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Josefine Bostner[1], Elin Karlsson[1], Cecilia Bivik Eding[2], Gizeh Perez-Tenorio[1], Hanna Franzén[1], Aelita Konstantinell[1], Tommy Fornander[3], Bo Nordenskjöld[1] and Olle Stål[1]

[1]Department of Clinical and Experimental Medicine, Department of Oncology [2]Department of Clinical and Experimental Medicine, Division of Dermatology, Linköping University, SE-58185 Linköping, Sweden
[3]Department of Oncology, Karolinska University Hospital, Karolinska Institute, SE-17176 Stockholm, Sweden

Correspondence should be addressed to J Bostner

Email
Josefine.Bostner@liu.se

Abstract

Detection of signals in the mammalian target of rapamycin (mTOR) and the estrogen receptor (ER) pathways may be a future clinical tool for the prediction of adjuvant treatment response in primary breast cancer. Using immunohistological staining, we investigated the value of the mTOR targets p70-S6 kinase (S6K) 1 and 2 as biomarkers for tamoxifen benefit in two independent clinical trials comparing adjuvant tamoxifen with no tamoxifen or 5 years versus 2 years of tamoxifen treatment. In addition, the prognostic value of the S6Ks was evaluated. We found that S6K1 correlated with proliferation, HER2 status, and cytoplasmic AKT activity, whereas high protein expression levels of S6K2 and phosphorylated (p) S6K were more common in ER-positive, and low-proliferative tumors with pAKT-s473 localized to the nucleus. Nuclear accumulation of S6K1 was indicative of a reduced tamoxifen effect (hazard ratio (HR): 1.07, 95% CI: 0.53–2.81, $P=0.84$), compared with a significant benefit from tamoxifen treatment in patients without tumor S6K1 nuclear accumulation (HR: 0.42, 95% CI: 0.29–0.62, $P<0.00001$). Also S6K1 and S6K2 activation, indicated by pS6K-t389 expression, was associated with low benefit from tamoxifen (HR: 0.97, 95% CI: 0.50–1.87, $P=0.92$). In addition, high protein expression of S6K1, independent of localization, predicted worse prognosis in a multivariate analysis, $P=0.00041$ (cytoplasm), $P=0.016$ (nucleus). In conclusion, the mTOR-activated kinases S6K1 and S6K2 interfere with proliferation and response to tamoxifen. Monitoring their activity and intracellular localization may provide biomarkers for breast cancer treatment, allowing the identification of a group of patients less likely to benefit from tamoxifen and thus in need of an alternative or additional targeted treatment.

Introduction

Breast cancer is the most common malignancy among women, with more than 75% of tumors positive for hormone receptors and thereby possibly responsive to adjuvant endocrine treatment. Active signaling in the phosphatidylinositol 3-kinase/mammalian target of rapamycin (PI3K/mTOR) cascade is a characterized mechanism

of endocrine resistance, with aberrant stimulation promoting estrogen receptor (ER) α (ESR1) activation and tumor growth, despite estrogen antagonizing- or estrogen restriction treatments (Miller *et al.* 2011). Inhibition with MABs-binding tyrosine kinase receptors, or mTOR inhibition with rapamycin analogues, has been shown clinically to sensitize tumors to endocrine therapy and prolonging time to progression (Baselga *et al.* 2009, 2012, Johnston *et al.* 2009, Bachelot *et al.* 2012). However, the pathway involves negative feedback regulatory mechanisms, which may provide unwanted activation of signals, such as AKT and ERK1/2, upon inhibition (Soares *et al.* 2013). Inhibitors targeting signals at more than one level have been promising *in vitro* and it is probably necessary in many tumors to prevent cells from finding alternative routes to proliferation. The best treatment combination for each tumor or subset of tumors has yet to be discovered, although finding large groups of patients responsive to similar therapy will encourage development of new biomarkers and treatments.

Everolimus (Afinitor) has been recently registered as a treatment for women recurring on endocrine therapy. It is a first-generation mTOR inhibitor, targeting the mTOR complex 1 (mTORC1). Two major downstream targets of mTORC1 are the S6 kinases (S6Ks) 1 and 2. Phosphorylation of these proteins reflects the activity of the mTORC1 (Hara *et al.* 1997). S6K1 and S6K2 are highly homologous proteins encoded by the *RPS6KB1* gene in chromosomal region 17q23, and by the *RPS6KB2* gene at 11q13, respectively. The S6Ks share several features, such as their ability to phosphorylate the 40S ribosomal protein S6 and the dependence of an activated mTORC1 for complete activation (Lee-Fruman *et al.* 1999, Park *et al.* 2002). The two genes are located in the commonly amplified regions in the genome, associated with poor prognosis in breast cancer (Barlund *et al.* 2000, Bostner *et al.* 2007, Perez-Tenorio *et al.* 2011). Gene expression of *RPS6KB1* is upregulated in various cancers (Ip *et al.* 2011, Sridharan & Basu 2011, Li *et al.* 2012), and we have observed the prognostic value of *RPS6KB2* gene expression in breast cancer (Karlsson *et al.* 2013). Results of studies have indicated increased protein expression of S6K1 and S6K2 in tumors when compared with normal or benign tissue (Filonenko *et al.* 2004, Ismail 2012, Li *et al.* 2012). S6K1 protein overexpression predicted poor prognosis in multiple studies (van der Hage *et al.* 2004, Perez-Tenorio *et al.* 2011, Ismail 2012).

Measuring phosphorylated (p) threonine (t) at the amino acid residue 389 of S6K has become a method to evaluate mTORC1 kinase activity *in vitro*. Castellvi *et al.* (2006) detected increased pS6K expression in malignant ovarian tumors compared with benign ovarian tumors, and high pS6K expression was associated with worse prognosis in two breast cancer cohorts (Noh *et al.* 2008, Kim *et al.* 2011).

S6K1 has been shown to phosphorylate the ER on serine (s) 167, inducing conformational changes in the receptor and making it less responsive to the inhibitory effects of tamoxifen (Yamnik *et al.* 2009). In addition, *RPS6KB1* is a target gene for ER transcription (Maruani *et al.* 2012). S6K2 has recently been brought to light as a potentially important oncoprotein, with fewer essential roles in normal tissue than S6K1, making it an interesting treatment target (Pardo & Seckl 2013).

