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Research Article

Phospho-Akt expression is high in a subset of Triple Negative Breast Cancer Patients

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Abstract. The most commonly used biomarkers to predict the response of breast cancer patients to therapy are oestrogen receptor (ER), progesterone receptor (PgR), and human epidermal growth factor receptor 2 (HER2). Patients positive for these biomarkers are eligible for specific therapies such as anti-oestrogen therapy in the event of ER and PgR positivity, and trastuzumab, a monoclonal antibody, in the case of HER2 positive patients. Patients who are negative for all these three biomarkers, the so-called triple negatives, however, derive little benefit from such therapies. The PI3K/Akt pathway is activated in triple negative breast cancer cases, providing a possible target for therapy.

The activation of Akt was investigated in Maltese triple negative breast cancer cases using an antibody detecting Akt phosphorylated at serine 473 (anti-Akt pS473). The study showed that 26% of triple negative breast cancer patients had an elevated level of Akt (pS473). Furthermore, FTY720, a pharmacological activator of the phosphatase PP2A, was shown to block Akt activation at a concentration of 1μM, in HCC1937 cells subjected to insulin-like growth factor 1 (IGF-1).

Our data defined a subset of triple negative breast cancer patients based on high activity of AKT (pS473). This subset would be eligible for treatment using therapies which target the PI3K/Akt pathway, such as kinase inhibitors or phosphatase activators. In support of this, the BRCA1 mutant cells (HCC1937) were sensitive to the PP2a activator, FTY720. This suggests that FTY720 is a potential drug for use in adjuvant therapy in breast cancer cases having a high Akt (pS473).

Keywords Triple Negative Breast Cancer - biomarkers - phosphatases - Akt - BRCA1.

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1 Introduction

Breast cancer accounts for approximately 23% of cancer cases in females and is responsible for 14% of cancer-related deaths in females (Jemal et al., 2011). Classification of breast cancers is based on morphological features (lobular or ductal) and on the expression of the oestrogen receptor (ER), progesterone receptor (PgR) and the human epidermal growth factor receptor 2 (HER2). Breast cancer patients found negative for ER, PgR, and HER2 (triple negative) tend to fall within the subset of cases which have a basal-like phenotype and have a worse overall and disease-free survival (Onitilo et al., 2009). Interestingly, this subset has a higher occurrence of phosphatidylinositol 3-kinase (PI3K) pathway activation (Umemura et al., 2007).

Receptor tyrosine kinases such as Her2 and insulin-like growth factor 1 (IGF-1) receptor activate the PI3K pathway, initiating a cascade of signals. Active PI3K generates phosphatidylinositol 3,4,5 triphosphate (PIP3), which serves as an anchor for Pleckstrin homology (PH)-domain containing proteins, both adaptor molecules such as Gab2 and Dok1 and kinases such as Tec, Btk, PDK1 and Akt (Leevers et al., 1999; Saito et al., 2001; Stokoe et al., 1997; Tang et al., 1994). Activation of Akt increases cell cycle progression and maintains mammalian target of rapamycin

(mTOR) signalling resulting in enhanced cell proliferation and survival. The PI3K pathway is attenuated by phosphatases, including PTEN, which dephosphorylates PIP3 (Russillo et al., 2011) and PP2A, which inactivates mTOR effectors (Liu et al., 2010). In addition, inactivation of PP2A by phosphorylation at Tyrosine 307 is significantly correlated with HER2 positive tumour progression (Wong et al., 2010).

PI3K/Akt activates mTOR/Frap1 through phosphorylation of the tumour suppressor complex Tsc1/Tsc2 (tuberous sclerosis protein 1/2). Tsc1/Tsc2 releases Rheb (Ras-homolog enriched in brain), a small GTPase that positively modulates mTOR function (Inoki et al., 2003). Activation of mTOR results in phosphorylation and activation of ribosomal protein S6 Kinase (S6K; Rps6kb1; p70S6Kinase) and hierarchical phosphorylation of 4EBP (4E-binding protein) (Wang et al., 2005). Rapamycin-induced dephosphorylation of 4EBP is dependent on the activity of protein phosphatases type 1 and 2 (Chen et al., 1998), suggesting that mTOR inhibition releases a phosphatase to act on its downstream targets. Interestingly, studies on breast cancer cell lines show an increased sensitivity of triple negative cells to mTOR inhibitors (Noh et al., 2004). This suggests that deregulation of the mTOR effectors and/or regulators plays an important role in the pathology of triple-negative breast cancers.

Increased Akt activation correlates with low PTEN expression (Lopez-Knowles et al., 2010) and PI3K mutations (Stemke-Hale et al., 2008). Gain of function mutations in the PIK3CA gene (encoding the p110 catalytic subunit of PI3K) are present in 25% of invasive breast cancers (Bachman et al., 2004) and low PTEN expression in approximately 30% of invasive breast cancers (Tsutsui et al., 2005).

