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PI3K pathway activation in breast cancer is associated with the basal-like phenotype and cancer-specific mortality

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Breast cancer is a common malignancy with current biological therapies tailored to steroid hormone (ER, PR) and HER2 receptor status. Understanding the biological basis of resistance to current targeted therapies and the identification of new potential therapeutic targets is an ongoing challenge. The PI3K pathway is altered in a high proportion of breast cancers and may contribute to therapeutic resistance. We undertook an integrative study of mutational, copy number and expression analyses of key regulators of the PI3K pathway in a cohort of 292 invasive breast cancer patients with known treatment outcomes. The alterations identified in this cohort included *PIK3CA* mutations (12/168, *i.e.* 7%), *PIK3CA* copy number gain (28/209, *i.e.* 14%), PTEN loss (73/258, *i.e.* 28%) and AKT activation (62/258, *i.e.* 24%). Overall at least 1 parameter was altered in 72% (139/193) of primary breast cancers. PI3K pathway activation was significantly associated with ER negative ($p = 0.0008$) and PR negative ($p = 0.006$) status, high tumor grade ($p = 0.032$) and a "basal-like" phenotype ($p = 0.01$), where 92% (25/27) of tumors had an altered pathway. In univariate analysis, PI3K pathway aberrations were associated with death from breast cancer; however, this relationship was not maintained in multivariate analysis. No association was identified between an activated pathway and outcome in tamoxifen- or chemotherapy-treated patients. We concluded that >70% of breast cancers have an alteration in at least 1 component of the PI3K pathway and this might be exploited to therapeutic advantage especially in "basal-like" cancers.

The phosphatidylinositol 3 kinase (PI3K) pathway regulates many cellular functions, mainly associated with cell proliferation, survival and migration.[1] Activation of PI3K can occur in response to a variety of extracellular signals through growth factor receptor- or integrin-mediated pathways. Upon receptor activation, the p85 regulatory subunit of Class 1a PI3K is recruited to phosphotyrosine residues within the receptor. Once recruited to the membrane, the p110α catalytic subunit, encoded by *PIK3CA*, phosphorylates phosphatidylinositol biphosphate to generate phosphatidylinositol triphosphate (PIP3), which recruits phosphoinositide-dependent kinase 1 (PDK1) and V-Akt murine thymoma viral oncogene homolog 1 (AKT) to the cell membrane. PDK1 and PDK2 (likely the TORC2 complex) then phosphorylate AKT leading to the regulation of an array of downstream pathways including forkhead rhabdomyosarcoma, GSK3β and BAD with consequent effects on cell proliferation, cell metabolism and apoptosis, respectively. PIP3 is a substrate for phosphatase and tensin homologue deleted on chromosome 10 (PTEN), a well-described tumor suppressor protein, which dephosphorylates PIP3, inactivating the PI3K pathway.

Alterations in the PI3K pathway have been documented in many human cancers.[2] In breast cancer, *PIK3CA* activating mutations, in exons 9 and 20, have been identified in ~24% (range, 12–40%) of patient specimens.[3–16] These mutations in the p110α catalytic subunit can activate the pathway and contribute to mammary tumor progression.[15] *PIK3CA* amplification has only been described in 2 breast cancer studies

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with a frequency of 1% and 9%, respectively.[12,16] Molecular alterations and loss of PTEN have also been described in ~24% (range, 4–48%) of breast cancers[5,6,14,17–23] and increased activation of AKT has been identified in ~41% (range, 9–76%) of breast cancer patients.[5,17,19,21,22,24–28] Despite the number of studies exploring the PI3K pathway in breast cancer, the association of specific molecular alterations with clinicopathological markers and outcome is often contradictory and unclear with a consensus yet to be reached. This may be due in part to the widely different frequencies of specific aberrations reported in published studies.

PIK3CA mutations have been associated with positive hormone receptor status in 2 studies,[3,14] but not in others,[7,11,15] and with both favorable[9] and poor patient outcome.[10] PTEN loss has been associated with high grade and negative hormone receptor status in some studies[17,20] but not in others,[22,23] whereas AKT activation has been associated with high tumor grade,[19] PR negativity and HER2 positive status[28] but other studies have identified no such associations.[12,17] Furthermore, there has been no published integrative study determining the combined effects of aberrations in these 3 pivotal members of the pathway.

Breast cancer remains the most common invasive cancer of women in industrialized nations and is second only to lung cancer as the leading cause of cancer-related death in women. Breast cancer is heterogenous but can be classified into at least 4 subtypes by gene expression profiling: Luminal A, Luminal B, HER2+ and "basal-like" tumors,[29,30] with each subgroup reflecting a different biology, behavior and outcome. A surrogate classification of these subgroups has been attempted using immunohistochemistry, whereby patients were subclassified according to hormone receptor (ER, PR), HER2, cytokeratin 5/6 (CK5/6) and epidermal growth factor receptor (EGFR) status.[31] Current breast cancer treatment is tailored in great measure by this subtype classification. Hormone receptor positive cancers are treated predominantly with endocrine therapy (ER+/PR+/HER2−) or Trastuzumab (HER2+), but despite the advances in treatment and outcome, understanding the development of therapeutic resistance and developing strategies to combat it is a continuing challenge. "Basal-like" cancers (triple negative for ER, PR and HER2 and positive for CK5/6 or EGFR) have no identified target for biological therapy and have recently been a focus of studies searching for potential new therapeutic targets. Thus, the aim of our study was to analyze the overall status of the PI3K pathway, by defining mutational status in exons 9 and 20, copy number and expression of key molecules within the pathway, in a cohort of breast cancer patients of known phenotype, treatment response and outcome status.

Material and Methods

Patients and tumors

Cases were drawn from the St. Vincent's Campus Outcome Cohort, which comprised 292 invasive ductal carcinomas of the breast from patients treated by a single surgeon (P.C.) between February 1992 and August 2002 at St. Vincent's Hospital, Sydney, Australia. Ethics approval was granted for the use of pathology specimens and cognate clinicopathological data (Human Research Ethics Committee of St. Vincent's Hospital, Sydney). A more detailed description of the clinicopathological characteristics of the cohort is published elsewhere.[32] Forty percent of tumors were >20 mm, 45% were Grade >2, 43% were lymph node positive, 68% were ER positive, 57% were PR positive and 18% were HER2 FISH positive (>2.2 ratio of HER2:chr.17 centrosome). Median age was 54 years and patients were treated with endocrine therapy (49%), chemotherapy (38%) or both (24%). Cases were prospectively followed-up for a median of 64 months and the outcome events measured were as follows: recurrence (local or distant) (25%), metastasis (23%) and all deaths were recorded but only breast cancer-related deaths (18%) were considered for survival analyses.

DNA extraction

One core from each tumor block, from an area previously selected by a specialist breast pathologist (S.A.O'T.) as the most representative of the tumor, was used to extract DNA. The Agencourt formapure 96-well format kit (Beckman Coulter, Beverly, MA), a paramagnetic bead-based technology, was used to extract DNA from formalin-fixed, paraffin-embedded tissue from 288 individual patients. A significant number of cases ($n = 120$ of 288, *i.e.* 42%) were unsuitable for the mutational analysis, and the main reasons were the poor quality of DNA caused by years of storage of these tissues, the fixation protocol employed or the small amount of tissue sometimes available for DNA extraction.