To further delineate the treatment-predictive role and prognostic value of the S6Ks, we studied protein expression of S6K1, S6K2, and pS6K-t389 in tumors of patients participating in trials comparing adjuvant tamoxifen with no tamoxifen or 2-year with 5-year tamoxifen treatment.

Material and methods

Patients and tumor samples

This study was designed and presented with regard to the reporting recommendations for tumor marker prognostic studies (REMARK) guidelines (McShane *et al.* 2006). Ethical approval for cohort 1 was from Karolinska Institute Ethics Board. Ethical approval for cohort 2 was obtained from Linköping University Ethics Board.

Cohort 1 (tamoxifen versus no tamoxifen)

The Stockholm trial included a cohort of breast carcinoma patients with node-negative disease and a tumor size not exceeding 30 mm, randomized to tamoxifen or no adjuvant treatment. Radiotherapy was used for patients receiving breast-conserving therapy. No adjuvant chemotherapy was given to this group of patients. Demographic data and detailed information on the cohort have been previously described (Rutqvist & Johansson 2007, Bostner *et al.* 2013). Tissue micro arrays (TMAs) with three individual cores were constructed from formalin-fixed paraffin-embedded (FFPE) tumors from 912 patients. ER, progesterone receptor (PgR), HER2, pAkt-s473, pER-s167, p-mTOR status, and mitosis were previously determined (Wrange *et al.* 1978, Rutqvist & Johansson 2007, Bostner *et al.* 2013).

Endocrine-Related Cancer

Cohort 2 (tamoxifen 5 years versus 2 years)

Tumor tissues of invasive primary breast carcinomas were obtained from a randomized trial of 5 years versus 2 years of adjuvant tamoxifen for postmenopausal early-stage breast cancer conducted by the Swedish Breast Cancer Group during 1983–1991 in five regional cancer centers in Sweden (Swedish Breast Cancer Cooperative Group 1996). The 130 tumors available for this study were represented into two TMA blocks, and patients had been diagnosed in the South-east Sweden region. The ER and PgR status had been determined previously through isoelectric focusing or an enzyme immune assay (Wrange et al. 1978, Fernö et al. 1986). In addition, S-phase fraction and HER2 status were later determined (Fernö et al. 2000, Stål et al. 2000). TMAs with three individual cores were constructed from FFPE tumors and 4 μm sections were used for immunohistochemistry.

Immunostaining

The two patient series were analyzed with the MAB against pS6K-t389 at a concentration of 1:100 (Cell Signaling Technology, Danvers, MA, USA; #9206). The tumors were additionally analyzed with the S6K1 antibody at a concentration of 1:100 (Cell Signaling Technology; #2708). The tumors in cohort 2 were stained with a newly synthesized S6K2 antibody at a concentration of 1:100 (kindly provided by Professor Filonenko) (Savinska et al. 2012). The TMAs of cohort 1 had been previously stained with an alternative S6K2 antibody (Perez-Tenorio et al. 2011).

The PT-link system (Dako, Glostrup, Denmark; PT10126) with the Envision FLEX Target Retreival Solution, Low pH, was used for deparaffinization, rehydration, and epitope retrieval of the TMAs. The slides were washed in PBS/0.05% Tween-20, subjected to endogenous peroxidase inactivation in 3% hydrogen peroxide, washed in PBS/0.5% BSA, blocked for 10 min in serum-free protein block (Spring Bioscience, Freemont, CA, USA), and incubated with primary antibody in a moisturized chamber at 4 °C overnight. On day 2, the slides were washed in PBS/0.5% BSA, incubated with secondary antibody (Dako Cytomation Envision+ HRP system; Dako) at room temperature for 30 min, washed, developed in PBS/3,3′-diaminobenzidine hydrochloride (DAB) for 8 min, counterstained with hematoxylin for 1 min, washed, dehydrated in an ethanol series, and mounted with Pertex (HistoLab, Västra Frölunda, Sweden). The images at 20× and 40× magnification were produced

with an Olympus BX21 microscope and an Olympus DP70 camera, and whole-slide images of the first cohort p-S6K staining were generated using the ScanScope aT at 200× magnification (Aperio, Vista, CA, USA).

Scoring

Before scoring, the tumors were evaluated for scoring cutoffs appropriate for each stain, with regard to intensity levels and percentage of tumor cells with positive staining, when applicable. Scoring was thereafter conducted by two individual observers, blinded to clinical data. A consensus on the score for each tumor was reached following the individual scoring.

In cohort 1, S6K1 intensity was graded for cytoplasm and nuclei individually as 0 (negative), 1 (weak), 2 (medium), and 3 (strong) (Supplementary Table 1, see section on supplementary data given at the end of this article). Cutoff for positive staining was set at strong intensity. In addition, a positive score for nuclear S6K1 expression being stronger than cytoplasmic S6K1 was generated. In cohort 2, S6K1 and S6K2 were evaluated for cytoplasmic and nuclear intensity 0 (negative), 1 (weak), 2 (medium), and 3 (strong). Cutoff was set at medium intensity as fewer than 10% received the highest score. In cohort 1, pS6K-t389 was graded according to cytoplasmic and nuclear intensity into four groups (negative, weak, medium, and strong), and cutoff was set at strong intensity. In cohort 2, pS6K-t389 expression was evaluated for cytoplasmic intensity (0 (negative), 1 (weak), 2 (medium), and 3 (strong)), and proportion (0 (<1%), 1 (1–25%), 2 (26–75%), and 3 (>75%)). A score was generated by adding intensity and proportion (0–6) and the cutoff was set at score >4 for high expression. No nuclear staining of pS6K-t389 was observed in cohort 2.

Statistical analysis

To compare high and low expression in two groups, the Pearson χ^2-test was performed. For rank order of expression levels in four groups, the Spearman's test was applied. Hazard ratios (HRs) with 95% CI were estimated using the Cox proportional hazards model and the time from diagnosis to any breast cancer recurrence was used as an endpoint. Recurrence-free survival (RFS), defined as time to the first of the following events, local or distant recurrence, or breast cancer-related death was compared with the log-rank test and Kaplan–Meier plots were drawn for visualization. Endocrine-treated patients were excluded from the prognostic analyses of the first cohort.

