Wwox and Ap2γ Expression Levels Predict Tamoxifen Response

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Abstract

Purpose: Assessment of expression levels of Wwox, Wwox-interacting proteins Ap2α, Ap2γ, and ErbB4, the Ap2γ transcriptional target protein Her2, and the possible Ap2α transcriptional target PrkaRIα, in breast cancers, to determine their roles in tamoxifen resistance. The hypothesis was that sequestration of Wwox interactors in the cytoplasm might control tamoxifen response.

Experimental Design: Tissue sections from 51 tamoxifen-sensitive and 38 tamoxifen-resistant, estrogen receptor α–positive breast cancers were stained for the above proteins, as well as progesterone receptor (PR). The relation of tamoxifen resistance and other clinical features, with level of expression of these proteins, and pairwise correlations among various immunohistochemical markers were determined.

Results: Menopausal status, tumor, node, and stage, loss of PR, lost or reduced expression of Wwox, and high level of expression of PrkaRIα, Ap2γ, and Her2 were significantly correlated with tamoxifen resistance. In multivariate analysis, Wwox, PrkaRIα, Ap2γ, and ErbB4 were found to be independent markers of tamoxifen resistance. Reduced Wwox expression was better than PR in prediction of resistance, especially in high-risk patients, and nuclear Ap2γ expression was better than Her2, especially in low-risk patients.

Conclusion: The results illustrate the complex relationships among the marker proteins assessed in this *in vivo* study and suggest new markers for prediction of response to tamoxifen treatment as well as possible new targets for treatment of breast cancer. Wwox and Ap2γ emerge as new biomarkers that may be superior to PR and Her2 in predicting tamoxifen response.

Tamoxifen is the oldest and most commonly used drug for estrogen receptor α (ERα)-positive (ER^+) breast cancers. Tamoxifen treatment in the adjuvant setting reduces recurrence rate and improves overall survival; when used for treatment of metastatic breast cancer, it provides remission in up to half of patients and is also used for prevention of breast cancer (1–3). However, *de novo* and acquired resistance to tamoxifen is an important clinical problem because almost all metastatic patients and up to 40% of patients receiving adjuvant tamoxifen treatment will relapse and die from breast cancer. Despite many studies of breast cancers and derived cell lines with acquired or selected tamoxifen resistance, mechanisms of resistance are not fully understood (4–6).

Progesterone receptor–negative (PR^-) status in ER^+ cases was shown to be an independent predictive factor for benefit from adjuvant tamoxifen treatment (7); it was suggested that growth factor signaling is enhanced when the PR level is low (8, 9). With Arimidex or Tamoxifen Alone or in Combination trial, a major benefit for anastrazole was reported in the ER^+/PR^- subgroup (10).

Patients with Her2/ErbB2-positive cancers ($Her2^+$) also failed to benefit from tamoxifen treatment (11–13). It was suggested that (*a*) increased growth factor signaling with overexpression of *epidermal growth factor receptor/Her2* genes may activate mitogen-activated protein kinase, in turn activating ERα by phosphorylation at Ser^{118}, and (*b*) AIB1 may be activated by signaling downstream of Her2, and in the presence of phosphorylated ERα and high AIB1, the agonistic activity of tamoxifen may be enhanced (14). It was shown that weak agonist activity of tamoxifen is enhanced by up-regulation of coactivators, such as AIB1 (SRC3). It has also been suggested that another coactivator, SRC1, may enhance agonistic activity of 4-hydroxytamoxifen (15). Stabilization of the interaction between ERα and SRC1 by cyclin D1 was reported to be related to resistance *in vitro* (16).

More recently, a correlation was reported between down-regulation of the inhibitory subunit of protein kinase A (PKA; PrkaRIα) and tamoxifen resistance (17). Activation of PKA by PrkaRIα down-regulation leads to phosphorylation of ERα at Ser^{305}, converting tamoxifen from an ERα inhibitor to a growth stimulator. The mechanisms by which PrkaRIα is down-regulated and Her2 is up-regulated in tamoxifen-resistant cases were unknown.

