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Molecular Pathways: PI 3-Kinase Pathway Phosphatases as Biomarkers for Cancer Prognosis and Therapy

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Abstract

Cancer research has seen tremendous changes over the past decade. Fast progress in sequencing technology has afforded us with landmark genetic alterations, which had immediate impact on clinical science and practice by pointing to new kinase targets, such as PI 3-Kinase, the EGF receptor or BRAF. The PI 3-Kinase pathway for growth control has emerged as a prime example for both oncogene activation and tumor suppressor loss in cancer.

Here, we discuss how therapy using PI 3-kinase pathway inhibitors could benefit from information on specific phosphatases, which naturally antagonize the kinase targets. This PI 3-Kinase pathway is found mutated in most cancer types, including prostate, breast, colon and brain tumors. The tumor suppressing phosphatases operate at two levels. Lipid level phosphatases, such as PTEN and INPP4b revert PI 3-kinase activity to keep the lipid second messengers inactive. At the protein level, PHLPP1/2 protein phosphatases inactivate AKT kinase, thus antagonizing mTOR complex 2 activity. However, in contrast to their kinase counterparts the phosphatases are unlikely drug targets. They would need to be stimulated by therapy and are commonly deleted and mutated in cancer. Yet, since they occupy critical nodes in preventing cancer initiation and progression, the information on their status has tremendous potential in outcome prediction, and in matching the available kinase inhibitor repertoire with the right patients.

1. Background

1.1. The PTEN/ PI 3-Kinase pathway

Phosphatase and Tensin homologue deleted on chromosome Ten (PTEN) was discovered in 1997 as the result of a chase for the candidate tumor suppressor in the frequently deleted chromosome 10q23 region (1, 2). The two teams immediately saw that the gene encodes a phosphatase, which launched a flurry of investigations for its substrate. In spite of the logical appeal for a phosphatase tumor suppressor to reverse the action of an oncogenic protein kinase, a landmark study identified the PTEN substrate to be the membrane phospholipid Phosphatidylinositol 3,4,5 trisphosphate, $PI(3,4,5)P_3$ (3). Since PTEN showed specificity for removing the phosphate at the 3-position of the inositol ring (creating $PI(4,5)P_2$) it immediately became clear that its activity antagonizes the previously identified

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Note: We aim to consistently use the convention for nomenclature rules of human genes (e.g. *PTEN*), proteins (PTEN), and mouse gene and protein homologues (*Pten*, Pten, respectively) wherever possible.

class I PI 3-Kinases, which conversely phosphorylate the PI(4,5)P_2 lipid at that position (4), (reviewed in (5)). These results gave birth to our current concept of PTEN and the class I PI 3-Kinases as top level communicators of growth control in cancer (see Figure). Today we know that this pathway constitutes the major oncogenic signaling axis next to the RAS/MAP Kinase pathway.

In this Review, we discuss how the lipid and protein level phosphatases cooperate to protect from cancer and how their use as biomarkers could assist outcome prediction and therapy approach.

Phosphoinositide 3-kinases (PI3Ks) constitute a conserved family of lipid kinases that phosphorylate phosphoinositides (PIs) at the 3-position of their inositol head group (5). The family is classified into several subtypes depending on the substrate PIs that they can phosphorylate, yet the class I PI3Ks are unique: only they can create the master growth control second messenger, PI(3,4,5)P_3 (below termed PIP_3). The class IA PI3Ks relay extracellular growth and survival signals into the cell by producing PIP_3 after activation by ligand bound receptor tyrosine kinases (RTKs). The PIP_3 lipid then attracts proteins such as AKT kinase and its activating kinase 3-Phosphoinositide-dependent protein kinase 1 (PDK1) via their Pleckstrin Homology (PH) domains, thus converting the lipid-phosphorylation code into protein signaling cascades (see Figure). Accordingly, tumor suppression by the pathway's phosphatases occurs at two fundamentally different levels: lipid level phosphatases convert the actively signaling PIP_3 lipids to their inactive isoforms, and protein level phosphatases inactivate the downstream phosphorylated proteins back to the non-phosphorylated state. Below, we discuss the functions and interactions among the pathway's major phosphatases, and their potential in predicting disease outcomes and therapy response.

