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## Clinical potential of the mTOR targets S6K1 and S6K2 in breast cancer

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## **Abstract**

*Aim:* The Mammalian Target of Rapamycin (mTOR) and its substrates S6K1 and S6K2 regulate cell growth, proliferation and metabolism through translational control. *RPS6KB1* (*S6K1*) and *RPS6KB2* (*S6K2*) are situated in the commonly amplified 17q21-23 and 11q13 regions. *S6K1* amplification and protein overexpression have earlier been associated with a worse outcome in breast cancer, but information regarding S6K2 is scarce. The aim of this study was to evaluate the prognostic and treatment predictive relevance of *S6K1/S6K2* gene amplification, as well as S6K2 protein expression in breast cancer.

*Material & Methods:* *S6K1/S6K2* gene copy number was determined by real-time PCR in 207 stage II breast tumors and S6K2 protein expression was investigated by immunohistochemistry in 792 node-negative breast cancers.

*Results:* *S6K1* amplification/gain was detected in 10.7%/21.4% and *S6K2* amplification/gain in 4.3%/21.3% of the tumors. S6K2 protein was detected in the nucleus (38%) and cytoplasm (76%) of the tumor cells. *S6K1* amplification was significantly associated with *HER2* gene amplification and protein expression. *S6K2* amplification correlated significantly with high *S6K2* mRNA levels, ER+ status and *CCND1* amplification. *S6K1* and *S6K2* gene amplification was associated with a worse prognosis independent of *HER2* and *CCND1*. *S6K2* gain and nuclear S6K2 expression was related to an improved benefit from tamoxifen among patients with ER+ respectively ER+/PgR+ tumors. In the ER+/PgR- subgroup, nuclear S6K2 rather indicated decreased tamoxifen responsiveness. *S6K1* amplification predicted reduced benefit from radiotherapy.

*Conclusions:* This is the first study showing that *S6K2* amplification and overexpression, like *S6K1* amplification, have prognostic and treatment predictive significance in breast cancer.

**Keywords:** mTOR, S6 kinase, 17q21-23, 11q13, gene amplification, tamoxifen response

## Introduction

The Mammalian Target of Rapamycin (mTOR) is a serine/threonine kinase, which in response to growth factors, hormones, nutrients, hypoxia and energy (ATP) regulates cell growth, proliferation and metabolism through translational control of essential proteins [1]. mTOR is a critical effector in several cellular functions commonly deregulated in cancer, and multiple alterations resulting in overstimulation of the pathway have been described [2]. Two major regulators of mTOR function, the RAS/MAPK and PI3K/AKT signaling pathways, are constitutively activated in many cancers. Mutations in the *PIK3CA* gene (encoding the p110 $\alpha$  subunit of the PI3K), PTEN loss and aberrant activation or expression of AKT are some of these alterations found in breast cancer [3-5]. Cross-talk between estrogen receptor (ER) signaling and the AKT/mTOR pathway is one suggested mechanism behind endocrine resistance in breast cancer [6-8] and mTOR inhibition has been shown to increase the effect of endocrine treatments in both preclinical and clinical settings [9-11]. Since multiple oncogenic cellular pathways converge on mTOR, an important prospect is a further dissection of the downstream signaling network of mTOR and to determine the clinical relevance of genetic alterations in the mTOR signaling pathway [2].

The ribosomal S6 kinases S6K1 and S6K2 are well-known mTOR substrates, involved in regulation of the translational machinery [1, 12-14]. S6K1 and S6K2 share 70 % overall amino acid identity, whereas the catalytic domains have even higher sequence homology with > 83 % overlapping residues. The domain structure and the several phosphorylation sites are also conserved and are found in the corresponding drosophila dS6K, indicating that the two S6K isoforms present in mammals result from gene duplication [13, 15]. Both kinases phosphorylate the 40S ribosomal protein S6 and are believed to have overlapping functions; however there are also data indicating divergence in their biological activities. In contrast to S6K1, S6K2 contains a proline-rich sequence, homologous to a

sequence in the p85 subunit of PI3K, allowing interactions with SH3 domain-containing proteins [13]. Knock-out of *S6K1* in mice, as well as drosophila *dS6K* has been connected to a reduction in animal body size during embryogenesis, as a result of a decrease in individual cell size [15, 16]. In contrast, *S6K2*<sup>-/-</sup> mice had normal or slightly increased body size [17]. Of note however, *S6K1*-deficient mice showed a significant upregulation of *S6K2* protein in several tissues, suggesting a compensatory mechanism, which may explain why the phenotype of size reduction was mostly overcome by adulthood [15]. Deletion of both *S6K1* and *S6K2* in mice, as well as *dS6K* in drosophila has been shown semilethal, severely reducing the viability. In contrast, no difference in lethality of *S6K1* or *S6K2* deficient mice have been seen, supporting the compensatory and essential roles for the kinases in normal development [16, 17].

The genes *RPS6KB1* (*S6K1*) and *RPS6KB2* (*S6K2*) are situated in the chromosomal regions 17q21-23 and 11q13, which are commonly amplified in several malignancies. In breast cancer, *HER2* and *CCND1* may be the most well-known oncogenes in these areas, where they are found amplified in 20-30% [18-20] and 10-15% [21-23] of cases, respectively.

*S6K1* amplification [24, 25] and *S6K1* protein overexpression [24-26] has earlier been associated with a worse outcome in breast cancer, but nothing has been reported about *S6K2* in this context. Due to the location of *S6K2* in a chromosomal region commonly amplified in malignancies, and the high homology between *S6K1* and *S6K2*, one may hypothesize that also *S6K2* could be of clinical importance. Consequently, the aim of this study was to evaluate the possible alterations of the mTOR targets *S6K1* and *S6K2* in postmenopausal breast cancer. *S6K1* and *S6K2* gene copy number was determined by fast real-time PCR in 207 stage II breast tumors; whereas *S6K2* protein expression was detected by immunohistochemistry in 792 node-negative breast cancers. The prognostic and treatment predictive value regarding tamoxifen and radiotherapy was explored.

## Materials and methods

In the following section the method procedures are briefly covered, and a detailed description can be found in Supplementary Methods. Study design and presentation of results are in line with the Reporting recommendations for tumor marker prognostic studies (REMARK) guidelines [27].