We noted that Wwox expression was reduced in a large fraction of breast cancers (18–20) and in a clone of MCF7 cells

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Table 1. Association of clinical and pathologic characteristics with tamoxifen sensitivity

		Tamoxifen sensitive, *n* (%)	Tamoxifen resistant, *n* (%)	*P*
Age	≤50	13 (44.8)	16 (55.2)	0.11
	>50	37 (62.7)	22 (37.3)	
Menopause	Pre	6 (31.6)	13 (68.4)	0.011
	Post	45 (64.3)	25 (35.7)	
T-stage	1	22 (78.6)	6 (21.4)	0.003
	2	24 (49.0)	25 (51.0)	
	3	2 (28.6)	5 (71.4)	
N-stage	0	33 (78.6)	9 (21.4)	<0.001
	1	13 (52.0)	12 (48.0)	
	2	4 (33.3)	8 (66.7)	
	3	0 (0.0)	9 (100.0)	
Stage	1	17 (65.4)	9 (34.6)	0.025
	2	24 (66.7)	12 (33.3)	
	3	7 (31.8)	15 (68.2)	
Grade	1	12 (63.2)	7 (36.8)	0.48
	2	18 (58.1)	13 (41.9)	
	3	15 (46.9)	17 (53.1)	
Metastatic lymph nodes	Absent	32 (78.0)	9 (22.0)	<0.001
	Present	18 (38.3)	29 (61.7)	
PR	≤10	20 (43.5)	26 (56.5)	0.017
	>10	27 (69.2)	12 (30.8)	
Wwox	Strong	18 (81.8)	4 (18.2)	0.013
	Reduced	33 (49.3)	34 (50.7)	
Ap2γ	≤10	32 (74.4)	11 (25.6)	<0.001
	>10	15 (35.7)	27 (64.3)	
Ap2α	≤10	34 (63.0)	20 (37.0)	0.11
	>10	15 (45.5)	18 (54.5)	
Her2	Negative	42 (63.6)	24 (36.4)	0.025
	Positive	8 (36.4)	14 (63.6)	
ErbB4	≤50	31 (51.7)	29 (48.3)	0.24
	>50	17 (65.4)	9 (34.6)	
PrkaRIα	≤10	26 (70.3)	11 (29.7)	0.015
	>10	21 (43.8)	27 (56.3)	

selected for tamoxifen resistance *in vitro*[5] and is often down-regulated in breast cancers due to DNA hypermethylation in its regulatory region (21, 22). Wwox, a 46-kDa tumor suppressor protein containing two WW domains that play roles in Wwox function (23–26), is encoded by the *WWOX* gene, encompassing common fragile site FRA16D, in a chromosome region involved in allelic loss in breast cancers (23). WW domains interact with proline-containing ligands and mediate protein-protein interactions (27, 28). The Wwox WW domains were predicted to interact with several proteins of interest in breast cancer, including p73, the cytoplasmic domain of ErbB4, and the Ap2 transcription factors, using the ProChart database (Cytogen Corp.; ref. 29), and interactions were confirmed through *in vitro* overexpression and coimmunoprecipitation studies (24–26). We have observed that Wwox protein, which binds and retains Ap2α and Ap2γ transcription factor proteins in the cytoplasm, seems to mediate tamoxifen sensitivity *in vitro*.[5] Wwox loss initiated tamoxifen resistance through release of Ap2 factors to the nucleus where Ap2γ up-regulated Her2 expression and Ap2α may influence expression of PrkaRIα. *In vitro* restoration of Wwox in tamoxifen-resistant breast cancer–derived cells restored tamoxifen sensitivity and abrogated Her2 expression.[5] We have now examined expression levels of PR, Her2, Ap2α, Ap2γ, PrkaRIα, and ErbB4, in addition to Wwox, in a panel of tamoxifen-sensitive and tamoxifen-resistant cancers to clarify their roles in tamoxifen resistance *in vivo*, in comparison with the *in vitro* findings in breast cancer–derived, tamoxifen-sensitive, and tamoxifen-resistant cells.

