Akt2 expression is associated with good long-term prognosis in oestrogen receptor positive breast cancer

Helena Fohlin, Gizeh Perez-Tenorio, Tommy Fornander, Lambert Skoog, Bo Nordenskjöld, John Carstensen and Olle Stål

Original Publication:

Helena Fohlin, Gizeh Perez-Tenorio, Tommy Fornander, Lambert Skoog, Bo Nordenskjöld, John Carstensen and Olle Stål, Akt2 expression is associated with good long-term prognosis in oestrogen receptor positive breast cancer, 2013, European Journal of Cancer, (49), 6, 1196-1204.
http://dx.doi.org/10.1016/j.ejca.2012.12.006

Akt2 expression is associated with good long-term prognosis in estrogen receptor positive breast cancer

Helena Fohlin[1,2], Gizeh Pérez-Tenorio[2], Tommy Fornander[3], Lambert Skoog[4], Bo Nordenskjöld[2], John Carstensen[5], Olle Stål[2]

[1]Regional Cancer Center, Southeast Sweden, University Hospital, Linköping, Sweden, [2]Division of Oncology, Department of Clinical and Experimental Medicine, Faculty of Health Sciences, Linköping University, Sweden, [3]Department of Oncology, Karolinska University Hospital, Karolinska Institute, Stockholm, Sweden, [4]Department of Pathology, Karolinska University Hospital, Karolinska Institute, Stockholm, Sweden, [5]Division of Health and Society, Department of Medical and Health Sciences, Linköping University, Sweden

Corresponding author:

Helena Fohlin
Regional Cancer Center, Southeast Sweden
University Hospital, Linköping, Sweden
SE-581 85 Linköping
Sweden

Fax: +46-10-103 2846
Telephone: +46-10-103 8009
E-mail: helena.fohlin@lio.se

Keywords: breast cancer, Akt, protein kinase B, estrogen receptor, long-term, prognostic factor

Abstract

Introduction: Akt is a signaling modulator for many cellular processes, including metabolism, cell proliferation, cell survival and cell growth. Three isoforms of Akt have been identified, but only a few studies have concerned the isoform-specific roles in the prognosis of breast cancer patients. The aim of this study was to investigate the prognostic value of Akt1 and Akt2 in estrogen receptor positive (ER+) and estrogen receptor negative (ER-) breast cancer with long-term follow-up.

Material and Methods: The expression of Akt in tumor tissue was analyzed with immunohistochemistry in a cohort of 272 postmenopausal patients with stage II breast cancer. The median follow-up time was 19 years. Hazard ratios and 95% confidence intervals were estimated using the Cox's proportional hazards model.

Results: The risk of distant recurrence was reduced for patients with ER+ tumors expressing Akt2 compared to patients with no Akt2 expression (HR = 0.49, 95% CI 0.29 – 0.82, p= 0.007). When adjusting for important clinical tumor characteristics and treatment, Akt2 was still an independent prognostic factor (HR= 0.38, 95% CI 0.21 – 0.68, p= 0.001) and the association remained long-term. The prognostic value of Akt2 increased with higher estrogen receptor levels from no effect among patients with ER- tumors to 68% risk reduction for the group with high ER-levels (P for trend= 0.042). Akt1 showed no significant prognostic information.

Conclusion: Our results indicate that Akt2 expression is associated with a lower distant recurrence rate for patients with ER+ tumors and that this association remains long-term. The prognostic value of Akt2 increases with higher estrogen receptor expression, motivating further mechanistic studies on the role of Akt2 in ER+ breast cancer.

Introduction

Akt is a signaling modulator for many cellular processes, including metabolism, cell proliferation, cell survival and cell growth (1). Three isoforms of Akt have been identified: Akt1, Akt2 and Akt3. Several studies have shown that Akt status influences the prognosis of breast cancer patients, but only a few are concerned with potential isoform-specific roles and there are conflicting results. A study on transgenic mice showed that Akt2 was a protective factor against tumor induction, whereas Akt1 had the opposite effect (2). Another study in mice showed that Akt2 promoted metastases, whereas Akt1 impaired metastases (3). This indicates not only that the different isoforms may have different functions, but also that the same isoform can take both protective and destructive roles possibly depending on the stage of breast cancer development. Studies on the potential different roles of the Akt isoforms have been reviewed (4).

Activated Akt (pAkt) has been associated with up to 40 % of breast cancers (5). The mechanisms behind enhanced Akt phosphorylation are several, including HER2 amplification, PI3K mutation and PTEN loss (4). Most results suggest that pAkt correlates with poor prognosis (6, 7) and is associated with other aggressive prognostic factors, such as HER2-positivity and lymph node positive breast cancer (8). In a recent study, activated Akt1 was shown to drive progression in early breast cancers, whereas activated Akt2 may reverse this effect in cases where both Akt1 and Akt2 are activated (9). Although pAkt expression is more closely related to Akt1 than to Akt2 in breast cancer (8, 10, 11), it has been reported that Akt2 is also frequently upregulated in HER2-positive breast tumors and may contribute to tumor aggressiveness (12). However, recent studies have shown that overexpressed Akt2 is a favorable prognostic factor (11, 13). In these studies, only patients with estrogen receptor positive tumors were included.

In this paper we have investigated the prognostic value of Akt1 and Akt2 for stage II patients from a randomized study including both patients with estrogen receptor-negative (ER-) and estrogen receptor-positive (ER+) tumors, where only half of the patients with ER+ tumors received hormonal treatment.

The aim was to compare the influence of the Akt isoforms in tumors with different hormonal status. We have also examined how the prognostic value of Akt1 and Akt2 changes over time.

Materials and Methods

Patients

Between November 1976 and April 1990 both premenopausal and postmenopausal patients were randomized in a trial with the aim to compare postoperative radiotherapy with adjuvant chemotherapy. Only patients with tumor size ≥ 30 mm and/or lymph node metastases were included. The postmenopausal patients were further randomized using a 2x2 factorial study design to one of four groups: adjuvant CMF (Cyclophosphamide Methotrexate Fluorouracil) chemotherapy, adjuvant chemotherapy plus tamoxifen, postoperative radiotherapy or postoperative radiotherapy plus tamoxifen. The data material was previously described (14). In this study we have used data from a subset of the postmenopausal patients consisting of 272 patients with median age 59 years (range: 45 - 71) for whom data on Akt1, Akt2 and pAkt was available from a previous study (10). The period of follow-up has now been extended.