All tamoxifen treatment prediction analyses were restricted to patients with ER-positive tumors, and in cohort 2 analyses were restricted to starting 2 years after diagnosis to avoid arbitrary differences in the two, up until 2 years, identically treated groups. A P value of <0.05 or <0.01 was considered significant. All statistical analyses of the patient cohorts were performed using Statistica 10 software.

Cell culture

The cell line ZR751 (Engel *et al*. 1978) (ATCC, Manassas, VA, USA; LGC standards, Teddington, Middlesex, UK) was cultured in Optimem without phenol red (Gibco, Life Technologies), supplemented with 4% heat-inactivated fetal bovine serum (Gibco) at 37 °C, 5% CO_2. Cell authentication was done at ATCC using Short-Tandem Repeat Profiling analysis. All experiments were conducted with cells in the exponential growth phase and cell passage number was kept low.

siRNA knockdown

The cells were transfected with siRNA using the Nucleofector Kit V with the Amaxa nucleofection system (Lonza, Basel, Switzerland). Briefly, cells were detached, resuspended in 100 μl nucleofector solution with 300 nM siRNA (Silencer Negative Control No. 1; AM4611, S6K1; 110802 and s12284, and S6K2; 471, Ambion by Life Technologies), transferred to cuvettes and transfected in an Amaxa Biosystems Nucleofector II, program P20. The cells were transferred to cell culture media, counted using a Countess Automated Cell Counter (Life Technologies), and seeded at 100 000 cells/ml. After 24 h, cell culture media was changed.

Western blotting analysis

For protein preparation, cells were rinsed in ice-cold PBS, lysed in RIPA buffer, containing 150 mM NaCl, 2% Triton, 0.1% SDS, 50 nM Tris pH 8.0, Complete Mini Protease Inhibitor Cocktail (1836153; Roche), PhosSTOP phosphatase inhibitor cocktail (Roche Pharma), and phosphatase inhibitor cocktail 2 and 3 (P5726 and P0044, Sigma–Aldrich). The cell lysates were incubated on ice for 30 min and centrifuged at 20 800 *g*. Protein concentration of the supernatants was determined by the colorimetric BCA Protein Assay (Thermo Scientific Pierce, Rockford, IL, USA) and the lysates were stored at −70 °C. The samples containing 15 μg protein were denatured with Laemmli

sample buffer (Bio-Rad), β-mercaptoethanol (Bio-Rad), and heating at 95 °C for 5 min, separated on mini-PROTEAN TGX precast gels, 4–15% (Bio-Rad) at 90 V for 15 min and 150 V for 45 min. Separated proteins were transferred onto a PVDF membrane in a Trans-Blot Turbo system (Bio-Rad), program Mixed-MW kDa, for 7 min. The membranes were blocked according to the specification of the primary antibody before incubation with primary antibodies in a blocking buffer overnight at 4 °C. The membranes were washed in Tris (2.5 mM)–Glycine (19.2 mM)–SDS (0.01%) (Bio-Rad) with 0.1% Tween-20, and incubated for 1 h at room temperature with secondary antibodies. The protein bands were visualized using an Amersham ECL Prime Western Blot detection system (GE Healthcare Life Sciences, Little Chalfont, Buckinghamshire, UK) and a charge-coupled camera with the software Image Reader LAS-1000 Pro v.2.6 for detection of chemiluminescent signals (Fujifilm LAS-1000, Stockholm, Sweden). The antibodies used for western blotting were S6K1, pS6K-t389, pAkt-s473 (Cell Signaling Technology), S6K2 (kindly provided by Professor Filonenko) (Savinska *et al*. 2012), and a GAPDH antibody (rabbit monoclonal; Epitomics, Cambridge, UK; #5632-1) was used as internal control. The ladder MagicMark XP Western Protein Standard was used for molecular weight estimations (Life Technologies).

Fixation and paraffin embedding of cells

The cells at 80% confluence were washed in PBS, dissociated from the surface with Tryple express (Life Technologies) for 5 min in 37 °C, and centrifuged at 200 *g* for 5 min. The cells were washed in PBS and centrifuged again to form a pellet, and fixed with 4% formaldehyde (Sigma–Aldrich) at room temperature for 4 h, visualized with hematoxylin, centrifuged, and dehydrated with an ethanol to xylene gradient overnight. The cell pellet was paraffin embedded at 56 °C for 4 h by the addition of new paraffin every hour. The embedded cells were cut in 3 μm sections using a rotation microtome (Microm International GmbH, Waldorf, Germany).

λ-phosphatase assay

Phosphorylation specificity of the pS6K-t389 antibody was determined by dephosphorylation of proteins in heregulin β1 (ImmunoTools, Friesoythe, Germany, 0.1 μM) (HRG)-treated FFPE ZR751 breast cancer cells by λ-phosphatase (New England Biolabs, Ipswich, MA, USA). The cells on slides were treated with 1000 U λ-phosphatase or

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water (control) for 2 h at 37 °C followed by immunohis-tochemical staining according to the protocol used for the pS6K-t389 antibody.

Results

Immunohistochemical staining of tumors

S6K proteins and phosphorylated kinase expression as potential biomarkers of tamoxifen benefit were analyzed in the two breast cancer cohorts. Distribution of protein expressions and cutoffs in the two cohorts are presented in a Supplementary Table 1 and a mechanistic scheme of the signaling pathways with treatment predictive and prognostic results are presented in Supplementary Figure 1, see section on supplementary data given at the end of this article. Successful staining for S6K1 was found in 849 tumors (cohort 1) and in 130 tumors (cohort 2), and detection of pS6K was possible in 807 tumors (cohort 1) and in 130 tumors (cohort 2).

S6K1 and S6K2 correlate with separate tumor characteristics

In cohort 1, pS6K was highly correlated with both high S6K1 and high S6K2 expression, supporting the antibody selectivity found *in vitro* toward the two homologs also in FFPE breast tissue (Table 1). In contrast, S6K1 and S6K2 were not significantly co-expressed. Nuclear pS6K correlated well with ER positivity ($P=0.001$), as did nuclear S6K2 in the two sets, whereas high S6K1 expression was connected with HER2 positivity (Tables 1 and 2).