Materials and Methods

Breast cancers. The panel of 89 breast cancers consisted of cases treated for primary breast cancer at Hacettepe University between 1985 and 2001. The patients received no neoadjuvant treatment but were all treated by modified radical mastectomy and then received adjuvant tamoxifen treatment. The cancer tissues of all the patients were tested for ER expression at the time of diagnosis by ligand-binding assay or immunohistochemistry. By ligand-binding assay (≥10 fmol/mg protein) and by immunohistochemical nuclear staining in ≥10% of invasive neoplastic cells were the criteria for ER positivity and all the cancers in this panel were ER^+ according to these criteria. The patients who relapsed during or in the 2 years after termination of tamoxifen treatment were considered tamoxifen resistant and cases that were tumor-free 2 years after tamoxifen termination were classified tamoxifen sensitive. Tamoxifen was given for 5 years, 2 × 10 mg, daily; 51 (57.3%) cases were tamoxifen sensitive and 38 (42.7%) cases were tamoxifen resistant. The ages of patients ranged from 31 to 79 (mean, 56.8). Nineteen (21.3%) were premenopausal and 70 (78.7%) were postmenopausal. Clinicopathologic features are listed in Table 1.

Immunohistochemistry. The primary antisera and detection kits used in immunohistochemical studies are listed in Table 2. Antigen retrieval was the same for all antisera; sections were boiled in pH 6 citrate buffer in pressure cooker for 3 min. The details of immunostaining methods

[5] D. Iliopoulos et al. Wwox tumor suppressor is a mediator of tamoxifen response, in preparation.

Table 2. Primary antisera and detection kits used in immunohistochemical studies

Primary antibody	Source	Description	Positive control	Dilution	Detection kit
PR	Lab Vision	Rabbit monoclonal (SP2)	Normal breast	1:200	DakoCytomation Universal LSAB 2 kit
Her-2	NeoMarkers	Rabbit monoclonal (SP3)	Breast tumor	1:200	DakoCytomation Universal LSAB 2 kit
Wwox	Huebner lab*	Rabbit polyclonal	Normal breast	1:1500	DakoCytomation Universal LSAB 2 kit
Ap2γ	Santa Cruz Biotechnology	Mouse monoclonal (6E4/4)	Normal breast	1:50	DakoCytomation Universal LSAB 2 kit
Ap2α	Santa Cruz Biotechnology	Mouse monoclonal (3B5)	Normal breast	1:50	UltraTek HRP Anti-Polyvalent Lab Pack
ErbB4	NeoMarkers	Rabbit polyclonal	Breast tumor	1:50	DakoCytomation Universal LSAB 2 kit
PrkaRIα	Calbiochem	Rabbit polyclonal	Thyroid follicular adenoma	1:500	UltraTek HRP Anti-Polyvalent Lab Pack

NOTE: Antigen retrieval was the same for all antisera; sections were boiled in pH 6 citrate buffer in pressure cooker for 3 min.
*The features of the antiserum were given in detail in Guler et al. (18) and Guler et al. (20).

were described previously (18, 20). Negative controls were involved in all studies. Stained sections were evaluated by two pathologists (G. Guler and C. Himmetoglu) who were blinded to clinical data. Wwox and Her2 were evaluated as described (18). Her2 2+ and 3+ cases were grouped together versus negative and 1+ cases as a Her2 overexpression group. PR was scored as positive (>10% stained) or negative (≤10% stained) according to the proportion of nuclear staining in tumor cells. Nuclear staining of Ap2α and Ap2γ was scored as >10% or ≤10% in tumor cells. We noted some cytoplasmic reaction with both Ap2α and Ap2γ, as have others (30, 31), but it was not possible to accurately detect and score specific cytoplasmic signaling of these transcription factor proteins by immunohistochemistry. Thus, we scored only the nuclear expression of the Ap2 proteins. ErbB4 is expressed in cell membrane, cytoplasm, and nuclei and scored as positive in ≤50% or >51% of neoplastic cells. PrkaRIα, an inhibitory subunit of the holoenzyme PKA, is expressed in the cytoplasm and was scored as >10% or ≤10% in tumor cells.