PIK3CA mutational analysis

Primers were designed to avoid amplification of a known *PIK3CA* pseudogene for exons 9 and 20 of the *PIK3CA* gene: 9F CTGTAAATCATCTGTGAATC, 9R ATTTTAGCACTTA CCTGTGAC; 20F CAATCTTTTGATGACATTGC, 20R TG GAATCCAGAGTGAGCTTT. These 2 exons contain the 3 hotspots for *PIK3CA* mutations in cancer. Codons 542 and 545 in exon 9 and codon 1047 in exon 20. PCR reactions were undertaken using 10–50 ng of DNA, 0.4 μmol/l of each primer, 200 μmol/l deoxynucleotide triphosphates, 3 mmol/l $MgCl_2$, 1× PCR II buffer and 1.5 U of Amplitaq Gold DNA Polymerase (Applied Biosystems, Foster City, CA). The PCR conditions used were as follows: 94°C (10 min) for 1 cycle, 94°C (30 sec), 50°C (30 sec), 72°C (30 sec) for 40 cycles and a final extension step of 72°C (10 min). All PCR products were sequenced with the ABI 3100 Genetic analyzer (Applied Biosystems, Foster City, CA). All mutations were confirmed by analyzing the product of a second, independent PCR.

PIK3CA gene copy number

A subset of tumors ($n = 209$) was analyzed for *PIK3CA* gene copy number by real time quantitative PCR (qPCR) on the

Roche Lightcycler 480 platform (Roche Diagnostics; F. Hoffmann-La Roche Ltd., Mannheim, Germany). *RPLPO*, located at 12q24, was used as an endogenous control, as previously described.[33] *RPLPO* yielded similar results as the use of a gene copy number control from an unaltered region on the same chromosome as *PIK3CA*. Primers for both genes were designed by the Roche primer software using the universal probe library system. qPCR reactions were completed using 50 ng of DNA, 0.2 μmol/l of each primer, 0.2 μl of probe and 5 μl of master mix. qPCR conditions used were as follows: 95°C (7 min) for 1 cycle, 95°C (5 sec), 50–60°C (0.4°C per second) (30 sec), 72°C (15 sec) for 50 cycles and a final extension step of 40°C (30 sec). The efficiency of both assays was analyzed from the slope of a standard curve; RPLPO and PIK3CA qPCR assays had an efficiency of 95% and 97%, respectively. Fold changes were calculated with the Delta Ct method. Fold changes above the mean ± 1 SD of all the samples analyzed, *i.e.* >1.381, were considered copy number gain. This is in agreement with a previously published copy number threshold based on a pool of normal breast tissue.[34,35] The coefficient of variation for triplicate estimates was <0.04.

Tissue specimens

A total of 18 tissue microarrays (TMA) containing 2 cores of each tumor sample were constructed from the formalin-fixed, paraffin-embedded tumor material from each patient in the cohort as previously reported.[32] TMAs were produced using the MTA-1 Manual Tissue Arrayer (Beecher Instruments, Woodland, CA, USA).

Immunohistochemistry

Three-micrometer sections of each TMA were cut, deparaffinized and used for immunostaining. The following antibodies were used: pAKT (Ser473) rabbit monoclonal antibody clone 736E11 (Biocare Medical, Concord, CA, USA) and PTEN mouse monoclonal antibody clone 6H2.1 (Dako, Denmark). For pAKT, antigen retrieval was performed using DAKO solution pH 6.0 (s1699; DAKO, Carpentaria, CA) at 125°C for 4 min in a Pascal pressure chamber (DAKO), the antibody was incubated for 90 min at a 1:50 dilution. For PTEN, antigen retrieval was performed using DAKO solution pH 9.0 (s2367; DAKO) at 125°C for 30 sec in a Pascal pressure chamber, and antibody incubation was 90 min at a 1:200 dilution. A DAKO autostainer was used for immunostaining (DAKO). As secondary antibody a FLEX envision system (DAKO) was used to improve staining. Reactions were developed using diaminobenzidine and sections were counterstained, dehydrated and mounted. For pAKT, sections incubated with nonimmune serum (IgG) and LNCAP prostate cancer cells treated with LY29402 were used as negative controls[36] and untreated LNCAP cells as positive controls. For PTEN, sections of human breast and prostate tumors were used as positive controls and the same sections incubated with IgG were used as negative controls. Immunohistochemistry protocols, scoring and analysis for $p27^{Kip1}$, $p21^{Cip1/Waf1}$, cyclin E1, cyclin D1 and Ki67 are described elsewhere[37] (McNeil C.M., unpublished data).

Immunohistochemical scoring

Scoring was completed by 4 independent investigators (E.L.-K., S.A.O'T., E.K.A.M., M.R.Q.) blinded to the clinical and pathological information, 3 of whom were specialist breast pathologists; in cases of discrepancy a consensus was reached by conferencing. All tissue areas were examined for both intensity (1+ to 3+) and the proportion of positive cells. A histoscore was calculated by multiplying the percentage of positively stained cells and each category of staining intensity (Fig. 1). PTEN showed cytoplasmic localization and loss of expression was defined as the lack of any tumor cell staining. The data were dichotomized as present or absent staining. pAKT demonstrated predominantly cytoplasmic staining, although in a few cases nuclear staining was apparent. For immunostaining of pAKT the mean histoscore value was used to determine the cutoff value of $H > 22$.

Statistical analyses

To assess the independence of 2 categorical variables, the chi-square test was applied when the variables were dichotomous, the Mann–Whitney test was applied when one variable was dichotomous and the other continuous and the Spearman Rank correlation was applied when both variables were continuous. Kaplan–Meier survival curves and Cox proportional hazard ratio (HR) were estimated to obtain risks of recurrence, metastasis and death, after adjusting for other confounder variables. The results were considered significant at the two-sided p of 0.05 level. Statview version 5.0 was used for the analysis (Abacus Systems, Berkeley, CA).

Results

PIK3CA genetic alterations and PTEN loss

To assess the frequency of genetic lesions in the *PIK3CA* gene or loss of PTEN protein, both central players in the PI3K pathway, we analyzed *PIK3CA* mutations by sequencing, *PIK3CA* copy number by qPCR and PTEN expression levels by immunohistochemistry.

Overall, 12 of 168 (7%) breast cancers assessed harbored *PIK3CA* mutations. All mutations were at previously reported hotspot codons in exons 9 and 20, *i.e.* E542K ($n = 1$), E545K ($n = 2$), Q546R ($n = 1$) and H1047R ($n = 8$).

Copy number gain was identified in 28 of 209 (14%) cancers analyzed. Mutations and copy number gains were almost exclusive events with only 1 cancer encompassing both mutation and copy number gain of the *PIK3CA* gene ($p = 0.748$). Neither mutations nor copy number gain were associated with any clinicopathological parameter or outcome.

PTEN, a negative regulator of the PI3K pathway, was lost in 73 of 258 (28%) cancers and was associated with high tumor grade ($p = 0.009$), tumor size >20 mm ($p = 0.009$), ER negativity ($p < 0.001$) and PR negativity ($p = 0.0002$) (Table 1).

