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AKT signaling in ERBB2-amplified breast cancer

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Abstract

The PI3K/AKT pathway is the focus of several targeted therapeutic agents for a variety of malignancies. In ERBB2-amplified breast cancer, the hyperactivation of this signaling cascade is associated with resistance to ERBB2-targeted therapy. This can occur through gain-of-function alterations or compensatory mechanisms that enter into play upon pharmacological pressure. The strong rationale in combining anti-ERBB2 agents with PI3K/AKT inhibitors, together with the identification of genomic alterations conferring sensitivity to targeted inhibition, are guiding the design of clinical studies aimed at preventing the emergence of drug resistance and achieving more durable response.

In the present review we describe the involvement of this pathway in breast cancer pathogenesis, with an emphasis on AKT kinases, and provide insight into currently available targeted agents for the treatment of ERBB2-amplified breast cancer. Finally, we provide preliminary data on a novel AKT3 mutation detected in the context of resistance to anti-ERBB2 therapy as an example of genomics-based approaches towards uncovering novel actionable targets in this setting.

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Conflicts of interest

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Keywords

PI3K; mTOR; trastuzumab; targeted therapy; drug resistance; AKT3

Introduction

With over 20 years of research on its shoulders, the phosphatidylinositol 3-kinase (PI3K)/ Protein kinase B (PKB/Akt) signaling pathway is one of the most intensely studied signaling networks in human cell biology and, in particular, in cancer (Chalhoub & Baker, 2009). Through a tightly coordinated cascade of phosphorylation events, the components of this pathway integrate the signals from cell membrane receptors – receptor tyrosine kinases (RTKs) and G-protein-coupled receptors (GCPRs), among others, to regulate numerous cellular functions including cell survival, proliferation, metabolism, protein synthesis, transcription and cell death (Figure 1). Soon after its discovery, hyperactivation of the PI3K/AKT pathway was identified as a potent contributor to tumorigenesis in different cancer types, and prompted investigators to elucidate its mechanisms of action and regulation. Accordingly, in recent years, The Cancer Genome Atlas (TCGA) and other cancer-genome sequencing studies have uncovered a variety of alterations affecting different components of the pathway. Many of these have been recognized as oncogenic drivers and thus represent validated targets for the development of targeted therapies.

In breast cancer, hyperactivation of this cascade can occur upon amplification or gain-of-function mutations of RTKs such EGFR and ERBB2. Moreover, activating mutations in the PI3K/AKT pathway are frequent in this disease and have been related with resistance to both endocrine and anti-ERBB2 targeted therapies. Despite the fact that most of these mutations have been identified in *PIK3CA*, the gene encoding the catalytic subunit of PI3K, less frequent but functionally relevant alterations in other components of the pathway, such as AKT1, are attracting attention as putative mediators of tumor progression and drug resistance. In line with this, efforts have been made in developing specific PI3K/AKT inhibitors for cancer treatment that can be used alone or in combination with existing drugs.

This article will review the involvement of the PI3K/AKT pathway in ERBB2-amplified breast cancer, with a focus on the role of AKT as a central player in this signaling cascade. Moreover, we will summarize the current status of PI3K/AKT inhibitors under clinical development as single agents and in combination with ERBB2-targeted therapy. Finally, we will provide preliminary findings on a novel AKT3 mutation discovered in the context of resistance to anti-ERBB2 therapy.

Structure and activity of the Protein Kinase B/AKT

AKT is an evolutionary conserved serine-protein kinase belonging to the AGC subfamily of protein kinases that acts as a hub integrating numerous signaling inputs and controlling downstream effectors that maintain cell homeostasis. There are three highly conserved homologous isoforms: AKT1 (PKBα), AKT2 (PKBβ) and AKT3 (PKBγ). Studies on isoform-specific functions have unveiled unique roles for each (Chen, et al., 2001; Cho, et al., 2001; Garofalo, et al., 2003; Tschopp, et al., 2005); however, phenotypic

characterization of isogenic *in vitro* models revealed a certain degree of redundancy among the three isoforms, with all contributing to cellular fitness (Dummler & Hemmings, 2007).