Loss of function of the phosphatases PTEN (Marty et al., 2008) and INPP4B (Gewinner et al., 2009) is associated with aggressive basal-like breast carcinoma. PTEN, INPP4B and PP2A are known antagonists of Akt phosphorylation, hence loss of phosphatase function leads to increased Akt activation. Interestingly, BRCA1 is known to activate PP2A, a phosphatase that dephosphorylates Akt at Threonine 308 (T308) and Serine 473 (S473) (Ma et al., 2007; Ugi et al., 2004). This is supported by the findings that loss of BRCA1 activity leads to increased Akt activity (Xiang et al., 2008) and reduced PP2A activity (Ma et al., 2007). In addition, BRCA1 is known to bind p-Akt and lead to its ubiquitination (Chen et al., 1998). In fact one finds an enhanced stability and higher expression of p-Akt in BRCA1 mutants, in which the mutant BRCA1 lacks the ability to bind to phosphorylated Akt (Xiang et al., 2008).

In vitro studies showed that serum starvation of phosphatase-depleted cells maintained a high pAkt signal (Fedele et al., 2010). In this study we tested the effect of PP2A activators on BRCA1 mutant cells which represent a subtype of triple negative breast cancer patients, potentially having a suppressed PP2A feedback mechanism. Interestingly, BRCA1 mutant cells (HCC1937) were sensitive to the PP2A activator, FTY720, resulting in enhanced dephosphorylation of Akt (pS473) upon 1 hour starvation. The ER positive cell line, MCF7, was less responsive to FTY720. In addition, restimulation of phosphorylated Akt (pS473) using IGF-1 was blocked by FTY720. A retrospective study of triple negative breast cancers showed a high activity of Akt (pS473) in 27% of the cases. These cases are eligible to pharmaceutical inhibition of the PI3K pathway and potentially activation of the phosphatase PP2A. Activation of PP2A will allow targeting of the deregulated PI3K pathway, including kinase mutants and cells with a low PTEN expression, but also BRCA1 mutants due to the sensitivity conferred by the lower PP2A activity.

2 Materials and Methods

Cell Lines Used and Culturing Conditions

Two adherent human breast cancer cell lines were used in the study: MCF-7 and HCC1937 (ATCC). MCF-7 and HCC1937 were both cultured in sterile T-25 flasks in an incubator with temperature set at 37°C, having an atmosphere of 5% CO_2 and 98% humidity. The MCF-7 cell line was cultured in high glucose Dulbecco's Modified Eagle's Medium (DMEM; Sigma-Aldrich) containing 10% Foetal Bovine Serum (FBS; Gibco, Invitrogen) and 1% Penicillin/Streptomycin (Gibco, Invitrogen). The HCC1937 cell line was cultured in RPMI-1640 (Sigma-Aldrich) containing 10% FBS and 1% Pen/Strep. Passaging was carried out when the cells reached around 90% confluence.

Dosage-Viability Experiments

The effect of different dosages of FTY720 (Cayman Chemical) on the viability of MCF-7 and HCC1937 cells was investigated before FTY720 was used in the experiments. MCF-7 and HCC1937 cells were seeded in 6-well plates and allowed to reach around 90% confluence. FTY720 was then added to each well such that the following concentrations were obtained: 0, 0.5, 1, 2.5, and 5 μM. These were incubated overnight (for 24 hours) and the percentage of viable cells measured. The percentage of viable cells was measured using a CASY Cell Counter and Analyser System (Roche).

Starvation and Starvation-Stimulation Experiments

MCF-7 and HCC1937 cells were seeded in a 96-well plate cultured for 3 days. The cells were first starved for 2 hours by serum deprivation. After the 2 hours elapsed, IGF-1 was added to selected wells to a final

concentration of 200ng/mL. Cells were stimulated for 30 or 60 minutes. FTY720 was added at a final concentration of 1μM. At the end of the procedure, the medium was removed from the wells by aspiration and the cells were fixed by the addition of 3.7% formaldehyde in 1xPhosphate Buffered Saline (PBS; Sigma-Aldrich) and an ICW assay was performed, using an AKt (pS473) antibody (Abcam).

Western Blotting (WB) Procedure
Western blotting was carried out on the cytoplasmic protein fraction of MCF-7 cells in order to confirm that the Akt (pS473) antibody to be used bound to the intended target specifically. The cytoplasmic protein lysate obtained from MCF-7 cells were separated using a 7.5% SDS-PAGE (Sodium Dodecyl Sulfate - PolyAcrylamide Gel Electrophoresis) procedure. Electrophoresis was run for 2 hours at 150V and 30mA. The separated proteins were then transferred using electroblotting (a semi-dry procedure) onto a nitrocellulose membrane using a tris-glycine transfer buffer. Blocking of the membrane was done for 1 hour at room temperature, using Odyssey blocking buffer (LI-COR Biosciences). The primary antibody was used at a dilution of 1 in 200 in blocking buffer and incubation was carried out at room temperature for 1 hour. The secondary antibody was an anti-rabbit antibody conjugated to IRDye680 (LI-COR Biosciences), and was used at a dilution of 1 in 15,000. Secondary antibody incubation was carried out for 45 minutes at room temperature. The membrane was then scanned using an Odyssey®Infrared Imaging System (LI-COR Biosciences), obtaining fluorescence at 700nm.