### *Patients*

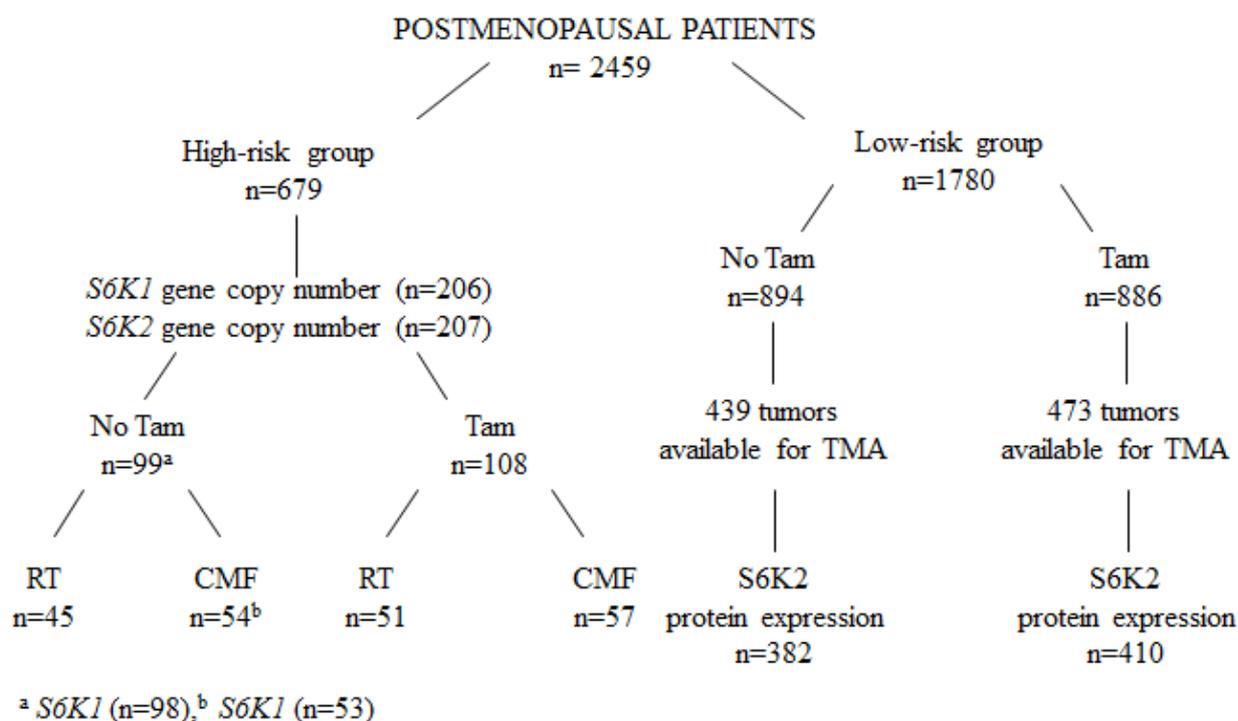
The patient materials used to study *S6K1/S6K2* gene amplification and S6K2 protein expression were previously reported in detail [28, 29]. Briefly, accrual of high-risk and low-risk postmenopausal patients started in November 1976 and ended in April 1990. The low-risk group included patients without positive lymph nodes and a tumor diameter  $\leq 30$  mm, while the high-risk group consisted of patients with either histological verified lymph node metastases or a tumor diameter  $> 30$  mm. Both patient cohorts were randomized to receive adjuvant tamoxifen or no endocrine treatment. Furthermore, the high-risk group was randomized to cyclophosphamide-methotrexate-5-fluorouracil (CMF) chemotherapy or radiotherapy (RT) (Fig. 1).

The *S6K1/S6K2* gene copy number analysis comprised a subset of patients from the high-risk group, from whom frozen tumor tissue was still available after hormone receptor assays and other biochemical analyses. Furthermore, all samples included were judged to contain  $> 50\%$  of malignant cells ( $n=207$ ). From these, 34 tumors with 11q13 amplification were selected, out of which 23 were available for *S6K2* mRNA expression analysis. Formalin-fixed and paraffin-embedded tumors from the low-risk group ( $n=912$ ) were used for S6K2 protein expression analysis (Fig.1).

The two subsets showed no major differences in comparison with all the postmenopausal patients in the trial in terms of tumor characteristics and treatment. Median

follow-up times were 18 years for the low-risk patients and 11 years for the high-risk patients.

This study was approved by the local ethical committee at the Karolinska Institute.



*Figure 1:* Representation of the patient flow through the study (TMA=tissue microarray, Tam=tamoxifen, RT=radiotherapy, CMF=cyclophosphamide-methotrexate-5-fluorouracil chemotherapy)

### *DNA and mRNA preparation*

Extraction of genomic DNA was performed as described before [30] and DNA concentration was estimated using a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies). For mRNA preparation, fresh frozen tumor tissue was homogenized with a microdismembrator (B Braun) and total RNA was isolated using the mirVana<sup>TM</sup> miRNA Isolation kit (Ambion), according to manufacturer's recommendations. Purified RNA was eluted in nuclease-free water and RNasin<sup>®</sup> Ribonuclease Inhibitor (Promega) was added before storage in  $-70^{\circ}\text{C}$ . RNA quantity and quality was assessed with an Agilent 2100 Bioanalyzer (Agilent biosystems).

### *Evaluation of S6K1/S6K2 gene copy number*

*S6K1* and *S6K2* gene copy number was determined in 206 respectively 207 available breast tumors, using quantitative real-time PCR. Details of the performance can be found in Supplementary Methods.

### *S6K2 mRNA quantification*

*S6K2* mRNA levels were measured in 23/34 available samples selected for amplification in the 11q13 area, using quantitative real-time PCR. Reverse transcription and mRNA quantitation is further described in Supplementary Methods.

### *Immunohistochemistry and immunoblotting*

Formalin-fixed and paraffin-embedded tumors from the low-risk group (n=912) were used for *S6K2* protein expression analysis. Procedures for immunochemical staining of *S6K2* as well as Cyclin D1, and evaluation of antibody specificity using immunoblotting are presented in detail in Supplementary Methods. Preparation of breast cancer tissue microarrays (TMA) and evaluation of ER, progesterone receptor (PgR) and HER2 protein expression have been described previously [31].

### *Statistical analysis*

Spearman's rank order correlation was used to determine the association between *S6K2* gene copy number and mRNA expression levels. The relationships between different grouped variables were assessed by the Chi-square test or Chi-square test for trend, when appropriate. The product-limit method was used for estimation of cumulative probabilities of

recurrence-free survival (RFS) and distant recurrence-free survival (DRFS). Differences in survival between groups were tested with the log-rank test. Univariate and multivariate analysis of event rates was performed with Cox proportional hazard regression. This was also applied for interaction analysis of different factors and treatment by including the variables X (potential predictive factor), treatment, and the interaction variable (X \* treatment). All the procedures were comprised in STATISTICA, version 8.0, StatSoft, Inc. (2007). The criterion for statistical significance was  $P < 0.05$ .

## Results

### *S6K1 and S6K2 gene amplification*

*S6K1* and *S6K2* gene amplification was analyzed in 206 and 207 high-risk breast tumors, respectively. Amplification ( $\geq 4$  copies) of the *S6K1* gene could be detected in 22/206 cases (10.7%) while the *S6K2* gene was amplified in 9/207 cases (4.3%). Gain ( $\geq 3$  copies) was observed in 44 cases for both *S6K1* (21.4%) and *S6K2* (21.3%). *S6K1* amplification varied from 4 to 21 estimated copies of the gene, while *S6K2* amplification was in the range from 4 to 9 copies. Amplification of *S6K1* and *S6K2* were mutually exclusive events in the cohort (Table 1), why *S6K1* or *S6K2* amplification was detected in 31/206 cases (15%). *S6K1* gain and/or *S6K2* gain occurred in 74/205 cases (36%).