The AKT protein is central in the PI3K/mTOR-pathway, with its activity being regulated upstream and downstream of the S6Ks. AKT stimulates S6K through mTORC1, and mTORC1 or S6K1 repression commonly results in AKT activation through the release of S6K1 inhibition of the IGF1 receptor regulator IRS1 (Um *et al.* 2004, Tabernero *et al.* 2008). A connection between active expression of AKT (pAKT-s473) and all analyzed S6K variants was evident in the first set, with strongest correlations detected when proteins were expressed in the same location: cytoplasm and nucleus respectively (Table 1). We detected strong coexpression of pAKT and pS6K in the nucleus and of pAKT and S6K2 in the nucleus. A pAKT correlation with S6K1 was observed in the cytoplasm. S6K1 and AKT are both known to phosphorylate the ER on serine 167. pS6K correlated well with pER-s167 in the cytoplasm and in the nucleus. pAKT and pER-s167 were strongly associated ($P<0.00001$),

as previously shown in cohort 1 (Bostner *et al.* 2013). The Spearman rank order test was applied for p-mTOR versus nuclear pS6K ($P=0.0002$) and for p-mTOR versus nuclear S6K2 ($P=0.00002$).

We found high nuclear pS6K and high nuclear S6K2 to correlate significantly with small tumor size, but no obvious relationship between S6K1 and tumor size in the first set (Table 1). In the second set the pS6K cytoplasmic expression was correlated with large size of tumors (Table 2). Results of a previous study indicated that overexpression of S6K2 but not S6K1 was important for cell proliferation in HEK293 cells (Goh *et al.* 2010). Proliferation in our two sets was represented by mitosis in the first set, and S-phase fraction in the second set (Tables 1 and 2). High levels of S6K1 expression indicated increased proliferation, while high levels of nuclear pS6K and S6K2 indicated reduced proliferation in the first set. In the second set, no significant correlations with proliferation were observed; however, a tendency toward increased proliferation was detected with high cytoplasmic S6K1, consistent with results from the first set.

High nuclear S6K1 and high pS6K predict groups that do not benefit from tamoxifen

Up to one-third of the tumors showed a distinct pattern of S6K1 nuclear staining with weaker cytoplasmic staining. A significant tamoxifen benefit was observed in cohort 1 for patients whose tumors did not show the S6K1 nuclear pattern ($P<0.00001$) (Fig. 1A). The benefit was not evident in patients with tumors showing nuclear dominance of S6K1 (Fig. 1B), and the difference in treatment efficacy between the groups was significant (Table 3). Similarly, patients in cohort 2 with tumors not showing the S6K1 nuclear pattern tended to benefit from prolonged tamoxifen therapy (Fig. 1C and D). No significant treatment-predictive role was identified for S6K2 alone in the two sets (Perez-Tenorio *et al.* 2011). The role of nuclear S6K2 seemed dependent on the PgR status, showing a loss of treatment response when tumors expressed high levels of nuclear S6K2 and no PgR.

High levels of expression of pS6K in the cytoplasm and nucleus simultaneously resulted in reduced response to tamoxifen compared with low expression in one or both compartments in the first set (Fig. 2A, B and Table 3). In the second set, a trend towards benefit from longer treatment was observed with low pS6K expression (Fig. 2C and D). With a high pS6K expression in the cytoplasm, the potential benefit from longer tamoxifen treatment disappeared.

Table 1 Cohort 1: clinicopathological characteristics and pathway-related phosphoprotein expression in correlation with activated pS6K-t389 (pS6K) and total S6Ks in cytoplasm (c) and in nuclear (n) compartments of tumors from postmenopausal breast cancer patients

	pS6Kc n (%)			pS6Kn n (%)			S6K1c n (%)			S6K1n n (%)			S6K2c n (%)			S6K2n n (%)		
	Low	High	P	Low	High	P	Low	High	P	Low	High	P	Low	High	P	Low	High	P
No tamoxifen	318 (80)	79 (20)		248 (63)	148 (37)		377 (90)	41 (10)		352 (84)	66 (16)		301 (79)	82 (21)		242 (63)	140 (37)	
Tamoxifen	326 (80)	84 (20)	0.8	256 (62)	154 (38)	1.0	383 (89)	48 (11)	0.5	362 (84)	69 (16)	0.9	321 (78)	88 (22)	1.0	247 (60)	163 (40)	0.4
Size <20 mm	485 (79)	126 (21)		357 (59)	253 (41)		574 (89)	68 (11)		537 (84)	105 (16)		471 (78)	135 (22)		352 (58)	253 (42)	
Size >20 mm	144 (81)	34 (19)	0.7	133 (75)	45 (25)	0.00009	165 (90)	19 (10)	0.9	156 (85)	28 (15)	0.7	138 (82)	31 (18)	0.3	128 (75)	42 (25)	0.00005
Mitosis low	368 (80)	94 (20)		272 (59)	190 (41)		440 (92)	36 (8)		412 (87)	64 (13)		333 (75)	112 (25)		226 (51)	219 (49)	
Mitosis high	189 (81)	43 (19)	0.6	163 (71)	68 (29)	0.003	203 (86)	34 (14)	0.004	189 (80)	48 (20)	0.02	205 (86)	33 (14)	0.0006	202 (85)	36 (15)	<0.00001
ER−	134 (77)	39 (23)		126 (73)	46 (27)		162 (89)	21 (11)		153 (84)	30 (16)		141 (84)	27 (16)		141 (84)	27 (16)	
ER+	495 (81)	117 (19)	0.3	364 (59)	248 (41)	0.001	573 (89)	68 (11)	0.7	538 (84)	103 (16)	0.9	466 (77)	138 (23)	0.05	339 (56)	265 (44)	<0.00001
PgR−	276 (79)	72 (21)		230 (66)	117 (34)		316 (88)	44 (12)		294 (82)	66 (18)		273 (80)	70 (20)		254 (74)	88 (26)	
PgR+	300 (80)	71 (20)	0.6	225 (61)	146 (39)	0.1	350 (91)	35 (9)	0.2	330 (86)	55 (14)	0.1	284 (77)	85 (23)	0.4	191 (52)	179 (48)	<0.00001
HER2−	528 (80)	130 (20)		419 (64)	239 (36)		626 (91)	62 (9)		595 (86)	93 (14)		516 (79)	139 (21)		396 (60)	259 (40)	
HER2+	70 (80)	18 (20)	0.9	50 (57)	37 (43)	0.3	68 (75)	23 (25)	<0.00001	60 (66)	31 (34)	<0.00001	66 (80)	17 (20)	0.8	62 (75)	21 (25)	0.01
pAkt c low	265 (86)	44 (14)		217 (70)	92 (30)		302 (93)	22 (7)		292 (90)	32 (10)		238 (81)	55 (19)		174 (59)	120 (41)	
pAkt c high	360 (76)	112 (24)	0.001	269 (57)	202 (43)	0.0002	417 (87)	62 (13)	0.005	384 (80)	95 (20)	0.0002	351 (76)	110 (24)	0.1	292 (63)	169 (37)	0.3
pAkt n low	470 (84)	89 (16)		396 (71)	162 (29)		518 (90)	60 (10)		496 (86)	82 (14)		433 (81)	103 (19)		361 (67)	176 (33)	
pAkt n high	155 (70)	67 (30)	0.00001	90 (41)	132 (59)	<0.00001	201 (89)	24 (11)	0.9	180 (80)	45 (20)	0.04	156 (72)	62 (28)	0.005	105 (48)	113 (52)	<0.00001
pER-s167 low	529 (83)	108 (17)		442 (70)	194 (31)		595 (90)	69 (10)		564 (85)	100 (15)		500 (81)	116 (19)		418 (68)	200 (32)	
pER-s167 high	115 (68)	54 (32)	0.0008	62 (37)	107 (63)	<0.00001	156 (89)	20 (11)	0.7	141 (80)	35 (20)	0.1	116 (69)	52 (31)	0.0007	66 (40)	100 (60)	<0.00001
pS6K c low				436 (68)	207 (32)		582 (91)	58 (9)		548 (86)	92 (14)		488 (81)	112 (19)		381 (63)	220 (37)	
pS6K c high				68 (42)	95 (58)	<0.00001	133 (83)	28 (17)	0.002	124 (77)	37 (23)	0.008	103 (66)	52 (34)	0.00006	86 (55)	69 (45)	0.07
pS6K n low							461 (92)	39 (8)		442 (88)	58 (12)		389 (83)	81 (17)		337 (71)	135 (29)	
pS6K n high							254 (85)	46 (15)	0.0008	229 (76)	71 (24)	0.00001	201 (71)	83 (29)	0.0001	129 (46)	154 (54)	<0.00001

