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PRECLINICAL STUDY

Activation of Akt, mTOR, and the estrogen receptor as a signature to predict tamoxifen treatment benefit

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Abstract The frequent alterations of the PI3K/Akt/mTOR-growth signaling pathway are proposed mechanisms for resistance to endocrine therapy in breast cancer, partly through regulation of estrogen receptor α (ER) activity. Reliable biomarkers for treatment prediction are required for improved individualized treatment. We performed a retrospective immunohistochemical analysis of primary tumors from 912 postmenopausal patients with node-negative breast cancer, randomized to either tamoxifen or no adjuvant treatment. Phosphorylated (p) Akt-serine (s) 473, p-mTOR-s2448, and ER phosphorylations-s167 and -s305 were evaluated as potential biomarkers of prognosis and tamoxifen treatment efficacy. High expression of p-mTOR indicated a reduced response to tamoxifen, most pronounced in the ER+/progesterone receptor (PgR) + subgroup (tamoxifen vs. no tamoxifen: hazard ratio (HR), 0.86; 95 % confidence interval (CI), 0.31–2.38; $P = 0.78$), whereas low p-mTOR expression predicted tamoxifen benefit (HR, 0.29; 95 % CI, 0.18–0.49; $P = 0.000002$). In addition, nuclear p-Akt-s473 as well as p-ER at -s167 and/or -s305 showed interaction with tamoxifen efficacy with borderline statistical significance. A combination score of positive pathway markers including p-Akt, p-mTOR, and p-ER showed significant association with tamoxifen benefit (test for interaction; $P = 0.029$). Cross-talk between growth signaling pathways and ER-signaling has been proposed to affect tamoxifen response in hormone receptor-positive breast cancer. The results support this hypothesis, as an overactive pathway was significantly associated with reduced response to tamoxifen. A clinical pre-treatment test for cross-talk markers would be a step toward individualized adjuvant endocrine treatment with or without the addition of PI3K/Akt/mTOR pathway inhibitors.

Keywords mTOR · Akt · Estrogen receptor phosphorylation · Tamoxifen resistance · Immunohistochemistry

Abbreviations

DAB	3,3-Diaminobenzidine tetrahydrochloride
C	Celcius
CI	Confidence interval
EGFR	Epidermal growth factor receptor
ER	Estrogen receptor α
ERE	Estrogen response element
Erk	Extracellular signal-regulated kinase
Gy	Gray
HR	Hazard ratio
HER2	Human epidermal growth factor receptor 2
IHC	Immunohistochemistry

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IGF	Insulin-like growth factor
IGFR1	Insulin-like growth factor 1 receptor
mTOR	Mammalian target of rapamycin
mTORC	Mammalian target of rapamycin complex
min	Minutes
MAPK	Mitogen-activated protein kinase
MEK	Mitogen-activated protein kinase extracellular signal-regulated kinase kinase
PI3K	Phosphatidylinositol 3-kinase
PIK3CA	Phosphatidylinositol 3-kinase catalytic alpha polypeptide gene
p	Phosphorylated
PgR	Progesterone receptor
Raf	Rapidly accelerated fibrosarcoma
RFS	Recurrence–free survival
RT	Room temperature
S6K1	S6 kinase 1
s	Serine
TMA	Tissue microarray
vs	Versus

Introduction

Membrane-bound growth factor receptors, such as the four epidermal growth factor receptors and the insulin-like growth factor 1 receptor, activate the pathway phosphatidylinositol 3-kinase (PI3K)/Akt/mammalian targeted of rapamycin (mTOR) and the ras-raf-MAP kinase axis to induce essential tumor cell promoting effects such as survival, proliferation, and translation. Cross-talk and feedback loops within the pathways make up a complex signaling network complicating development of targeted treatments and the establishment of reliable biomarkers [1].

Human epidermal growth factor receptor 2 (HER2) overexpressed simultaneously with the estrogen receptor α (ER) is a proposed mechanism of endocrine resistance [2–4]. Activation of downstream pathways, frequently represented by *PIK3CA* mutations in clinically HER2-negative breast cancer, leading to Akt activation, seems to play an important role for breast cancer patients relapsing after adjuvant endocrine treatment [5]. Growth factor signals promote phosphorylation of the ER, thereby altering the receptor conformation, its affinity to coregulators, and the transcriptional activity [6–8]. Ligand-independent ER phosphorylation in vitro resulted in activation of the ERE-promoter region, leading to an altered sensitivity to the selective ER modulator tamoxifen [9]. mTORC1, a highlighted protein complex regulating ER phosphorylation at serine 167 through S6 kinase 1 (S6K1), has been reported to play a central role in oncogenic maintenance by controlling growth signaling, translation, metabolism, and autophagy [10–12].

Double inhibition of ER and mTOR signaling shows promising results for patients who have progressed during endocrine treatment. For adjuvant therapy, there is a need for new biomarkers for selection of patients who may benefit from the combined therapy and those who may have excellent prognosis with endocrine monotherapy. We evaluated the p-mTOR-s2448, p-Akt-s473, and p-ER-s167/s305 status in a large series of tumors from women diagnosed with breast cancer, randomized to either adjuvant tamoxifen or local treatment alone. The single and combined targets served as markers for pathway activation and the expression was evaluated with regard to prognosis and tamoxifen response.