Immunohistochemistry

The expression of Akt1, Akt2 and pAkt was analyzed by immunohistochemistry as previously reported (10). Goat polyclonal antibodies against Akt1 and Akt2 (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), and a sheep polyclonal antibody against the phosphorylated serine residue in position 473 of human Akt1 (Upstata Biotechnology, Lake Placid, NY, USA) were used for immunostaining.

The isoforms had different staining patterns. In the immunopositive tumors, Akt1 was frequently expressed in a high percentage of cells, whereas the staining of Akt2 was mostly sparse. Patients

whose tumors showed staining of at least 1% of the cells were defined as Akt1+ and Akt2+, respectively.

Estrogen receptor determination was performed using ER cytosol assays at the time of primary diagnosis (14). Tumors with receptor content of at least 0.1 fmol/µg DNA were classified as ER+ as in the previous study (10).

HER2 protein expression was quantified with an immunocytochemical method using flow cytometry (10).

Mutations of the PIK3CA gene were detected by a single-stranded conformational analysis followed by sequencing (15).

Statistical methods

To compare the association between Akt and clinical parameters, the Pearson chi-squared test was applied.

Time for follow-up was defined as the time from diagnosis until the first event of distant recurrence, death or last observation (December 31, 2006). Median follow-up time for all patients was 9.1 years (range 0.04 to 28.5 years) and for distant recurrence-free patients 18.9 years.

The distant recurrence-free survival was estimated using the Kaplan-Meier method. The end-point was defined as the first distant recurrence from the patient's primary breast cancer as described in (14). Nine patients' cause of death was breast cancer, but no date of distant recurrence was registered. For these patients the date of death was used as event of distant recurrence. Hazard ratios and 95% confidence intervals were estimated using the Cox's proportional hazards model. The proportional hazards assumption was verified applying Schoenfeld residuals. Interactions between clinically important tumor parameters and Akt1 and Akt2, respectively, were examined using the likelihood ratio test. A p-value of <0.05 was considered to be statistically significant. The statistical analyses were performed using STATA/SE 10.0.

Our study was reported according to the Reporting Recommendations for Tumor Marker Prognostic Studies (REMARK) criteria (16) .

Results

Expression of Akt was found in 121 tumors. Eighty tumors (29%) were Akt1+ and 81 tumors (30%) Akt2+ (Table 1). Among tumors which expressed Akt2 there was a higher percentage of ER-negativity (38%) compared to those with no Akt2 expression, where 26% of the tumors were ER- (p= 0.040). There was no statistically significant correlation between Akt2 and other clinical factors investigated. Akt1 had a significant positive correlation with PIK3CA mutation (p= 0.036).

Univariate analysis of distant recurrence-free survival

The risk of distant recurrence was reduced for patients with tumors expressing Akt2 compared to patients with no Akt2 expression (HR = 0.66, 95% CI 0.45 – 0.97, p= 0.033). Stratifying on estrogen-receptor status showed that the risk reduction was seen for patients with ER+ tumors (HR= 0.49, 95% CI 0.29 – 0.82, p= 0.007) (Fig. 1a), whereas there was no difference for those with ER- tumors (HR=1.00, 95% CI 0.53 – 1.87, p= 1.00) (Fig. 1b). The difference in effect of Akt2 in ER+ and ER- tumors showed a tendency to statistical significance (p= 0.11).

The ER+ tumors were further divided into two groups with intermediate levels of ER (between 0.1 and 1.0 fmol/µg DNA) and high levels (≥1.0 fmol/µg DNA). The prognostic value of Akt2 was more pronounced for patients with tumors expressing high levels of ER. The relative risk in this subgroup was 0.32 (95% CI 0.14 – 0.77, p= 0.010) for Akt2+ compared with Akt2- (Fig 2a). For patients with intermediate ER-expression the relative risk for Akt2+ versus Akt2- was not statistically significant, HR= 0.67 (95% CI 0.35 – 1.27, p= 0.22) (Fig. 2b). A test for trend showed that the prognostic significance of Akt2 increased with higher estrogen receptor expression (P for trend= 0.042).

The better prognosis for patients with ER+ tumors and expression of Akt2 was more pronounced in the combination of Akt2+ and activated Akt (pAkt+) (HR= 0.31, 95% CI 0.13 – 0.73, p= 0.007) (Fig. 3a). The distant recurrence-free survival after 20 years was 76% (95% CI 0.51 – 0.90) for patients with Akt2+/pAkt+ tumors compared to 42% (95% CI 0.33 – 0.49) for patients with other combinations of Akt2 and pAkt. There was no statistically significant association of Akt2 with distant recurrence-free

survival if the tumor was pAkt- (HR= 0.71, 95% CI 0.37 – 1.37, p= 0.30) (Fig. 3b). A test for interaction between ER status and the combined Akt2/pAkt status showed statistical significance (p= 0.015) with respect to prognosis.

For patients with Akt1+ tumors we could not show prolonged or shortened distant recurrence-free survival compared with patients with Akt1- tumors, irrespective of ER status (ER- tumors, HR= 0.98 (95% CI 0.50 – 1.91, p= 0.95); ER+ tumors, HR= 0.89 (95% CI 0.58 – 1.38, p= 0.61)).

In the subgroup of Akt1- tumors, the prognostic significance of Akt2 was increased. Patients with Akt2+ tumors had 52% risk reduction for distant recurrence compared to patients with no Akt2 expression (HR= 0.48, 95% CI 0.27 – 0.85, p= 0.012) and for patients with ER+ tumors and absent Akt1 the risk reduction was 64% (HR= 0.36, 95% CI 0.16 – 0.79, p= 0.011) (Fig. 4a). For the other subgroup of patients having ER+ tumors expressing Akt1, the corresponding hazard ratio was not statistically significant (HR= 0.69, 95% CI 0.33 – 1.47, p= 0.34) (Fig. 4b). There was a tendency of interaction between Akt1 and Akt2 (p= 0.094).

For patients with ER+ tumors receiving tamoxifen, the distant recurrence risk reduction for Akt2+ versus Akt2- was 60% (HR= 0.40, 95% CI 0.12 – 1.37, p= 0.15) during the first five years after diagnosis, whereas it was 66% (HR= 0.34, 95% CI 0.12 – 0.98, p= 0.045) for patients with ER+ tumors receiving no hormonal treatment. The difference in hazard ratio was not statistically significant.

Multivariate analysis of distant recurrence-free survival

In multivariate analysis, when adjusting for important clinical tumor parameters and treatment, Akt2 was an independent prognostic factor (HR= 0.60, 95% CI 0.39 – 0.93, p= 0.022). In the period following five years since diagnosis, the prognostic value of Akt2 was still evident (HR = 0.42, 95% CI 0.20 – 0.90, p= 0.026). No statistically significant association with prognosis was found for Akt1 or pAkt. The only factors that had a long-term effect on distant recurrence were Akt2 and presence of

more than three lymph node metastases at the time of diagnosis. Mutation of the PIK3CA gene had no influence on the prognostic significance of Akt2.