Pearson χ^2 P values <0.01 were considered to be statistically significant.

S6K1 expression indicates worse prognosis

High S6K1 protein expression, independent of localization, predicted a poor prognosis in the tamoxifen-untreated subgroup (Fig. 3A and B). This was also true in multivariate analysis, adjusting for tumor size, grade, HER2-, ER-, and PgR status ($P=0.00041$ for S6K1 in the cytoplasm and $P=0.016$ for S6K1 in the nucleus). The tamoxifen-treated group did not differ in recurrence rate in relation to cytoplasmic S6K1 expression (data not shown). The second cohort did not include tamoxifen-untreated patients and no significant relationship between recurrence rate and S6K1 expression was observed in the analysis of all patients (Fig. 3C). S6K2 amplification has previously been shown to be a marker of worse prognosis in stage II breast cancer (Perez-Tenorio et al. 2011). Strong S6K2 expression did not associate with prognosis in either of the two cohorts. Thus, pS6K did not qualify as a prognostic marker irrespective of intracellular localization, cutoff, HER2 status, or cohort. A tendency towards improved breast cancer survival was found for high levels of pS6K in the nucleus (HR: 0.72, 95% CI: 0.44–1.17, $P=0.18$).
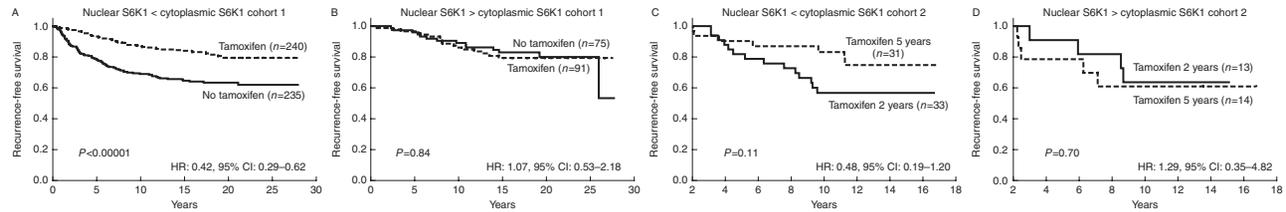
In vitro validation shows antibody specificity

The ER-positive breast cancer cell line ZR751 was transfected with siRNA to knockdown the S6K1 and S6K2 mRNAs, separately and in combination. Results of western blotting indicated almost complete knockdown of proteins at 72 h, indicating an efficient transfection and specific target detection with the antibodies (Fig. 4). The S6K2 antibody showed a weak non-specific band slightly larger than the specific band, which was not S6K1 as the band remained after S6K1 knockdown. The pS6K-t389 (pS6K) antibody detected both pS6K1 and pS6K2. The specific bands of the two phosphorylated homologs were distinguishable by western blotting. pS6K1 appeared at 70 and 85 kDa, whereas pS6K2 (p54 and p56) appeared as one non-separated band at 60 kDa, which was consistent with the sizes stated in the literature. AKT was additionally phosphorylated after S6K1 knockdown. This was not evident after S6K2 knockdown.

FFPE cells treated with HRG showed increased expression of pS6K compared with control, indicating the antibody's validity on FFPE tissue (Supplementary Figure 2A, B, and C, see section on supplementary data given at the end of this article). The phosphorylation specificity of the p-S6K antibody was validated with FFPE cells treated with λ-phosphatase. The λ-phosphatase test