Statistics. Factors associated with tamoxifen resistance were analyzed using χ^2 or Fisher tests, where appropriate, for univariate analyses and multiple logistic regression for multivariate analysis. Pairwise correlations of biomarkers were assessed using Spearman's correlation test. A P value of 0.05 was considered to indicate statistical significance.

Results

Association of clinical features and biomarkers with tamoxifen response. Menopausal status, tumor size, axillary nodal metastasis and stage, loss of PR, lost or reduced expression of Wwox, and high level of expression of PrkaRIα, Ap2γ, and Her2 were significantly correlated with tamoxifen resistance. In multivariate analysis, Wwox, PrkaRIα, Ap2γ, and ErbB4 were found to be independent markers of tamoxifen resistance (Table 3). Examples of immunostains are shown in Fig. 1. The variables related significantly with tamoxifen resistance are noted in Table 1.

The risk groups and tamoxifen resistance. When cases were stratified as postmenopausal and stage 1 and 2 (low-risk group) versus premenopausal and stage 3 (high-risk group), Wwox was a reliable marker of tamoxifen resistance, especially in high-risk cases. In the high-risk group, tamoxifen resistance risk was 22% in cases with high Wwox level and 83% in cases with reduced Wwox expression. In the low-risk group, when Wwox was reduced, the probability of tamoxifen resistance was 33%, and when the Wwox level was normal, the probability of tamoxifen resistance was 15%. This interaction of Wwox with the risk groups in terms of predicting tamoxifen resistance persisted in multivariate analysis.

When Wwox was reduced, the probability of tamoxifen resistance increased 4.6 times (odds ratio; 95% confidence interval, 1.4-15.2); loss of PR increased tamoxifen resistance probability 2.9 times (odds ratio; confidence interval, 1.2-7.2). In univariate analysis, PR loss was more frequent in cases with reduced Wwox expression (P = 0.002; Table 4). After adjustment for other risk factors, Wwox, as opposed to PR, remained in the multivariate model as an independent predictor of tamoxifen resistance (Table 3).

In low-risk cancers with Her2 overexpression, the probability of tamoxifen resistance was 54%, and when negative for Her2

Table 3. Multivariate analyses predict factors significantly associated with tamoxifen resistance

	P	Risk ratio (95% CI)	Value predicting*	
			Sensitivity (%)	Resistance (%)
Risk group (high vs low)	0.122	3.3 (0.7-15.1)	71.2	65.6
Wwox				
Reduced vs normal	0.002	14.6 (2.7-79.1)		
With Wwox/risk group interaction	0.043	30.8 (1.1-856.8)	72.1	82.6
PrkaRIα (>10 vs ≤10)	0.003	10.8 (2.3-51.3)	81.1	67.4
Ap2γ (>10 vs ≤10)	0.002	9.7 (2.3-41.3)	77.1	80.6
ErbB4 (>50 vs ≤50)	0.029	6.0 (1.2-29.5)	77.1	80.6

Abbreviation: 95% CI, 95% confidence interval.
*Shows changes in predictive values with additional information of the variable on the given line.