When applying a multivariate analysis with the same factors, but only for ER+ tumors, the prognostic value of Akt2 increased (Table 2). For this subgroup the risk reduction was 62% for patients with Akt2+ tumors compared with Akt2- (HR= 0.38, 95% CI 0.21 – 0.68, p= 0.001). For patients with ER- tumors there was no difference in distant recurrence-free survival (HR= 1.24, 95% CI 0.56 – 2.73, p= 0.59). To investigate if Akt2 had a long-term effect on prognosis for patients with ER+ tumors we performed a multivariate analysis dividing the follow-up period into two different time intervals, before and after 5 years since diagnosis (Table 3). While tumor size and tamoxifen treatment failed to provide long-term prognostic information, Akt2 remained statistically significant even after 5 years (HR= 0.43, 95% CI 0.19 – 0.97, p= 0.043).

Discussion

Since breast cancer therapy continuously improves, and over 74% of the patients are still alive ten years after diagnosis (17), it is important to identify factors to distinguish between good and poor prognosis even several years after diagnosis. Besides conventional prognostic factors, such as age and tumor size, several biomarkers are claimed to provide prognostic information, but many of them need further follow-up to confirm long-term effects (18). We have shown that, for patients with ER+ tumors, Akt2 is an independent prognostic factor for distant recurrence-free survival. In contrast to many well-known prognostic factors, the effect of Akt2 remained several years after diagnosis.

For patients with ER- tumors there was no statistical difference in recurrence-free survival in relation to Akt2, whereas for the ER+ group the prognostic value of Akt2 increased with higher levels of estrogen receptor expression. This shows that the estrogen receptor levels are important for the effect of Akt2 expression. Since recurrences occurring several years after diagnosis are more associated with ER+ tumors, a possible explanation for the fact that the effect of Akt2 is limited to this group of

patients could be that Akt2 by some means attenuates estrogen signaling and thereby inhibits recurrence. One such pathway could involve the Forkhead box transcription factor 3a (FoxO3a). Akt inactivates FoxO3a which in turn influences ER expression. Morelli et al. showed that, among the Akt isoforms, only Akt2 seems to have a key role in the regulation of ER expression (19). Although the authors conclude that Akt2 induces ER expression by inhibiting FoxO3a, other studies have shown the reverse. Guo et al. suggested that activated Akt reduces ER expression by inactivation of FoxO3a (20) and in the present study Akt2 negatively correlated to ER.

Van Agthoven et al. showed that lymph node negative patients with ER+ tumors containing high EGFR and low Akt2 mRNA expression had a worse prognosis compared to the other groups (13). It has also been shown that overall survival was improved for tamoxifen treated patients with high Akt2 expression, and that patients who had a recurrence more often had a low Akt2 expression (11). Recently, Spears et al. considered the impact of isoform-specific Akt1 and Akt2 expression (9). The authors concluded that expression of high pAkt1 alone is associated with poorer outcome, whereas pAkt2 may act protective in cases where both isoforms are activated. To our knowledge, we have for the first time described the prognostic significance of Akt2 in a cohort with stage II patients containing both ER+ and ER- tumors in a randomized study with long follow-up.

Half of the patients with ER+ tumors had not received any hormonal treatment, making it possible to investigate a more pure prognostic value of Akt2 expression. The relative risk for distant recurrence-free survival between Akt2+ and Akt2- was 66% during the first five years after diagnosis for patients with ER+ tumors receiving no hormonal therapy. For patients who were treated with tamoxifen and had ER+ tumors the relative risk was similar but not statistically significant, suggesting that Akt2 is a prognostic rather than a treatment predictive factor. A limitation is that the cohort contains only postmenopausal stage II patients. However, as data suggests that the treatment does not influence the prognostic significance of Akt2, we believe that Akt2 will have the same impact in stage I breast

cancer, which is the majority of the breast cancer patients. This is also supported by the multivariate analysis where the significance for Akt2 remained when adjusting for tumor size and nodal status.

In our cohort, Akt1 was frequently expressed in a high percentage of cells, whereas the staining of Akt2 was mostly sparse. The cut-off for positivity was set to 1%. It is hard to point out the consequences of the choice of cut-point. In the study by Kirkegaard et al., the cut-off was set to the median protein expression in the cytoplasm (11). The expression was evaluated using a semi-quantitative weighted histoscore method. The authors found that the median of Akt2 expression was higher than for Akt1. This highlights that the distribution of Akt1 and Akt2 may differ due to different technical methods. However, independently of the method used, both studies conclude that high Akt2 expression has a favorable prognostic value. In the future, it could be valuable to determine an optimal cut-point, but it requires data from large cohorts, where different methods are compared.

The antibody used for immunostaining of pAkt is likely not specific for any of the isoforms and might react with activated forms of both Akt1 and Akt2. The effect of Akt2 in distant recurrence-free survival was stronger among pAkt+ and weaker among Akt1+ tumors, so it is probable that the more pronounced effect among Akt2+/pAkt+ tumors is due to activated Akt2.

Although the Akt isoforms are similar in protein structure and several functions, we could show prognostic significance of Akt2 but not for Akt1. This could be due to lack of statistical power, but the result is supported by other studies. The prognostic significance of Akt1 has been examined among premenopausal patients with receptor positive tumors, but in contrast to pAkt, no association with distant recurrence-free survival was found (21). Our understanding of the distinct roles of Akt isoforms in tumor development is still limited, but several studies report isoform-specific functions of Akt kinases. The role of Akt2 is suggested to be more important than the other isoforms regarding cell cycle progression, cell proliferation (22) and metabolism (23). The molecular mechanisms that lead to the different functions of Akt isoforms are unclear. It has been suggested that the specific functions of Akt1 and Akt2 are regulated through distinct subcellular localization (4). Akt2 has been shown to be

primarily expressed in the stroma of the mammary gland (2). The authors suggested that Akt2 may influence the expression of stroma-derived molecules that regulate the growth of the mammary epithelium and that ablation of Akt2 may accelerate tumor induction and enhance tumor growth.

Other possible explanations for the different mechanisms of Akt1 and Akt2 could be due to interaction with different proteins. Although both isoforms phosphorylate Plenty of SH3 domains (POSH) which induces apoptotic cell death, only Akt2 interacts directly with POSH (24, 25).