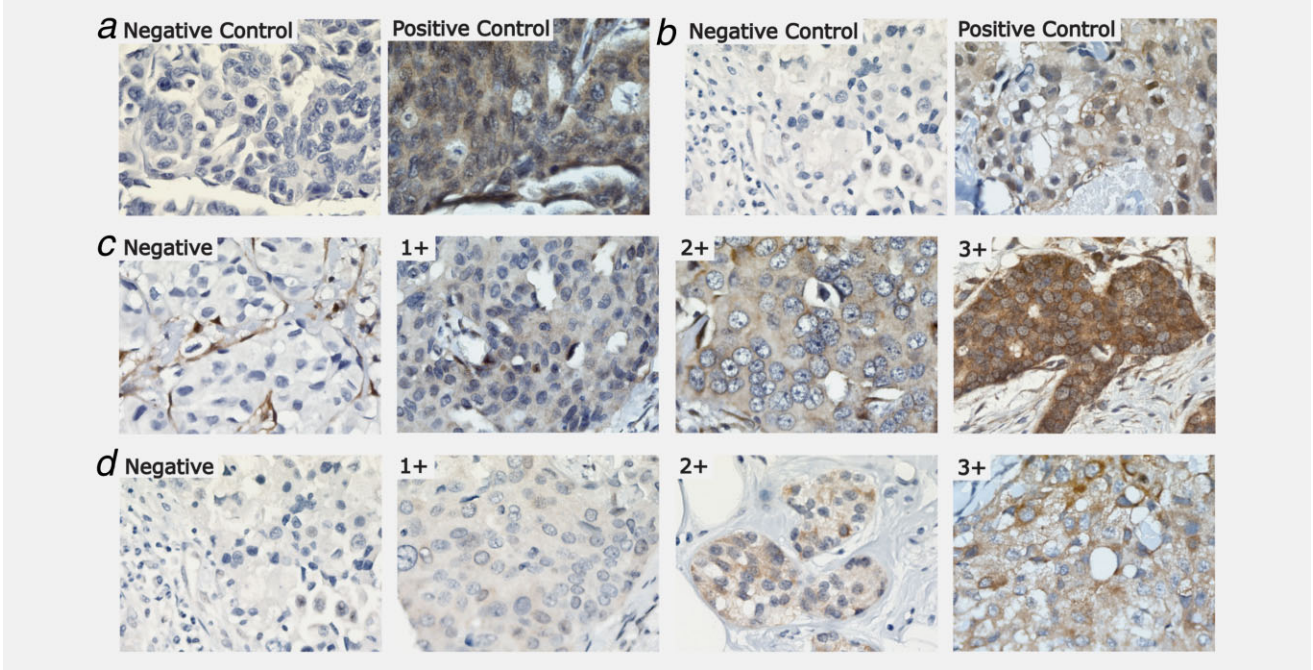

Figure 1. Immunohistochemical staining of paraffin-embedded breast cancer tissue. Photomicrographs for PTEN expression: negative (IgG) and positive controls (*a*) and tumor scoring ranging from negative to intensities 1+, 2+ and 3+ (*c*). Photomicrographs for pAKT expression: negative (IgG) and positive controls (*b*) and tumor scoring ranging from negative to intensities 1+, 2+ and 3+ (*d*).

PTEN loss was not associated with recurrence, metastasis or breast cancer-specific death.

AKT activation

As a measure of PI3K pathway activation, we also examined pAKT expression levels by immunohistochemistry. pAKT was positive in 62 of 258 (24%) specimens and was positively associated with high tumor grade ($p = 0.011$), ER negativity ($p = 0.040$), PR negativity ($p = 0.014$) and HER2 positivity ($p = 0.016$). pAKT was also associated with Ki67, a marker of cell proliferation ($p = 0.005$) (Table 1) and breast cancer-specific death ($p = 0.03$).

PI3K pathway activation and clinicopathological parameters

To assess the overall status of the PI3K pathway, we considered *PIK3CA* mutational and copy number data, PTEN expression and AKT activation together. PTEN loss and *PIK3CA* mutations were mutually exclusive events ($p = 0.0368$); all *PIK3CA* mutations were identified in PTEN-expressing tumors. No association was identified between PTEN loss and *PIK3CA* amplification ($p = 0.982$). A negative correlation was identified between expression of PTEN and activation of AKT by the Spearman Rank correlation ($p = 0.0001$). Low PTEN expression was associated with high AKT activation. When all 4 parameters were combined (*PIK3CA* mutations, *PIK3CA* copy number gain, PTEN loss and AKT activation) 139 of 193 (72%) breast cancer patients had an alteration in 1 or more components of the PI3K pathway. These 193 patients had similar clinicopathological characteristics as the overall 292 tumors and therefore were representative of the whole cohort (data not shown). Figure 2 describes the overlap between the alterations in the 4 parameters analyzed. Over 75% (105/139) of cancers had only 1 altered parameter, 23% (32/139) had 2 alterations and only 1% (2/139) had 3 altered parameters. This measure of pathway activation was associated with high tumor grade ($p = 0.032$), ER negative ($p = 0.0008$) and PR negative ($p = 0.006$) status (Table 1).

Of the 193 patients, with at least 1 altered parameter or wild type for all four, 124 had data for all 4 parameters analyzed. The clinicopathological characteristics of this subgroup of 124 patients were not statistically significantly different to the 193 group of patients (data not shown). All statistical analysis were then undertaken for this group to determine relationships with clinicopathological parameters, subtypes and outcome. The results and conclusions remained unchanged when the analysis was confined to this subgroup of 124 patients (data not shown).

PI3K pathway activation and breast cancer subtypes

When cases were distributed into 5 phenotypes according to ER/PR/HER2, CK5/6 and EGFR status,[31,38] *i.e.* Luminal A (ER+ and/or PR+, HER2−), Luminal B (ER+ and/or PR+, HER2+), HER2 (ER− and PR−, HER2+), "basal-like" (ER−, PR−, HER2−, CK5/6+ and/or EGFR+) and unclassified (negative for all markers), there was no significant relationship with *PIK3CA* mutation or amplification. PTEN loss

Table 1. Association between the PI3K pathway and clinicopathological parameters

	PIK3CA sequence			*PIK3CA* copy number			PTEN expression			AKT expression			PI3K pathway		
	WT	MUT	*p*-Value*	NC	GAIN	*p*-Value*	$H = 0$	$H > 0$	*p*-Value*	$H < 22$	$H > 22$	*p*-Value*	Inactive	Active	*p*-Value*
All cohort	156	12		181	28		73	185		196	62		54	139	
Tumor size															
<20 mm	93	5		96	16		33	116		112	37		34	72	
>20 mm	62	7	0.214	84	12	0.707	40	68	0.009	84	24	0.627	20	66	0.176
Tumor grade															
≤2	89	6		90	13		29	107		112	24		33	61	
>2	66	6	0.617	91	15	0.746	44	78	0.009	84	38	0.011	21	78	0.032
LN status															
0	95	7		94	14		36	101		104	33		31	70	
>0	58	5	0.796	84	14	0.782	37	81	0.371	89	29	0.928	20	69	0.202
Age (years)															
<50	60	3		75	10		27	71		76	22		24	48	
>50	96	9	0.353	106	18	0.566	46	114	0.835	120	40	0.642	30	91	0.201
ER															
Negative	43	5		59	12		38	39		52	25		10	61	
Positive	106	7	0.351	114	16	0.369	32	144	<0.0001	140	36	0.040	43	74	0.0008
PR															
Negative	63	4		77	14		43	64		73	34		17	74	
Positive	88	8	0.570	97	14	0.571	28	119	0.0002	120	27	0.014	36	62	0.006
HER2															
Negative	113	8		136	21		59	142		157	43		42	103	
Positive	29	4	0.295	35	6	0.834	11	35	0.442	29	18	0.016	10	30	0.621
Ki67															
Negative	71	5		84	13		30	101		109	22		30	60	
Positive	64	6	0.649	84	14	0.858	40	83	0.086	84	39	0.005	24	74	0.181

WT, wild type; MUT, mutated; NC, no change; H, histoscore; LN, lymph node status. Thresholds of positivity: ER and PR, $H > 10$; HER2 FISH ratio, >2.2 HER2:chr.17 centromere; Ki67 staining >5%; *PIK3CA* copy number is considered gain $2^{dCt} > 1.38$. PI3K pathway status is defined as active if tumors present an alteration in at least one of the studied parameters. Inactive tumors are wild type for the parameters analyzed.