1. 2. The lipid level phosphatases

The phosphatidylinositol membrane lipids (PIPs) constitute only a few percent of total membrane lipid mass, consistent with the notion that they do not define physical membrane properties but instead serve as top level intracellular second messengers for signaling (6). Of the seven naturally occurring PIP_n phospho-isoforms, PIP_3 executes the major known signaling function in cancer. However, PIP_3 is only present at very low levels in cell membranes (6) reflecting the transient nature of signaling at the level of the lipids. Keeping these second messenger levels low is the major known function of the phosphatase PTEN (7).

PTEN—PTEN occupies a unique position in antagonizing PI-3 Kinases (**see** Figure) by dephosphorylating PI(3,4,5)P_3 to PI(4,5)P_2. To date, it represents the most efficient suppressor of the PI 3-Kinase pathway. *In vitro*, it inhibits cell growth, survival and proliferation so efficiently that stable expression in cancer cells is very hard to achieve. The *PTEN* gene has been mutated in heritable cancer syndromes, which are now collectively referred to as the PTEN Hamartoma Tumor Syndromes (PTHS), with Cowden Disease, Proteus-like- and Bannayan-Riley-Ruvalcaba syndromes showing the highest PTEN germline mutation frequency (80%, 60% and 60%, respectively, (8). Somatic *PTEN*-

alterations are seen in such a high number of human epithelial cancers that they rival those of p53 (9), see also COSMIC database, http://cancer.sanger.ac.uk/cosmic/gene/analysis?ln=PTEN). However, deregulation of PTEN that does not involve gene alterations is also of critical importance in cancer (10, 11). Primary human prostate cancers for example, frequently present with partial reduction of the PTEN protein but rarely with complete gene inactivation (12-14). Animal models have shown that *Pten* is haploinsufficient in prostate and other cancer types (15-18). Given the prominent role of PTEN in cancer signaling, this led to the realization that the classical 'two-hit-hypothesis' for tumor suppression (19) needed to be expanded (reviewed in (20)). Analysis of PTEN alteration in surgically removed prostate cancers has revealed that 86% of tumors retain the gene while protein loss or strong reduction is found at 75% percent frequency (13). Similar observations were made in colorectal and lung cancer (21, 22) where PTEN protein loss is far more frequent than gene/ RNA loss, and in thyroid cancer, endocrine pancreatic tumors, and melanoma where PTEN is often lost from cell nuclei (23-25) (reviewed in (26). These cases mostly exhibit normal RNA levels, suggesting that PTEN protein degradation is a common cause of cancer formation (reviewed in (26). The finding that homozygous *PTEN*-loss triggers p53-mediated senescence explained why the partial loss is widespread in cancer cells with functional p53 (27). In prostate for example, p53-deletion with concurrent deletion of *PTEN* is frequently observed in metastasis, but not in primary disease (12, 13).

INPP4b—The inositol polyphosphate 4-phosphatase (INPP4) activity was first identified in brain lysates (28) and cloning of the human cDNA revealed that it was widely expressed and responsible for most of the cell's phosphatase activity towards the 4' position on PIPs (29). A second human gene sharing 42% identity at the protein level was soon identified and classified as Type II phosphatase (30) with the gene name *INPP4b*. *In vitro* analysis showed that the enzyme primarily hydrolyzes $PI(3,4)P_2$ at the 4-position through its C-terminal catalytic domain to generate PI(3)P (29), a result that was confirmed in cells (31). Through this action, INPP4B suppresses $P1(3,4)P_2$, which can still serve as a platform for AKT recruitment and activation (31). In agreement, knockdown of *INPP4b* was found to phenocopy hallmarks of *PTEN*-suppression, such as increased AKT signaling triggered by insulin (31, 32) and showed an increased p53-dependent cellular senescence response upon co-suppression with *PTEN*. Taken together, PTEN and INPP4b have emerged as the most strongly cooperating lipid level PI 3-Kinase pathway phosphatases.