In-Cell Western (ICW) Assays
The LI-COR®In-Cell Western™Assay Kit was used in the ICW protocol. ICW assays were carried out on MCF-7 and HCC1937 cells after the starvation and starvation-stimulation experiments. Cells were first fixed (20 minutes in 150μl of 3.7% formaldehyde in 1x PBS per well) and then permeabilised (5 washes of 5 minutes each in 200μl of 0.1% Triton X-100 (Sigma-Aldrich) in 1x PBS per well). Blocking (for 1.5 hours with continuous shaking) was then performed using 150μl of Odyssey®blocking buffer per well. Primary antibody incubation was carried out using 50μl of a 1:100 dilution of Akt (pS473) antibody per well. Secondary antibody incubation was carried out for 1hr on a plate shaker at room temperature. The secondary antibody (anti-rabbit and conjugated to IRDye800) was used at a dilution of 1:800 (50μl per well) and was supplemented with two dyes which fluoresce at 700nm (DRAQ5 at 1:2000 and Sapphire700 at 1:1000). The plate was scanned using an Odyssey®Infrared Imaging System with detection in both 700nm and 800nm channels. The two dyes (DRAQ5 and Sapphire700) were added in order to be able to normalise the fluorescence obtained due to the Akt (pS473) to the number of cells present in a given well. This is important since variation in cell number could lead to inaccurate results.

Immunohistochemical (IHC) Staining of FFPE Tumour Samples
A total of 47 Formalin-Fixed Paraffin-Embedded (FFPE) triple negative breast cancer samples from 2001 to 2009 were chosen for use in the study. The FFPE material was subjected to immunohistochemical staining using the Vectastain Elite ABC kit (Vector Laboratories). 3μm sections were cut from the FFPE samples and placed on APES coated slides for IHC staining. FFPE tissue were dewaxed using xylene and rehdydrated in decreasing concentration of alcohol. No antigen retrieval was necessary in the procedure and endogenous peroxidase activity was quenched by immersion in 3% hydrogen peroxide for 20 mins. Non-specific binding of the primary antibody was blocked by incubating the sections with diluted normal swine serum (DAKO - 1/20). The primary antibody used in the procedure (1/ 40) was a rabbit polyclonal antibody raised against Akt phosphorylated at Serine-473 (hereafter referred to as Akt (pS473)) (Abcam), where sections were incubated at 6°C overnight. After washing with PBS, the slides were incubated in diluted biotinylated goat anti-rabbit antibody (Dako - 1/200) for 60mins at room temperature. Following washing with PBS, the signal produced was amplified by incubating the sections with Avidin-Biotin Complex (ABC) containing horse radish peroxidase (HRP) for 60mins at room temperature. Visualisation was performed by the use of 3,3'-diaminobenzidine (DAB), a substrate of HRP which gives a permanent brown colour. Slides were counterstained with haematoxylin and dehydration was carried out in increasing concentration of alcohol. After clearing in xylene, slides were mounted with DPX. The stained sections were scored according to the intensity of the stain. The score ranged from 0 to 3, with 0 being negative and 3 being intense. Samples scoring 2 or higher were considered positive for the purposes of this study.

3 Results

In this study, BRCA1 mutant cells (HCC1937) were sensitive to the PP2a activator, FTY720. Re-stimulation of AKT (pS473) by IGF-1 was blocked by FTY720. A subset of triple negative breast cancer patients showed a high activity of AKT (pS473), with potential benefit for PI3K-pathway targeted intervention.