Akt1 and Akt2 have also been shown to have opposing roles regarding activation of p21-activated kinase 1 (Pak1). While Akt1 is a weak activator of Pak1, Akt2 is a strong inhibitor (26). Since high expression of Pak1 is associated with decreased recurrence-free survival (27) and disease progression (28), this could be one explanation why Akt2 is associated with prolonged distant recurrence-free survival in breast cancer.

Phosphorylated Akt has been shown to correlate with poor survival and several clinical trials are concerned with inhibiting the PI3K/Akt /mTOR pathway (29-32). In this work we have shown that Akt2 may play a protecting role regarding breast cancer relapse. If the results are confirmed and explained mechanistically, it could indicate that anticancer drugs should target only Akt1 and not Akt2 in ER+ breast cancer. There are other advantages of inhibiting the pathway through Akt1. It has been reported that deletion of Akt2 in both mice and humans can provoke insulin resistance (33, 34). Furthermore, isoform-specific, rather than complete inhibition of all isoforms, could lead to reduced toxicity to the patients.

Conclusion

Our results indicate that Akt2 expression is associated with a lower distant recurrence rate for patients with ER+ tumors and that this association remains long-term. In this cohort we could not show statistical significance of Akt1 expression, but the results suggest that the positive influence of Akt2 is more pronounced in combination with Akt1- status or pAkt+ status. The prognostic significance of

Akt2 increases with higher estrogen receptor expression, motivating further mechanistic studies on the role of Akt2 in breast cancer.

List of abbreviations used

Akt1	v-akt murine thymoma viral oncogene homolog 1
Akt2	v-akt murine thymoma viral oncogene homolog 2
CI	confidence interval
CMF	cyclophosphamide methotrexate fluorouracil
ER	estrogen receptor
FoxO3a	Forkhead box transcription factor 3a
HER2	human epidermal growth factor receptor 2
HR	hazard ratio
mTOR	mammalian target of rapamycin
Pak1	p21-activated kinase 1
pAkt	phosphorylated Akt
PI3K	phosphatidylinositol 3’ kinase
PIK3CA	phosphatidylinositol 3’ kinase catalytic subunit
POSH	Plenty of SH3 domains
PTEN	phosphatase and tensin homologue
wt	wildtype

Acknowledgements

The work was supported by grants from the Swedish Cancer Society and the Swedish Research Council.

Role of funding source

The funding source did not have any role in the study design, in the collection, analysis, interpretation of data, in the writing of the manuscript or in the decision to submit the manuscript for publication.

Conflict of interests: None declared.

Figure legends

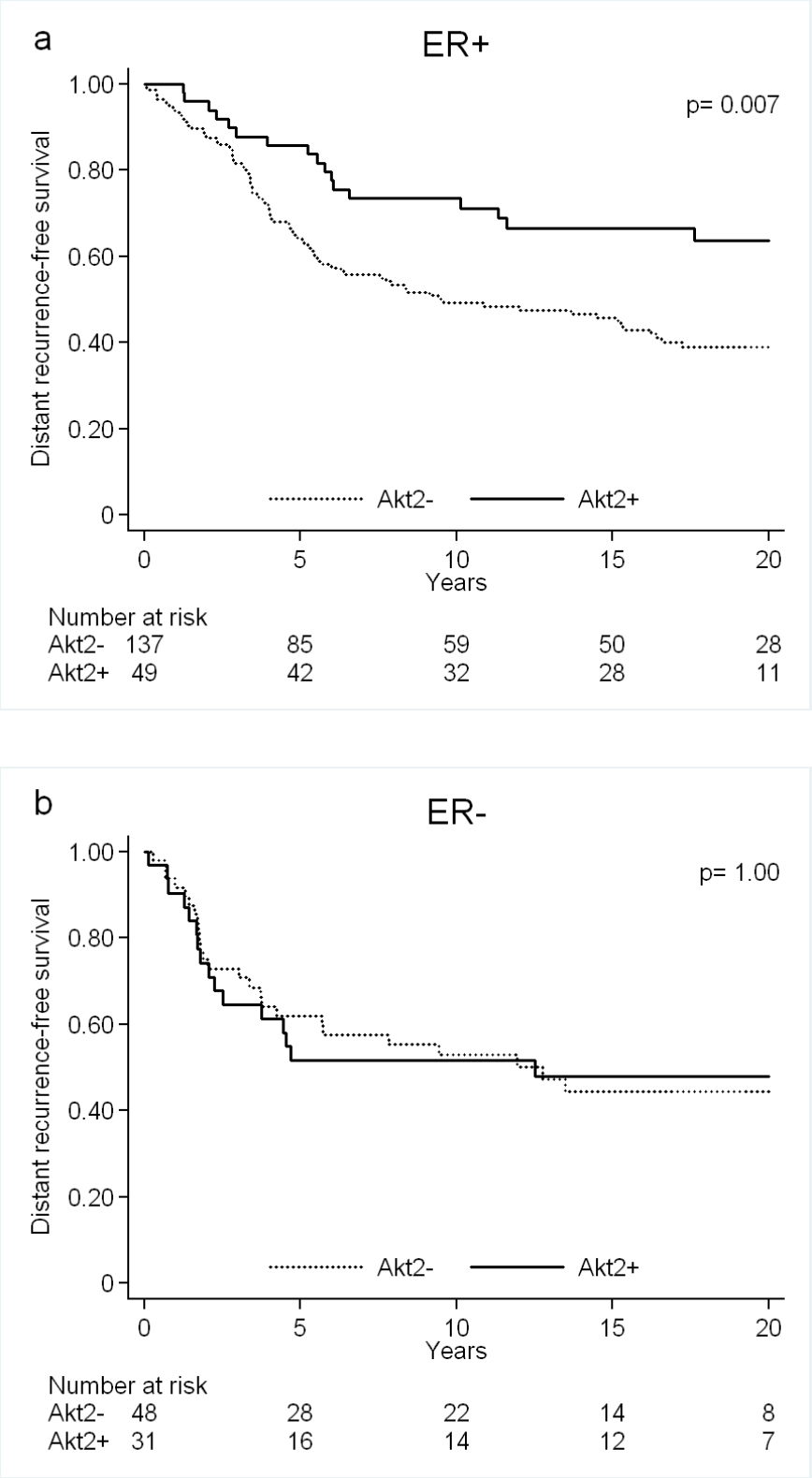
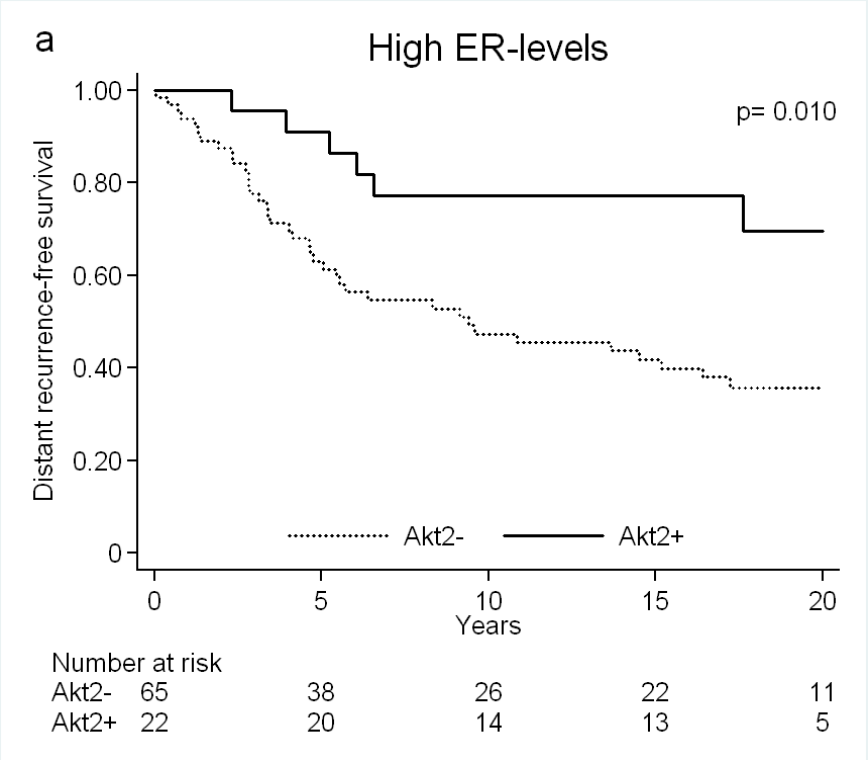