*p-Values were obtained by chi-square analysis.

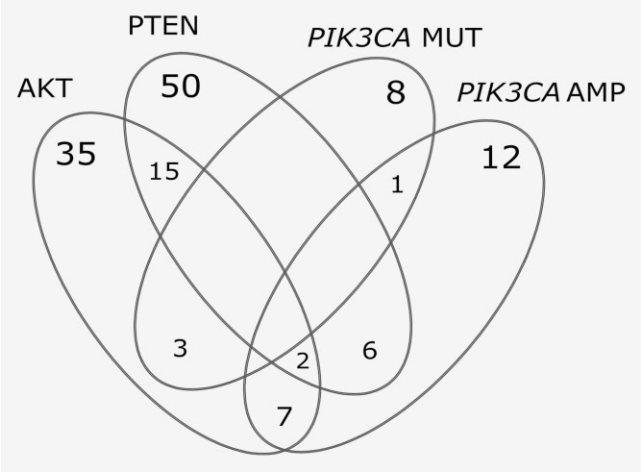

Figure 2. Venn diagram representing the overlap between AKT activation measured by pAKT positive staining, PTEN loss measured by PTEN negative staining, *PIK3CA* mutation analyzed by sequencing and *PIK3CA* copy number gain analyzed by qPCR. The figure shows that although there is a slight overlap between the alterations (24%), many cancers have only 1 altered parameter.

was associated with the "basal-like" phenotype ($p < 0.001$) and high PTEN expression with Luminal A cancers. High pAKT levels were associated with the HER2 phenotype ($p = 0.04$) and low pAKT levels with the Luminal A phenotype ($p = 0.01$). The activated pathway as measured by *PIK3CA* mutations, *PIK3CA* copy number gain, AKT activation or PTEN loss was associated with "basal-like" tumors ($p = 0.01$) and the inactive pathway with Luminal A cancers ($p = 0.005$) (Table 2).

PI3K pathway activation and cell cycle markers

We next analyzed the relationship between components of the PI3K pathway and key cell cycle regulators that had previously been assessed in these cancers as downstream markers of pathway activation. PTEN loss was associated with increased cyclin E1 expression ($p = 0.019$) and low expression of cyclin D1 ($p = 0.019$), $p21^{Cip1/Waf1}$ ($p = 0.019$) and $p27^{Kip1}$ ($p = 0.024$). AKT activation was associated with increased cyclin E1 ($p = 0.008$) and $p21^{Cip1/Waf1}$ expression ($p = 0.0005$). An activated PI3K pathway was associated with high expression of cyclin E1 ($p = 0.009$), but not with expression of cyclin D1 ($p = 0.158$) or the Cdk inhibitors $p27^{Kip1}$ ($p = 0.112$) and $p21^{Cip1/Waf1}$ ($p = 0.724$) (Fig. 3).

PI3K pathway activation, disease outcome and treatment

In a univariate analysis an aberrant PI3K pathway was associated with breast cancer-specific death (HR = 4.17, 95% CI = 1.28–13.59, $p = 0.017$) (Fig. 4*a*). However, when the cohort was stratified on ER status the relationship approached significance for ER+ tumors ($n = 117$) (HR = 6.61, 95% CI = 0.86–50.38, $p = 0.068$) but not ER− tumors ($n = 71$) (HR = 1.40, 95% CI = 0.32–6.04, $p = 0.649$). In a

Table 2. Association between the PI3K pathway and breast cancer subtypes

	PIK3CA sequence			*PIK3CA* copy number			PTEN expression			AKT expression			PI3K pathway		
	WT	MUT	*p*-Value*	NC	GAIN	*p*-Value*	*H* = 0	*H* > 0	*p*-Value*	*H* < 22	*H* > 22	*p*-Value*	Inactive	Active	*p*-Value*
"Basal like"	16	1	0.74	25	3	0.62	19	10	<0.0001	19	10	0.19	2	25	0.01
HER2	13	2	0.41	16	4	0.38	6	15	0.95	12	9	0.04	4	15	0.49
Luminal A	89	7	0.71	101	15	0.70	30	125	<0.001	125	30	0.01	37	66	0.005
Luminal B	16	2	0.59	19	2	0.55	5	21	0.28	17	9	0.22	6	15	0.93
Unclassified	6	0	0.46	9	3	0.24	9	6	0.65	12	3	0.65	2	12	0.24

WT, wild type; MUT, mutated; NC, no change; H, histoscore. PI3K pathway status is defined as active if tumors present an alteration in at least one of the studied parameters. Inactive tumors are wild type for the parameters analyzed.
*p-Values were obtained by chi-square analysis.

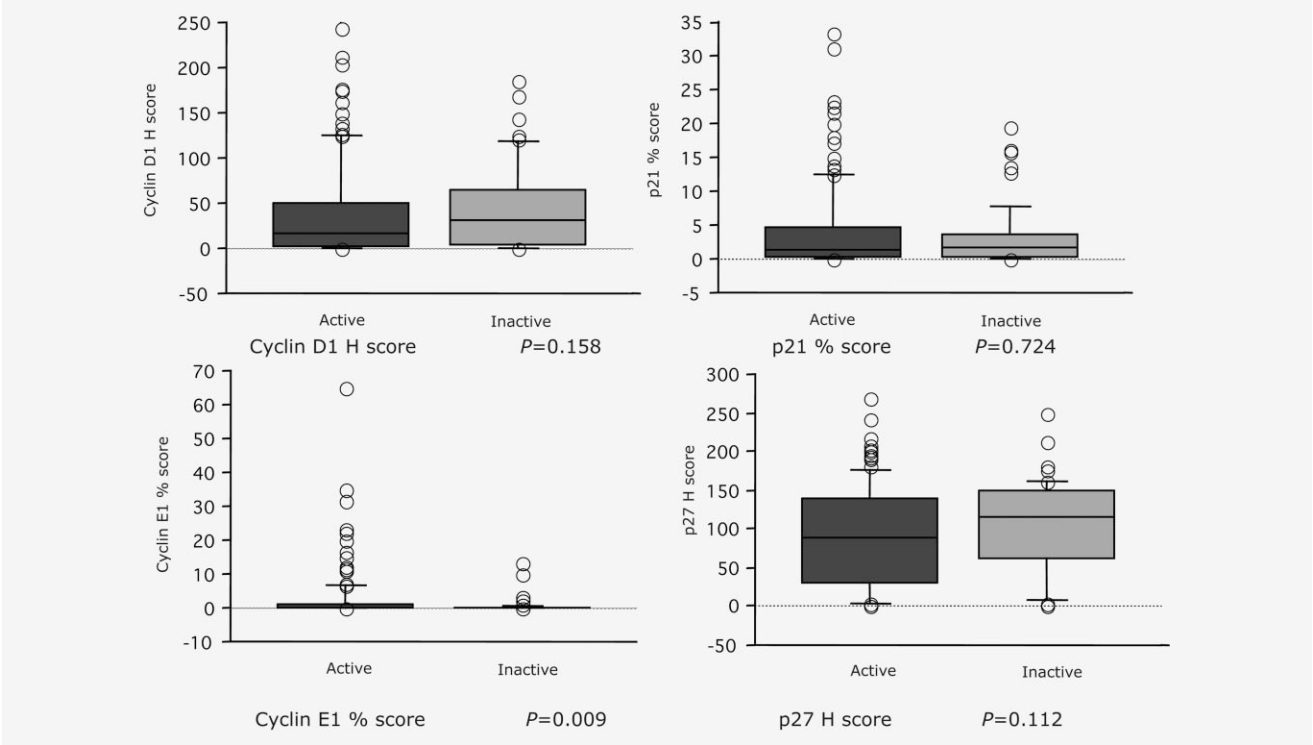

Figure 3. Correlation between expression of cell cycle markers cyclin D1, cyclin E1, $p21^{Cip1/Waf1}$ and $p27^{Kip1}$ and PI3K pathway status. Box plots from a Mann–Whitney analysis of the association between an active or inactive PI3K pathway and expression, measured by immunohistochemistry, are shown.