AKT consists of three conserved domains, an N-terminal pleckstrin homology (PH) domain connected through a linker region with a central kinase catalytic domain, and a short C-terminal tail containing a regulatory hydrophobic motif (HM). Following recruitment to the plasma membrane –stimulated by RTK activation, for example, PI3K catalyzes the conversion of phosphatidylinositol 4, 5-bisphosphate (PI (4,5) P_2) to the second messenger phosphatidylinositol 3, 4, 5-triphosphate (PIP_3). Accumulation of PI (3,4,5) P_3 and, to a lesser extent, PI (3,4) P_2, at the membrane induces the recruitment of AKT via its PH domain, and stimulates its catalytic activity through the activation of two regulatory sites: a threonine located in the activation T-loop (Franke, et al., 1995) – Thr308 (Akt1), Thr309 (Akt2) and Thr305 (Akt3) and phosphorylated by Phosphoinositide-Dependent Kinase 1 (PDK1), and a serine in the C-terminal hydrophobic motif – Ser473 (Akt1), Ser474 (Akt2) and Ser472 (Akt3) (Alessi, Andjelkovic, et al., 1996; Sarbassov, Guertin, Ali, & Sabatini, 2005) and phosphorylated by the mammalian Target of Rapamycin (TOR) Complex mTORC2. Studies based on AKT crystal structures showed that the conformational change occurring upon dual phosphorylation unmasks the catalytic core and leads to an increase in its kinase activity by 1000-fold (Yang, et al., 2002). These phosphorylation events are required for full activity of the kinase. However, three complementary studies reported that PDK1 and mTORC2-mediated phosphorylation of the activation loop and the hydrophobic motif, respectively, are independent from each other and determine AKT substrate specificity (rather than its activity) (Frias, et al., 2006; Jacinto, et al., 2006; Shiota, Woo, Lindner, Shelton, & Magnuson, 2006). These reports showed that AKT Thr308 phosphorylation was sustained in the absence of Ser473 phosphorylation. Interestingly, most of AKT downstream substrates remained phosphorylated in the absence of mTORC2 activity, indicating that mTORC2 was not upstream mTORC1. However, inhibitory phosphorylation of FoxO1 and FoxO3 transcription factors was impaired, suggesting a role for AKT phosphorylation status in determining its substrate selectivity.

AKT activity is modulated by lipid and protein phosphatases. Accumulation of lipid second messenger molecules (PI(3,4,5)P_3 and PI(3,4)P_2) is opposed by the activity of phosphatase and tensin homolog (PTEN) (Myers, et al., 1998) and inositol polyphosphate 4-phosphatase type II (INPP4B) (Gewinner, et al., 2009) lipid phosphatases that catalyze PIP dephosphorylation and thus limit AKT recruitment to the plasma membrane. A second type of serine/threonine protein phosphatases directly controls AKT dual phosphorylation. Protein phosphatase 2A (PP2A) terminates AKT signaling by directly dephosphorylating the T308 residue in the activation loop (Andjelkovic, et al., 1996; Kuo, et al., 2008), whereas PH domain and leucine rich repeat protein phosphatases (PHLPP) 1 and 2 catalyze the dephosphorylation of S473 in the hydrophobic motif (Brognard, Sierecki, Gao, & Newton, 2007). The activity of these phosphatases restraining PI3K/AKT activation is frequently lost or inactivated in human cancer, consistent with their tumor-suppressive function, causing an excessive accumulation of lipid second messenger at the inner membrane that in turn results in AKT hyperactivation (Figure 1).

Regulatory functions downstream of AKT

Following membrane localization and phosphorylation by PDK1 and mTORC2, AKT is locked in the active conformation and diffuses across the cytoplasm and nucleus to phosphorylate a multitude of effectors involved in diverse functions. Studies on AKT isoform-specific knockout mice indicate that ubiquitously expressed AKT 1 and AKT 2 might promote cell survival and boost metabolism, respectively, whereas tissue-restricted AKT 3 expression would play a pivotal role in brain development (Chen, et al., 2001; Garofalo, et al., 2003; Tschopp, et al., 2005). A consensus motif R-X-R-X-X-S/T-F/L - X being any amino acid - is shared by direct substrates of AKT and provides specificity over other protein kinases within the same subfamily (Alessi, Caudwell, Andjelkovic, Hemmings, & Cohen, 1996). To date, more than 100 AKT substrates involved in glucose metabolism, cell proliferation, survival and protein synthesis have been identified (reviewed in (Manning & Cantley, 2007).