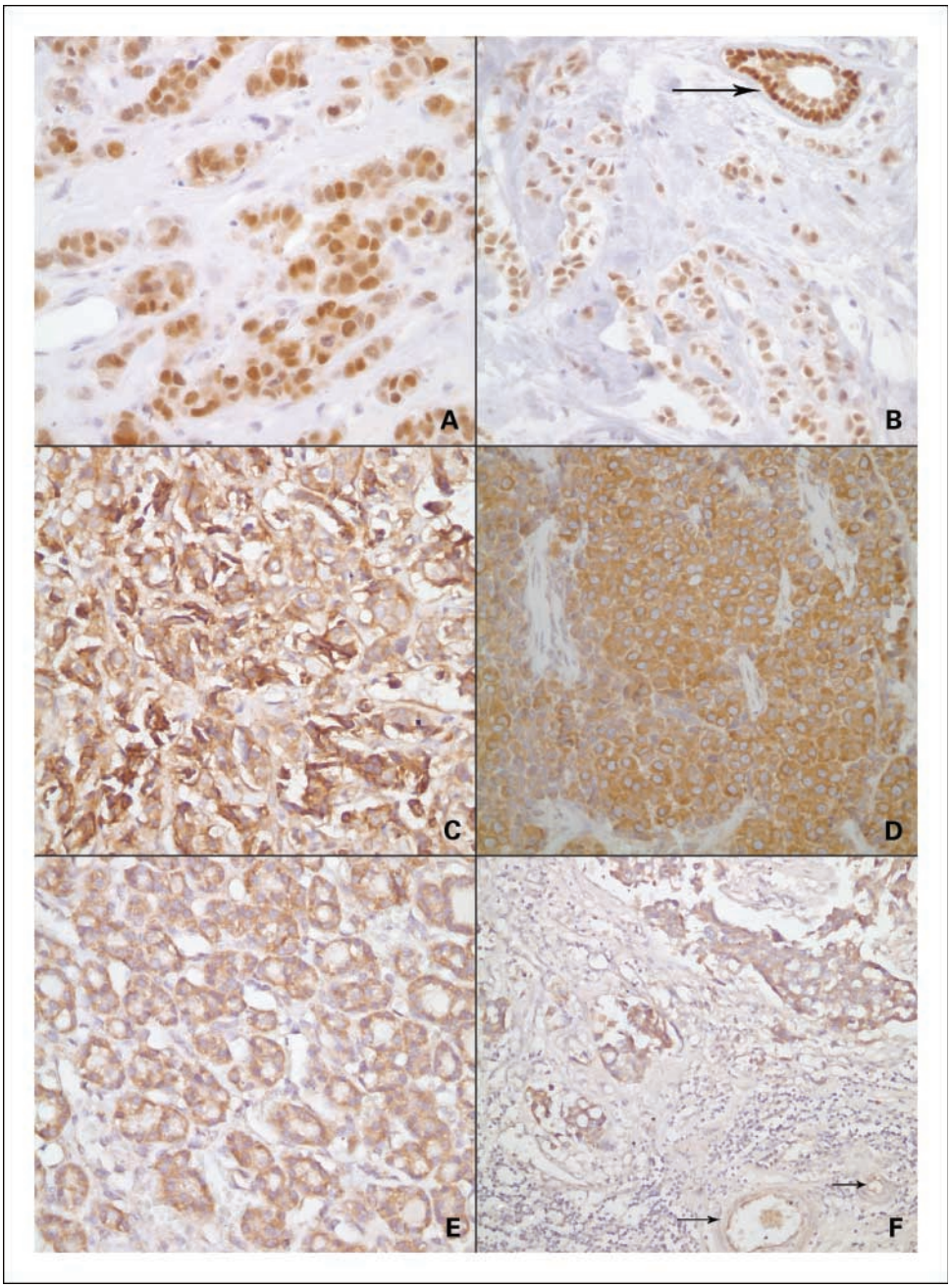

Fig. 1. Representative photographs of immunohistochemical staining of breast cancer sections for Wwox and interacting proteins. *A,* mainly nuclear staining of Ap2α in neoplastic cells. Magnification, ×200. *B,* mainly nuclear staining of Ap2γ in tumor cells. Arrow, in a residual breast duct, staining in myoepithelial layer is more prominent. Magnification, ×100. *C,* cytoplasmic, membranous, and nuclear positivity for ErbB4. Magnification, ×200. *D,* strong cytoplasmic positivity for Wwox. Magnification, ×200. *E,* a follicular adenoma case used as positive control for PrkaRIα, expressed in cytoplasm of neoplastic cells. Magnification, ×200. *F,* cytoplasmic staining for PrkaRIα. Arrow, positive staining in vascular endothelial cells serves as internal positive control. Magnification, ×100.

expression, the probability of tamoxifen resistance was 21%. Her2 was a good marker of tamoxifen resistance in the low-risk group but not in high-risk patients.

In the low-risk group, when nuclear Ap2γ expression was >10%, the risk of tamoxifen resistance was 52.2%, and when nuclear Ap2γ expression was ≤10%, 11.5% of cases were resistant. In the high-risk group, when Ap2γ nuclear expression was >10%, tamoxifen resistance risk was 78%, and in cases with ≤10% of nuclei positive for Ap2γ, it was 54%. Thus, Ap2γ nuclear expression was a good marker of tamoxifen resistance, especially in the low-risk group.

Her2 overexpression was observed more frequently in cases with nuclear Ap2γ expression ($P = 0.041$; Table 4), and nuclear Ap2γ was one of the independent indicators of tamoxifen resistance. When Her2 was overexpressed, the probability of tamoxifen resistance was increased 3.1 times (odds ratio; confidence interval, 1.1-8.4), and when Ap2γ was expressed in >10% of tumor cell nuclei, the probability of tamoxifen resistance increased 5.2 times (odds ratio; confidence interval, 2.1-13.3). Likewise, multivariate analysis revealed Ap2γ to be a better predictor of tamoxifen resistance than Her2 (Table 3).