*S6K1* amplification (Table 1) was significantly associated with *HER2* gene amplification ( $P=0.025$ ) and *HER2* protein expression ( $P=0.014$ ) and tended to be inversely correlated to *CCND1* amplification ( $P=0.065$ ). Also *S6K1* gain correlated significantly to *HER2* amplification ( $P=0.007$ ) and was borderline associated with high S-phase fraction ( $P=0.062$ ) and large tumor size ( $P=0.067$ ). *S6K2* gene copy number was significantly associated with *S6K2* mRNA expression levels ( $P=0.0001$ ). Amplification of *S6K2* (Table 1) correlated to positive ER status ( $P=0.046$ ) whereas both *S6K2* amplification and *S6K2* gain correlated to *CCND1* amplification ( $p<0.00001$  and  $P=0.00003$ ). *S6K2* gain was also significantly associated with a high S-phase fraction ( $P=0.027$ ). The combination variable *S6K1* or *S6K2* amplification, as well as *S6K1* and/or *S6K2* gain was inversely correlated to *PIK3CA* mutations ( $P=0.012$  and  $P=0.029$ ), whereas the latter combination variable also correlated significantly to high S-phase fraction ( $P=0.016$ ).

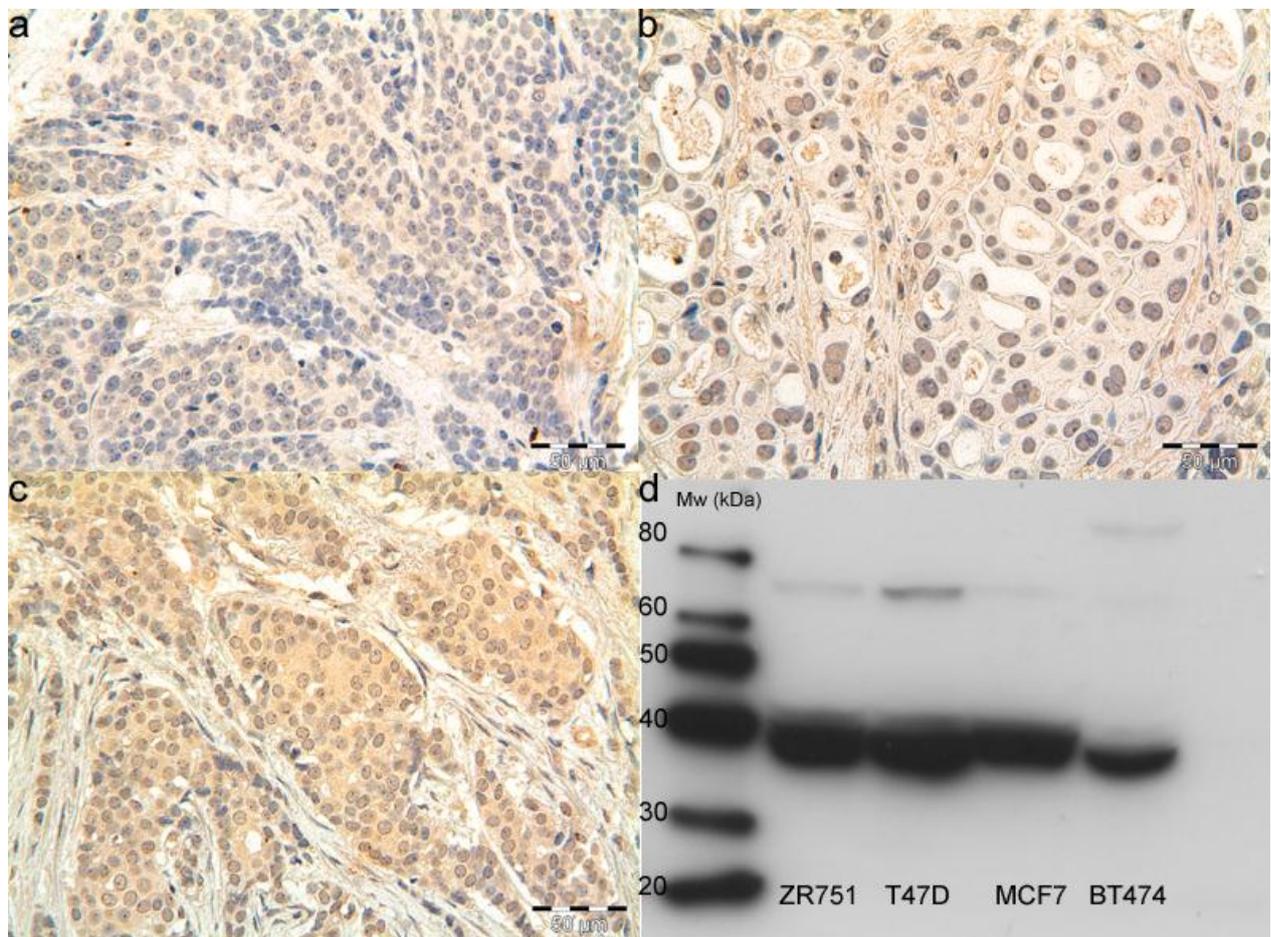
**Table 1.** *S6K1* and *S6K2* gene amplification ( $\geq 4$  copies) in relation to clinicopathological factors and the PI3K/AKT pathway

	<i>S6K1</i> amplification n (%)			<i>S6K2</i> amplification n (%)		
	-	+	Test for significance	-	+	Test for significance
<b>Nodes</b>						
-	18 (81.8)	4 (18.2)	P=0.23	20 (90.9)	2 (9.1)	P=0.25
+	166 (90.2)	18 (9.8)		178 (96.2)	7 (3.8)	
<b>Tumor Size</b>						
$\leq 20$ mm	73 (90.1)	8 (9.9)	P=0.76	80 (96.4)	3 (3.6)	P=0.67
$> 20$ mm	111 (88.8)	14 (11.2)		118 (95.2)	6 (4.8)	
<b>ER</b>						
-	55 (90.2)	6 (9.8)	P=0.78	61 (100)	0 (0)	P=0.046
+	127 (88.8)	16 (11.2)		135 (93.8)	9 (6.2)	
<b>S-phase fraction<sup>a</sup></b>						
$< 10\%$	96 (90.6)	10 (9.4)	P=0.26	103 (97.2)	3 (2.8)	P=0.26
$\geq 10\%$	69 (85.2)	12 (14.8)		76 (93.8)	5 (6.2)	
<b><i>PIK3CA</i> mutation<sup>b</sup></b>						
-	134 (87.1)	20 (12.9)	P=0.081	146 (94.2)	9 (5.8)	P=0.084
+	47 (95.9)	2 (4.1)		49 (100)	0 (0)	
<b>pAKT(Ser 473)<sup>c</sup></b>						
- (0%)	86 (88.7)	11 (11.3)	P=0.82	92 (94.9)	5 (5.1)	P=0.87
+ (1-10%)	46 (93.9)	3 (6.1)		47 (95.9)	2 (4.1)	
++ (>10%)	50 (86.2)	8 (13.8)		57 (96.6)	2 (3.4)	
<b><i>HER2</i> amplification<sup>d</sup></b>						
-	141 (91.6)	13 (8.4)	P=0.025	147 (94.8)	8 (5.2)	P=0.42
+	35 (79.5)	9 (20.5)		43 (97.7)	1 (2.3)	
<b><i>HER2</i> protein<sup>a</sup></b>						
-	134 (92.4)	11 (7.6)	P=0.014	138 (94.5)	8 (5.5)	P=0.25
+	45 (80.4)	11 (19.6)		55 (98.2)	1 (1.8)	
<b><i>CCND1</i> amplification<sup>e</sup></b>						
-	145 (87.4)	21 (12.6)	P=0.065	165 (98.8)	2 (1.2)	P< 0.00001
+	24 (100)	0 (0)		17 (70.8)	7 (29.2)	
<b><i>S6K2</i> amplification</b>						
-	175 (89.3)	21 (10.7)	P=0.30			
+	9 (100)	0 (0)				