The first direct substrate identified was the serine/threonine glycogen synthase kinase 3 (GSK3β) (Cross, Alessi, Cohen, Andjelkovich, & Hemmings, 1995), described as a negative regulator of glycogen biosynthesis. In addition, inhibitory phosphorylation of GSK3β was shown to enhance proliferation by abating the inhibitory effect on pro-mitotic oncoprotein cyclin D1 (Diehl, Cheng, Roussel, & Sherr, 1998). AKT-dependent phosphorylation has been shown to promote cell proliferation by means of inactivation of cell cycle checkpoint proteins such as p27 and p21 (Liang, et al., 2002; Shin, et al., 2002; Zhou, et al., 2001), and by enhancing mTORC1-dependent control of mRNA translation of oncoproteins that stimulate cell cycle progression (Gera, et al., 2004). mTORC1 activation is also central to anabolic processes underlying cell growth. Biosynthesis of proteins, lipids and nucleotides is increased by enhanced translation initiation and ribosome biogenesis (Chan, et al., 2011; Raught, et al., 2004; Thoreen, et al., 2012). AKT-dependent mTORC1 activation induces transcription machineries driving the expression of lipogenic enzymes involved in the synthesis of sterols and fatty acids (Sundqvist, et al., 2005) and promotes the production of ribose-5-phosphate required for nucleotide generation (Ben-Sahra, Howell, Asara, & Manning, 2013). Furthermore, AKT signaling negatively regulates apoptosis by blocking the activity of pro-apoptotic factors such as Bax, Bad and Forkhead box O (FoxO) transcription factors (Brunet, et al., 1999), and activates pro-survival signaling pathways (Dan, et al., 2008).

Molecular Alterations of AKT in Breast Cancer

About 25% of breast cancers exhibit mutations in *PIK3CA*. These frequently involve hotspots that are characterized by mutations on the helical (E545K, E542K) and kinase (H1047R) domains of the p110α catalytic subunit of PI3K. Data from 825 breast tumor samples analyzed by the TCGA showed that ERBB2-enriched samples, with a prevalence of ERBB2-amplified tumors, have a frequency of *PIK3CA* mutations of 39% (Cancer Genome Atlas, 2012; Stephens, et al., 2012), causing hyperactivation of the PI3K enzyme and aberrant PI3K/AKT signaling. Of note, a recent report analyzing mutations and copy number alterations at single-cell resolution in ERBB2-amplified breast tumor samples has revealed that cells carrying mutations in *PIK3CA* often represent different clonal populations

than those having ERBB2-copy gain within the same heterogeneous tumor (Janiszewska, et al., 2015). Despite these mutations were found to coexist within the same tumors, the frequency at which this occurs and the diversity of cellular subpopulations are not detected by bulk tumor sequencing at the coverage attained in the TCGA studies (Figure 2). The application of *in situ* sequencing techniques, therefore, may be a step forward to elucidate tumor heterogeneity and evolution under therapeutic pressure. In ERBB2-amplified breast cancer, AKT is also directly activated upon loss of PTEN and INPP4B lipid phosphatases (Gewinner, et al., 2009; Juric, et al., 2015) or mutations and copy-number alterations of AKT isoforms (Figure 2). The most frequent AKT mutation is found in the PH domain of AKT1 where a glutamic acid is substituted with a lysine residue at amino acid 17 (E17K) (Carpten, et al., 2007), resulting in enhanced activity of the kinase. In general, these mutations tend to be mutually exclusive with *PIK3CA* alterations or PTEN loss, suggesting functional redundancy in activating downstream signaling and inducing oncogenic transformation. Less common non-hotspot mutations in AKT1 with varying transforming potential have been reported in human breast cancers (Yi, Axtmayer, Gustin, Rajpurohit, & Lauring, 2013). AKT3 is the most frequently amplified AKT isoform in breast cancer, and has been mostly studied in the triple-negative subtype in the context of resistance to therapy (Chin, et al., 2014) (Figure 2). Its role in ERBB2-amplified disease remains only partially understood.

Rationale for the inhibition of the PI3K/AKT pathway in ERBB2-amplified breast cancer

Fifteen years ago ERBB2 amplification/overexpression, present in ~20% of breast cancers, correlated with a very poor prognosis (Slamon, et al., 1987; Slamon, et al., 1989). The discovery and clinical development of ERBB2-targeted therapies have radically changed the management of this malignancy, now curable in the majority of cases diagnosed with local disease. Once spread to distant metastases, however, these tumors show high rates of drug resistance and, even if initially sensitive to anti-ERBB2 therapy, almost invariably recur.

As a common denominator, factors associated with resistance to ERBB2-targeted agents have been invariably associated with a reactivation of the PI3K/AKT/mTOR signaling cascade. Decreased levels of the tumor suppressor PTEN or activating mutations in PIK3CA have been shown to limit the response to ERBB2-targeted agents in pre-clinical models (Berns, et al., 2007; Eichhorn, et al., 2008; Kataoka, et al., 2010; Nagata, et al., 2004). In the clinic, while *PIK3CA* mutations were confirmed to be predictive of response (Juric, et al., 2015; Majewski, et al., 2015), loss of PTEN expression failed to do so (Nuciforo, et al., 2015).