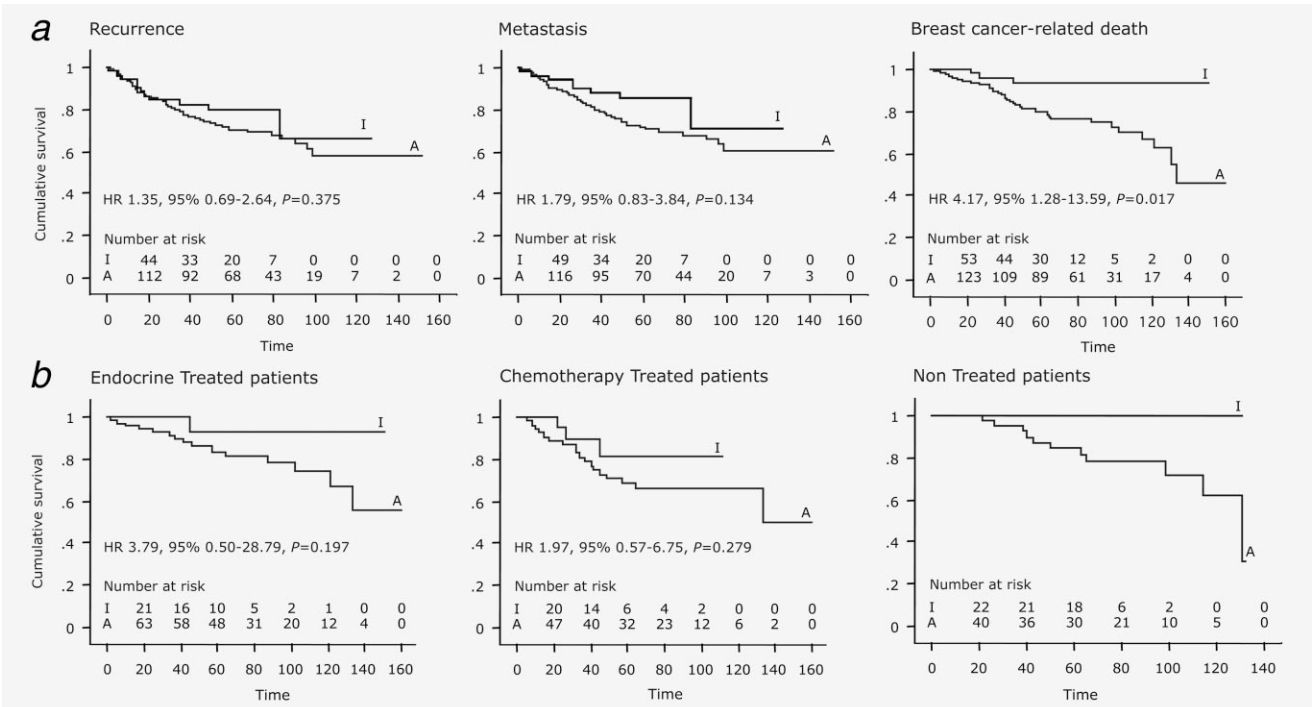

Figure 4. Kaplan–Meier curves illustrating the relationship between an activated PI3K pathway and disease progression and treatment. (*a*) Recurrence, metastasis and cancer-specific death stratified by an active (A: alteration of *PIK3CA*, PTEN or AKT) or inactive (I: wild type for parameters studied) pathway. Number of patients at risk, hazard ratios, 95% CI and *p* value of the association by log rank testing are shown. (*b*) Breast cancer-specific death in patients treated with adjuvant endocrine (tamoxifen) or chemotherapy and untreated patients, stratified by an active or inactive pathway.

Table 3. Multivariate analysis for clinicopathological parameters, hormone receptor status and PI3K pathway

	Variable	Hazard ratio	95% confidence interval	*p*-Value
Univariate	Grade >2	3.520	1.930–6.418	<0.0001
	Size >20 mm	2.468	1.415–4.304	0.0015
	Lymph node >0	3.691	2.025–6.729	<0.0001
	Age >50 years	1.427	0.799–2.551	0.2297
	ER+	0.300	0.172–0.524	<0.0001
	PR+	0.170	0.087–0.333	<0.0001
	HER2 FISH+	3.491	1.956–6.229	<0.0001
	AKT activation	1.891	1.065–3.356	0.029
	Activated pathway	4.174	1.281–13.598	0.017
Multivariate				
Model 1	Grade >2	2.053	0.952–4.429	0.066
	Lymph node status >0	3.309	1.601–6.841	0.001
	PR+	0.273	0.119–0.628	0.002
	HER2 FISH+	2.037	1.017–4.078	0.045
	Activated pathway	2.612	0.796–8.570	0.113
	Activated pathway	3.738	1.092–12.793	0.035
Model 2	AKT activation	1.293	0.669–2.499	0.444

multivariate analysis incorporating all clinicopathological and molecular parameters reported in Table 3, PI3K pathway activation was not an independent predictor of breast cancer-related death in a model that included lymph node status, tumor grade, PR and HER2 status (HR = 2.61, 95% CI = 0.80–8.57, $p = 0.113$). However, when tested in a multivariate model against pAKT the activated pathway was a better prognostic marker than pAKT alone (Table 3, model 2).

Next, patients were distributed into 3 treatment categories, *i.e.* endocrine therapy, chemotherapy and no adjuvant treatment, and the associations between an active PI3K pathway and breast cancer-specific death were assessed. In patients treated with endocrine therapy ($n = 93$), an activated pathway was not associated with breast cancer-specific death (HR = 3.79, 95% CI = 0.50–28.79, $p = 0.197$). A similar result was apparent in the chemotherapy-treated patients ($n = 75$) (HR = 1.97, 95% CI = 0.58–6.76, $p = 0.279$). However, in the group of patients that received no adjuvant treatment ($n = 67$), an activated pathway was associated with a markedly inferior outcome to patients with no pathway aberrations where no patient relapsed. There is no p value as there are no events in the inactive pathway group (Fig. 4*b*).

The same association between an active PI3K pathway and breast cancer-specific death was then calculated as crude 5-year survival rate. For endocrine treated patients the crude 5-year survival rate was 69% (11/16), for chemotherapy treated patients it was 89% (16/18) and for patients with no adjuvant treatment it was 54% (6/11), of all breast cancer-specific death events recorded in each therapy group of patients.