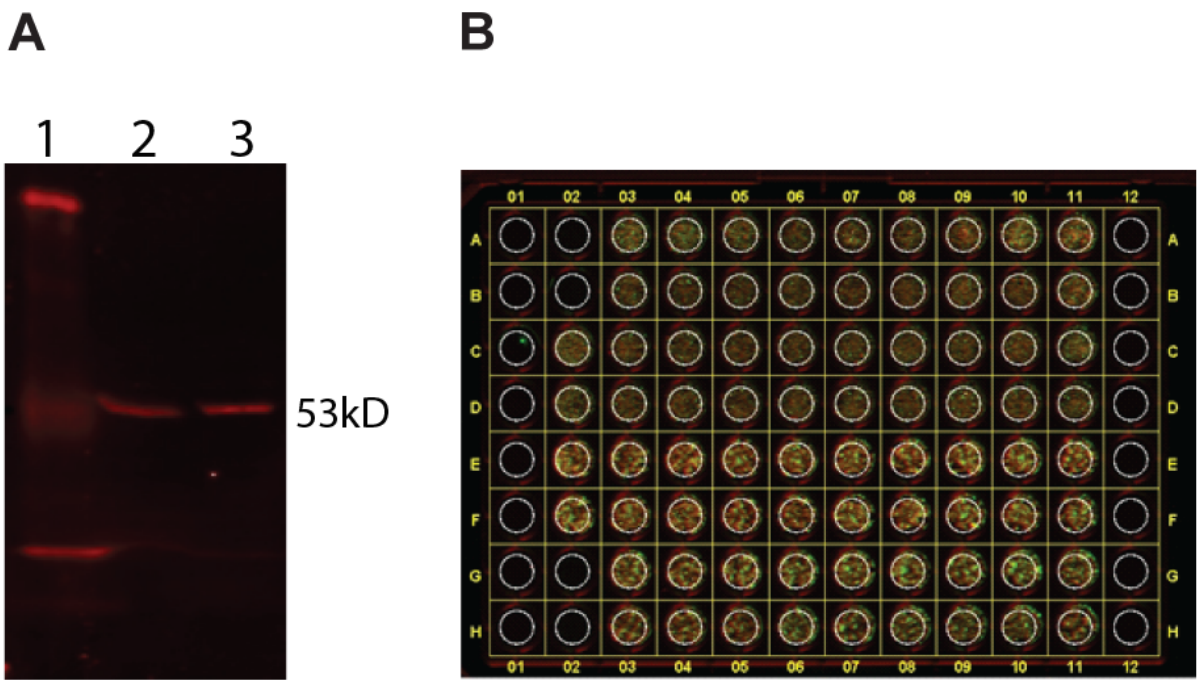

Figure 1: Specificity of Akt (pS473) staining and ICW analysis. A: Western Blot analysis staining using MCF-7 protein lysates stained for Akt (pS473). Lane 1 contains the ladder while lanes 2 and 3 contain the MCF-7 protein lysates. B: Cells were seeded in 96 well plates and stained according to the ICW protocol. Scanned image with rows A to D containing HCC1937 cells while rows E to H contain MCF-7 cells. Columns 3, 6, 9 contain cells which have been starved for 2 hours. Columns 4, 7, 10 contain cells which have been starved for 2 hours and stimulated for 30 minutes while columns 5, 8, 11 contain cells which have been starved then stimulated for 60 minutes. Column 2 contains the controls, A2, B2, G2, H2 being negative controls, while C2, D2, E2, F2 contain positive controls. Columns 1 and 12 are empty.

Akt (pS473) Antibody Staining is Specific

The specificity of the Akt (pS473) antibody to be used was first investigated by Western blotting carried out on cytoplasmic protein lysates taken from cultured MCF-7 cells. The Akt (pS473) antibody was expected to bind to phosphorylated Akt at a molecular weight of 56kDa. The bands visualised in lanes 2 and 3 are close to the band in the ladder (lane 1), which corresponds to a molecular weight of 60kDa (Figure 1A). This confirmed that the antibody indeed binds to the intended target and that it is quite specific since no other bands are visible on the blot.

Cell Viability Unaffected by FTY720

FTY720 is an activator of PP2A, a phosphatase known to inhibit Akt activity. Before performing the starvation and stimulation experiments, it was necessary to ensure that the FTY720 concentrations used in the study do not affect the viability of these cells. MCF-7 and HCC1937 cells were incubated for 24 hours in their respective mediums containing FTY720 at a concentration ranging from 0μM to 5μM. Concentrations of FTY720 used were 0, 0.5, 1, 2.5, and 5μM. This range of concentrations was chosen based on the fact that FTY720 causes significant growth inhibition of MCF-7 cells, when used in concentrations of 5μM or higher, over a period of 48 hours (Nagaoka et al., 2008).

The percentage of viable cells was found using a cell counter which is able to distinguish the viable cells from the dead cells, and hence give the viability as the percentage of cells in a suspension which are alive. From Table 1 one can note that FTY720 does not affect cell viability at the concentrations used since the percentage of viable cells remained at around 90% throughout. FTY720 was used at a concentration of 1μM in the experiments carried out.

Table 1: Percentage viability of cultured cells when exposed to a range of FTY720 concentrations over 24 hours. MCF-7 and HCC1937 cells remained viable up to a concentration of 5μM FTY720. FTY720 can be used to investigate phosphorylation events without affecting viability.

FTY720 Concentration (μM)	Percentage Viability (%)	
	MCF-7	HCC1937
0	91.92	91.23
0.5	90.87	90.50
1	89.72	91.45
2.5	90.95	92.49
5	91.68	91.39

Serum Deprivation Results in Akt (pS473) Dephospho-

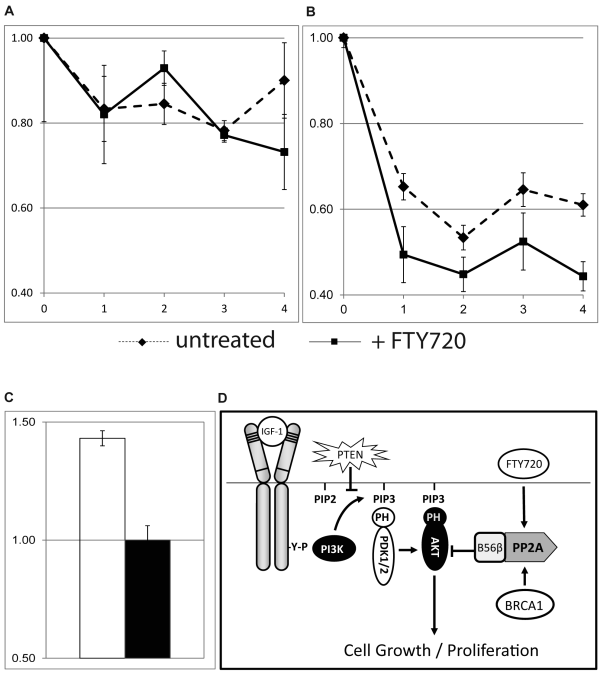

Figure 2: HCC1937 are sensitive to serum starvation and FTY720 addition. Cells were serum starved for 4 hours in the presence (solid line) or absence (dashed line) of 1μM FTY720. Fold change in Akt (pS473) during starvation was measured for the MCF-7 (A) and HCC1937 (B) cell lines. Each point represents a mean taken over three values, with the error bars representing the standard error. C: Following 2 hours starvation, HCC1937 cells were stimulated for 1 hour with 200ng/mL IGF-1 in the presence (black bar) or absence (open bar) of 1μM FTY720. D: Schematic diagram showing the attenuation mechanism of the PP2A holoenzyme complex. BRCA1 activates PP2A activity, hence supporting the sensitivity of the BRCA1-mutant HCC1937 cells to FTY720 (which is an activator of PP2A activity).