Moreover, inhibition of ERBB2, PI3K or AKT was shown to lead to compensatory FOXO-dependent RTK expression (Chandarlapaty, et al., 2011; Garrett, et al., 2011; Sergina, et al., 2007). A genetic functional approach in ERBB2-overexpressing cell lines showed that PTEN loss or gain-of-function E545K and H1047R PIK3CA mutations resulted in sustained AKT activation despite the presence of the ERBB2 kinase inhibitor lapatinib; yet combination with the dual PI3K/mTOR inhibitor NVP-BEZ235 was able to abrogate AKT phosphorylation and tumor growth (Eichhorn, et al., 2008). Moreover, similar results were

obtained when combining the dual PI3K/mTOR inhibitor NVP-BEZ235 with trastuzumab in trastuzumab-resistant xenograft models (Serra, et al., 2011).

As a result, the PI3K/AKT pathway became an attractive target for therapeutic intervention with a plethora of agents currently under investigation and clinical development. The first compounds targeting this pathway were everolimus and temsirolimus (Table 1), mTOR allosteric inhibitors that, by interfering with mTOR complex 1 (mTORC1) activity, result in inhibition of cell cycle progression and survival. Everolimus was tested in ERBB2-amplified advanced breast cancer patients progressing to trastuzumab (Morrow, et al., 2011). In combination with trastuzumab, everolimus resulted in improved clinical benefits and increased progression-free survival (PFS), suggesting that mTOR inhibition could delay the emergence of resistance to anti-ERBB2 therapy. Confirmation of these early encouraging results was sought in two randomized clinical trials in HER2-positive metastatic breast cancer patients (Andre, et al., 2014; Hurvitz, et al., 2015). In the BOLERO-3 trial everolimus was added to trastuzumab and vinorelbine in patients with trastuzumab-resistant disease and yielded a statistically significant but small and transient PFS advantage. In the more recently published BOLERO-1 trial, everolimus was added to paclitaxel and trastuzumab as first-line treatment and resulted in no efficacy on the co-primary end-point of PFS in the overall population. No survival advantage was seen in patients receiving everolimus in these two trials. A potential treatment by hormone-receptor status on PFS was consistently reported, suggesting meaningful activity of everolimus in patients with hormone-receptor negative disease. Furthermore, combined biomarker analysis of the BOLERO-1 and 3 trials confirmed that the activity of everolimus seems to be confined to tumors showing evidence of “hyperactivity” of the PI3K pathway (Slamon, et al., 2015). These data suggest that targeting mTOR is potentially effective in a subset of HER2-positive patients, but also that compensatory pathways that have been well documented experimentally may limit the efficacy of this strategy.

Although treatment with allosteric inhibitors effectively target mTORC1 activity, it relieves a feedback inhibition of AKT leading to compensatory pathway activation and loss of therapeutic effectiveness (O’Reilly, et al., 2006). Aiming to overcome this problem, a number of mTOR ATP-competitive inhibitors have been developed; some of which are under clinical development (U. Banerji, 2012; Varga, 2013).

In ERBB2-amplified breast cancer, multiple PI3K or AKT inhibitors are currently under pre-clinical or clinical investigation. Isoform-specific or pan-PI3K inhibitors show activity in inhibiting *PIK3CA*-mutated cell lines, and responses have been documented in early phase clinical trials in solid tumors (Bendell, et al., 2012). Class I PI3K inhibitor BKM120 has shown activity in ERBB2-positive cell lines and xenograft models (Nanni, et al., 2012). GDC0941, also a pan PI3K inhibitor, suppresses proliferation in ERBB2-amplified *in vitro* models (Wallin, et al., 2012) and is currently under clinical evaluation in ERBB2-amplified metastatic breast cancer progressing to trastuzumab-based therapy (Table 1).

PI3K p110α specific inhibitor BYL719 exhibited enhanced activity on a large panel of *PIK3CA*-mutated and ERBB2-amplified breast cancer cell lines (Huang, 2012), and is being tested in combination with anti-ERBB2 and anti-HER3 therapy in patients with metastatic

ERBB2-positive breast cancer (Shah, 2015). Similarly, breast cancer cell lines and xenograft models harboring mutations in *PIK3CA* or amplification of *ERBB2* were found to be sensitive to p110β-sparing PI3K inhibitor GDC-0032 in combination with trastuzumab or T-DM1 (Sampath, 2013), and provided the rationale for its ongoing clinical evaluation in combination with anti-ERBB2 therapies.