Materials and methods

The present study was designed and presented with regard to the reporting recommendations for tumor marker prognostic studies (REMARK) guidelines [13].

Patients and TMA construction

During the years 1976 through 1990, a cohort of Swedish postmenopausal breast cancer patients was included in a controlled trial to evaluate tamoxifen as adjuvant treatment [14]. Patients in the tamoxifen arm were treated for 2 years, and thereafter, randomized to continued treatment for three more years or no more treatment. In the present study, women with low-risk tumors, defined as node negative and with tumor diameter ≤30 mm, were included. Patients were treated either with modified radical mastectomy or breast-conserving therapy and radiation therapy to the breast with a total dose of 50 Gy with 2 Gy per fraction, 5 days weekly, for about 5 weeks. Patient demographic data are presented in Supplementary Fig. 1. Median period of follow-up was 18 years. Methods for tissue microarray (TMA) construction and determination of ERα status, progesterone receptor (PgR) status, and HER2 status were previously described [15]. ER- and PgR positivity was defined as 10 % or more positive tumor cells.

Immunohistochemistry

Immunohistochemistry was performed on TMAs consisting of samples from 912 tumors with three cores from each tumor, using the PT-link rinse station for deparaffinization and antigen retrieval for 20 min at 96 °C (DakoCytomation, Glostrup, Denmark). Sections were placed in 3 % H_2O_2 in methanol for 5 min to inactivate endogenous peroxidase, incubated with serum-free protein block (Spring Bioscience, Freemont, CA) for 10 min, and incubated with primary antibodies; p-Akt-s473 (1:33 dilution),

p-mTOR-s2448 (1:300), p-ER-s167 (1:400) (Cell Signaling Technologies, Danvers, MA), and p-ER-s305, as previously described [16] (1:300) (Bethyl laboratories, Montgomery, TX) overnight at 4 °C in a moisturized chamber. All slides were washed, incubated with an anti-rabbit antibody DakoCytomation Envision + system labeled with horse radish peroxidase (DakoCytomation) for 30 min at RT. Positive staining was visualized using 3, 3-diaminobenzidine tetrahydrochloride (DAB; Vector Laboratories, CA). Nuclei were counterstained with hematoxylin before mounting. Images were generated using an Olympus SC20 camera with a Leica ×20 and ×40 objective. P-Akt whole slide images with ×200 magnification were generated from a Scanscope AT (Aperio, Vista, CA).

Scoring

Staining intensity was evaluated on three separate core biopsies for each tumor. Protein expression in tumor cells was scored by two independent observers. For dissimilar scoring, consensus was reached after a joint reevaluation of the tumor. P-mTOR was visible in the cytoplasm. Expression intensity was evaluated as negative, weak, medium, or strong. As many of the tumors showed more than one intensity grade, each intensity grade was evaluated according to percentage. P-mTOR positivity was defined as strong staining in >25 % of cells. P-Akt was visible in the cytoplasmic and in the nuclear compartments. Expression intensity in each compartment was evaluated as negative, weak, medium, or strong (0, 1, 2, and 3, respectively), and the tumors were additionally evaluated for percentage stained cells where score 0 was <1 %, score 1 was 1–25 %, score 2 was 26–75 %, and score 3 was >75 %. A histological score was calculated by adding intensity to percentage score, with a final score of 0–6. P-Akt positivity was defined as a histological score >3 in the cytoplasm and >4 in the nucleus. The intensity for p-ER-s167 was scored as negative, weak, and strong nuclear staining and nuclear positivity was defined as strong staining in >75 % of cells. P-ER-s167 cytoplasmic staining was evaluated as positive or negative. P-ER-s305 nuclear positivity was defined as visible staining in >1 % of cells and cytoplasmic staining was evaluated as negative or positive.

Antibody validation

The optimal antibody titers were assessed by staining TMA slides and choosing the concentration with the most discriminatory power, with the intensity ranging from negative-to-strong staining among different cases on the same slide. Antibody phospho-specificity was validated by dephosphorylation of proteins using λ-phosphatase (New England Biolabs). Slides were treated with 1,000 units of λ-phosphatase for 2 h at 37 °C followed by immunohistochemical staining according to the protocol used for the respective antibodies. All antibodies were phospho-specific and have been used previously in several studies [16–20].

Statistical analysis

All statistical analyses were performed using Statistica 10. Kaplan–Meier curves were plotted to describe recurrence-free survival (RFS) and differences between groups were evaluated with log-rank tests. Cox regression was used to assess hazard ratios, in univariate and multivariate models, and Pearson Chi-square tests were performed to investigate the interrelations between biological markers. A value of $P < 0.05$ was considered statistically significant with the exception of the test in Table 1 where the significance limit was set to $P < 0.01$ to compensate for the multiple testing.

Results

Expression analysis of phosphorylated mTOR and phosphorylated Akt was successful in 821 and 816 tumors, respectively. Activation of mTOR was evaluated with an antibody targeting the mTOR serine 2448 phospho-site, predominantly represented by the mTORC1, the indirect downstream target of Akt [21]. P-mTOR was visible in the extranuclear compartment, and strongly expressed in 11.8 % of tumors. Akt activation was measured by targeting the phosphorylated serine 473 residue of Akt, which is required for full activity of the kinase [1]. A high expression of p-Akt in the cytoplasm and in the nucleus was found in 59.7 and 56.1 % of tumors, respectively. Cross-talk between the PI3K/Akt/mTOR pathway and ER-signaling has been suggested as a mechanism of endocrine resistance in breast tumors; therefore, we added two phosphorylation sites on the ER to the analyses. P-mTOR correlated with nuclear expression of p-ERs167 and with PgR positivity. High cytoplasmic p-Akt was more frequent in tumors with a positive HER2 status, and with cytoplasmic p-ER. P-Akt in the nucleus correlated with small tumor size, ER-positive status, and with nuclear p-ER (Table 1).