Table 2 Cohort 2: clinicopathological characteristics in correlation with active pS6K-t389 (pS6K) and total S6Ks in cytoplasm (c) and in nucleus (n) in tumors from postmenopausal breast cancer patients

	pS6Kc n (%)			S6K1c n (%)			S6K1n n (%)			S6K2c n (%)			S6K2n n (%)		
	Low	High	P	Low	High	P	Low	High	P	Low	High	P	Low	High	P
Tamoxifen 2 years	45 (71)	18 (29)		46 (73)	17 (27)		46 (52)	43 (48)		43 (80)	11 (20)		32 (45)	39 (55)	
Tamoxifen 5 years	50 (75)	17 (25)	0.7	46 (69)	21 (31)	0.6	17 (41)	24 (59)	0.3	51 (82)	11 (18)	0.7	22 (49)	23 (51)	0.7
Size <20 mm	39 (89)	5 (11)		34 (79)	9 (21)		33 (37)	56 (63)		32 (80)	8 (20)		21 (30)	50 (70)	
Size >20 mm	56 (65)	30 (35)	0.004	58 (67)	29 (33)	0.1	10 (24)	31 (76)	0.2	62 (82)	14 (18)	0.8	19 (42)	26 (58)	0.2
S phase low	48 (72)	19 (28)		49 (72)	19 (28)		47 (70)	20 (30)		47 (77)	14 (23)		34 (62)	21 (38)	
S phase high	27 (79)	7 (21)	0.4	18 (53)	16 (47)	0.06	21 (60)	14 (40)	0.3	25 (81)	6 (19)	0.7	27 (73)	10 (27)	0.3
ER−	19 (63)	11 (37)		25 (83)	5 (17)		24 (27)	65 (73)		22 (81)	5 (19)		22 (31)	49 (69)	
ER+	76 (76)	24 (24)	0.2	67 (67)	33 (33)	0.08	6 (15)	35 (85)	0.1	72 (81)	17 (19)	0.9	5 (11)	40 (89)	0.01
PgR−	31 (58)	22 (42)		39 (72)	15 (28)		37 (42)	52 (58)		40 (82)	9 (18)		32 (45)	39 (55)	
PgR+	64 (83)	13 (17)	0.002	53 (70)	23 (30)	0.8	17 (41)	24 (59)	1.0	54 (81)	13 (19)	0.9	17 (38)	28 (62)	0.4
HER2−	79 (73)	29 (27)		79 (72)	30 (28)		75 (89)	9 (11)		76 (78)	21 (22)		63 (91)	6 (9)	
HER2+	10 (77)	3 (23)	0.8	8 (67)	4 (33)	0.7	34 (92)	3 (8)	0.7	10 (91)	1 (9)	0.3	34 (87)	5 (13)	0.5
pS6K low				65 (69)	29 (31)		64 (73)	24 (27)		71 (85)	13 (15)		54 (77)	16 (23)	
pS6K high				26 (74)	9 (26)	0.6	30 (73)	11 (27)	1.0	22 (71)	9 (29)	0.1	30 (67)	15 (33)	0.2

Endocrine-Related Cancer

Figure 1

Benefit of tamoxifen in ER-positive patients grouped according to location of S6K1 expression. (A versus B) S6K1 accumulation in the nucleus predicted loss of tamoxifen benefit when compared with endocrine-untreated patients in cohort 1 (n=641), P value for interaction was 0.025, and (C versus D) a reduction in the benefit from 5 years of tamoxifen treatment compared with 2 years of treatment in cohort 2 (n=91), P value for interaction was 0.25.

Discussion

Signaling of the S6Ks downstream of mTORC1 was retrospectively evaluated in two randomized sets of breast cancer patients. In this study, we have demonstrated S6K protein expression, activity, and location to be the useful markers for prediction of response to endocrine treatment, and for prognosis.

Resistance to endocrine treatments remains the reality for a large group of patients with recurring breast cancer, and validated biomarkers for treatment prediction in primary tumors are scarce besides ER and PgR status. Aberrant PI3K/mTOR-signaling is a highlighted cascade in endocrine therapy resistance, leading to maintained proliferation despite inhibition of hormonal-stimulatory effects. Therefore, we examined S6K1 and S6K2 as markers for activity in the pathway. The protein pER-s167, a downstream target for phosphorylation by AKT, S6K1, and RSK, has been shown previously to increase ER transcriptional activity, and consequently affect the proliferation rate (Yamnik & Holz 2010). The mTORC1 inhibitor rapamycin as well as S6K1 knockdown retained osteosarcoma cells in G1 phase (Fingar et al. 2004), and S6K1 indirectly induced proliferation through ER stimulation in epithelial cells (Yamnik et al. 2009). A mutant active S6K2 in immune cells increased proliferation (Cruz et al. 2005), and in embryonic cells from S6K1/2-knockout mice mild proliferation defects were observed (Kawasome et al. 1998, Pende et al. 2004). Results of several studies have suggested diverse roles of the S6K isoforms in cell-cycle progression (Lane et al. 1993, Reinhard et al. 1994, Fingar et al. 2004, Boyer et al. 2008). Consequently, the involvement of the S6Ks in proliferation is established in a variety of cells and systems. The common and separate roles of the two homologues for breast cancer patients are yet to be elucidated.

This is the first study to our knowledge to show that a dominant pattern of nuclear S6K1 is associated with reduced benefit from tamoxifen in breast cancer patients,

resulted in a decreased epitope binding of the antibody. The S6K1 antibody showed high specificity on FFPE cells, with almost complete loss of staining after S6K1 siRNA transfection (Supplementary Figure 2D and E).

Table 3 Cox proportional hazard analysis of the benefit from tamoxifen in patients with ER-positive tumors in relation to S6K1 nuclear (n) and cytoplasmic (c) location, and phosphorylated S6K-t389 (pS6K) expression

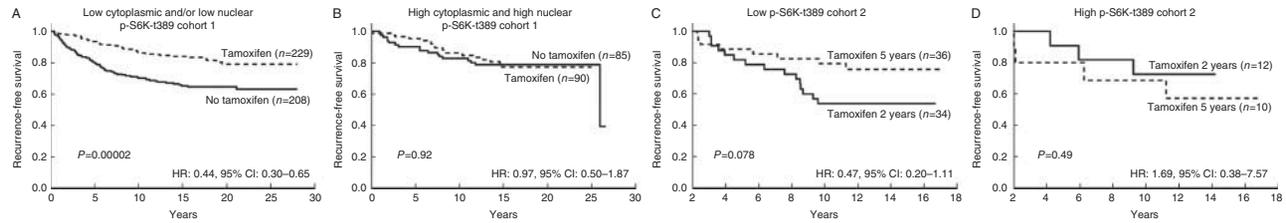|	Figure	Cohort	HR	95% CI	P (cox)	P (interact)	P (interact) adjusted for tumor size
S6K1 n<c	1A	1	0.42	0.29–0.62	**0.000007**		
S6K1 n>c	1B	1	1.07	0.53–2.18	0.84	**0.025**	**0.016**
S6K1 n<c	1C	2	0.48	0.19–1.20	0.12		
S6K1 n>c	1D	2	1.29	0.35–4.82	0.70	0.25	0.25
pS6K low	2A	1	0.44	0.30–0.65	**0.000037**		
pS6K high	2B	1	0.97	0.50–1.87	0.92	0.060	**0.040**
pS6K low	2C	2	0.47	0.20–1.11	0.085		
pS6K high	2D	2	1.69	0.38–7.57	0.50	0.13	0.13