<sup>a</sup>[50], <sup>b</sup>[3], <sup>c</sup>[51], <sup>d</sup>[52], <sup>e</sup>[22]

### *S6K2 protein expression*

S6K2 protein expression was analyzed with immunohistochemistry in 792/912 low-risk breast tumors. Nuclear and cytoplasmic S6K2 were detected in 38% and 76% of the tumors respectively (Fig. 2 a-c). The S6K2 antibody was evaluated by immunoblotting in order to disregard the presence of unspecific bands or cross-reaction with the S6K1 protein (Fig. 2 d). Nuclear S6K2 was positively correlated with ER+ ( $P < 0.00001$ ), PgR+ ( $P < 0.00001$ ) status and nuclear Cyclin D1 protein expression ( $P < 0.00001$ ), whereas it was inversely correlated with HER2 protein expression ( $P = 0.013$ ). Cytoplasmic S6K2 correlated with ER+ status ( $P = 0.009$ ) and nuclear Cyclin D1 protein expression ( $P < 0.00001$ ).



**Figure 2:** Immunostaining of the S6K2 protein; examples of a tumor scored negative (**a**), a nuclear positive tumor (**b**) and a nuclear and cytoplasmic positive tumor (**c**). The anti-S6K2 antibody was validated by immunoblotting, using lysates from ZR751, T47D, MCF7 and BT474 breast cancer cell lines (**d**)

### *Survival analysis*

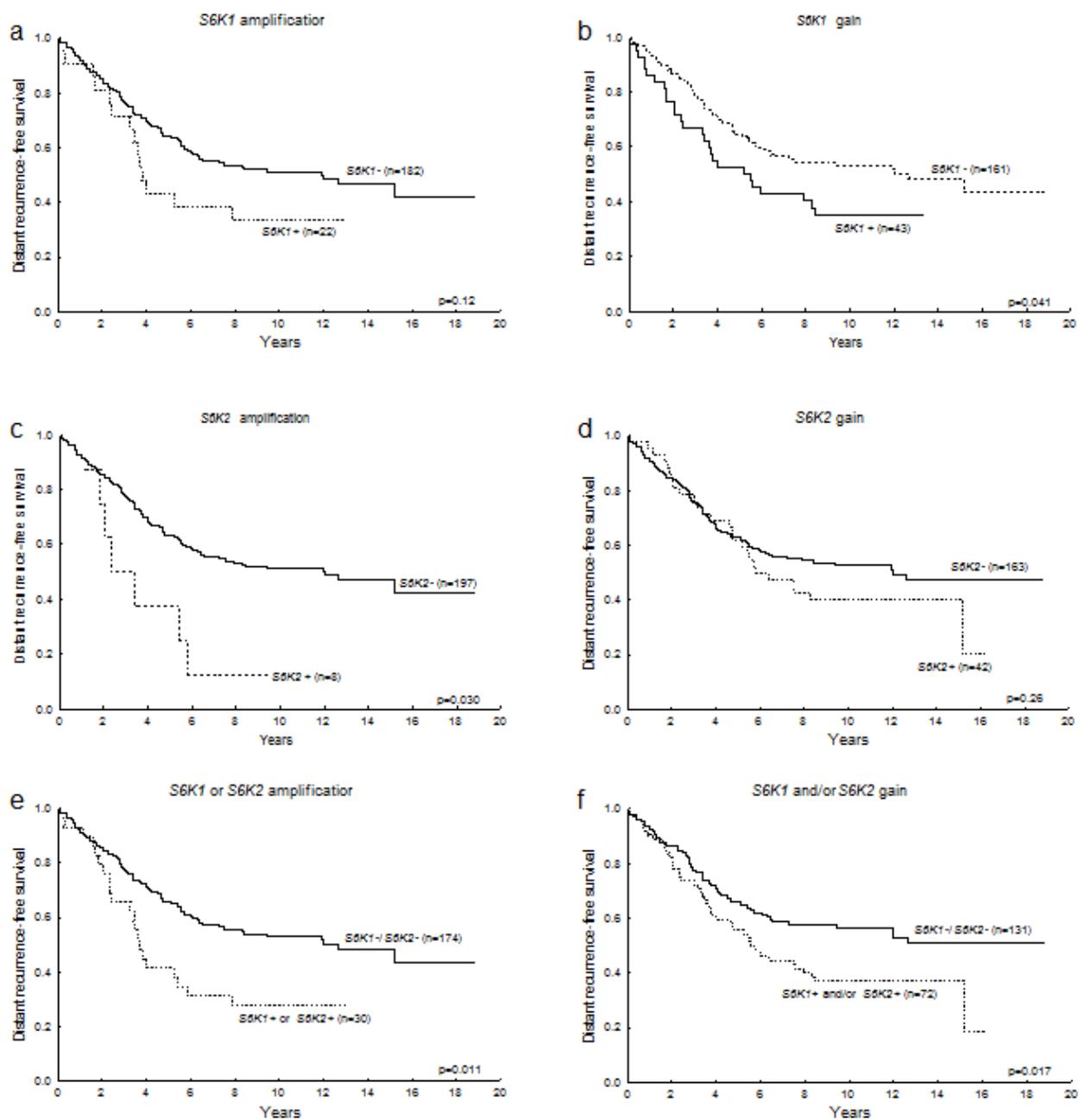
In an univariate analysis including all high-risk patients, *S6K1* gene amplification tended to confer a higher risk of developing distant metastasis (HR=1.63, 95% CI, 0.92-2.85, P=0.092, Fig. 3 a) whereas *S6K1* gain was significantly associated with increased risk of distant recurrence (HR=1.62, 95% CI, 1.05-2.52, P=0.031, Fig. 3 b).

Amplification of *S6K2* significantly predicted a higher risk of distant recurrence in breast cancer (HR=2.70, 95% CI, 1.24-5.83, P=0.012, Fig. 3 c), whereas this could not be seen for *S6K2* gain (HR=1.29, 95% CI, 0.83-2.01, P=0.26, Fig. 3 d).

The combination variable *S6K1* or *S6K2* amplification was significantly associated with poor DRFS (HR=1.98, 95% CI, 1.22-3.20, P=0.006, Fig. 3 e) and this was also true for the combination variable *S6K1* and/or *S6K2* gain (HR=1.61, 95% CI, 1.09-2.37, P=0.016, Fig. 3 f). Among patients with ER positive tumors, the combination variables tended to have an even stronger prognostic value in terms of DRFS (*S6K1* or *S6K2* amplification: HR=2.23, 95% CI, 1.29-3.88, P=0.0044; *S6K1* and/or *S6K2* gain: HR=1.90, 95% CI, 1.18-3.05, P=0.008).

In a multivariate analysis, including *HER2* and *CCND1* amplification as well as treatment, among other common variables, *S6K2* amplification remained an independent prognostic factor of increased risk for distant recurrence, whereas *S6K1* gene amplification reached borderline significance (Table 2). The combination variables *S6K1* or *S6K2* amplification (HR=2.11, 95% CI, 1.27-3.50, P=0.004) as well as *S6K1* and/or *S6K2* gain (HR=1.54, 95% CI, 1.00-2.38, P=0.049) also resulted as independent prognostic factors in an analogous multivariate analysis.