Prognosis

For patients who received no systemic therapy, no prognostic value was detected for either p-mTOR or p-Akt, no matter of cut-off value and subgroup classification, analyzed with the end-point recurrence-free survival (RFS).

Table 1 Clinicopathological characteristics and pathway-related protein expression and activation in correlation with mTORC1 activity in extra nuclear compartment and Akt activity in nuclear (nu) and cytoplasmic (cyto) compartment of tumors from postmenopausal breast cancer patients

	Cytoplasmic p-mTOR-s2448			Nuclear p-Akt-s473		*P*	Cytoplasmic p-Akt-s473		*P*
	N (%)			*N* (%)			*N* (%)		
	−	+	*P*	−	+		−	+	
Total	726	95		358	458		329	487	
Tamoxifen									
−	365 (89)	43 (11)	0.36	294 (74)	106 (26)	0.41	156 (39)	244 (61)	0.45
+	361 (87)	52 (13)		295 (71)	121 (29)		173 (42)	243 (58)	
Size									
<20 mm	554 (89)	69 (11)	0.51	430 (69)	190 (31)	**0.001**	253 (41)	367 (59)	0.57
≥20 mm	156 (87)	23 (13)		145 (82)	32 (18)		68 (38)	109 (62)	
ER 10 %									
−	164 (93)	13 (7)	0.059	143 (81)	34 (19)	**0.0061**	63 (36)	114 (64)	0.16
+	542 (88)	77 (12)		432 (70)	182 (30)		255 (42)	359 (58)	
PR 10 %									
−	320 (92)	26 (8)	**0.0037**	262 (75)	87 (25)	0.26	133 (38)	216 (62)	0.37
+	324 (86)	54 (14)		264 (71)	106 (29)		156 (41)	217 (59)	
p-mTOR s2448 cyto									
−				501 (72)	192 (28)	0.29	285 (41)	408 (59)	0.036
+				61 (67)	30 (33)		27 (30)	64 (70)	
p-Akt s473 nu									
−	501 (89)	61 (11)	0.29				278 (47)	311 (53)	**<0.00001**
+	192 (86)	30 (14)					51 (22)	176 (78)	
p-Akt s473 cyto									
−	285 (91)	27 (9)	0.036	278 (85)	51 (15)	**<0.00001**			
+	408 (86)	64 (14)		311 (64)	176 (36)				
HER2									
−	588 (89)	76 (11)	0.33	478 (72)	186 (28)	0.018	281 (42)	383 (58)	**0.0023**
+	81 (92)	7 (8)		73 (84)	14 (16)		22 (25)	65 (75)	
p-ER s167 nu									
−	580 (90)	63 (10)	**0.0027**	493 (77)	147 (23)	**<0.00001**	267 (42)	373 (58)	0.14
+	141 (82)	31 (18)		90 (53)	79 (47)		60 (36)	109 (64)	
p-ER s167 cyto									
−	195 (92)	16 (8)	0.037	174 (84)	32 (16)	**<0.00001**	138 (67)	68 (33)	**<0.0001**
+	526 (87)	78 (13)		409 (68)	194 (32)		189 (31)	414 (69)	
p-ER s305 nu									
−	444 (88)	60 (12)	0.79	391 (78)	111 (22)	**<0.00001**	193 (38)	309 (62)	0.46
+	252 (89)	32 (11)		178 (62)	109 (38)		118 (41)	169 (59)	
p-ER s305 cyto									
−	299 (87)	45 (13)	0.28	255 (74)	89 (26)	0.27	159 (46)	185 (54)	**0.00058**
+	397 (89)	47 (11)		314 (71)	131 (29)		152 (34)	293 (66)	

P values ≤0.01 were considered significant after multiple analyses correction. Significant *P* values are shown in bold

A better prognosis for patients with high expression of nuclear p-ER-s167 was seen (RFS: HR, 0.71; 95 % CI, 0.45–1.13; $P = 0.14$) and (breast cancer survival: HR, 0.49; 95 % CI, 0.26–0.96; $P = 0.037$). This was not evident for cytoplasmic p-ER-s167 expression (data not shown).

Tamoxifen treatment prediction

For patients with ER-positive breast cancer, phospho-protein levels in the tumors were taken into account when comparing recurrence-free survival for tamoxifen-treated patients and patients receiving no endocrine treatment.

Low p-mTOR expression was associated with treatment benefit ($P = 0.00003$; Fig. 1a), whereas high p-mTOR expression indicated reduced response to treatment ($P = 0.55$; Fig. 1b). Restricting the analysis to patients with PgR-positive tumors further strengthened the results (Fig. 1c, d) and the interaction between p-mTOR and tamoxifen efficacy showed borderline significance in the latter analysis ($P = 0.064$; Table 2). High expression of p-Akt in the nucleus predicted reduced response to treatment compared with low expression (Table 2; Fig. 1e, f). No treatment predictive value was detected for cytoplasmic expression of p-Akt (low p-Akt, $P = 0.0036$ vs. high p-Akt, $P = 0.0089$).