Fig. 1 Distant recurrence-free survival in relation to Akt2 for patients with ER+ tumors (a) and ER- tumors (b)



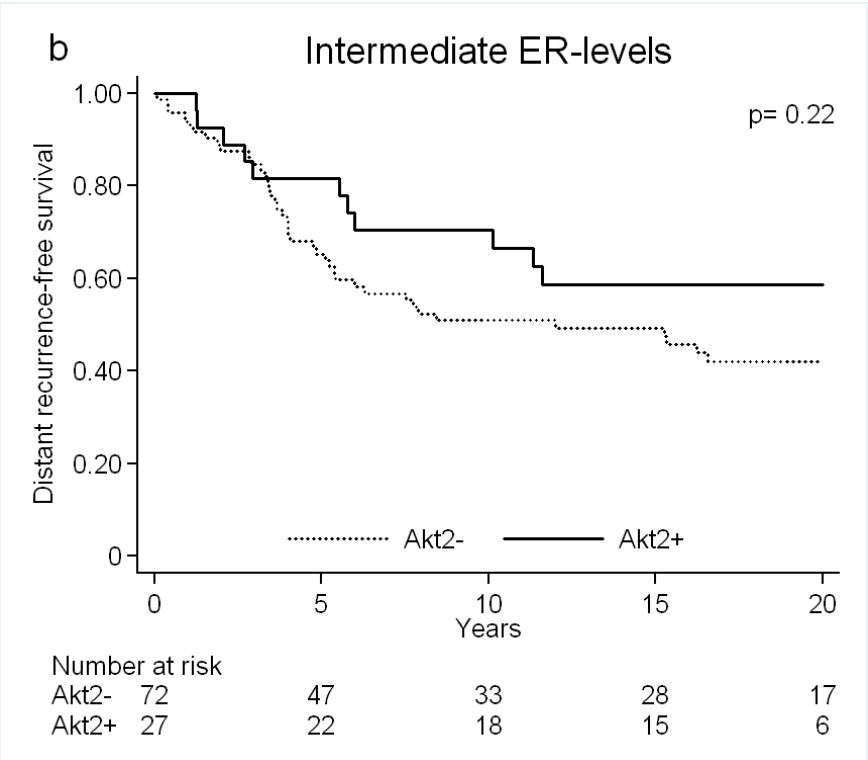
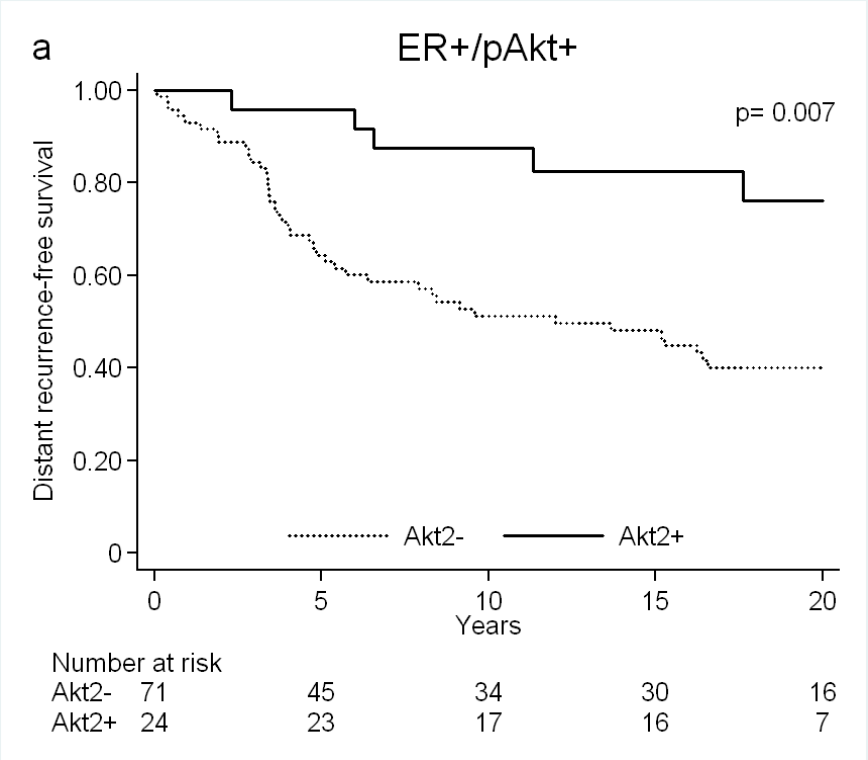


Fig. 2 Distant recurrence-free survival in relation to Akt2 for patients with tumors with high ER-levels (≥ 1.0 fmol/µg DNA) (a) and intermediate ER-levels (0.1 ≤ ER < 1.0 fmol/µg DNA) (b)