Finally, catalytic (e.g. AZD5363, GDC-0068) and allosteric (MK-2206) inhibitors of AKT are also being investigated in preclinical and clinical settings (Table 1). *In vitro*, these compounds have shown enhanced activity in *PIK3CA*-mutant and PTEN-deficient experimental models, and showed growth inhibition in xenografts (Davies, et al., 2012; Sangai, et al., 2012; Tao, et al., 2014). Catalytic AKT inhibitors have shown activity in cell lines harboring AKT1 activating mutations (Carpten, et al., 2007) or oncogenic AKT3-MAGI gene fusions (S. Banerji, et al., 2012). These experimental evidences have fostered the design of trials aimed to test their efficacy in patients as single agents (U. Banerji, 2013; Yan, et al., 2013) or in combination with other therapeutic regimes (H. S. Han, 2011; Michalarea, 2015).

Because of the involvement of the PI3K/Akt pathway in several physiological processes, including energy metabolism, it is no surprise that the potential clinical benefits of these drugs come at the cost of increased side effects (reviewed in (Chia, et al., 2015)). Some of these (e.g. hyperglycemia and cutaneous rash) are common to different drugs targeting the pathway; others are more typical of specific classes of compounds. For example, pan-PI3K inhibitors are associated with neutropenia, gastrointestinal side effects and mood disorders whereas stomatitis and non-infectious pneumonitis are more commonly observed in patients treated with mTOR inhibitors. The increasing clinical experience with these agents, however, resulted in a more proactive attitude of oncologists towards specific toxicities and the proportion of patients who need to discontinue treatment because of safety issue is reasonably small.

AKT Alterations in breast cancer and resistance to anti-ERBB2 therapy

Molecular profiling of tumor samples in recent years has uncovered a series of alterations affecting the AKT family members in breast cancer. Specifically, a hot-spot oncogenic somatic mutation (E17K) affecting the region encoding the pleckstrin homology domain (PHD) of AKT1 was identified in breast, colorectal and ovarian cancers (Carpten, et al., 2007). Structurally, the lysine (K) substitution for glutamic acid (E) at amino acid 17 alters the conformation of the phosphoinositide-binding pocket resulting in an increased affinity for $PI(4,5)P_2$ and $PI(3,4,5)P_3$. This leads to a constitutive membrane localization of the kinase and increased phosphorylation on T308 and S473 in a PI3K-independent manner (Carpten, et al., 2007; Kumar & Purohit, 2013; Landgraf, Pilling, & Falke, 2008). At the functional level, the augmented localization of AKT at the plasma membrane is sufficient to transform cells *in vitro* and induce leukemia in mice transduced with the human $AKT1^{E17K}$ allele (Cancer Genome Atlas, 2012; De Marco, et al., 2015). Subsequent analyses of cancer genomes indicated that the $AKT1^{E17K}$ mutation is present in other tumor types, but is more frequently detected in invasive breast carcinoma with an overall somatic mutation rate of 2.5% (TCGA results from 1098 patients). This mutation is restricted to the ductal and

lobular subtypes and is (usually) mutually exclusive with the presence of activating mutations in *PIK3CA*, (TCGA, 2015 in preparation; (Bleeker, et al., 2008).

Moreover, several additional somatic mutations involving the PHD-kinase domain interface of AKT1 (L52R and D323H) detected in clinical specimens were shown to mediate tumor formation *in vivo* (Parikh, et al., 2012). Previous studies have shown that AKT allosteric inhibitors (e.g.: MK2206) bind to the closed PHD conformation, and thus genomic mutations altering this configuration reduce sensitivity to these compounds. However catalytic AKT inhibitors (e.g.: GDC0068, AZD5363) retain their activity and effectively inhibit AKT signaling. Accordingly, the TCGA data shows a prevalence of mutations involving the PHD as compared to other protein domains suggesting that genomic alterations perturbing the native conformation of this domain might be a mechanism of oncogenic AKT1 activation. These findings indicate that the mutational status of AKT has crucial clinical implications when selecting for appropriate therapeutic regimes.

Although infrequently (<0.5%) mutated in breast tumors, a recent study identified an equivalent mutation on AKT2 (Stephens, et al., 2012). Unlike AKT1, AKT2 copy number variations are relatively frequent in breast cancer and other cancer types (Bellacosa, et al., 1995). Results from the TCGA indicate that this gene is amplified in 1.7–2.8% of invasive breast carcinomas – co-occurring with ERBB2-amplification in some cases, and has been correlated with an increased incidence of pulmonary dissemination using either cell line models (Arboleda, et al., 2003) or an engineered mouse model of ERBB2-driven breast cancer (Dillon, et al., 2009).