Phosphorylations of the ER and their role in tamoxifen response and ligand-independent receptor activation have been discussed during the past few years. Previously, we showed an association of p-ER-s305 with a decreased tamoxifen efficacy [16]. In addition, the serine 167 was evaluated in the present study. Alone, the p-ER-s167 did not render significant interaction with tamoxifen efficacy comparing low and high expression. However, the data pointed toward a decreased treatment response, rather than the opposite (P (low) $= 0.00018$ vs. P (high) $= 0.16$; Table 2)). As both ER phospho-sites were associated with reduced response to tamoxifen, a p-ER variable, including either one or both of the sites, was constructed. The combined p-ER variable exhibited stronger treatment predictive value than both sites separately, showing borderline significance in the test for interaction (Table 2). The p-ER variable was also combined with the p-mTOR-s2448 and the p-Akt-s473 markers, respectively, and the two variables were further analyzed in relation to tamoxifen benefit. Both combinations indicated less benefit when both markers were positive, most evident when p-Akt was combined with p-ER (Table 2; Fig. 2).

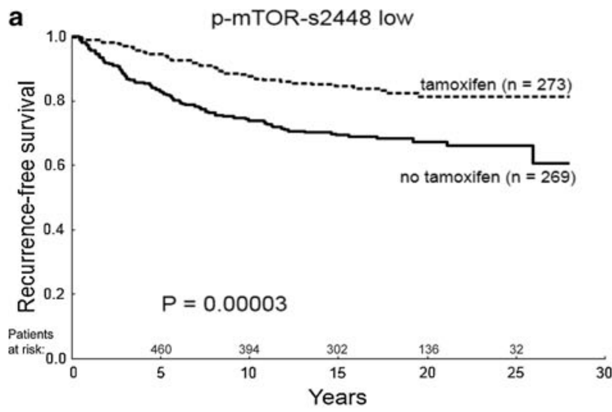
All studied biomarkers contributed more or less to the prediction of tamoxifen resistance. Therefore, we tested a combined score, where the sum of p-Akt, p-mTOR, and p-ER status was used. The score (0–3) showed significant interaction with tamoxifen efficacy ($P = 0.029$; Table 2) and a comparison of patients with no positive marker, one positive marker, and those with two or three positive markers is shown in Fig. 3.

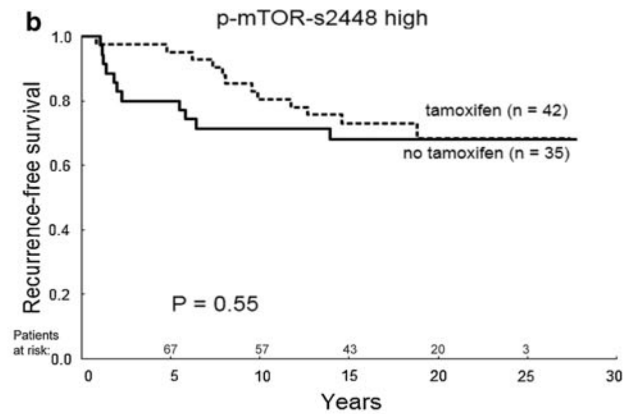
Discussion

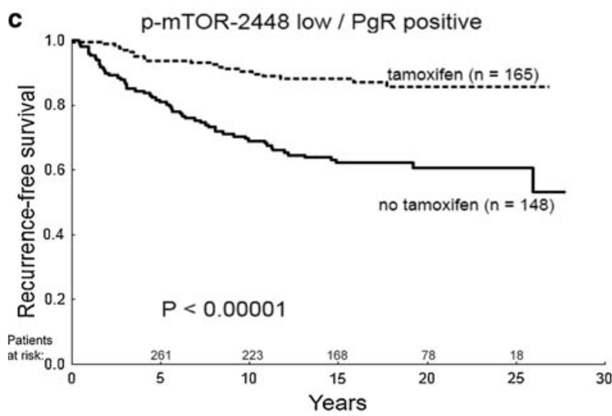
In this study, we evaluated markers for cross-talk signaling between the PI3K/Akt/mTOR and ER-pathways and their role for prognosis and tamoxifen response in a large randomized cohort of breast cancer patients with long-term follow-up. We observed strong expression of nuclear p-Akt-s473, cytoplasmic p-mTOR-s2448, and nuclear p-ER-s167 in 56, 12, and 20 % of the tumors, respectively. Correlations between the growth signaling markers and ER phosphorylations were mostly observed as intracellular location specific. P-Akt showed stronger association with the receptor phosphorylations than p-mTOR did, possibly as a result of direct ER interaction with p-Akt and indirect with mTOR. P-mTOR was closer correlated with PgR than with ER status, supporting a functional connection between the two markers.