Endocrine-Related Cancer

Figure 2

Benefit of tamoxifen in ER-positive patients grouped according to pS6K-t389 expression. High levels of expression of pS6K-t389 in the cytoplasm and in the nucleus predicted reduced response to tamoxifen in the two cohorts, P value for interaction was 0.060 in (A versus B) cohort 1 ($n=612$), and 0.13 in (C versus D) cohort 2 ($n=92$).

indicating that nuclear accumulation of S6K1 in primary biopsies may serve as a potential target to prevent treatment resistance. In addition, cytoplasmic and nuclear overexpression of S6K1 significantly indicated worse prognosis independent of *HER2* gene amplification, consistent with results of previous studies on S6K1 protein expression and *RPS6KB1* gene amplification and expression (van der Hage *et al.* 2004, Perez-Tenorio *et al.* 2011). The S6K1 antibody demonstrated high specificity and could serve as a clinical biomarker.

Overactivated S6K has been suggested an upcoming prognostic and treatment predictive marker in ER-positive breast cancer (Noh *et al.* 2008, Kim *et al.* 2011, Beelen *et al.* 2014). Here, we did not detect worse prognosis with high activity of the S6Ks, instead a somewhat improved prognosis was observed in the tamoxifen-untreated subset of patients when pS6K was highly expressed in the nucleus. Recently, Beelen *et al.* (2014) have shown an improved prognosis with high levels of pS6K expression in the cytoplasm. It has been suggested that the p31 short isoform of S6K1 is the primary oncogenic protein in the S6K family (Rosner & Hengstschlager 2011). The pS6K-t389 antibody did not detect this short isoform, which could be an explanation as to why its prognostic value was not seen in our study. Regarding the treatment-predictive value of pS6K-t389, we found high simultaneous expression in cytoplasmic and nuclear compartments to indicate loss of benefit from tamoxifen in the two cohorts. These results highlight pS6K as one of the potential markers in the PI3K/ER crosstalk interfering with response to tamoxifen treatment. The antibody showed high specificity on western blots as well as on FFPE cells. Staining with the phosphorylation-specific antibody was evaluated in the two cohorts, separately. Slightly different staining patterns were observed between the cohorts, with nuclear staining only in the first set and variations in staining intensity, although the antibody concentration

and method were identical. Therefore, we recommend the phosphorylation-specific S6K antibody to be further investigated before it is taken into consideration for clinical use. Instead, we suggest that this antibody may serve as a validated marker in cohort studies for further evaluation of mTOR inhibition and endocrine treatment response.

S6K2 was found in protein complexes by the centrosome in the nuclear membrane (Rossi *et al.* 2007), and the results of an *in silico* study indicated domains of the S6K2 to connect to chromatin (Ismail *et al.* 2014). *In vitro* data indicated that S6K2, not S6K1 or AKT, binds histone 3 and that this was dependent on the C-terminal nuclear localization signal in the S6K2 protein. On the other hand, growth factor stimulation of S6K2 induced phosphorylation of the C-terminal nuclear localizing signal, retaining active S6K2 in the cytoplasm (Valovka *et al.* 2003). This indicates that S6K2 has active roles in the nuclear and in the cytoplasmic compartment, with possible involvement in proliferation. In contrast, mTOR-dependent growth factor stimulation led to nuclear localization of S6K1 in G1 phase, indicating that a high level of expression of S6K1 in the nucleus is a marker of extracellular growth stimulation (Rosner & Hengstschlager 2011). The S6Ks proliferative roles may also act through S6 in concert with 4EBP1, and consequently through translational upregulation of the key G1-to-S phase transition regulator, cyclin D1 (Averous *et al.* 2008).

We observed a strong correlation of S6K1 with markers of high proliferation, and S6K2 correlation with markers of low proliferation. In contrast, Lyzogubov *et al.* (2005) reported that S6K2, not S6K1, correlated with the proliferation markers Ki67 and proliferating cell nuclear antigen (PCNA). We found that S6K1 correlated with HER2-positive tumors and S6K2 correlated with ER-positive status. This is probably in part a consequence of co-amplification, as *RPS6KB1* is located in the 17q23
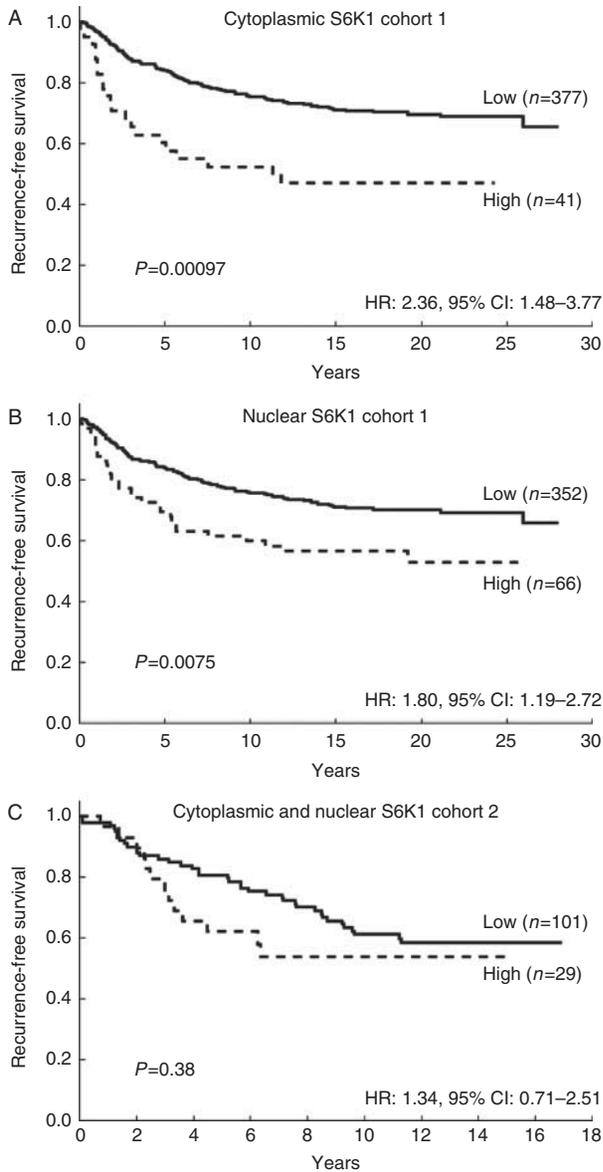
Figure 3