In the cohort of low-risk patients, *S6K2* protein expression did not show any prognostic value (data not shown).



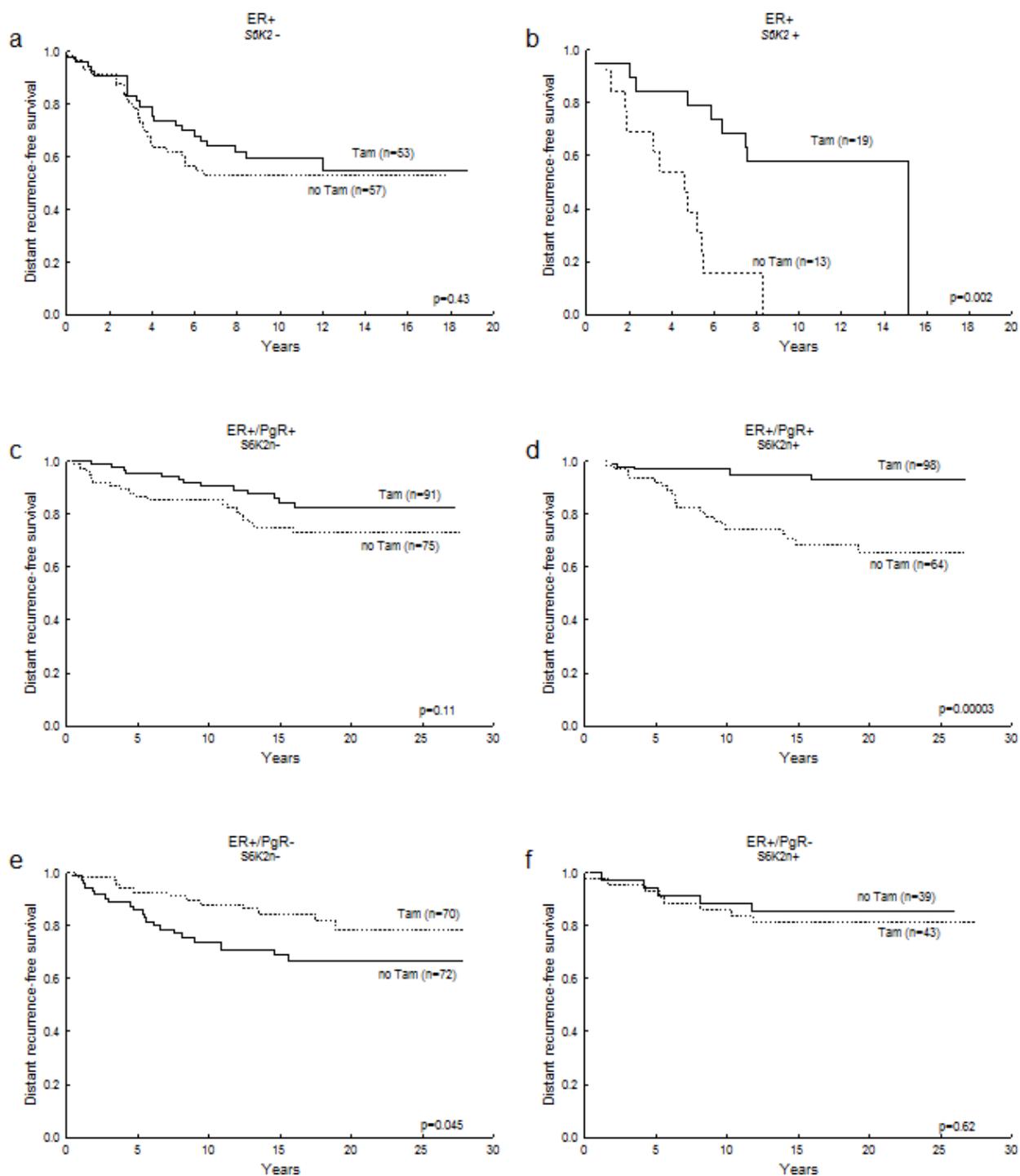
*Figure 3:* Distant recurrence-free survival among all high-risk patients, in relation to *S6K1* amplification (a), *S6K1* gain (b), *S6K2* amplification (c), *S6K2* gain (d), the combination variables *S6K1* or *S6K2* amplification (e) and *S6K1* and/or *S6K2* gain (f). (amplification  $\geq 4$  gene copies, gain  $\geq 3$  gene copies)

**Table 2.** Multivariate analysis of distant recurrence using Cox proportional hazard regression

	HR (95% CI)	Test for significance
Lymph node status N+ vs. N-	2.59 (1.09-6.15)	P=0.032
Tumor size > 20 mm vs. ≤ 20 mm	1.77 (1.13-2.75)	P=0.012
ER status ER+ vs. ER-	0.82 (0.51-1.29)	P=0.39
<i>HER2</i> gene amplification Amplified vs. nonamplified	1.63 (1.01-2.64)	P=0.045
<i>CCND1</i> gene amplification Amplified vs. nonamplified	0.95 (0.46-1.99)	P=0.90
Tamoxifen vs. no Tamoxifen	0.77 (0.51-1.17)	P=0.22
Chemotherapy vs. Radiotherapy	1.16 (0.76-1.76)	P=0.49
<i>S6K1</i> gene amplification Amplified vs. nonamplified	1.78 (0.98-3.22)	P=0.059
<i>S6K2</i> gene amplification Amplified vs. nonamplified	3.65 (1.40-9.54)	P=0.008

### *Treatment prediction*

As a result of the low number of cases with *S6K2* amplification, *S6K2* gain was considered in analyses of treatment prediction. The benefit from tamoxifen was evident for high-risk patients having ER positive tumors with *S6K2* gain regarding DRFS, whereas no significant tamoxifen response could be seen in the *S6K2* negative group (Fig. 4 a, b). In the low-risk group, nuclear *S6K2* protein expression was associated with an increased benefit from tamoxifen among patients with ER+/PgR+ tumors (Fig. 4 c, d). However in the ER+/PgR- group, nuclear *S6K2* expression was rather an indicator of decreased tamoxifen responsiveness (Fig. 4 e, f). In an interaction test, *S6K2* gain had borderline significance as a



**Figure 4:** Distant recurrence-free survival for breast cancer patients treated with tamoxifen (Tam) vs. no tamoxifen (no Tam) in relation to S6K2 status; S6K2- (<3 gene copies) **(a)**, S6K2+ ( $\geq 3$  gene copies) **(b)**, S6K2n- (no nuclear S6K2 staining), ER+/PgR+ tumors **(c)**, S6K2n+ (positive nuclear S6K2 staining), ER+/PgR+ tumors **(d)**, S6K2n- (no nuclear S6K2 staining), ER+/PgR- tumors **(e)** and S6K2n+ (positive nuclear S6K2 staining), ER+/PgR- tumors **(f)**. **(a, b):** stage II tumors; **c-f:** node-negative breast cancers

predictor of increased tamoxifen efficacy, using DRFS as the end-point (Table 3) and the interaction reached significance in terms of RFS (P=0.026, data not shown). Also nuclear S6K2 protein expression interacted significantly with the benefit from tamoxifen among the low-risk ER+/PgR+ patients, whereas a trend for a negative interaction between nuclear S6K2 and tamoxifen efficacy could be seen in the ER+/PgR- group (Table 4). *S6K1* gene amplification alone did not show any predictive value regarding tamoxifen treatment (Table 3), however a trend was seen for the combination variable *S6K1* amplification and/or *S6K2* gain to predict increased benefit from tamoxifen (Table 3), and the test for interaction reached significance using RFS as the primary end-point (P=0.046, data not shown).