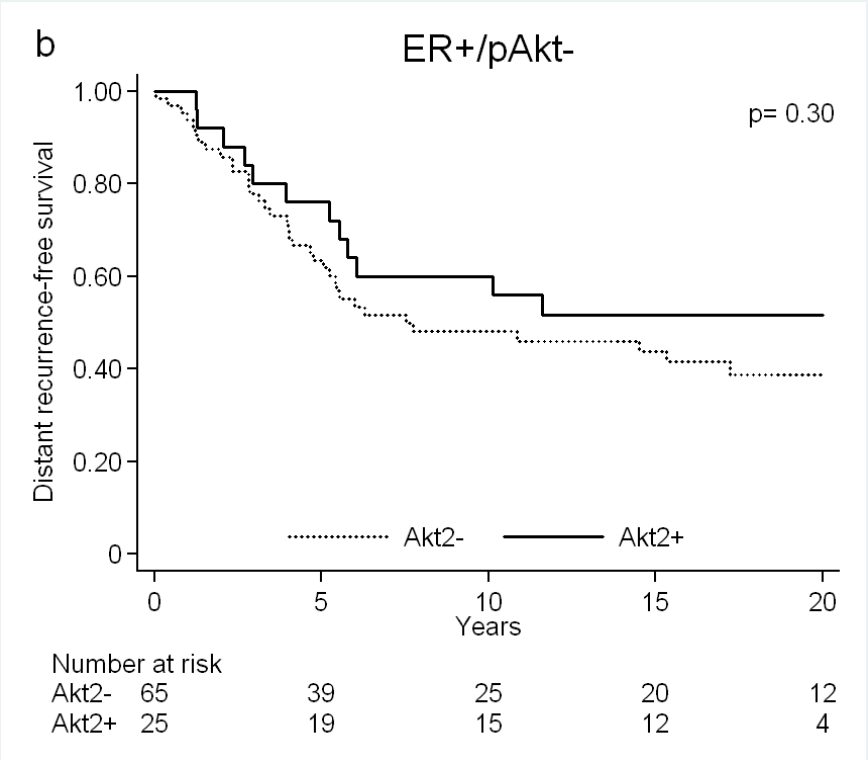
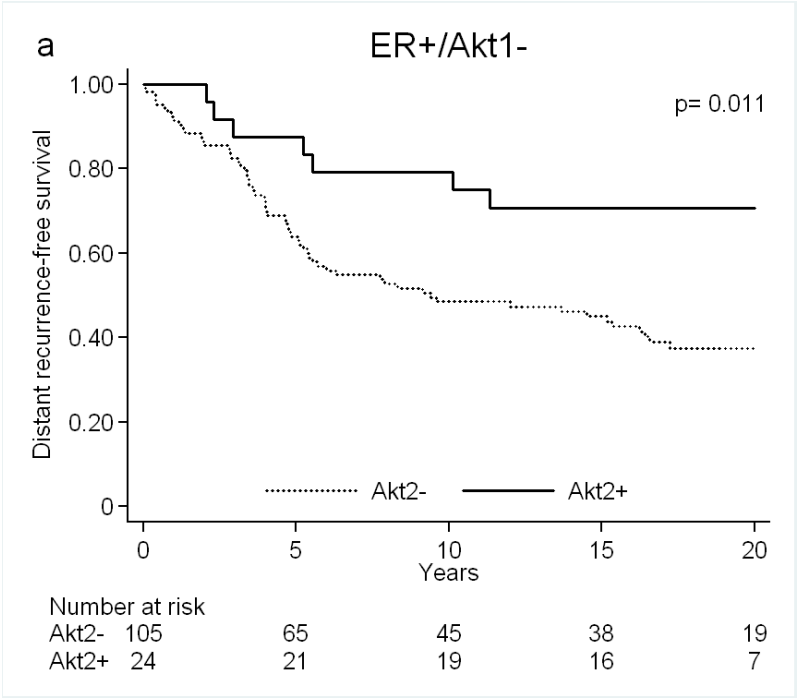


Fig. 3 Distant recurrence-free survival in relation to Akt2 for patients with ER+/pAkt+ tumors (a) and ER+/pAkt- tumors (b)



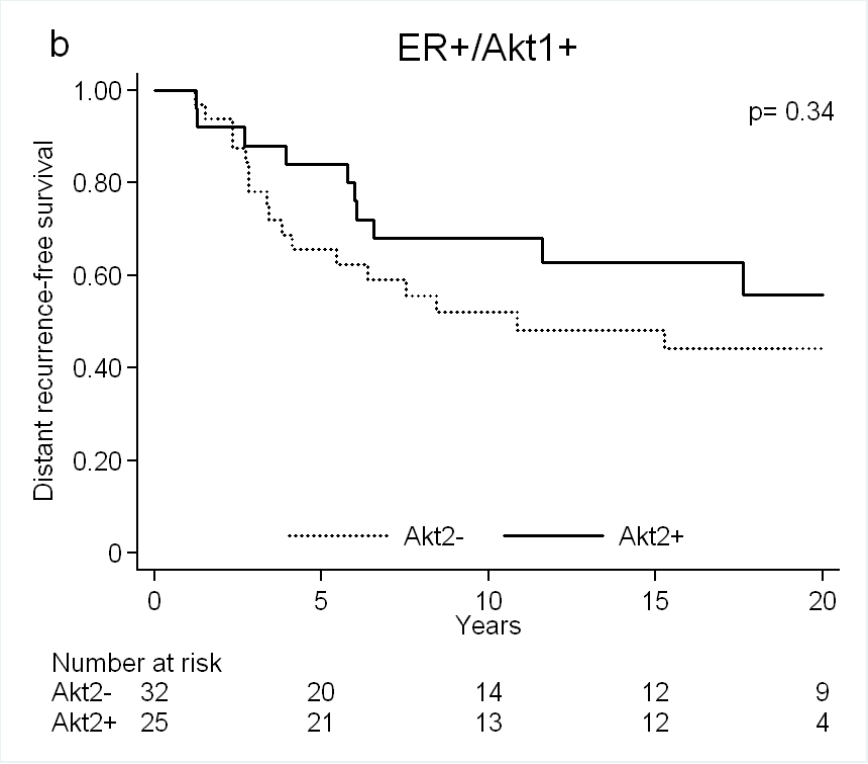


Fig. 4 Distant recurrence-free survival in relation to Akt2 for patients with ER+/Akt1- tumors (a) and ER+/Akt1+ tumors (b)

References

1. Vivanco I, Sawyers CL. The phosphatidylinositol 3-Kinase AKT pathway in human cancer. Nat Rev Cancer. 2002;2(7):489-501.