rylation

During the starvation experiment the phosphorylated Akt (pS473) level was suppressed in MCF-7 and HCC1937 cells (Figure 2A, B). In order to better analyse the observed starvation pattern, the data was subjected to the ANOVA statistical test at a significance level of 0.05. It was found that there is a statistical difference between the mean values obtained in the case of both MCF-7 ($p=0.011$) and HCC1937 ($p=0.000$), showing that starvation significantly affected the level of Akt (pS473). In particular, it decreases significantly in the first hour ($p=0.017$ for MCF-7, and $p=0.000$ for HCC1937) but any changes beyond the first hour were not statistically significant. Dephosphorylation of Akt in HCC1937 was stronger, indicating that the BRCA1 mutant cells are more sensitive to starvation.

The use of FTY720 at a concentration of 1μM resulted in the same pattern: a statistically significant decrease in Akt (pS473) occurred within the first hour ($p=0.005$ for MCF-7, $p=0.000$ for HCC1937). FTY720 did not produce a statistically significant difference in Akt (pS473); however, in the case of HCC1937 the level of Akt (pS473) was slightly lower when FTY720 was used compared to starvation in the absence of FTY720 (Figure 2B).

IGF-1 stimulation of p-Akt is suppressed by FTY720. From the previous starvation experiment a 2 hour starvation time point was selected since during starvation p-Akt decreases significantly during the first hour. MCF-7 and HCC1937 cells were first subjected to starvation (2hrs) followed by stimulation with 200ng/mL IGF-1 (a known stimulator of Akt) for 30 or 60 minutes. FTY720 was also used at a concentration of 1μM to investigate whether this affects the stimulation.

The effect of FTY720, on Akt activation (measured as the level of phosphorylated Akt (pS473)) in the two human breast cancer cell lines (MCF-7 and HCC1937) under conditions of starvation and stimulation was investigated. ICW assays were carried out to measure the level in Akt (pS473) in these cells which were either starved by serum deprivation, or stimulated using IGF-1 following a period of starvation. The Akt (pS473) antibody used in IHC analysis was also used in the ICW assays. Akt (pS473) in HCC1937 increased by a 1.4 fold change, upon IGF-1 stimulation, when compared to the level at 2 hrs starvation. The addition of 1μM of FTY720 abrogated Akt activation (Figure 2C). Stimulation of MCF-7, following starvation, was marginal (fold change < 1.2) and hence the effect of FTY720 on stimulation was not possible (data not shown).

Akt (pS473) Activity is High in a Subset of Triple Negative Breast Cancer Patients

Triple negative breast cancer samples were analysed using immunohistochemistry to investigate the fraction which is characterised by elevated Akt activation. Of the 47 chosen triple negative FFPE samples, 2 samples could not be adequately analysed by immunohistochemistry due to insufficient amounts of tumour tissue. The other 45 were successfully stained with the Akt (pS473) antibody. The staining pattern obtained with the p-Akt antibody was, as expected, cytoplasmic and granular in appearance (Figure 3). The staining intensity from sample to sample varied and a range of scores were obtained from the triple negative breast cancer samples.

Triple negative breast cancer samples having an intensity score of 0 or 1 were considered as having a low expression of Akt (pS473) and consist of 73% of analysed samples. Samples having an intensity score of 2 or 3 were considered to have a high level of Akt (pS473). This subset accounts for 27% of stained triple negative breast cancer samples.