1. 3. The Protein level phosphatases

PHLPP1—The Pleckstrin Homology domain Leucine-rich repeat Protein Phosphatase 1 (PHLPP1) was discovered in a logical search for AKT antagonists that link a phosphatase- to a PH-domain (33). PHLPP1, previously implicated in circadian rhythms (34), fulfilled these criteria and was confirmed to directly dephosphorylate and inactive AKT at the serine 473 activation site (33) in addition to other targets (reviewed in (35)). The gene locates to chromosome 18q21 and expresses two alternatively spliced isoforms. PHLPP1b differs from PHLPP1a by a 50Kd N-terminal extension, containing a RAS-association domain of still unclear function. Both isoforms share the same features: the PH domain, leucine-rich repeats (LRR), a PP2C catalytic domain and a PDZ-ligand domain (reviewed in (35)). *PHLPP1* has been well-studied in cancer models (13, 36-38), development and function of T cells (39),

cardiac cell survival (40), and circadian rhythms (41). In the mouse prostate, *Phlpp1*-loss leads to Akt-driven neoplasia and synergizes with partial *Pten*-loss to accelerate tumor proliferation, onset and incidence. Importantly, loss of *Phlpp1*, just like loss of *Pten*, triggers activation of p53 which causes cellular senescence. This response acts as a barrier against disease progression and is spontaneously overcome by p53-inactivation in the mutant mice (13).

PHLPP2—*PHLPP2* (on chromosome 16q21) shares its domain structure with the longer *PHLPP1b* splice isoform (42). Just like PHLPP1, PHLPP2 is also ubiquitously expressed in most tissues and shows highest abundance in the brain. PHLPP2 also acts predominantly on the serine 473 site of AKT and differential specificities of PHLPP1/2 for AKT1/2/3 have been reported *in vitro* (43, 44) and are being investigated further in animal knockout models. One critical distinction between Phlpp1 and Phlpp2 is their differential response to PI 3-Kinase pathway activation in genetically controlled experiments: while Phlpp1 levels remain constant, Phlpp2 is surging to antagonize Akt (13). Thus, after *Pten*-loss, Phlpp2 is critically attenuating pathway output. It remains to be seen if the two PHLPP isoforms show differential tissue specific-roles for this function. Mechanistically, the PHLPP protein levels are regulated downstream of mTORC1, thus linking levels and activity to PI 3-kinase pathway output in a negative feedback (13, 45) (reviewed in (46)). This role of PHLPP2 in partially substituting for PTEN may explain the frequent co-deletion of *PTEN* and *PHLPP2* in lethal prostate cancers.

2. Clinical-Translational Advances

2.1 Phosphatases as prognostic biomarkers

Single gene associations—PTEN has been extensively investigated as biomarker for prediction of disease outcome across many cancer types (47). As a single factor, low *PTEN* gene expression is associated with prostate metastasis (48), faster rising PSA levels after surgery, (13, 49), and with castration-resistance (50). In breast cancer, signatures of *PTEN*-loss have been associated with poor prognosis (51), similar to findings in colon (52). Several studies showed the correlation between disease progression and/ or relapse after intervention, when low or absent PTEN protein levels were detected in a prostate tumor (53-55). Similarly, PTEN protein status has been associated with better response to HER2 target therapy in breast cancer (56-59), although the straightforward correlation has been called into question by a recent large-scale study (60), which highlighted a major issue with pathway biomarkers. How much reduction of a tumor suppressor is called to be functionally relevant ((61), see also Conclusions)?

Gene loss and reduction of INPP4b protein has been frequently seen in breast and ovarian cancer and correlated with increased progression of disease and shorter overall survival (31, 32). In basal-like breast cancer furthermore, loss of *INPP4b* (and *PTEN*) strongly correlated with PI 3-Kinase pathway activation as confirmed through the TCGA consortium ((32, 62) reviewed in (63)). INPP4b protein is also frequently lowered in prostate cancer, an event that was associated with shorter times to biochemical recurrence ((64), reviewed in (65)). Intriguingly, the study found that the androgen receptor (AR) positively regulated INPP4b transcription and protein levels, consistent with an emerging pattern of AR-mediated

suppression of AKT: two recent studies demonstrated that AR also positively regulates PHLPP1 to suppress AKT signaling (37, 38). These findings suggest that anti-hormone therapy could come at the price of increased AKT activity, when INPP4b (and PHLPP1) are intact. Decreased INPP4b expression was furthermore found to correlate with disease progression in melanocytic tumors (66). Collectively, these results confirm the key pathway position of INPP4b and point to its usefulness as pathway biomarker.