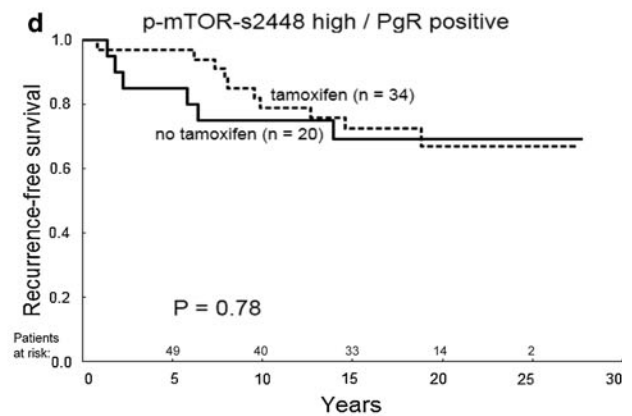
Bakarakos and collaborators implied that p-mTOR was related to an aggressive phenotype in invasive breast cancer [22]. In our hands, no prognostic value of p-mTOR-s2448 could be detected. The early stage of breast cancer in the present cohort may explain the distinctions between the studies. P-mTOR, as a single biomarker for tamoxifen response, showed close to significant interaction in the ER/PgR-positive subgroup. The PgR has for long been used as a marker of estrogen-dependent tumor growth and an indicator of a functional ER, even though the clinical value of PgR as an endocrine treatment predictive marker has been questioned recently [23, 24]. In vitro, mTORC1 inhibition restored tamoxifen sensitivity in Akt-induced tamoxifen resistant cell lines [25]. The mTORC1 inhibitor rapamycin induced a modest reduction of ER transcriptional activity, while a combinatorial inhibition of mTOR and MEK more significantly reduced ER activity, implying that both the PI3K- and the MAPK-pathways regulate ER driven cell growth [26]. Ongoing clinical studies, most recently the phase III BOLERO-2 trial, investigate the possibility to combine endocrine treatment with mTOR inhibitors [27–29]. Results are promising, with higher response rate in combination-therapy arms; however, it is not known what magnitude of benefit from the combined therapy that would be achieved in the adjuvant setting. To further delineate which patients have the best treatment benefit and who should be spared the side effects of a non-functional therapy, the signaling pathways need to be further investigated.

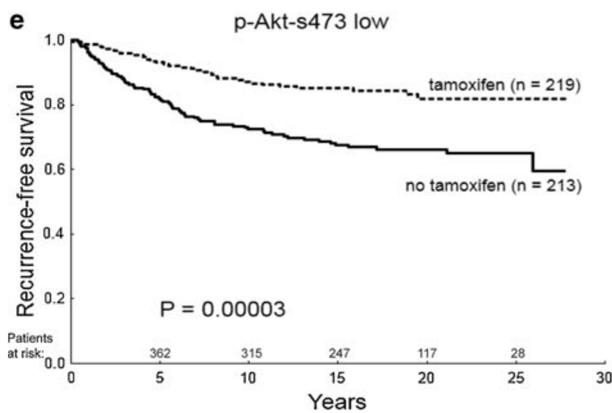
Nuclear p-Akt was associated with ER positivity, as shown in a previous publication also reporting a better prognosis for high p-Akt in the nucleus but not in the cytoplasm [30]. Spears et al. [31] recently reported a prognostic value of p-Akt1-t308 but not of p-Akt2-t309. Hence, the lack of prognostic value for p-Akt-s473 expression in the present study may reflect the balanced prognostic value of activated Akt1 and Akt2. Akt-induced tamoxifen resistance was shown in vitro and in vivo to partly depend on mTOR signaling [25]. To completely activate Akt, the serine 473 is crucial. Threonine 308 phosphorylation in the absence of serine 473 phosphorylation rendered 60 % Akt activity, seen after double inhibition of mTORC1 and mTORC2 [1]. We, therefore, chose to analyze the serine 473 phospho-expression. As a single marker, nuclear localized p-Akt tended to predict resistance to treatment. Fifteen years after surgery, the tamoxifen-treated group showed similar RFS as the untreated

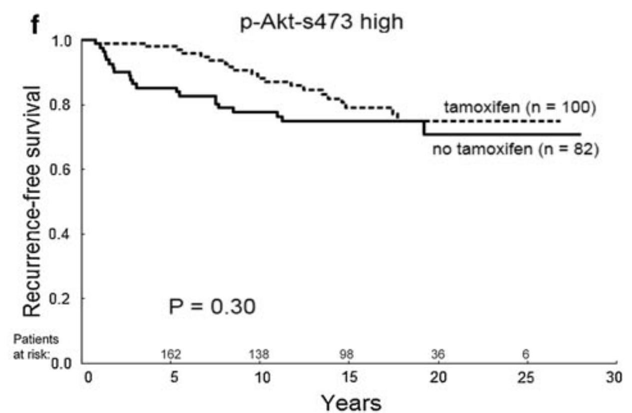

a
p-mTOR-s2448 low
tamoxifen (n = 273)
no tamoxifen (n = 269)
P = 0.00003
Recurrence-free survival
Patients at risk:
460 394 302 136 32
Years