2. Maroulakou IG, Oemler W, Naber SP, Tsichlis PN. Akt1 ablation inhibits, whereas Akt2 ablation accelerates, the development of mammary adenocarcinomas in mouse mammary tumor virus (MMTV)-ErbB2/neu and MMTV-polyoma middle T transgenic mice. Cancer Res. 2007;67(1):167-77.

3. Dillon RL, Marcotte R, Hennessy BT, Woodgett JR, Mills GB, Muller WJ. Akt1 and akt2 play distinct roles in the initiation and metastatic phases of mammary tumor progression. Cancer Res. 2009;69(12):5057-64.

4. Dillon RL, Muller WJ. Distinct biological roles for the akt family in mammary tumor progression. Cancer Res. 2010;70(11):4260-4.

5. Liu W, Bagaitkar J, Watabe K. Roles of AKT signal in breast cancer. Front Biosci. 2007;12:4011-9.

6. Cicenas J, Urban P, Vuaroqueaux V, Labuhn M, Kung W, Wight E, et al. Increased level of phosphorylated akt measured by chemiluminescence-linked immunosorbent assay is a predictor of poor prognosis in primary breast cancer overexpressing ErbB-2. Breast Cancer Res. 2005;7(4):R394-401.

7. Tokunaga E, Kimura Y, Oki E, Ueda N, Futatsugi M, Mashino K, et al. Akt is frequently activated in HER2/neu-positive breast cancers and associated with poor prognosis among hormone-treated patients. Int J Cancer. 2006;118(2):284-9.

8. Wu Y, Mohamed H, Chillar R, Ali I, Clayton S, Slamon D, et al. Clinical significance of Akt and HER2/neu overexpression in African-American and Latina women with breast cancer. Breast Cancer Res. 2008;10(1):R3.

9. Spears M, Cunningham CA, Taylor KJ, Mallon EA, Thomas JS, Kerr GR, et al. Proximity ligation assays for isoform-specific Akt activation in breast cancer identify activated Akt1 as a driver of progression. J Pathol. 2012;227(4):481-9.

10. Stal O, Perez-Tenorio G, Akerberg L, Olsson B, Nordenskjold B, Skoog L, et al. Akt kinases in breast cancer and the results of adjuvant therapy. Breast Cancer Res. 2003;5(2):R37-44.

11. Kirkegaard T, Witton CJ, McGlynn LM, Tovey SM, Dunne B, Lyon A, et al. AKT activation predicts outcome in breast cancer patients treated with tamoxifen. J Pathol. 2005;207(2):139-46.

12. Bacus SS, Altomare DA, Lyass L, Chin DM, Farrell MP, Gurova K, et al. AKT2 is frequently upregulated in HER-2/neu-positive breast cancers and may contribute to tumor aggressiveness by enhancing cell survival. Oncogene. 2002;21(22):3532-40.

13. van Agthoven T, Sieuwerts AM, Meijer-van Gelder ME, Look MP, Smid M, Veldscholte J, et al. Relevance of breast cancer antiestrogen resistance genes in human breast cancer progression and tamoxifen resistance. J Clin Oncol. 2009;27(4):542-9.

14. Rutqvist LE, Johansson H. Long-term follow-up of the Stockholm randomized trials of postoperative radiation therapy versus adjuvant chemotherapy among 'high risk' pre- and postmenopausal breast cancer patients. Acta Oncol. 2006;45(5):517-27.

15. Perez-Tenorio G, Alkhori L, Olsson B, Waltersson MA, Nordenskjold B, Rutqvist LE, et al. PIK3CA mutations and PTEN loss correlate with similar prognostic factors and are not mutually exclusive in breast cancer. Clin Cancer Res. 2007;13(12):3577-84.

16. McShane LM, Altman DG, Sauerbrei W, Taube SE, Gion M, Clark GM. REporting recommendations for tumor MARKer prognostic studies (REMARK). Breast Cancer Res Treat. 2006;100(2):229-35.

17. Tejler G, Norberg B, Dufmats M, Nordenskjöld B. Survival after treatment for breast cancer in a geographically defined population. British Journal of Surgery. 2004;91:1307-12.

18. Soerjomataram I, Louwman MW, Ribot JG, Roukema JA, Coebergh JW. An overview of prognostic factors for long-term survivors of breast cancer. Breast Cancer Res Treat. 2008;107(3):309-30.

19. Morelli C, Lanzino M, Garofalo C, Maris P, Brunelli E, Casaburi I, et al. Akt2 inhibition enables the forkhead transcription factor FoxO3a to have a repressive role in estrogen receptor alpha transcriptional activity in breast cancer cells. Mol Cell Biol. 2010;30(3):857-70.

20. Guo S, Sonenshein GE. Forkhead box transcription factor FOXO3a regulates estrogen receptor alpha expression and is repressed by the Her-2/neu/phosphatidylinositol 3-kinase/Akt signaling pathway. Mol Cell Biol. 2004;24(19):8681-90.

21. Perez-Tenorio G, Stal O. Activation of AKT/PKB in breast cancer predicts a worse outcome among endocrine treated patients. Br J Cancer. 2002;86(4):540-5.

22. Santi SA, Lee H. Ablation of Akt2 induces autophagy through cell cycle arrest, the downregulation of p70S6K, and the deregulation of mitochondria in MDA-MB231 cells. PLoS One. 2011;6(1):e14614.

23. Tan SX, Ng Y, James DE. Next generation Akt inhibitors provide greater specificity-effects on glucose metabolism in adipocytes. Biochem J. 2011.

24. Figueroa C, Tarras S, Taylor J, Vojtek AB. Akt2 negatively regulates assembly of the POSH-MLK-JNK signaling complex. J Biol Chem. 2003;278(48):47922-7.

25. Lyons TR, Thorburn J, Ryan PW, Thorburn A, Anderson SM, Kassenbrock CK. Regulation of the Pro-apoptotic scaffolding protein POSH by Akt. J Biol Chem. 2007;282(30):21987-97.

26. Zhou GL, Tucker DF, Bae SS, Bhatheja K, Birnbaum MJ, Field J. Opposing roles for Akt1 and Akt2 in Rac/Pak signaling and cell migration. J Biol Chem. 2006;281(47):36443-53.

27. Bostner J, Ahnstrom Waltersson M, Fornander T, Skoog L, Nordenskjold B, Stal O. Amplification of CCND1 and PAK1 as predictors of recurrence and tamoxifen resistance in postmenopausal breast cancer. Oncogene. 2007;26(49):6997-7005.