Discussion

Previous articles indicate that the PI3K pathway is altered in a high proportion of breast cancer patients.[3,17,39] Here, we report the first combined analysis of several pivotal molecules within the PI3K pathway in a single cohort of breast cancer patients in an attempt to derive an overall measure of pathway activation and relate this to clinicopathological features and outcome of the disease. We detected *PIK3CA* mutations or copy number gain, PTEN loss or AKT activation in >70% of breast cancer patients, an association between an active pathway and worse outcome and a high prevalence of alterations in the "basal-like" phenotype, which identifies the PI3K pathway as a potential therapeutic target in this subset of breast cancers.

We detected a relatively low frequency of *PIK3CA* mutations, *i.e.* 7% compared to earlier estimates of 12–40%.[3–16] The use of sequencing is the standard method to detect mutations and although pyrosequencing has a higher analytical sensitivity which could aid in the mutation detection in DNA from tumor tissue contaminated with normal cells, all previous breast cancer studies have used sequencing as their detection method. Some additional mutations could be present in other exons not analyzed but exons 9 and 20 contain the highest frequency of *PIK3CA* mutations in cancer.[14] Each mutation was confirmed in an independent PCR reaction. Our Australian cohort is representative of previously published population-based breast cancer cohorts, with respect to clinicopathological parameters such as tumor grade, tumor size, ER, PR and HER2 status and outcome (McNeil C.M., unpublished data). Therefore, neither the methodology nor

the cohort characteristics explain this lower proportion of *PIK3CA* mutations. However, the frequency of copy number gain was 14%, higher than that detected in previous studies (1–9%).[12,16] *PIK3CA* mutation and amplification were almost mutually exclusive events and since they represent independent mechanisms for elevating PI3K kinase activity, a higher frequency of copy number gain could perhaps explain the lower prevalence of *PIK3CA* mutations in this cohort compared to those in other reports. Also, the poor quality of the DNA derived from paraffin embedded samples and the failure to enrich for tumor cells before sequencing could be a reason for the low frequency of mutations reported here. We identified AKT activation in 24% of patients, slightly lower levels that some publications although the range of published data is highly variable[5,17,19,21,22,24–28] and a frequency of PTEN loss of 28%, similar to the average of previous reports on breast cancer patients.[5,6,14,17–23]

In our study, PTEN loss was exclusive of *PIK3CA* mutations, as described previously,[14] suggesting that these alterations represent redundant mechanisms for activating the PI3K pathway. Recent data have shown that the enhanced proliferation and survival resulting from PTEN loss are unaffected by *PIK3CA* knockdown and highlight an important role for the p110β isoform.[40,41] This may reflect different basal activities for p110α and β. However, it should be noted that in a recent paper, PTEN loss and *PIK3CA* mutation were often concordant in breast cancer patients,[3] indicating additional work is required to clarify the functional contribution of these pathway aberrations as recently highlighted in the review of Yuan and Cantley.[42] Furthermore, these workers[3] identified that PTEN loss, but not *PIK3CA* mutation, was significantly associated with AKT activation. We report similar findings, where *PIK3CA* mutation and/or copy number gain was not associated with AKT S473 phosphorylation, in contrast to PTEN loss. These findings raise the possibility that alternative PI3K effectors to AKT, such as Rac, play an important role in *PIK3CA* mutated cancers.

To examine the overall activation status of the pathway, *PIK3CA* mutational status, *PIK3CA* copy number gain, PTEN loss and activation of AKT as measured by pAKT levels were combined and by this definition >70% of cancers had an activated pathway. Only 25% of cancers had overlapping alterations, emphasizing the need of an integrative analysis of the PI3K pathway. This activation was associated with a worse patient outcome although it was not an independent predictor of relapse or death from breast cancer implying that the reported associations with tumor grade (data not shown) and hormone receptor status were likely confounding. Pathway activation was identified in half of the hormone receptor-positive patients and was associated with an adverse outcome, although this did not reach statistical significance. In hormone receptor-negative disease pathway activation was identified in a higher percentage of cases (85%) but was not associated with outcome.

Confirmation of the activated pathway was measured by studying selected downstream cell cycle targets. The strongest association was between AKT activation, PTEN loss and pathway activation with increased cyclin E expression. This could be a rate limiting molecule in cell cycle progression due in part to a decrease of GSK3β phosphorylation, which also leads to stabilized cyclin E protein levels.[43]

The PI3K pathway was not predictive of response to endocrine therapy in our cohort of breast cancer patients; however, an association was identified when only *PIK3CA* status and AKT activation were combined (data not shown). This latter relationship is in agreement with previous data from *in vitro* studies which show that activation of PI3K/AKT pathway in breast cancer cells activates ERα and protects breast cancer cells from tamoxifen-induced apoptosis.[44] Other studies have shown an association between individual members of the PI3K pathway and tamoxifen resistance.[6,26,45] Despite 1 report showing PTEN loss was associated with relapse in patients treated with tamoxifen,[6] we found no association, possibly because in our cohort PTEN is commonly lost in hormone receptor negative tumors and was thus less common in endocrine-treated patients. Analysis of pAKT and *PIK3CA* may be beneficial in identifying tamoxifen-resistant patients and this requires further testing in the context of a randomized treatment trial where treatment and outcome data are more robust. However, a poor outcome was also observed in patients not treated with adjuvant therapy, suggesting that aberrations in the PI3K pathway are associated with poor outcome independent of treatment, *i.e.* they are prognostic rather than predictive markers.

Another important discovery from the study was the association between an activated PI3K pathway and the "basal-like" phenotype of breast cancers. In HER2 cancers an activated pathway described by *PIK3CA* mutations or PTEN loss has been previously associated with a poor prognosis after trastuzumab therapy,[46,47] confirming the importance of the PI3K pathway in this subgroup of cancers and pointing to the need for further characterization of PI3K pathway inhibitors as complementary treatment strategies for trastuzumab-resistant disease. In "basal-like" cancers the PI3K pathway is emerging as an attractive candidate for targeted therapy. Previous studies have shown that PTEN inactivation leads to the development of mammary tumors that display pathological and molecular features associated with the "basal-like" subtype of breast cancer[48,49] and 2 further studies have shown an association of cyclin E overexpression and $p27^{Kip1}$ loss with a basal phenotype.[50,51] Furthermore, a recent publication has reported that PTEN alterations and an active PI3K pathway are associated with "basal-like" breast cancers.[52] These data, together with the results presented here, point to the importance of further analyzing PI3K pathway aberrations in "basal-like" cancers and the effect of specific PI3K inhibitors on this cancer subtype.

In conclusion, over 70% of breast cancers in our study had an altered PI3K pathway which was associated with breast-cancer specific death, and we identified this pathway as a potential therapeutic target in "basal-like" breast cancers.

Further studies of the integrated PI3K pathway need to be undertaken in larger cohorts of breast cancer patients, preferably in the context of randomized treatment trials, to confirm these findings.