**Table 3.** Cox proportional hazard regression of distant recurrence rate for patients with stage II, ER+ tumors, treated or not with adjuvant tamoxifen, in relation to *S6K1* amplification ( $\geq 4$  gene copies), *S6K2* gain ( $\geq 3$  gene copies) and the combination variable *S6K1* amplification and/or *S6K2* gain

	No. of patients	Tamoxifen vs. no tamoxifen HR (95% CI)		Test for interaction
<i>S6K1</i> amplification				
-	125	0.66 (0.40-1.10)	P=0.11	
+	16	0.62 (0.16-2.40)	P=0.49	P=0.94
<i>S6K2</i> gain				
-	110	0.80 (0.45-1.41)	P=0.44	
+	32	0.21 (0.08-0.53)	P=0.001	P=0.065
<i>S6K1</i> amplification and/or <i>S6K2</i> gain				
-	96	0.81 (0.43-1.52)	P=0.52	
+	46	0.34 (0.26-0.74)	P=0.006	P=0.16

In terms of loco-regional control, the patients with normal *S6K1* gene copy number responded significantly better to radiotherapy compared to chemotherapy in contrast to the patients harboring tumors with *S6K1* amplification (Supplementary Table 1). Genomic amplification on 17q21-23 including *S6K1* and/or *HER2* gene amplification, also indicated

poor response to radiotherapy (Supplementary Fig. 1). Both 17q21-23 and *S6K1* amplification interacted significantly with the benefit from radiotherapy (Supplementary Table 1). A similar trend was seen for *S6K2*, where a normal copy number was associated with a significant benefit from radiotherapy compared to chemotherapy, whereas *S6K2* gain was not (Supplementary Table 1). Though, no significant interaction between *S6K2* or the combination variable *S6K1* amplification and/or *S6K2* gain, and radiotherapy, was evident (Supplementary Table 1).

**Table 4.** Cox proportional hazard regression of distant recurrence rate for patients with node-negative breast cancers, and ER+, ER+/PgR+ or ER+/PgR- tumors, respectively, treated or not with adjuvant tamoxifen, in relation to nuclear S6K2 protein expression

	No. of patients	Tamoxifen vs. no tamoxifen HR (95% CI)		Test for interaction
ER+				
S6K2n-	337	0.54 (0.33-0.88)	P=0.013	
S6K2n+	265	0.44 (0.21-0.78)	P=0.007	P=0.52
ER+/PgR+				
S6K2n-	165	0.60 (0.29-1.22)	P=0.16	
S6K2n+	163	0.17 (0.07-0.42)	P=0.0001	P=0.034
ER+/PgR-				
S6K2n-	142	0.49 (0.24-1.00)	P=0.049	
S6K2n+	80	1.33 (0.43-4.06)	P=0.62	P=0.13

## Discussion

Genomic amplifications occur frequently and non-randomly in tumors and are expected to be essential for the development and progression of malignancy. In breast cancer, 17q21-23 and 11q13 are commonly amplified chromosomal regions where *HER2* and *CCND1* may be the most well-known oncogenes [18-23]. The present study suggests a role for *S6K1* and *S6K2* as clinically valuable in these amplicons.

This is the first study to report amplification/gain of *S6K2* and its correlation to an increased *S6K2* mRNA expression in primary breast tumors. Amplification of the homologous *S6K1* was detected in about 10 % of the tumors, which is in agreement with earlier studies where amplification of *S6K1* also has been correlated to increased expression of the corresponding protein [24, 25, 32]. *S6K1* and *S6K2* amplification were mutually exclusive events in the cohort, suggesting compensatory roles as tumor driving oncogenes. The joint value of *S6K1* and/or *S6K2* gene copy number alterations was explored and appeared to be of clinical relevance.

Amplification and gain of *S6K1* was significantly associated with *HER2* gene amplification and *HER2* protein overexpression. The possibility of *S6K1* and *HER2* coamplification has been discussed before due to their physical proximity [24]. *S6K1* was identified as the first candidate oncogene in the 17q23 region [33] and *S6K1* or *S6K1/HER2* amplification have been associated to a poor outcome in breast cancer [24].

*S6K2* amplification and gain were strongly correlated to *CCND1* amplification. The physical proximity of these two genes (2.2 Mb) suggests that they belong to the same amplicon or to frequently coamplified cores within the 11q13 area [34]. 11q13 amplification has in several studies been connected to positive ER status in breast cancer [35, 36], which could also be confirmed here for amplification of *S6K2*. However, the mechanisms behind a possible interaction between 11q13 and ER remain to be elucidated.

Of note, amplification or gain of *S6K1* or *S6K2* were inversely correlated with the presence of *PIK3CA* mutations, indicating that deregulation of the S6 kinases may be an alternative and compensatory mechanism for PI3K/AKT stimulation in breast tumors.

*S6K1* and *S6K2* share structural homology although they exhibit differences in the C and N terminal domains [14]. The S6 kinases have earlier been observed in both cytoplasmic and nuclear compartments of malignant cells [37] where different *S6K1* and 2 isoforms have been reported. *S6K1* exists as p70 and p85 isoforms. Likewise, the two *S6K2* isoforms p60/ $\beta$ I and p54/ $\beta$ II have been found in the cytoplasm and the nucleus [38]. Since the known function of these proteins is so far coupled to phosphorylation of a ribosomal protein present in the cytoplasm, the role of the nuclear *S6K1/2* is intriguing, suggesting the possibility of other substrates. *S6K1* protein expression have earlier been correlated to *S6K1* gene amplification [24, 25] and associated with a worse outcome in breast cancer [24-26] but very little is known about *S6K2* protein expression. In the present study, *S6K2* protein could be detected in the nuclear and cytoplasmic compartments of breast tumor cells. In accordance with *S6K2* amplification, *S6K2* protein expression also correlated to Cyclin D1 expression and ER positive status, but also to PgR expression, implying a functional connection at the cellular level between *S6K2* and ER signaling.

The current data confirm a role for both *S6K1* and *S6K2* amplification/gain as prognostic factors in breast cancer, possibly of greatest significance in the ER-positive subgroup. *S6K2* amplification remained as an independent prognostic factor and *S6K1* reached borderline significance in a multivariate analysis including *HER2* and *CCND1* amplification as well as treatment, among other common variables, demonstrating the individual contribution of the S6 kinases as potential oncogenes in the amplicons. The combination variables *S6K1* or *S6K2* amplification, as well as *S6K1* and/or *S6K2* gain, also remained independent prognostic factors in analogues analysis.