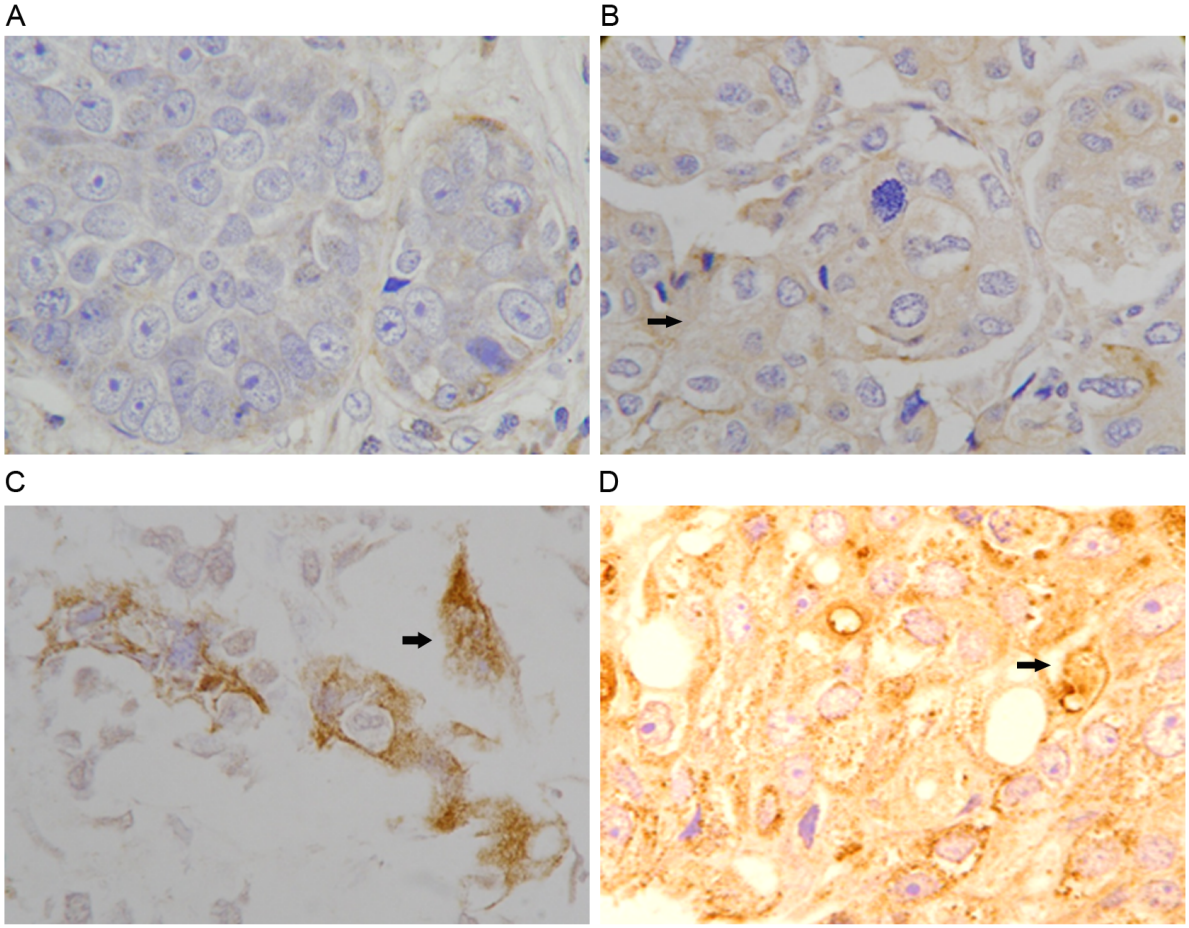

Figure 3: FFPE breast cancer patient sample sections stained with the Akt (pS473) antibody, showing different staining intensities. In all cases magnification was by a factor of 600, and arrows point towards a region of tumour with the appropriate intensity of stain. A: Intensity=0 (Negative); B: Intensity=1; C: Intensity=2; D: Intensity=3.

4 Discussion

This study revealed that a subset of triple negative breast cancer samples stained high for Akt (pS473). FTY720, a pharmacological activator of PP2A, was shown to abrogate Akt activation by IGF-1, in the BRCA1 mutant cell line, HCC1937.

IGF-1 Stimulation of p-Akt abrogated by FTY720
The effect of FTY720 on Akt activity in the human breast cancer cell lines, MCF-7 and HCC1937 was investigated. The HCC1937 cell line is negative for ER, PgR, and HER2 and bears mutant PTEN and BRCA1 (5382insC mutation) genes. One would anticipate that the BRCA1 mutant cells, HCC1937, have a low PP2A activity. This is based on the studies showing that knockdown of the BRCA1 gene leads to reduced PP2A activity (Ma et al., 2007). In the case of MCF-7, there is a significantly lower expression of PP2A Aα subunits (Suzuki and Takahashi, 2003). Lowered expression of this subunit is associated with enhanced activation of Akt with higher expression of Akt (pS473) (Chen et al., 2005).

MCF-7 and HCC1937 cells were subjected to starvation in the form of serum deprivation followed by stimulation using IGF-1 (at 200ng/ml), both in the presence and absence of FTY720. IGF-1 was chosen as a stimulant since it is known to increase Akt activity by phosphorylation at T308 and S473 (Alessi et al., 1996). FTY720 is a pharmacological activator of PP2A, and was used to investigate its effect on Akt activity during serum deprivation and IGF-1 stimulation in the MCF-7 and HCC1937 cell lines. PP2A is known to inhibit Akt activity by inhibiting phosphorylation at both T308 and S473 (Rodgers et al., 2011). Figure 2D shows the pathway involved in the starvation-stimulation including the action of FTY720. FTY720 was used at a concentration of 1μM since it was shown to significantly inhibit growth in MCF-7 cells at concentrations of 5μM and higher (Nagaoka et al., 2008).

FTY720 at a concentration of 1μM had minimal effect during starvation. In the case of HCC1937, a lower Akt

(pS473) level was observed when FTY720 was added; however, this was not statistically significant except at 4 hours of starvation. In the case of MCF-7 there was almost no difference in Akt (pS473) activity with and without FTY720 during starvation. FTY720 had a considerably more pronounced effect during IGF-1 stimulation, where it suppressed Akt (pS473) stimulation in the HCC1937 cell line. Of particular interest is the fact that the HCC1937 cell line is triple negative and is known to have an enhanced Akt activity. This implies that FTY720 is a potential therapeutic agent in the treatment of triple negative breast cancer patients having an elevated Akt activity. This is especially important since there are few therapies which are effective in treating triple negative breast cancers.