Recent sequencing studies, however, revealed that the most frequently amplified isoform of AKT is AKT3 (TCGA Nature 2015, in preparation). AKT3 overexpression has been previously associated with metastatic dissemination in triple-negative breast cancers (Nakatani, et al., 1999) and was proposed as a therapeutic target in this subtype (Chin, et al., 2014). The effects of AKT3 activation in ERBB2-positive breast cancer has been recently examined using Balb-neuT ERBB2-driven transgenic mouse model (Grabinski, et al., 2014). In this study, the authors conclude that AKT3 is a crucial mediator of cell proliferation, and its genetic depletion causes FOXO3a-mediated estrogen receptor (ER)α upregulation and tamoxifen sensitivity, consistent with a crosstalk between the ERBB2 and ER pathways (Grabinski, et al., 2014; Hurtado, et al., 2008). Concurring with these findings, tumor-sequencing data has also revealed that AKT3 amplification is found in a subset of ERBB2-amplified breast cancer patients, suggesting that this isoform could represent a valid therapeutic target in this setting.

A novel AKT3 mutation involved in acquired resistance to ERBB2-targeted therapy

The emergence of AKT mutations as a mechanism of pathway reactivation in response to therapeutic agents has not yet been documented. In our laboratory we have recently identified a novel AKT3 mutation in a lesion resected from an ERBB2-amplified breast cancer patient treated with trastuzumab monotherapy (Carmona, 2014). After complete and durable tumor remission during trastuzumab monotherapy, the patient progressed in a

subclavicolar lymph node, which was surgically removed and submitted for genomic analysis. Targeted exome-sequencing of this sample revealed that the acquisition of drug resistance coincided with the appearance of a missense mutation in AKT3 causing a single amino acid substitution in position 247 (R247C) that was not present in the lesion prior to treatment initiation. Importantly, given that the patient did not receive concomitant chemotherapy, the emergence of this mutation is only imputable to the pharmacological pressure associated with the anti-ERBB2 therapy. The substitution of an arginine with a cysteine within the kinase domain of the protein appears to affect the structure of the protein (Figure 3A–C) in a way that favors a conformational shift towards its active state that in turn may stimulate activation of the pathway (Figure 3D). Molecular modeling and simulations (details to be presented elsewhere) suggest that the mutation from R247 to C247 leads to loss of stabilizing interactions made by R247. This results in the destabilization of the inactive conformational state and shifts the population of the kinase conformations towards the active state. Cell proliferation assays show that ERBB2-overexpressing cells transduced with an $AKT3^{R247C}$ mutant cDNA exhibit enhanced mTORC1 activity and increased tolerance to trastuzumab when compared with untransfected or wild-type AKT3-transduced cells (Figure 3C), further suggesting that $AKT3^{R247C}$ could have causative role in the acquisition of resistance to anti-ERBB2 therapy. Immunostaining of histologic sections from the pre-treatment and post-treatment biopsies shows strong phospho-S6 staining (Figure 3D), indicative of mTOR pathway activation that could be attributed to increased AKT activity. Further experimental evidences are needed to assess the causal role of $AKT3^{R247C}$ in promoting mTORC1 activity. These preliminary results entail important clinical implications since, if confirmed in additional patients, they would provide the rationale to administer a catalytic AKT inhibitor (e.g.: GDC-0068 or AZD5363) in this setting. Combined inhibition of AKT and ERBB2 has already been tested and showed promising activity in an unselected patient population (H. S. Han, et al., 2011). However, the potential of this therapeutic approach would be more effective if $AKT3^{R247C}$ and/or other biomarkers can identify a subset of patients exquisitely susceptible to dual AKT/ERBB2 blockade.

Conclusions

Molecular screening of patient samples using high-throughput profiling platforms is starting to guide the choice of therapeutic intervention. The criteria for establishing therapy regimes are switching from histopathologic biomarkers to dominant oncogenic alterations. Given the validity of the PI3K/AKT/mTOR axis as a pharmacological target in ERBB2-positive breast cancer, it is expected that patients with tumors harboring specific alterations in this pathway would benefit from PI3K/AKT inhibitors combined with anti-ERBB2 therapy. Ongoing and future clinical studies will likely provide insights for patient stratification and clinical management of HER2-positive breast cancer.