Anti-estrogen treatments are corner stones in the management of ER positive breast cancer, however *de novo* and acquired endocrine resistance remains a substantial problem. Identifying new biomarkers for prediction of responsiveness to endocrine treatments is therefore of great importance [39]. Results from this study indicate that the S6 kinases, in particular S6K2, may be relevant in this context. Increased *S6K2* gene copy number and nuclear S6K2 expression was shown related to a better response to tamoxifen among patients with ER positive tumors. Interestingly, the ability of S6K2 to predict benefit from tamoxifen was restricted to the ER+/PgR+ subgroup among the low-risk cohort in the present study. Among patients with ER+/PgR- tumors, nuclear S6K2 expression was rather connected to a worse response to endocrine treatment. This allows for the speculation that ER signaling in this subgroup may be driven in a hormone-independent manner, via cross-talk to intracellular signaling pathways including mTOR/S6K. mTOR inhibitors have been shown effective in combination with endocrine therapies in both clinical and preclinical studies [9-11]. In the light of the present findings, S6K2 may have a role in predicting when this combination therapy is useful. S6K1 has earlier been implicated in the regulation of ER signaling by phosphorylating ER $\alpha$ -Ser 167, leading to increased ER transcriptional activity and cell growth *in vitro* [40]. In addition, phosphorylation of ER $\alpha$ -Ser 167 has been associated with better response to tamoxifen [41, 42], and a similar role for S6K2 in ER phosphorylation may be conceivable. The proline-rich motif found in S6K2 may support this speculation, since a proline rich, SH3 binding domain in certain ER coactivators have been shown essential for their function and interactions with ER $\alpha$  [43].

The HER2/PI3K/AKT signaling pathway has earlier been implicated in resistance to radiation-induced apoptosis in breast tumors [44], and this can be reversed by the HER2 inhibitor trastuzumab [45]. Results from the present study reveal that *S6K1* may also be of interest in this context, in particular in connection to *HER2* coamplification. A similar role

for *S6K2* cannot be excluded, however, the impact on radiosensitivity appear to be mainly connected to the 17q21-23 amplicon. Of note, the *RAD51C* gene is located about 1 Mb from *S6K1*, and the RAD51 DNA repair family has in both *in vivo* and *in vitro* studies been related to a poor sensitivity of radiation-induced apoptosis [46-49].

In conclusion, this study shows for the first time that *S6K2* is amplified and overexpressed in breast tumors, which like *S6K1* amplification may have prognostic significance. Resulting data demonstrate a role for the S6 kinases in predicting response of tamoxifen as well as radiotherapy treatment, but further studies are needed to uncover underlying mechanisms. The mTOR targets *S6K1* and *S6K2* may possess both compensatory and non-redundant functions associated with malignancy and therefore have potential as new prognostic and predictive markers in breast cancer.

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### **Disclosure/conflict of interest**

The authors have no conflicts of interest do disclose.

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## Supplementary Methods

### *Evaluation of S6K1/S6K2 gene copy number using real-time PCR*

*S6K1* and *S6K2* gene copy number was assessed in 206 respectively 207 breast tumors using real-time PCR. The gene encoding the Amyloid Precursor Protein (*APP*) served as endogenous control since no amplifications or deletions have been reported in breast cancer [1]. Primers and probes were designed using the software Primer Express version 1.5a (Applied Biosystems) and specificity was controlled by blasting to other sequences available at [www.ncbi.nlm.nih.gov/BLAST](http://www.ncbi.nlm.nih.gov/BLAST). The *APP* probe was coupled to the dye FAM and the quencher TAMRA (Sigma-Aldrich) and the *S6K1/2* MGB probes were attached to FAM and a non-fluorescent quencher (Applied Biosystems). Primers and probes sequences were as follows:

*S6K1* Forward: 5' AATATTTATGGAAGACACTGCCTGGTA3'

*S6K1* Reverse: 5' TGGCTGCCCATAGTGGGAA3'

*S6K1* Probe: 5' TTGTGGTTGCATAGATT3'

*S6K2* Forward: 5' ACTGGAGCCTTGTCCTCATTA ACT3'

*S6K2* Reverse: 5' TCAGTCAGCTCCACCTCTTCATAGT3'

*S6K2* Probe: 5' ACAGGCCTACGGACACA3'

*APP* Forward: 5' TTTGTGTGCTCTCCCAGGTCT3'

*APP* Reverse: 5' TGGTCACTGGTTGGTTGGC3'

*APP* Probe 5' CCCTGAACTGCAGATCACCAATGTGGTAG3'

Quantitative real-time PCR was performed with 20 ng DNA in a 15 µl reaction mixture, with TaqMan Fast Universal PCR Master Mix 1X final concentration (Applied

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Biosystems) supplemented with 0.1  $\mu\text{M}$  (*S6K1*) or 0.15  $\mu\text{M}$  (*S6K2*) of each primer and probe. The plates were loaded using the liquid handling workstation epMotion 5070 (Eppendorf AG). The absolute quantification assay was performed in the 7500 Fast Real Time PCR system (Applied Biosystems). The thermal cycling conditions were: 95°C for 20 s, followed by 40 cycles of 95°C for 3 s, and 60°C for 30 s. Reactions were analyzed in triplicates using the 7300 Sequence Detection Software version 1.3.1 (Applied Biosystems). A five point's standard curve (240 ng/ $\mu\text{l}$ -0.94 ng/ $\mu\text{l}$ ) was constructed using fourfold dilutions of DNA from the mammary breast cancer cell line T47D. *S6K1* and *S6K2* gene copy number was quantified using the standard curve, and each sample was normalized by calculating the ratio C (gene)/C (*APP*). Cut-off levels for amplification ( $\geq 4$  gene copies) were based on the frequency distribution of the gene copy ratios and were set to  $>1.38$  for *S6K1* and  $> 2.8$  for *S6K2*. Two gene copies were expected at the modal peak in the frequency distribution, being 0.69 for *S6K1* and 1.4 for *S6K2*. A cut off for 3 gene copies was defined as 1.04 for *S6K1* and 2.3 for *S6K2*. Five non-amplified and five amplified tumors were rerun on five separate occasions to validate the reproducibility of the method. The resultant coefficient of variation was less than 10% for both genes.

### *S6K2 mRNA quantification*

mRNA was reverse transcribed into cDNA using the high-capacity cDNA reverse transcription kit (Applied Biosystems), following manufacturer's instructions. For each reaction, 200 ng RNA was added to a final reaction volume of 20  $\mu\text{l}$ . To confirm that no gDNA was detected, reactions without reverse transcriptase (-RT) were included for five samples. Quantitative fast real time PCR was performed on an ABI Prism 7900ht (Applied

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Biosystems), using the thermal conditions: 95°C for 20 s, followed by 40 cycles of 95°C for 1 s, and 60°C for 20 s. TaqMan assays (Applied Biosystems) for *S6K2* (Hs00177689\_m1) and the endogenous control *ACTB* (part no 4310881E) were handled according to manufacturer's instructions, using the reaction volume 10 µl. Relative expression of the gene was calculated with the standard curve method, using SKBR3 cDNA to construct the standard curve. Briefly, all samples were run in triplicates and the median Ct-values were used to calculate a relative expression value (C) for each gene, based on the standard curves. Final mRNA quantitation was performed by calculating the ratio  $C(S6K2)/C(ACTB)$  for each sample.