S6K1 protein expression as a prognostic marker in endocrine-untreated patients. (A) High S6K1 cytoplasmic (n=418) and (B) nuclear expression indicated a significantly worse prognosis in cohort 1 (n=418). (C) S6K1 cytoplasmic and nuclear expression in all tumors from cohort 2 had no significant prognostic value, although the trend mirrored the results from cohort 1 (n=130).

respectively, with HER2 positivity being associated with high proliferation and ER with low proliferation. However, the prognostic value of S6K1 overexpression found in this study was independent of HER2 status.

In glioblastoma, pS6K and pS6 correlated with AKT activation, supporting our findings (Riemenschneider et al. 2006). High levels of expression of pAKT in this study correlated with pS6K in the cytoplasm, mainly S6K1, and in the nucleus, mainly S6K2. However, knockdown of S6K1, but not S6K2, increased the active form of AKT. Inhibition of S6K with rapamycin in pancreatic cancer cells upregulated pAKT, and mTORC1/2 inhibition abrogated the AKT phosphorylation, thus instead the MAPK pathway was activated (Soares et al. 2013). Metformin, on the other hand, inhibited mTOR without the regulatory feedback effects on AKT and MAPK. An effect of mTORC1/2 inhibition was also detected in multiple myeloma cells, with a RAF-dependent activation of ERK (Hoang et al. 2012). This indicates that the regulatory networks connecting the AKT with the S6Ks can be modulated, and with fine-tuned inhibitors the growth stimulation could be restrained. Upregulation of the HER family RTKs is a common resistance response to endocrine treatment in breast cancer. To circumvent this mechanism the RTK/PI3K/mTOR-pathways have been inhibited in various combinations. Axelrod et al. (2014) have recently described S6K as a critical node and a potential single target by describing similar responses to S6K1 inhibition alone as to double-targeting RTKs and PI3K/mTOR. We
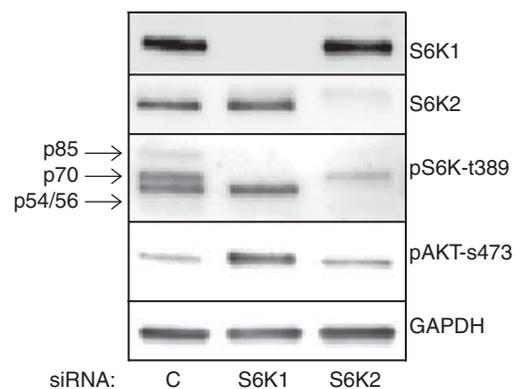

Figure 4

Antibodies showed specific epitope recognition in western blotting. S6K1 and S6K2 protein was downregulated upon siRNA transfection of ZR75-1 breast cancer cells. The pS6K-t389 antibody detected phosphorylated residues of S6K1 (70 and 85 kDa) and of S6K2 (60 kDa). AKT phosphorylation was induced by S6K1 knockdown. Removal of S6K2 did not induce AKT phosphorylation. GAPDH was used as a control for equal protein loading. The figure is a representative of three independent experiments.

chromosomal region close to the HER2 gene, and RPS6KB2 is located to 11q13 close to the CCND1 gene coding for the cyclin D1 protein, an amplicon strongly connected with ER expression, reported in this study and others (Bostner et al. 2007, Aaltonen et al. 2009). The opposing correlations of the two S6Ks with proliferation markers may come from their strong connections with HER2 and ER,

suggest that detection of S6K1 and pS6K1/2 could be used to identify patients with *de novo* resistance to tamoxifen, thus in need of additional targeted inhibition.

Phosphorylated epitopes are known to be unstable and highly dependent on fixation methods, which could be one reason that yet none has reached clinical use for prognostic or treatment predictive purposes. Therefore, we sought to thoroughly validate a well-known antibody targeting a potentially important site within the PI3K/mTOR cascade; the pS6K-t389 antibody from Cell Signaling Technology. Results from western blot analyses indicate that the antibody detected phosphorylated residues seen as three separated bands, interpreted as the two isoforms of S6K1, p70 and p85, and a single band from the two S6K2 isoforms, p54 and p56. The corresponding bands disappeared in an expected pattern upon knockdown of the two proteins, separately. For clinical use, an antibody should be validated on fixed tissue. Therefore, we used formalin-fixed and paraffin embedded-cells after stimulation with HRG. We observed increased staining after HRG stimulation and the results of a phosphatase assay indicated phosphorylation specificity of the antibody. To test the S6K1 antibody, we induced down-regulation of protein using siRNA transfection and showed high specificity of the S6K1 antibody on formalin-fixed and paraffin-embedded cells.

In conclusion, it is a matter of urgency to identify subgroups of breast cancer patients who fail to respond to endocrine treatment. Biomarkers predicting the response to endocrine treatment and identification of patients likely to benefit from mTOR inhibition will improve survival rates and prolong time to recurrence. In this study, we provide data on the involvement of S6K in effects of tamoxifen treatment and resistance, with S6K1 protein localization as a potential clinical biomarker. Patients with tumors showing high expression of the biomarker may respond well to an additional targeted treatment along with endocrine treatment in the neoadjuvant or adjuvant setting, as this may reduce recurrence of endocrine-treated breast cancer.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Author contribution statement

J Bostner, E Karlsson, and O Stål were involved in establishment of the project; J Bostner, E Karlsson, C B Eding, G Perez-Tenorio, and O Stål were involved in scientific directions of the project; T Fornander, and B Nordenskjöld contributed to the materials and clinical data; J Bostner, E Karlsson, H Franzén, and A Konstantinell performed immunohistochemical analysis and scoring; J Bostner, E Karlsson, C B Eding, G Perez-Tenorio, and O Stål performed statistical analysis and data evaluation; J Bostner, C B Eding, and H Franzén performed *in vitro* antibody validation procedures; and all authors have read and approved the final manuscript.

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