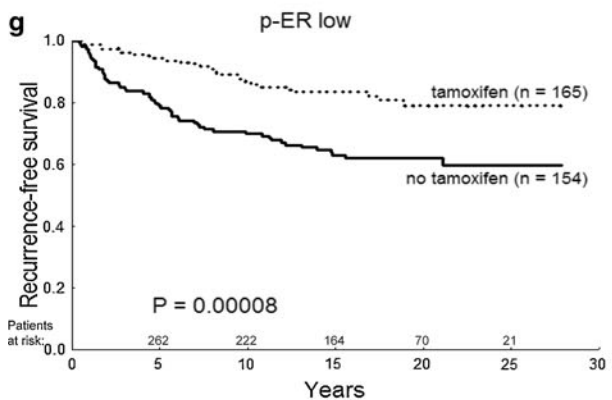

b
p-mTOR-s2448 high
tamoxifen (n = 42)
no tamoxifen (n = 35)
P = 0.55
Recurrence-free survival
Patients at risk:
67 57 43 20 3
Years


c
p-mTOR-2448 low / PgR positive
tamoxifen (n = 165)
no tamoxifen (n = 148)
P < 0.00001
Recurrence-free survival
Patients at risk:
261 223 168 78 18
Years


d
p-mTOR-s2448 high / PgR positive
tamoxifen (n = 34)
no tamoxifen (n = 20)
P = 0.78
Recurrence-free survival
Patients at risk:
49 40 33 14 2
Years


e
p-Akt-s473 low
tamoxifen (n = 219)
no tamoxifen (n = 213)
P = 0.00003
Recurrence-free survival
Patients at risk:
362 315 247 117 28
Years


f
p-Akt-s473 high
tamoxifen (n = 100)
no tamoxifen (n = 82)
P = 0.30
Recurrence-free survival
Patients at risk:
162 138 98 36 6
Years


g
p-ER low
tamoxifen (n = 165)
no tamoxifen (n = 154)
P = 0.00008
Recurrence-free survival
Patients at risk:
262 222 164 70 21
Years

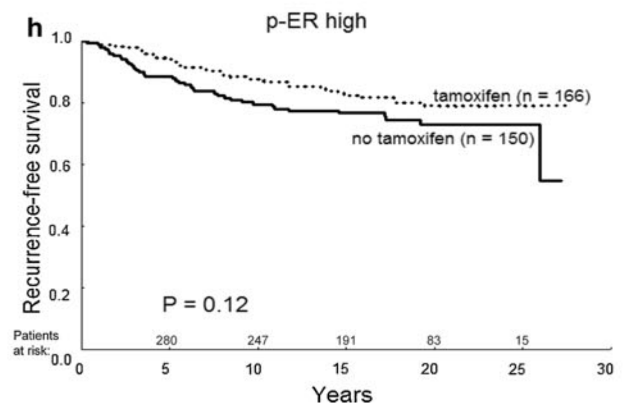

h
p-ER high
tamoxifen (n = 166)
no tamoxifen (n = 150)
P = 0.12
Recurrence-free survival
Patients at risk:
280 247 191 83 15
Years

Fig. 1 Tamoxifen efficacy in ER-positive patients grouped according to single biomarker expression, p-mTOR-s2448 (**a–b**), p-mTOR-s2488 in PgR-positive subgroup (**c–d**), nuclear p-Akt-s473 (**e–f**), and nuclear p-ER (s167 and/or s305) (**g–h**). Treatment response was reduced in case of high expression of either of the biomarkers, individually

group, indicating that tamoxifen treatment no longer improved RFS in the p-Akt high-expressing group but remained important for the low p-Akt-expressing group. Most previous studies of activated Akt and hormone treatment resistance lack untreated control groups, complicating the search for the actual treatment effect as the prognostic value of the biomarker may bias the result [32–34]. Combining p-Akt with p-mTOR rendered similar results as p-Akt alone, with initial treatment response. Akt has mainly been studied as a kinase upstream of mTORC1. Notably, the mTORC2 is necessary for p-Akt-s473 [35]. The p-mTOR-s2448, used in this study, is mainly a marker of mTORC1 [21]. Further studies involving p-mTORC2 and p-Akt will assess whether their connection is involved in treatment response.

An improved breast cancer survival in the systemically untreated group was detected for patients with high expression of p-ER-s167 in the tumor, compared with lower expression. This finding was in line with previous studies [36, 37]. In addition, several smaller studies found p-ER-s167 to provide an improved clinical outcome in tamoxifen-treated patients [20, 38]. Skliris et al. [39] proposed p-ER-s167 to be a good factor in a cohort of tamoxifen-treated breast cancer patients, suggesting an intact estrogen-dependent targetable growth. To our knowledge, the present study was the first based on a randomized trial to analyze p-ER-s167 in the context of predicting tamoxifen response. Abundant expression of p-ER-s167 was not a marker for increased tamoxifen sensitivity. As a single marker, p-ER-s167 showed no significant interaction. An additive role in

Table 2 Percent decrease with tamoxifen treatment in absolute risk at 15 years and numbers needed to treat (NNTT) to prevent one recurrence