Pairwise correlations between immunohistochemical markers. Wwox expression was positively associated with PR ($P = 0.002$), and there was a trend toward positive association of PrkaRIα and ErbB4 with Wwox ($P = 0.167$ and 0.103, respectively). We did not observe a correlation of nuclear Ap2α or Ap2γ with Wwox expression ($P = 0.623$ and 0.842, respectively; Table 4), but nuclear Ap2γ was related to nuclear Ap2α expression ($P = 0.011$) and there was a positive trend toward association of Ap2γ and PrkaRIα expression ($P = 0.118$).

The expression of ErbB4 was positively associated with Ap2α and showed a positive trend toward association with Wwox expression (P = 0.015 and 0.103, respectively; Table 4). In multivariate analysis, ErbB4 loss emerged as one of the independent markers of tamoxifen resistance when adjusted for other significant predictors (Table 3).

PrkaRIα expression was not significantly associated with other markers in univariate analysis. Yet, there was a positive trend toward association with Ap2α, Ap2γ, ErbB4, and Wwox (P = 0.190, 0.118, 0.118, and 0.167, respectively; Table 4). In multivariate analysis, high expression of PrkaRIα was one of the independent indicators of tamoxifen resistance (Table 3).

Discussion

The clinical features of this panel of breast cancers show complex associations of these proteins with tamoxifen response. Loss of Wwox was an independent and most powerful indicator of tamoxifen resistance, especially in premenopausal and advanced stage patients. Wwox seems to play roles in three known pathways of tamoxifen resistance.

Wwox loss or reduced expression is related to loss of PR (P = 0.002). When compared with PR, Wwox was the better predictor of tamoxifen resistance; loss of PR increased the risk of tamoxifen resistance 2.9-fold, whereas reduced Wwox level increased the probability of tamoxifen resistance 4.6 times. Wwox was also one of the independent markers of tamoxifen resistance, whereas PR did not remain in multivariate system when compared with other significant indicators. PR loss in ER^+ breast cancer is an accepted factor suggesting tamoxifen resistance clinically (32). Our results show that Wwox expression is a good candidate marker of tamoxifen resistance, especially in high-risk patients.