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References

1. Bader AG, Kang S, Zhao L, Vogt PK. Oncogenic PI3K deregulates transcription and translation. *Nat Rev Cancer* 2005;5: 921–9.
2. Hennessy BT, Smith DL, Ram PT, Lu Y, Mills GB. Exploiting the PI3K/AKT pathway for cancer drug discovery. *Nat Rev Drug Discov* 2005;4:988–1004.
3. Stemke-Hale K, Gonzalez-Angulo AM, Lluch A, Neve RM, Kuo WL, Davies M, Carey M, Hu Z, Guan Y, Sahin A, Symmans WF, Pusztai L, et al. An integrative genomic and proteomic analysis of PIK3CA, PTEN, and AKT mutations in breast cancer. *Cancer Res* 2008;68: 6084–91.
4. Liedtke C, Cardone L, Tordai A, Yan K, Gomez HL, Figureoa LJ, Hubbard RE, Valero V, Souchon EA, Symmans WF, Hortobagyi GN, Bardelli A, et al. PIK3CA-activating mutations and chemotherapy sensitivity in stage II-III breast cancer. *Breast Cancer Res* 2008;10:R27.
5. Lerma E, Catasus L, Gallardo A, Peiro G, Alonso C, Aranda I, Barnadas A, Prat J. Exon 20 PIK3CA mutations decreases survival in aggressive (HER-2 positive) breast carcinomas. *Virchows Arch* 2008; 453:133–9.
6. Perez-Tenorio G, Alkhori L, Olsson B, Waltersson MA, Nordenskjold B, Rutqvist LE, Skoog L, Stal O. PIK3CA mutations and PTEN loss correlate with similar prognostic factors and are not mutually exclusive in breast cancer. *Clin Cancer Res* 2007;13:3577–84.
7. Benvenuti S, Sartore-Bianchi A, Di Nicolantonio F, Zanon C, Moroni M, Veronese S, Siena S, Bardelli A. Oncogenic activation of the RAS/RAF signaling pathway impairs the response of metastatic colorectal cancers to anti-epidermal growth factor receptor antibody therapies. *Cancer Res* 2007;67:2643–8.
8. Barbareschi M, Buttitta F, Felicioni L, Cotrupi S, Barassi F, Del Grammastro M, Ferro A, Dalla Palma P, Galligioni E, Marchetti A. Different prognostic roles of mutations in the helical and kinase domains of the PIK3CA gene in breast carcinomas. *Clin Cancer Res* 2007;13: 6064–9.
9. Maruyama N, Miyoshi Y, Taguchi T, Tamaki Y, Monden M, Noguchi S. Clinicopathologic analysis of breast cancers with PIK3CA mutations in Japanese women. *Clin Cancer Res* 2007;13:408–14.
10. Li SY, Rong M, Grieu F, Iacopetta B. PIK3CA mutations in breast cancer are associated with poor outcome. *Breast Cancer Res Treat* 2006;96:91–5.
11. Buttitta F, Felicioni L, Barassi F, Martella C, Paolizzi D, Fresu G, Salvatore S, Cuccurullo F, Mezzetti A, Campani D, Marchetti A. PIK3CA mutation and histological type in breast carcinoma: high frequency of mutations in lobular carcinoma. *J Pathol* 2006;208:350–5.
12. Wu G, Xing M, Mambo E, Huang X, Liu J, Guo Z, Chatterjee A, Goldenberg D, Gollin SM, Sukumar S, Trink B, Sidransky D. Somatic mutation and gain of copy number of PIK3CA in human breast cancer. *Breast Cancer Res* 2005;7:R609–16.
13. Levine DA, Bogomolniy F, Yee CJ, Lash A, Barakat RR, Borgen PI, Boyd J. Frequent mutation of the PIK3CA gene in ovarian and breast cancers. *Clin Cancer Res* 2005; 11:2875–8.
14. Saal LH, Holm K, Maurer M, Memeo L, Su T, Wang X, Yu JS, Malmstrom PO, Mansukhani M, Enoksson J, Hibshoosh H, Borg A, et al. PIK3CA mutations correlate with hormone receptors, node metastasis, and ERBB2, and are mutually exclusive with PTEN loss in human breast carcinoma. *Cancer Res* 2005;65:2554–9.
15. Bachman KE, Argani P, Samuels Y, Silliman N, Ptak J, Szabo S, Konishi H, Karakas B, Blair BG, Lin C, Peters BA, Velculescu VE, et al. The PIK3CA gene is mutated with high frequency in human breast cancers. *Cancer Biol Ther* 2004;3: 772–5.
16. Campbell IG, Russell SE, Choong DY, Montgomery KG, Ciavarella ML, Hooi CS, Cristiano BE, Pearson RB, Phillips WA. Mutation of the PIK3CA gene in ovarian and breast cancer. *Cancer Res* 2004;64: 7678–81.
17. Bose S, Chandran S, Mirocha JM, Bose N. The Akt pathway in human breast cancer: a tissue-array-based analysis. *Mod Pathol* 2006;19:238–45.
18. Depowski PL, Rosenthal SI, Ross JS. Loss of expression of the PTEN gene protein product is associated with poor outcome in breast cancer. *Mod Pathol* 2001;14:672–6.
19. Janssen EA, Soiland H, Skaland I, Gudlaugson E, Kjellevold KH, Nysted A, Soreide JA, Baak JP. Comparing the prognostic value of PTEN and Akt expression with the Mitotic Activity Index in adjuvant chemotherapy-treated node-negative breast cancer patients aged <55 years. *Cell Oncol* 2007;29:25–35.
20. Lee JS, Kim HS, Kim YB, Lee MC, Park CS, Min KW. Reduced PTEN expression is associated with poor outcome and angiogenesis in invasive ductal carcinoma of the breast. *Appl Immunohistochem Mol Morphol* 2004;12:205–10.
21. Noh WC, Kim YH, Kim MS, Koh JS, Kim HA, Moon NM, Paik NS. Activation of the mTOR signaling pathway in breast cancer and its correlation with the clinicopathologic variables. *Breast Cancer Res Treat* 2008;110:477–83.
22. Panigrahi AR, Pinder SE, Chan SY, Paish EC, Robertson JF, Ellis IO. The role of PTEN and its signalling pathways, including AKT, in breast cancer; an assessment of relationships with other prognostic factors and with outcome. *J Pathol* 2004;204:93–100.
23. Tsutsui S, Inoue H, Yasuda K, Suzuki K, Higashi H, Era S, Mori M. Reduced expression of PTEN protein and its prognostic implications in invasive ductal carcinoma of the breast. *Oncology* 2005;68: 398–404.
24. Akcakanat A, Sahin A, Shaye AN, Velasco MA, Meric-Bernstam F. Comparison of Akt/mTOR signaling in primary breast tumors and matched distant metastases. *Cancer* 2008;112:2352–8.
25. Andre F, Nahta R, Conforti R, Boulet T, Aziz M, Yuan LX, Meslin F, Spielmann M, Tomasic G, Pusztai L, Hortobagyi GN, Michiels S, et al. Expression patterns and predictive value of phosphorylated AKT in early-stage breast cancer. *Ann Oncol* 2008; 19:315–20.
26. Kirkegaard T, Witton CJ, McGlynn LM, Tovey SM, Dunne B, Lyon A, Bartlett JM. AKT activation predicts outcome in breast cancer patients treated with tamoxifen. *J Pathol* 2005;207:139–46.
27. Rojo F, Najera L, Lirola J, Jimenez J, Guzman M, Sabadell MD, Baselga J, Ramon y Cajal S. 4E-binding protein 1, a cell signaling hallmark in breast cancer that correlates with pathologic grade and prognosis. *Clin Cancer Res* 2007;13:81–9.
28. Tokunaga E, Kimura Y, Oki E, Ueda N, Futatsugi M, Mashino K, Yamamoto M, Ikebe M, Kakeji Y, Baba H, Maehara Y. Akt is frequently activated in HER2/neu-positive breast cancers and associated with poor prognosis among hormone-treated patients. *Int J Cancer* 2006;118:284–9.