	Recurrence tamoxifen versus no tamoxifen				
	Decrease in absolute risk at 15 years (%)	NNTT at 15 years	HR (95 % CI)	*P*	*P* interaction[a]
p-mTOR-s2448					
−	15.1	6.6	0.46 (0.32–0.67)	**0.000043**	
+	4.8	21	0.78 (0.34–1.76)	0.55	0.25
p-mTOR-s2448 (PgR+)					
−	25.8	3.9	0.29 (0.18–0.49)	**0.000002**	
+	2.5	40	0.86 (0.31–2.38)	0.78	0.064
p-Akt-s473					
−	17.3	5.8	0.43 (0.28–0.65)	**0.00005**	
+	4.6	21.7	0.72 (0.39–1.33)	0.23	0.054
p-ER-s167					
−	15.7	6.4	0.50 (0.35–0.72)	**0.00018**	
+	4.7	21.3	0.60 (0.30–1.22)	0.16	0.77
p-ER					
−	20.5	4.9	0.41 (0.26–0.65)	**0.00012**	
+	5.7	17.5	0.68 (0.42–1.1)	0.12	0.067
p-mTOR and p-ER					
−	13.9	7.2	0.45 (0.36–0.70)	**0.000067**	
+	12.9	7.8	0.60 (0.18–2.0)	0.40	0.25
p-Akt and p-ER					
−	17.5	5.7	0.45 (0.31–0.65)	**0.000021**	
+	−3.2	–	1.0 (0.46–2.4)	0.91	**0.024**
p-mTOR, p-Akt, and p-ER					
0 positive	24.5	4.1	0.30 (0.16–0.55)	**0.00017**	
1 positive	11.6	8.6	0.60 (0.35–1.03)	0.062	
2 or 3 positive	2.9	34.5	0.76 (0.37–1.56)	0.45	**0.029***

Cox proportional hazard analysis of the benefit from tamoxifen in patients with ER-positive tumors in relation to p-mTOR-s2488 expression, p-Akt-s473 nuclear expression, p-ER-s167, p-ER-s167, and/or p-ER-s305 (p-ER), and biomarkers in combination. Significant *P* values are shown in bold

* Test for trend

[a] Adjusted for established prognostic factors; size and HER2-status

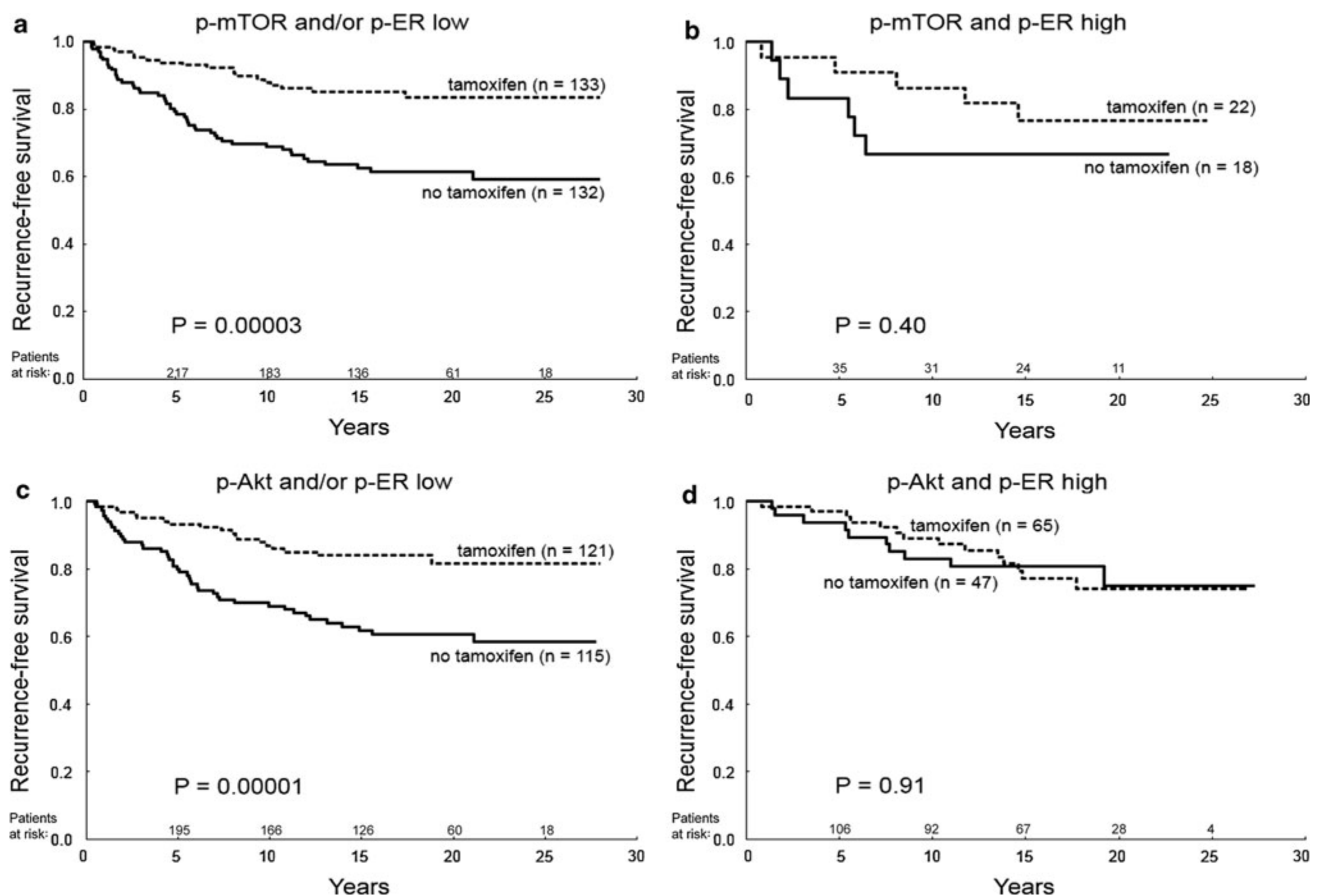


Fig. 2 Tamoxifen efficacy in ER-positive patients grouped according to combined biomarkers p-mTOR-s2448, p-Akt-s473, and p-ER (s167 and/or s305)

combination with other markers to detect cross-talk dependent tamoxifen resistance was noted.