Wwox level was not associated with Her2 expression using the scoring method adopted for this study (in which high Wwox expression was scored when there was intense cytoplasmic staining in more than half of the neoplastic cells). However, when the cases were regrouped as very high expressors of Wwox (high intensity in >75% of tumor cells versus all other cases scored as reduced), there was a significant inverse association between Her2 and Wwox expression, in line with results obtained with breast cancer–derived cells *in vitro*.[5] In cell lines, very high Wwox levels were associated with very low Her2 levels. In *in vitro* studies of breast cancer–derived cells, we have also observed that Wwox interacts with Ap2γ in the cytoplasm; when Wwox is down-modulated or lost, Ap2γ is released from the cytoplasm, moves to the nucleus (25), and leads to overexpression of Her2.[5] We did not find an inverse correlation of nuclear Ap2γ and Ap2α expression with cytoplasmic Wwox expression in immunohistochemical studies. We noted some cytoplasmic reaction, in addition to nuclear staining with antisera for both Ap2 factors, but it was not possible to score specific cytoplasmic staining of these proteins by immunohistochemistry. It may be necessary to do subcellular fractionation analyses using breast cancer epithelial tissues, coupled with immunoblot detection with individual specific Ap2 antisera, to clarify the apparent differences between Ap2α location and activity *in vitro* and *in vivo*.

The Ap2 genes are expressed in many human breast cancer cell lines, and critical Ap2-binding sites are described in the Her2, ER, and insulin-like growth factor I receptor promoters (33). Ap2α protein is reported to activate the *E-cadherin* gene (34) for maintenance of homotypic cell-cell adhesion and the *CDKN1* gene for mediation of growth arrest (35) and is implicated in promotion of cell apoptosis through interaction with the *MYC* gene (36). Reduced levels of nuclear Ap2 and Ap2α expression have been reported in association with aggressive behavior in human cancer specimens (30, 31). A significant correlation between the presence of the Ap2α protein and ERα expression (33, 37) and between Ap2α and Ap2γ proteins and Her2 expression was reported (33, 38) and confirmed in our experiments. In this study, a significant positive association was seen between Ap2γ and Her2 and a positive trend between Ap2α and Her2 (P = 0.041 and 0.064, respectively). Nuclear Ap2γ expression was another independent marker of tamoxifen resistance. Its overexpression predicted tamoxifen resistance sensitively, especially in low-risk patients (postmenopausal and stage 1 and 2 cases). For the first time, we have determined that Ap2γ is a better predictor of tamoxifen resistance than Her2. The risk of tamoxifen resistance was increased 3.1 times when Her2 was overexpressed and 5.2 times when Ap2γ was overexpressed.

There was a trend toward positive association between Wwox and PrkaRIα expression (P = 0.167). PrkaRIα protein is an important regulator of serine-threonine kinase activity catalyzed by PKA holoenzyme. It has been reported to have multiple interactions with major signaling pathways and opposing effects on critical cellular functions (39, 40). Overexpression of PrkaRIα has been reported for many tumor tissues in association with aggressive behavior (40). However, Carney complex, a multiple neoplasia syndrome, results from loss of wild-type PrkaRIα expression (39). Currently, the status of PrkaRIα among cancer-related genes is not clear, but it is apparently not a classic tumor suppressor gene (39, 40). Down-regulation of PrkaRIα has been reported to be associated with tamoxifen resistance (17), presumably through inhibition of PKA expression, a finding our *in vivo* study of this panel of breast cancers did not confirm. In these breast cancers, PrkaRIα expression was associated with tamoxifen resistance; 67.6% of cases with high PrkaRIα and low Wwox expression were tamoxifen resistant. On the other hand, tamoxifen resistance was also observed in 35.5% of cases with low expression of