29. Sorlie T, Perou CM, Tibshirani R, Aas T, Geisler S, Johnsen H, Hastie T, Eisen MB, van de Rijn M, Jeffrey SS, Thorsen T, Quist H, et al. Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications. *Proc Natl Acad Sci USA* 2001;98:10869–74.
30. Sotiriou C, Neo SY, McShane LM, Korn EL, Long PM, Jazaeri A, Martiat P, Fox SB, Harris AL, Liu ET. Breast cancer classification and prognosis based on gene expression profiles from a population-based study. *Proc Natl Acad Sci USA* 2003; 100:10393–8.
31. Cheang MC, Voduc D, Bajdik C, Leung S, McKinney S, Chia SK, Perou CM, Nielsen TO. Basal-like breast cancer defined by five biomarkers has superior prognostic value than triple-negative phenotype. *Clin Cancer Res* 2008;14:1368–76.
32. Millar EK, Anderson LR, McNeil CM, O'Toole SA, Pinese M, Crea P, Morey AL, Biankin AV, Henshall SM, Musgrove EA, Sutherland RL, Butt AJ. BAG-1 predicts patient outcome and tamoxifen responsiveness in ER-positive invasive ductal carcinoma of the breast. *Br J Cancer* 2009;100:123–33.
33. de Cremoux P, Tran-Perennou C, Brockdorff BL, Boudou E, Brunner N, Magdelenat H, Lykkesfeldt AE. Validation of real-time RT-PCR for analysis of human breast cancer cell lines resistant or sensitive to treatment with antiestrogens. *Endocr Relat Cancer* 2003;10:409–18.
34. Kita D, Yonekawa Y, Weller M, Ohgaki H. PIK3CA alterations in primary (de novo) and secondary glioblastomas. *Acta Neuropathol* 2007;113:295–302.
35. Jung V, Kindich R, Kamradt J, Jung M, Muller M, Schulz WA, Engers R, Unteregger G, Stockle M, Zimmermann R, Wullich B. Genomic and expression analysis of the 3q25-q26 amplification unit reveals TLOC1/SEC62 as a probable target gene in prostate cancer. *Mol Cancer Res* 2006;4:169–76.
36. Carson JP, Kulik G, Weber MJ. Antiapoptotic signaling in LNCaP prostate cancer cells: a survival signaling pathway independent of phosphatidylinositol 3′-kinase and Akt/protein kinase B. *Cancer Res* 1999;59:1449–53.
37. Millar EK, Dean JL, McNeil CM, O'Toole SA, Henshall SM, Tran T, Lin J, Quong A, Comstock CE, Witkiewicz A, Musgrove EA, Rui H, et al. Cyclin D1b protein expression in breast cancer is independent of cyclin D1a and associated with poor disease outcome. *Oncogene* 2009;28: 1812–20.
38. Millar EK, Graham PH, O'Toole SA, McNeil CM, Browne L, Morey AL, Eggleton S, Beretov J, Theocharous C, Capp A, Nasser E, Kearsley JH, et al. Prediction of local recurrence, distant metastases and death following breast-conserving therapy in early-stage invasive breast cancer using a five biomarker panel. *J Clin Oncol* 2009;27:(in press).
39. Tokunaga E, Kimura Y, Mashino K, Oki E, Kataoka A, Ohno S, Morita M, Kakeji Y, Baba H, Maehara Y. Activation of PI3K/Akt signaling and hormone resistance in breast cancer. *Breast Cancer* 2006;13: 137–44.
40. Jia S, Liu Z, Zhang S, Liu P, Zhang L, Lee SH, Zhang J, Signoretti S, Loda M, Roberts TM, Zhao JJ. Essential roles of PI(3)K-p110beta in cell growth, metabolism and tumorigenesis. *Nature* 2008;454:776–9.
41. Wee S, Wiederschain D, Maira SM, Loo A, Miller C, deBeaumont R, Stegmeier F, Yao YM, Lengauer C. PTEN-deficient cancers depend on PIK3CB. *Proc Natl Acad Sci USA* 2008;105:13057–62.
42. Yuan TL, Cantley LC. PI3K pathway alterations in cancer: variations on a theme. *Oncogene* 2008;27:5497–510.
43. Liang J, Slingerland JM. Multiple roles of the PI3K/PKB (Akt) pathway in cell cycle progression. *Cell Cycle* 2003;2:339–45.
44. Campbell RA, Bhat-Nakshatri P, Patel NM, Constantinidou D, Ali S, Nakshatri H. Phosphatidylinositol 3-kinase/AKT-mediated activation of estrogen receptor alpha: a new model for anti-estrogen resistance. *J Biol Chem* 2001;276: 9817–24.
45. Tokunaga E, Kataoka A, Kimura Y, Oki E, Mashino K, Nishida K, Koga T, Morita M, Kakeji Y, Baba H, Ohno S, Maehara Y. The association between Akt activation and resistance to hormone therapy in metastatic breast cancer. *Eur J Cancer* 2006;42:629–35.
46. Berns K, Horlings HM, Hennessy BT, Madiredjo M, Hijmans EM, Beelen K, Linn SC, Gonzalez-Angulo AM, Stemke-Hale K, Hauptmann M, Beijersbergen RL, Mills GB, et al. A functional genetic approach identifies the PI3K pathway as a major determinant of trastuzumab resistance in breast cancer. *Cancer Cell* 2007;12: 395–402.
47. Ginestier C, Adelaide J, Goncalves A, Repellini L, Sircoulomb F, Letessier A, Finetti P, Geneix J, Charafe-Jauffret E, Bertucci F, Jacquemier J, Viens P, et al. ERBB2 phosphorylation and trastuzumab sensitivity of breast cancer cell lines. *Oncogene* 2007;26:7163–9.
48. Dourdin N, Schade B, Lesurf R, Hallett M, Munn RJ, Cardiff RD, Muller WJ. Phosphatase and tensin homologue deleted on chromosome 10 deficiency accelerates tumor induction in a mouse model of ErbB-2 mammary tumorigenesis. *Cancer Res* 2008;68:2122–31.
49. Saal LH, Gruvberger-Saal SK, Persson C, Lovgren K, Jumppanen M, Staaf J, Jonsson G, Pires MM, Maurer M, Holm K, Koujak S, Subramaniyam S, et al. Recurrent gross mutations of the PTEN tumor suppressor gene in breast cancers with deficient DSB repair. *Nat Genet* 2008;40:102–7.
50. Foulkes WD, Brunet JS, Stefansson IM, Straume O, Chappuis PO, Begin LR, Hamel N, Goffin JR, Wong N, Trudel M, Kapusta L, Porter P, et al. The prognostic implication of the basal-like (cyclin E high/p27 low/p53+/glomeruloid-microvascular-proliferation+) phenotype of BRCA1-related breast cancer. *Cancer Res* 2004;64: 830–5.
51. Voduc D, Nielsen TO, Cheang MC, Foulkes WD. The combination of high cyclin E and Skp2 expression in breast cancer is associated with a poor prognosis and the basal phenotype. *Hum Pathol* 2008;39:1431–7.
52. Marty B, Maire V, Gravier E, Rigaill G, Vincent-Salomon A, Kappler M, Lebigot I, Djelti F, Tourdes A, Gestraud P, Hupe P, Barillot E, et al. Frequent PTEN genomic alterations and activated phosphatidylinositol 3-kinase pathway in basal-like breast cancer cells. Breast. *Cancer Res* 2008;10:R101.