A Subset of Triple Negative Breast Cancers Associated with High Akt (pS473)
To investigate the activation of Akt among triple negative (ER, PgR, and HER2 negative) breast cancer cases, a number of triple negative FFPE samples were chosen and stained with the Akt (pS473) antibody. Phosphorylation of Serine 473 and Threonine-308 are required for full activation of Akt (Bellacose et al., 2005). A wide range of scores were obtained (Table 2), with 16% (7/45) samples showing no staining, 58% (26/45) showing weak staining (score 1), 22% (10/45) showing intermediate staining (score 2), and 4% (2/45) showing strong staining (score 3).

One could split the group of triple negatives into two subgroups: those that had a low Akt (pS473) score (0-1, 74%) and those that had a high Akt (pS473) score (2-3, 26%). The subset of triple negatives having a high activation of Akt would be eligible for therapy targeted at the PI3K/Akt pathway. This is of great importance since triple negative breast cancer patients derive little benefit from current therapies.

Table 2: Distribution of Intensity scores following Immunohistochemistry staining with Akt (pS473). The range of Akt (pS473) scores and the respective frequency of each among triple negative breast cancer (TNBC) FFPE samples. 45 samples were stained in all. Intensity of pAkt staining is high in 12/45 (26%) of TNBC patients.

Intensity Score	Frequency
0	7
1	26
2	10
3	2

5 Conclusion

This study has shown that a subset of triple negative breast cancer (TNBC) cases in Malta, consisting of 26% of cases, have a moderate to high activation of Akt. Staining for phosphorylated Akt can be introduced in the clinic to classify TNBC patients and predict the potential use of targeted therapies. This subset would be eligible for therapies targeting the PI3K/Akt pathway. This is of great importance due to a current lack of effective therapies against triple negative breast cancer cases. Potential therapies which target the PI3K/Akt pathway include kinase inhibitors of mTOR and Akt, such as Palomid529 (Xue et al., 2008), and phosphatase activators such as FTY720 (Ugi et al., 2004). The potential use of FTY720 as a therapy in breast cancer cases having elevated levels of Akt (pS473) is supported by the finding that at a concentration of 1μM it suppressed stimulation of Akt activity by IGF-1 in HCC1937 cells *in vitro*. Interestingly, a correlation between a high level of Akt (pS473) and low expression of BRCA1 was found in Breast Cancer patients (Xiang et al., 2011). This supports our observations that breast cancer cases bearing a mutant BRCA1 gene or reduced BRCA1 expression could also be eligible for therapy targeting the PI3K/Akt pathway.

Competing interests

The authors declare that they have no competing interests.

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References

Alessi D., Andjelkovic M., Caudwell B., Cron P., Morrice N., Cohen P., Hemmings B. (1996). Mechanism of activation of protein kinase B by insulin and IGF-1. *Embo. J.*, 15(23), 6541–6551.

Bachman K., Argani P., Samuels Y., Silliman N., Ptak J., Szabo S., Konishi H., Karakas B., Blair B., Lin C., Peters B., Velculescu V., Park B. (2004). The PIK3CA gene is mutated with high frequency in human breast cancers. *Cancer Biol. Ther.*, 3(8), 772–5.

Bellacose A., Kumar C., Di Cristofano A., Testa J. (2005). Activation of AKT kinases in cancers: implications for therapeutic targeting. *Adv. Cancer Res.*, 94, 29–86.

Chen J., Peterson R., Schreiber S. (1998). Alpha 4 associates with protein phosphatases 2A, 4 and 6. *Biochem. Biophys. Res. Commun.*, 3(827-832).

Chen W., Arroyo J., Timmons J., Possemato R., Hahn W. (2005). Cancer-associated PP2A Aalpha subunits induce functional haploinsufficiency and tumorigenicity. *Cancer Res.*, 65(18), 8183–8192.

Fedele C., Ooms L., Ho M., Vieusseux J., O'Toole S.,

Millar E., Mitchell C. (2010). Inositol polyphosphate 4-phosphatase II regulated PI3K/Akt signaling and is lost in human basal-like breast cancers. *Proc. Natl. Acad. Sci U.S.A.*, 107(51), 22231–22236.

Gewinner C., Wang Z., Richardson A., Teruya-Feldstein J., Etemadmoghadam D., Bowtell D., Cantley L. (2009). Evidence that inositol polyphosphate 4-phosphatase type II is a tumor suppressor that inhibits PI3K signaling. *Cancer Cell*, 16(2), 115–125.

Inoki K., Li Y., Xu T., Guan K. (2003). Rheb GTPase is a direct target of TSC2 GAP activity and regulated mTOR signalling. *Genes Dev.*, 17(15), 1829–1834.

Jemal A., Bray F., Center M. M., Ferlay J., Ward E., Forman D. (2011). Global cancer statistics. *CA Cancer J. Clin.*, 61(2), 69–90.

Leevers S., Vanhaesebroeck B., Waterfield M. (1999). Signalling through phosphoinositide 3-kinases: the lipids take centre stage. *Curr. Opin. Cell Biol.*, 11(2), 219–225.

Liu L., Chen L., Luo Y., Chen W., Zhou H., Xu B., Huang S. (2010). Rapamycin inhibits IGF-1 stimulated cell motility through PP2A pathway. *PLoS One*, 5(5), e10578.

Lopez-Knowles E., O'Toole S., McNeil C., Millar E., Qiu M., Crea P., Sutherland R. (2010). PI3K pathway activation in breast cancer is associated with the basal-like phenotype and cancer-specific mortality. *Int. J. Cancer*, 5(1121-1131).