Table 4. Pairwise correlations between immunohistochemical markers

		PR	Wwox	Ap2α	Ap2γ	PrkaRIα	Her2
Wwox	*r*	0.32					
	P	**0.002**					
Ap2α	*r*	-0.14	-0.05				
	P	0.193	0.623				
Ap2γ	*r*	-0.13	-0.02	0.27			
	P	0.234	0.842	**0.011**			
PrkaRIα	*r*	-0.03	0.15	0.14	0.17		
	P	0.821	0.167	0.190	0.118		
Her2	*r*	-0.17	-0.09	0.20	0.22	0.09	
	P	0.127	0.400	0.064	**0.041**	0.437	
ErbB4	*r*	0.04	0.18	0.26	0.11	0.17	-0.10
	P	0.744	0.103	**0.015**	0.316	0.118	0.380

NOTE: Bold P values indicate statistically significant correlations.
Abbreviation: r, correlation coefficient.

Table 5. Combined effects of PrkaRIα and Wwox expression level on tamoxifen sensitivity

PrkaRIα	Wwox	Tamoxifen sensitive, *n* (%)	Tamoxifen resistant, *n* (%)	Total
≤10	Reduced	20 (64.5)	11 (35.5)	31
	Normal	6 (100.0)	0 (0.0)	6
	Total	26 (70.3)	11 (29.7)	37
>10	Reduced	11 (32.4)	23 (67.6)	34
	Normal	10 (71.4)	4 (28.6)	14
	Total	21 (43.8)	27 (56.3)	48

both Wwox and PrkaRIα (Table 5). In a similar analysis with PrkaRIα and Ap2γ, we noted that 74.1% of cases with high PrkaRIα were tamoxifen resistant when associated with high Ap2γ expression. In these cases, the Ap2γ-Her-2 pathway is probably responsible for tamoxifen resistance of at least some cases with high PrkaRIα levels, possibly suggesting that in some breast cancer cells, if the Her2 pathway to tamoxifen resistance is activated, the PKA pathway is not needed. It would be interesting to determine if there are breast cancers that become tamoxifen resistant through the PKA pathway in the absence of activation of the Her2 pathway. However, high PrkaRIα expression was an independent indicator of tamoxifen resistance, a result that suggests that PKA activity is not involved in tamoxifen resistance.

In contrast to other epidermal growth factor receptor family members, ErbB4 expression is reported to be associated with increased survival and lower proliferation indices in breast cancer (41, 42); however, there are also conflicting reports associating ErbB4 with adverse prognostic significance (43). ErbB4 expression was reported in correlation with good prognostic indicators, such as a lower grade of tumor (44, 45), ER positivity (46), and low proliferation indices and ER^+ phenotype (47). In this study, ErbB4 expression was not related with tamoxifen resistance significantly in univariate analysis, but in multivariate analysis, low ErbB4 expression emerged as an independent variable; probably, the effect of its correlations with other biomarkers was masking its effect in univariate analysis.

The results of this study describe new reliable markers of tamoxifen resistance; Wwox and Ap2γ, in particular, seem to predict tamoxifen resistance better than the two known biomarkers, PR and Her2. This study also revealed complex interrelationships among Wwox, Ap2α, Ap2γ, PrkaRIα, ErbB4, and Her2 in tamoxifen resistance. It is likely that this complexity is at least partly related to the fact that the Wwox WW domains can interact with many proteins and it is likely that other WW domain proteins can also interact with at least a subset of the same ligands (26). Thus, predicting the hierarchy of Wwox interactions in a specific cancer tissue is not yet possible. Continuing analyses of these signal pathways in a wider selection of breast cancer–derived, tamoxifen-sensitive, and tamoxifen-resistant cell lines, in parallel with confirmatory analyses of these markers *in situ* in larger breast cancer panels, will further define the pathways leading to tamoxifen resistance and further define markers of resistance and targets for therapy.

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