The connection between growth signaling pathways and the ER is a proposed mechanism for tamoxifen resistance [40, 41]. The ER is phosphorylated at serine 167 by the mTORC1 substrate S6K1 and by the MAPK target p90 ribosomal protein S6kinase (RSK) [12]. IGF-regulated signaling phosphorylated ER at serine 167 through Akt, and the signal was rapamycin sensitive, suggesting a signal from Akt, via mTOR to ER activation [42]. Our clinical data are in line with the results from the in vitro studies and demonstrated an association between Akt/mTOR signaling and ER phosphorylation. The phosphorylations have previously in retrospective clinical studies and in vitro been reported to reduce response to tamoxifen and activate the receptor ligand-independently [3]. Combining either p-mTOR or p-Akt with p-ER led to prediction of decreased tamoxifen response. Using all the available biomarkers in combination increased the size of the subgroup populations with reduced benefit. For patients with tumors expressing no markers, the response to tamoxifen was clearly significant, while in the group expressing one marker the response to treatment tended to be reduced; and in the group with two or three positive markers, the response was non-significant. The interaction was significant when analyzing all groups together.

In conclusion, our data support the model of cross-talk between growth factor signaling and ER phosphorylation and its association with tamoxifen resistance. The PI3K/Akt pathway is frequently deregulated in breast cancer, providing a mechanism for cells to sustain growth despite endocrine treatment. Phosphorylated mTOR, as a single predictive marker of reduced tamoxifen response, may be applicable in the ER/PgR-positive subgroup. However, we suggest a multiple phospho-marker test including p-Akt-s473, p-mTOR-s2448, and p-ER-s167/s305. With at least two positive markers, untreated patients showed similar recurrence-free survival as the tamoxifen-treated patients. We suggest this pathway to be further evaluated considering PI3K/mTOR inhibitors in addition to endocrine treatment.

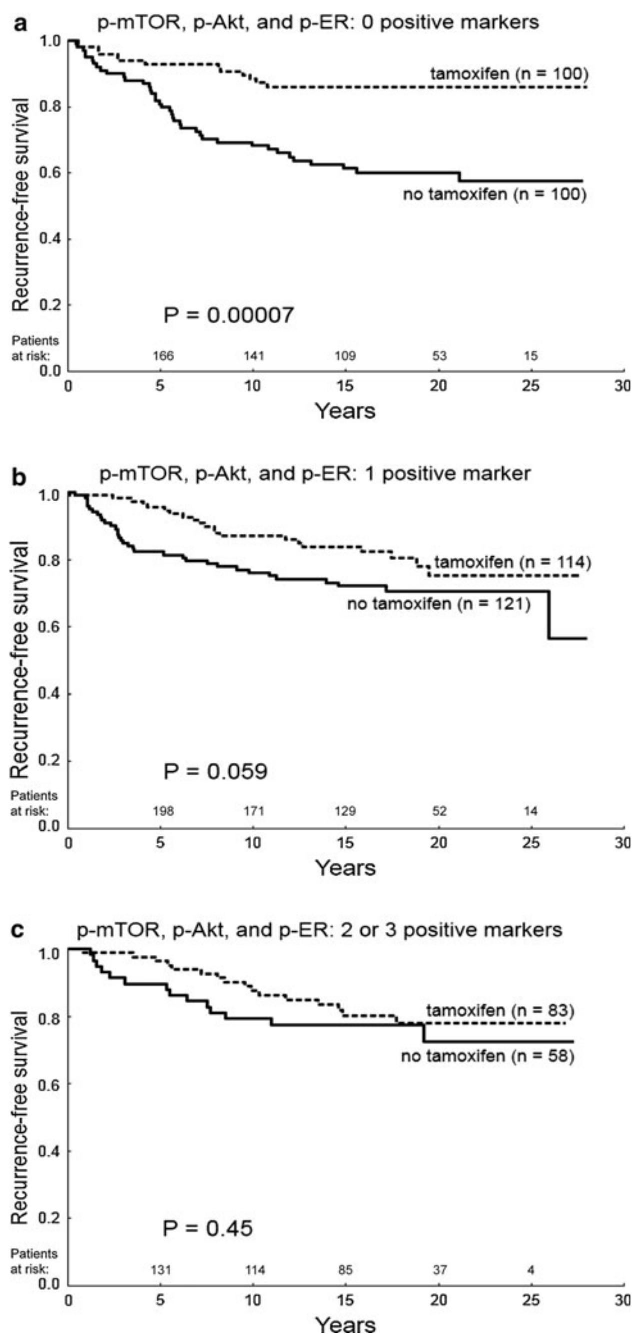


Fig. 3 Tamoxifen efficacy in ER-positive patients according to score, defined by no (**a**), one (**b**), and two or three (**c**) positive markers in the p-Akt, p-mTOR, and p-ER axis. Treatment response decreased with the number of positive markers

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Conflict of interest The authors declare that they have no conflict of interest.

Ethical standards The authors declare that the study comply with the current laws of Sweden.

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