Ma Y., Hu C., Riegel A., Fan S., Rosen E. (2007). Growth factor signaling pathways modulate BRCA1 repression of estrogen receptor-alpha activity. *Mol. Endocrinol.*, 21(8), 1905–1923.

Marty B., Maire V., Gravier E., Rigaill G., Vincent-Salomon A., Kappler M., Dubois T. (2008). Frequent PTEN genomic alterations and activates phophatidylinositol 3-kinase pathway in basal-like breast cancer cells. *Breast Cancer Res.*, 10(6), R101.

Nagaoka Y., Otuski K., Fujita T., Uesato S. (2008). Effects of phosphorylation of immunomodulatory agent FTY720 (fingolimod) on antiproliferative activity against breast and colon cancer cells. *Biol. Pharm. Bull.*, 1(6), 1177–1181.

Noh W., Mondesire W., Peng J., Jian W., Zhang H., Dong J., Meric-Bernstam F. (2004). Determinants of rapamycin sensitivity in breast cancer cells. *Clin. Cancer Res.*, 10(3), 1013–1023.

Onitilo A., Engel J., Greenlee R., Mukesh B. (2009). Breast cancer subtypes based on ER/PR and Her2 expression: comparison of clinicopathologic features and survival. *Clin. Med. Res.*, 7(1-2), 4–13.

Rodgers J., Vogel R., Puigserver P. (2011). Clk2 and B56beta mediate insulin-regulated assembly of the PP2A phosphatase holoenzyme on Akt. *Mol. Cell.*, 41(4), 471–479.

Russillo M., Di Benedetto A., Metro G., Ferretti G., Papaldo P., Cognetti F., Fabi A. (2011). Assessment of PTEN and P13K status in primary breast cancer and corresponding metastases: is it worthwhile? *J. Clin. Oncol.*, 29(20), 2834–2835.

Saito K., Scharenberg A., Kinet J. (2001). Interaction between the Btk PH domain and phospatidylinositol-3, 4, 5-triphosphate directly regulates Btk. *J. Biol. Chem.*, 276(19), 16201–16206.

Stemke-Hale K., Gonzalez-Angulo A., Lluch A., Neve R., Kuo W., Davies M., Hennessy B. (2008). An integrative genomic and proteomic analysis of PIK3CA, PTEN, and AKT mutations in breast cancer. *Cancer Res.*, 68(15), 6084–6091.

Stokoe D., Stephens L., Opeland T., Gaffney P., Reese C., Painter G., Hawkins P. (1997). Dual role of phosphatidylinositol-3, 4, 5-trisphosphate in the activation of protein kinase B. *Science*, 277(5325), 567–570.

Suzuki K., Takahashi K. (2003). Reduced expression of the regulatory A subunit of serine/threonine protein phosphatase 2A in human breast cancer MCF-7 cells. *Int. J. Oncol.*, 23(5), 1263–1268.

Tang B., Mano H., Yi T., Ihle J. (1994). Tec kinase associates with c-kit and is tyrosine phosphorylated and activated following stem cell factor binding. *Mol. Cell Biol.*, 14(12), 8432–8437.

Tsutsui S., Inoue H., Yasuda K., Suzuki K., Higashi H., Era S., Mori M. (2005). Reduced expression of PTEN protein and its prognostic implications in invasive ductal carcinoma of the breast. *Oncology*, 68(4-6), 398–404.

Ugi S., Imamura T., Maegawa H., Egawa K., Yoshizaki T., Shi K., Olefsky J. (2004). Protein phosphatase 2A negatively regulates insulin's metabolic signaling pathway by inhibiting Akt (protein kinase B) activity in 3T3-L1 adipocytes. *Mol. Cell Biol.*, 24(19), 8778–8789.

Umemura S., Yoshida S., Ohta Y., Naito K., Osamura R., Tokuda Y. (2007). Increased phosphorylation of Akt in triple-negative breast cancers. *Cancer Sci.*, 98(12), 1889–1892.

Wang X., Beugnet A., Murakami M., Yamanaka S., Proud C. (2005). Distinct signaling events downstream of mTOR cooperate to mediate the effects of amino acids and insulin on initiation factor 4E-binding proteins. *Mol. Cell Biol.*, 7(2558-2572).

Wong L., Zhang D., Chang C., Koay E. (2010). Silencing of the PP2A catalytic subunit causes HER-2/neu positive breast cancer cells to undergo apoptosis. *Exp. Cell Res.*, 316(20), 3387–3396.

Xiang T., Jia Y., Sherris D., Li S., Wang H., Lu D., Yang Q. (2011). Targeting the Akt/mTOR pathway in Brca1-deficient cancers. *Oncogene*, 30(21), 2443–

2450.

Xiang T., Ohashi A., Huang Y., Pandita T., Ludwig T., Powell S., Yang Q. (2008). Negative Regulation of AKT Activation by BRCA1. *Cancer Res.*, 68(24), 10040–10044.

Xue Q., Hopkins B., Perruzzi C., Udayakumar D., Sherris D., Benjamin L. (2008). Palomid 529, a novel small-molecule drug, is a TORC1/TORC2 inhibitor that reduces tumor growth, tumor angiogenesis and vascular permeability. *Cancer Res.*, 68(22), 9551–9557.