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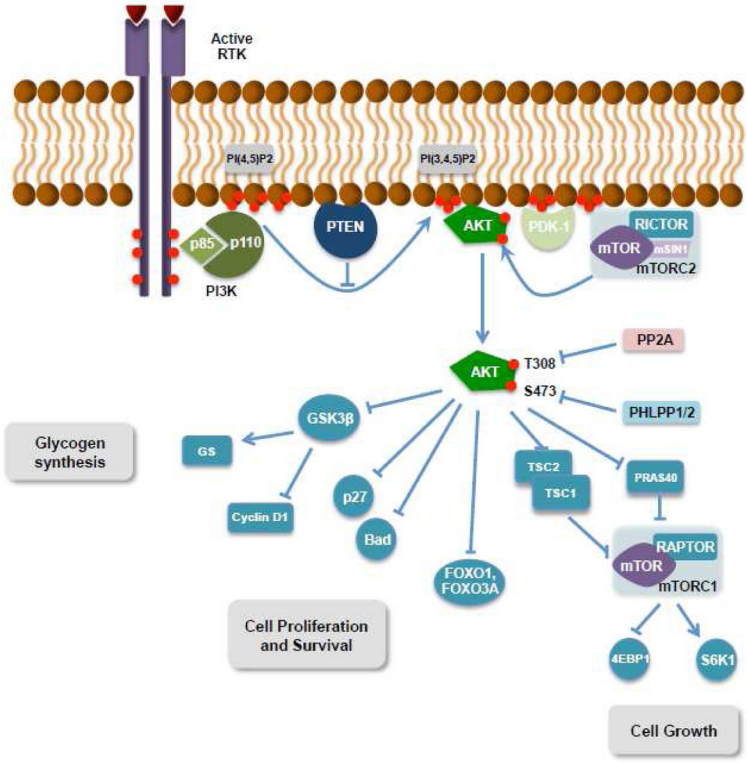

Figure 1.

Overview of the PI3K/AKT/mTOR pathway and cellular processes controlled by downstream signaling. RTK, tyrosine-kinase receptor; 4EBP1, eukaryotic initiation factor 4E-binding protein 1; TSC, tuberous sclerosis protein; GS, glycogen synthase. Red circles represent phosphate groups.

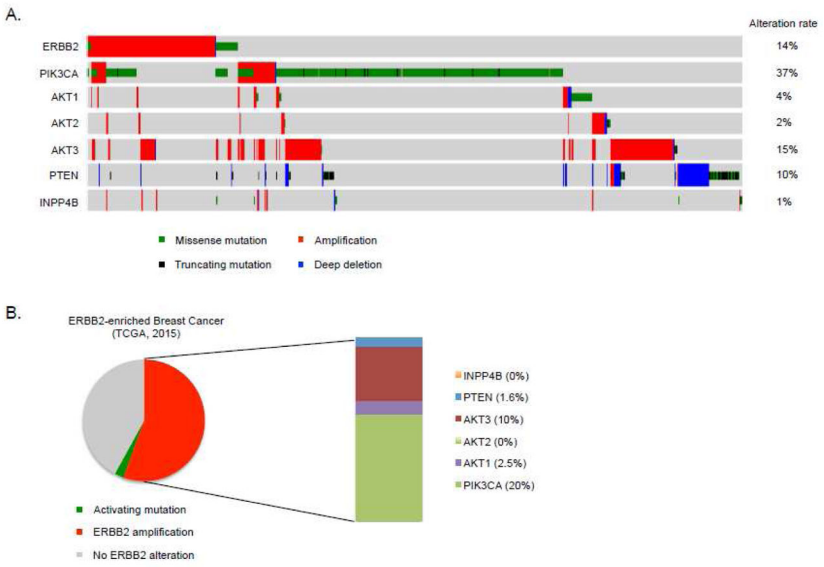

Figure 2.

Mutational landscape of regulators of the PI3K/AKT pathway in breast cancer. A. Copy-number and mutational data on ERBB2, PIK3CA, AKT 1–3 and phosphatases PTEN and INPP4B in breast cancer (TCGA, 2015). B. Mutation rates of the genes encoding these components in ERBB2-amplified breast cancer show that, after PI3K coding-gene, the gene encoding AKT3 is the most frequently altered, mainly by means of copy number gain.

