; 95% CI, 0.09–0.79; $P = 0.02$) patients showed the poorest rate of responses.

Discussion

Breast cancer is a multifactorial disease, and genetic alterations lead to transformation of normal cells into cancer cells. Human breast cancer is an angiogenesis-dependent tumor that initially depends on estrogens for development and progression. The ER-α, which is expressed in about 75% of breast tumors, is the target for estrogen antagonists, such as tamoxifen. In the present study, we evaluated the predictive value of combined *TP53* genotype and VEGF expression status in a series of 160 ER-α-positive breast cancer patients who were treated with tamoxifen for advanced disease.

Our study demonstrates a statistically significant association between *TP53* mutations and higher VEGF expression levels in human breast tumor samples. Moreover, tumors with nonmissense mutations, which are not likely to be detected by protein-based methods, were found to have the highest VEGF expression levels. These correlations had been reported earlier in breast tumors by Linderholm *et al.* (12) and are consistent with the reported up-regulation of VEGF by mutant TP53 (11). The association between loss of wild-type TP53 and increased VEGF levels indicates that angiogenic activity may, at least in part, depend on altered *TP53* function in breast tumors. In the present study, the median level was chosen as a cutoff for VEGF because otherwise subsets, particularly the combined *TP53*/VEGF subset, would have been too small for meaningful statistical analyses.

With respect to the predictive value, we and others have shown that only TP53 genotype, and not immunohistochemical results, is predictive for the type of response to (adjuvant) tamoxifen treatment in breast cancer patients [as shown in seven studies with a total of approximately 1500 patients (reviewed in Ref. 5; see Ref. 12)]. Few studies have shown that VEGF status is a significant predictor of poor outcome in patients receiving (adjuvant) endocrine therapy with tamoxifen (8, 12, 13). These effects were stronger in the ER-α-positive subset. Our present study shows for the first time that in patients with advanced breast cancer, the combined *TP53* gene and VEGF status gives additional predictive information for disease outcome in the ER-α-positive subset. In multivariate analysis for response, the relationship between combined factors and response to tamoxifen in the ER-α-positive tumors remained significant, even after correction for the traditional predictive factors. Thus, by using the *TP53*-VEGF status, one can basically predict that 70% of the ER-α-positive patients with wild-type *TP53* and low VEGF levels will respond to tamoxifen treatment. Similarly, the longest PFS and PR-OS were observed for patients with wild-type *TP53* and low VEGF levels, and the shortest PFS and PR-OS were observed for those with mutant *TP53* and high VEGF levels, both in univariate and multivariate analysis. No other investigations have yet evaluated the type of response to tamoxifen as a function of VEGF levels and the complete *TP53* gene status.

At present, there is no clear mechanism that explains the association between VEGF, TP53, and poor outcome of tamoxifen in ER-α-positive breast tumors. However, several preclinical and clinical studies may provide a basis. There is evidence that VEGF production is regulated by estrogens *in vitro* and *in vivo*, although data are conflicting. Whereas Hyder *et al.* (14) showed the presence of estrogen response elements in the VEGF gene, they have not been able to show estrogen response in various breast cancer cell types (15). Others have reported estrogen induction of VEGF (16). The estrogen induction of VEGF in DMBA tumors was shown by Nakamura *et al.* (17).

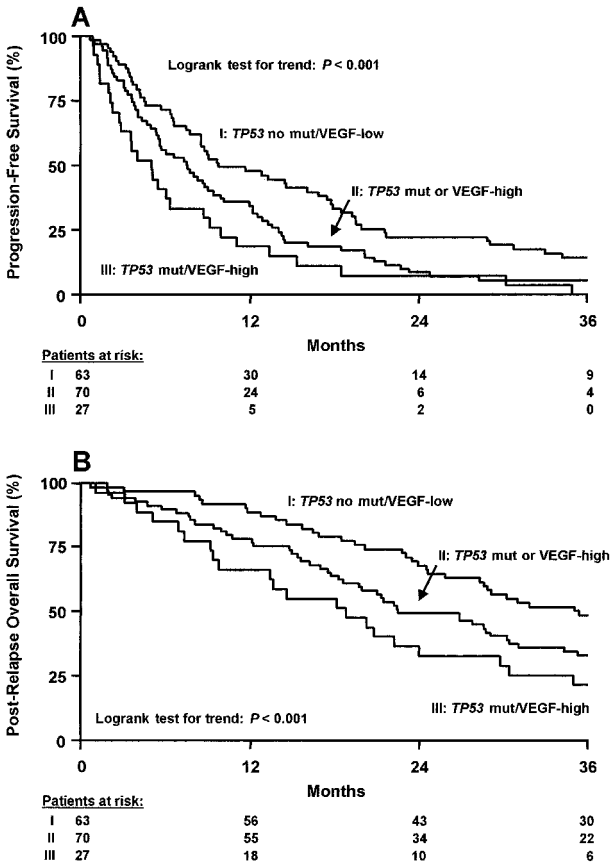

Fig. 1 PFS (*A*) and PR-OS (*B*) after start of tamoxifen treatment as a function of *TP53* gene status and VEGF levels. The number of patients below the *X* axis represents the number at risk over total patients in the three different subsets.

This supports the hypothesis that estrogens regulate VEGF expression, at least in part, via transcriptional effects of ER. In contrast to the previous study, Schafer *et al.* (18) reported that VEGF expression was not increased in MCF-7 tumors after estradiol stimulation but was increased only after tamoxifen treatment. However, this tamoxifen-induced expression of VEGF in MCF-7 cells was reported not to be mediated by ER (16). Furthermore, Adams *et al.* (19) showed that tamoxifen treatment of breast cancer patients resulted in higher circulating and platelet-derived VEGF levels in those patients with ER-positive breast cancer in remission. Thus, a stimulating effect of estrogens or tamoxifen on VEGF production is possible, but additional studies are needed to elucidate the mechanism(s).

In summary, the present study shows that knowledge of the geno- and phenotype of breast tumors is important for finding targets for treatment. Treatments aimed at combining (pure) antiestrogens with inhibitors of angiogenesis (*e.g.*, drugs blocking VEGF or its receptor) could be beneficial for those patients with wild-type TP53, ER-α-positive, and VEGF-positive tumors. However, based on the findings of Yu *et al.* (20), those breast tumors that have mutant *TP53* instead might be better treated with angiogenesis inhibitors and drugs that specifically target hypoxic cells. Nonetheless, only if validated in homogeneous, multicenter, prospective studies, the clinical relevance of the combined ER-α, *TP53*, and VEGF status can be assessed for novel therapeutic strategies.

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