28. Ong CC, Jubb AM, Haverty PM, Zhou W, Tran V, Truong T, et al. Targeting p21-activated kinase 1 (PAK1) to induce apoptosis of tumor cells. Proc Natl Acad Sci U S A. 2011;108(17):7177-82.

29. Caffarel MM, Andradas C, Mira E, Perez-Gomez E, Cerutti C, Moreno-Bueno G, et al. Cannabinoids reduce ErbB2-driven breast cancer progression through Akt inhibition. Mol Cancer. 2010;9:196.

30. deGraffenried LA, Friedrichs WE, Russell DH, Donzis EJ, Middleton AK, Silva JM, et al. Inhibition of mTOR activity restores tamoxifen response in breast cancer cells with aberrant Akt Activity. Clin Cancer Res. 2004;10(23):8059-67.

31. Luo J, Manning BD, Cantley LC. Targeting the PI3K-Akt pathway in human cancer: rationale and promise. Cancer Cell. 2003;4(4):257-62.

32. Baselga J, Campone M, Piccart M, Burris HA, Rugo HS, Sahmoud T, et al. Everolimus in postmenopausal hormone-receptor-positive advanced breast cancer. N Engl J Med. 2011.

33. Cho H, Mu J, Kim JK, Thorvaldsen JL, Chu Q, Crenshaw EB, 3rd, et al. Insulin resistance and a diabetes mellitus-like syndrome in mice lacking the protein kinase Akt2 (PKB beta). Science. 2001;292(5522):1728-31.

34. George S, Rochford JJ, Wolfrum C, Gray SL, Schinner S, Wilson JC, et al. A family with severe insulin resistance and diabetes due to a mutation in AKT2. Science. 2004;304(5675):1325-8.

Table 1 Akt1 and Akt2 in relation to other tumor characteristics and treatment[a]

	Akt1 n (%)			Akt2 n (%)		
	Neg.	Pos.	P value	Neg.	Pos.	P value
Total no. of patients	192	80		189	81	
Tumor size (mm)						
≤ 20	76 (40)	39 (49)	0.16	86 (46)	29 (36)	0.14
> 20	114 (60)	40 (51)		101 (54)	51 (64)	
Nodal status						
0	26 (14)	6 (8)	0.26	19 (11)	13 (16)	0.15
1 – 3	107 (59)	47 (59)		112 (63)	40 (50)	
4 –	49 (27)	26 (33)		48 (27)	27 (34)	
ER status						
Negative	58 (31)	22 (28)	0.60	48 (26)	31 (38)	0.040
Positive	131 (69)	58 (73)		138 (74)	50 (62)	
HER2						
Negative	143 (76)	56 (72)	0.47	141 (76)	57 (72)	0.53
Positive	45 (24)	22 (28)		45 (24)	22 (28)	
pAkt						
Negative	99 (52)	36 (45)	0.29	91 (48)	43 (54)	0.42
Positive	91 (48)	44 (55)		97 (52)	37 (46)	
PIK3CA						
Wildtype	147 (79)	50 (67)	0.036	136 (76)	59 (74)	0.70
Mutated	39 (21)	25 (33)		43 (24)	21 (26)	
Tamoxifen						
No	98 (51)	38 (48)	0.60	98 (52)	37 (46)	0.35
Yes	94 (49)	42 (53)		91 (48)	44 (54)	
CMF						
No	80 (42)	36 (45)	0.61	82 (43)	32 (40)	0.55
Yes	112 (58)	44 (55)		107 (57)	49 (61)	

a) Number of tumors with missing values: ER= 3, pAkt= 2, Tumor size= 3, Nodal status= 11, PIK3CA= 11, HER2= 5 for Akt1 och HER2= 4 for Akt2

Table 2 Multivariate analysis of distant recurrence-free survival for the whole follow-up period[b]

	ER-		ER+	
	HR	95% CI	HR	95% CI
Akt1 (pos vs. neg)	1.05	0.43 – 2.57	1.06	0.65 – 1.72
Akt2 (pos vs. neg)	1.24	0.56 – 2.73	0.38**	0.21 – 0.68
pAkt (pos vs. neg)	0.86	0.39 – 1.89	0.95	0.61 – 1.48
Tumor size (mm)(>20 vs. ≤20)	1.58	0.69 – 3.64	1.68*	1.06 – 2.65
Nodal status (1-3 vs. 0)	2.55	0.67 – 9.64	2.09	0.85 – 5.17
(4+ vs. 0)	5.15*	1.34 – 19.8	4.18**	1.63 – 10.7
HER2 (pos. vs. neg.)	1.10	0.50 – 2.41	1.17	0.70 – 1.97
PIK3CA (mutated vs. wt)	0.56	0.18 – 1.76	1.06	0.65 – 1.71
Tamoxifen (yes vs. no)	1.02	0.51 – 2.06	0.47**	0.30 – 0.73
CMF (yes vs. no)	1.27	0.60 – 2.68	0.93	0.60 – 1.42

*p< 0.05, ** p< 0.01

b) Multivariate analysis was performed for patients where we had data on all variables included (71 patients for the ER- group and 163 patients for the ER+ group)

Table 3 Multivariate analysis of distant recurrence-free survival for patients with ER+ tumors by follow-up period[c]

	<5 years		≥5 years	
	HR	95% CI	HR	95% CI
Akt1 (pos. vs. neg.)	1.08	0.55 – 2.10	1.03	0.50 – 2.13
Akt2 (pos. vs. neg.)	0.32*	0.13 – 0.78	0.43*	0.19 – 0.97
pAkt (pos. vs. neg.)	0.94	0.52 – 1.70	1.00	0.51 – 1.98
Tumor size (>20 vs. ≤20)	2.09*	1.12 – 3.87	1.22	0.59 – 2.52
Nodal status (1-3 vs. 0)	3.11	0.70 – 13.7	1.33	0.40 – 4.46
(4- vs. 0)	4.64*	1.02 – 21.0	3.94*	1.08 – 14.4
HER2 (pos. vs. neg.)	1.14	0.59 – 2.19	1.22	0.52 – 2.87
PIK3CA (mutated vs. wt)	1.06	0.55 – 2.02	1.09	0.53 – 2.25
Tamoxifen (yes vs. no)	0.41**	0.22 – 0.76	0.54	0.28 – 1.07
CMF (yes vs. no)	0.87	0.49 – 1.55	0.89	0.46 – 1.72

*p< 0.05, ** p< 0.01

c) Multivariate analysis was performed for patients where we had data on all variables included (163 patients for the first time period and 110 patients for the second time period)