### *Immunohistochemistry*

For staining of S6K2 protein, the tissue microarrays were deparaffinized and rehydrated by several passages in xylene, ethanol and distilled water and the technique proceeded as described before [2] with slight variations. Antigen retrieval was carried out in citrate buffer, pH 6.0, using a decloaking chamber (BioCare Medical) and the default program (SP1=125°C for 30 s, SP2=90°C for 10 s, at a pressure of 23-25 psi). After 30 min at room temperature, the samples were incubated with a protein block (Spring Bioscience) for 10 min, followed by 3 h incubation with a mouse monoclonal antibody against human S6K2 (cat. no MAB2987, R&D systems) diluted 1:100 in PBS-0.5% BSA. The anti-mouse Envision+ system conjugated with horse radish peroxidase (Dako) was used as secondary reagent. The color was developed with 3,3'-diaminobenzidine hydrochloride (DAB)/H<sub>2</sub>O<sub>2</sub> for 10 min at room temperature, and cell nuclei were counterstained with haematoxylin. All slides were evaluated by two independent observers blinded to the clinical data and the tumors scored

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according to the intensity of nuclear staining (negative or positive) and cytoplasmic staining (negative, weak, moderate or strong).

Cyclin D1 protein expression was assessed using immunohistochemistry as described above, with a few exceptions. For antigen retrieval the slides were boiled in citrate buffer, pH 6.0, for 12 min using a pressure cooker, and cooled in room temperature for 30 min. A protein block (Dako) was applied for 10 min and the slides were incubated with a rabbit polyclonal antibody against Cyclin D1 (Cyclin D1 ab3, Neomarkers, dilution 1:300) at 4°C for 21 hours. The secondary antibody (Envision, anti-rabbit, Dako) was applied and visualized according to above. Nuclear staining was evaluated by two observers and graded according to frequency of positive nuclei (<1%, 1-25%, 25-75% or >75%).

#### *Immunoblotting*

ZR751, T47D, MCF7 and BT474 cell lysates (30 µg per well) were loaded on a 4-15% gradient precast gel (Criterion, Bio-Rad). Proteins were transferred to a PVDF membrane, which was blocked with 5% milk in TBS+0.1% Tween-20 and probed with the anti-S6K2 antibody (0.5µg/ml) for incubation overnight at 4°C. The membranes were incubated with the secondary antibody (polyclonal anti-mouse, Dako, P0447, 1:1000) for one hour at room temperature. Signal was detected with the Amersham ECL Plus detection reagents (GE Healthcare).

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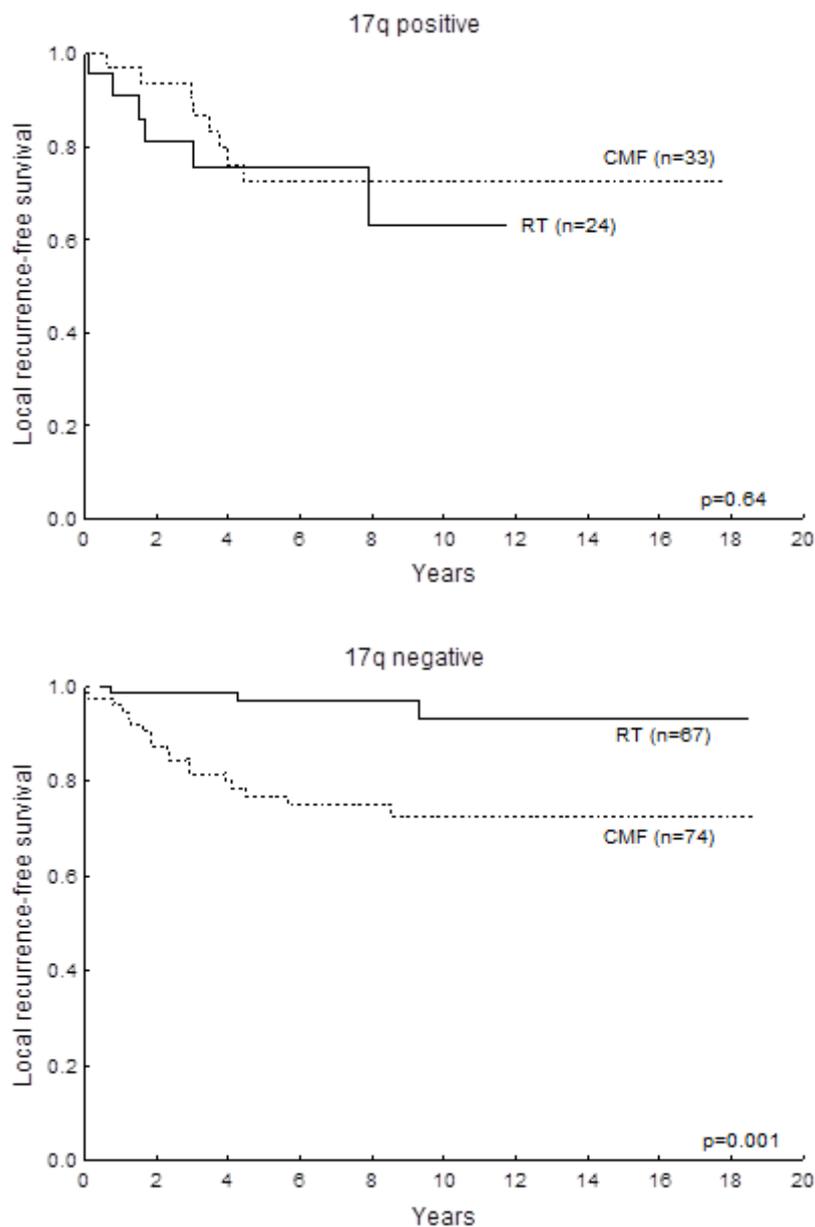
**Supplementary Table 1** Cox proportional hazard regression models of local recurrence to test the interaction between *S6K1* amplification, 17q amplification, *S6K2* gain respectively *S6K1* amplification and/or *S6K2* gain, and the benefit from radiotherapy

	No. of patients	Radiotherapy vs. Chemotherapy HR (95% CI)	Test for interaction	
<i>S6K1</i> amplification				
-	184	0.27 (0.11-0.66)	P=0.0038	
+	22	2.00 (0.40-10.0)	P=0.39	P=0.035
17 q amplification ( <i>S6K1</i> and/or <i>HER2</i> )				
-	141	0.17 (0.05-0.56)	P=0.0039	
+	57	1.30 (0.45-3.74)	P=0.63	P=0.013
<i>S6K2</i> gain				
-	163	0.41 (0.17-0.96)	P=0.040	
+	44	0.30 (0.06-1.43)	P=0.13	P=0.74
<i>S6K1</i> amplification and/or <i>S6K2</i> gain				
-	143	0.26 (0.09-0.77)	P=0.015	
+	64	0.58 (0.20-1.70)	P=0.32	P=0.29

Pérez-Tenorio G, Karlsson E, Marie Ahnström Waltersson M, Olsson B, Holmlund B, Nordenskjöld B, Fornander T, Skoog L and Stål O; Clinical potential of the mTOR targets *S6K1* and *S6K2* in breast cancer

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**Supplementary Figure 1.** Local recurrence-free survival for patients treated with radiotherapy (RT) vs. CMF chemotherapy in relation to 17q21-23 status: 17q positive (*S6K1* and/or *HER2*  $\geq 4$  copies) (a); 17q negative (*S6K1* and *HER2*  $<4$  copies) (b)

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