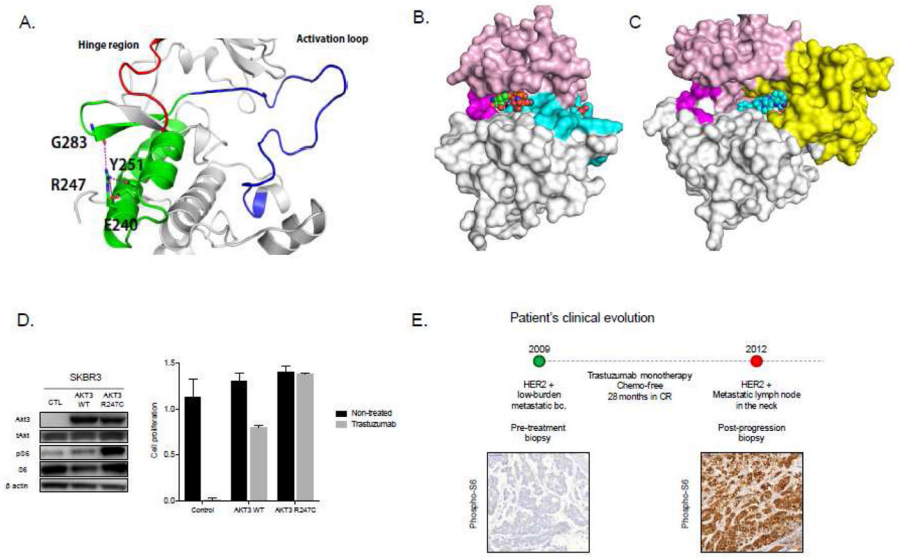

Figure 3.

A novel AKT3 *bona fide* gain-of-function mutation involved in resistance to trastuzumab monotherapy. A. Cartoon representation of the structure of AKT3 with regions involved in modulating functional conformational changes; residues R247 and those involved in establishing hydrogen bonds (dashed lines) are highlighted in stick representation. B. Structural model of AKT3 built based on homology with AKT1 (88% sequence similarity with the kinase domain and 85% sequence similarity with the PH domain) using the crystal structures of the active state (PDB code 3CQU resolved at 2.2A; the active state does not contain the PH domain) and the inactive state (PDB code 3O96 resolved at 2.7A). AKT3 in its active state with the ATP shown on the left and the phosphorylated Tyr shown on the right. C. AKT3 in its inactive state with the allosteric inhibitor ICQ shown in spheres bound between the kinase and the PH domains. The domains are colored as: pink for the N-terminal lobe, grey for the C-terminal lobe, magenta for the hinge region, cyan for the activation loop and yellow for the PH domain. The activation loop is not highlighted in the inactive state for clarity. D. Transduction of $AKT3^{R247C}$ and $AKT3^{WT}$ coding vectors in ERBB2-amplified SkBr-3 cells. $AKT3^{R247C}$ enhances mTORC1 signaling (left panel) and increases resistance to trastuzumab as seen in colony-formation assays comparing cell proliferation of control, $AKT3^{WT}$ and $AKT3^{R247C}$ transfected cells in the presence of the drug over 7 days. E. The ERBB2-amplified breast cancer patient experienced tumor progression after 28 months in complete response and showed re-activation of mTORC1 activity concurrent with the acquisition of the $AKT3^{R247C}$ mutation.

Table 1

Inhibitors of the PI3K/AKT/mTOR pathway currently under clinical development for breast cancer. HR, hormone receptor; MBC, metastatic breast cancer; TNBC triple negative breast cancer; AR, androgen receptor. Information retrieved from clinicaltrials.gov.

Agent	Activity	Phase of clinical development
Everolimus	mTORC1 allosteric inh.	Approved
Temsirolimus	mTORC1 allosteric inh.	Approved
BEZ235	PI3K/mTOR	Phase II in HER2+ patients failing to prior trastuzumab, and in HR+ in combination with endocrine treatment
GDC-0980	PI3K/mTOR	Phase II in combination with endocrine therapy
Buparlisib (BKM120)	Pan-class I PI3K	Phase III in combination with endocrine treatment in HR+/HER2−; in combination with BYL719 in MBC; in TNBC; and in combination with neoadjuvant trastuzumab in HER2+ patients
Pictilisib (GDC-0941)	Pan-class I PI3K	Phase II in HR+ in combination with endocrine treatment
GDC-0032	PI3K p110α, δ, and γ inhibitor	Phase I with anti-HER2 treatment in HER2+ patients; Ib/II with enzatulamide in AR + TNBC patients; and III in combination with endocrine therapy in HR+/HER2− patients
BYL719	PI3K p110α	Phase I in combination with T-DM1 in HER2+ patients progressing to trastuzumab; and II in monotherapy in patients harboring alterations on the PI3K pathway, or in combination with endocrine therapy
MK-2206	AKT	Phase II in combination with endocrine therapy in HR+ patients
GDC-0068	AKT	Phase II in TNBC
AZD5363	AKT	Phase I/II in combination with endocrine therapy in HR+ patients