The PHLPP phosphatases have quickly moved into the spotlight of tumor suppressor studies by virtue of their ability to directly dephosphorylate AKT kinase. Strong evidence to confirm the mouse tumor suppressor function of Phlpp1 in human has come from studies on prostate cancer where the gene is frequently deleted (12). The expression analysis of clinically annotated patient samples from this study revealed significant association of low *PHLPP1/2* expression with disease recurrence after surgery (13). In colon cancer, reduced protein levels have been described (67) and PHLPP1-status has also been linked to treatment response after chemo- and hormone therapy (37, 38, 68). A cancer associated polymorphism in the *PHLPP2* gene that reduces AKT-suppressing activity has been identified (69) and validated in breast and ovarian cancer (70, 71). Furthermore, several studies have shown a compensatory role for PHLPP proteins after pathway activation through e.g. *PTEN*-loss. This failsafe response is triggered by aberrant mTOR activation and serves to limit cell proliferation (13, 45). Thus, on the one hand, the PHLPP phosphatases are emerging as critical pathway breaks at the protein level, and on the other hand they serve as rheostats that actively dampen the malfunction of lipid level phosphatases.

2.2 Recovering phosphatase function through target therapy

At the lipid level, several candidate drugs against PI 3-Kinases have shown success and are currently in advanced clinical trials (see Figure, Lipid kinase inhibitors). Functionally, this approach supports or recovers PTEN or INPP4b activity, or it reverts activity of mutant PI 3-Kinase. The BKM120 inhibitor (Buparlisib), which has activity against all four isoforms of the class I PI 3-Kinase catalytic subunit (p110 alpha, beta, gamma delta), is currently in a Phase III trial for metastatic (HR^+, $HER2^-$) breast cancer. Other approaches include isoform specific inhibitors, such as CAL101 (Idelalisib), a p110-delta inhibitor, which Phase III trials showed a significant improvement in overall survival for Chronic Lymphocytic Leukemia (CLL) (72), in this type of cancer PTEN LOH has been observed at 20% frequency (73).

The PI 3-kinase pathway has been successfully targeted at the protein level after the discovery of the naturally occurring mTORC1 inhibitor rapamycin and its derivatives, the rapalogs. They are used as immunosuppressants after organ transplantation as they inhibit T-cell activation (74). The RAD001 derivative (Everolimus) has been FDA approved in 2010 for the treatment of Tuberous Sclerosis (TSC) syndrome, which predis-poses patients with inherited TSC mutations (see Figure) to precancerous lesions. In cancer, the drug has been approved for advanced kidney cancer, a TSC associated astrocytoma, a HR^+, $HER2^-$ breast cancer subtype and for treatment of pancreatic neuroendocrine tumors (PNETs) (reviewed in (75)). AKT inhibitors are targeting the protein by two mechanisms. Allosteric inhibitors, such as MK2206 or Perifosine prevent translocation of AKT to the plasma membrane, thus it cannot be activated by phosphorylation. The ATP-competitive inhibitor (e.g

GSK2110183, Afuresertib) in contrast targets the AKT active site, which results in hyperphosphorylation of the kinase. Both MK2206 and GSK2110183 are currently in Phase II trials against blood and solid cancers (see Figure), while the Phase III trials of Perifosine in colon cancer and multiple myeloma have not shown significant results.

Finally, several promising compounds exhibit so-called dual specificity, due to the close evolutionary relationship between the kinase domains of PI 3-kinases and mTOR– in spite of the diversification into lipid and protein specific kinases (76). Although no dual specificity inhibitor has so far been FDA approved, several of them are currently used in Phase 2 trials (see Figure) against PNET and other advanced cancers.

3. Conclusions

The lipid and protein level phosphatases of the PI 3-Kinase pathway form a tight natural network against cancer, which should be routinely monitored at the genetic and protein level to assist outcome prediction. In addition, successful drug discovery programs have afforded us with many compounds that can support or replace core functions of these phosphatases when they are lost. The challenge now consists in matching molecular genetic events on the phosphatase side with therapeutic strategies on the inhibitor side. It remains unclear if alterations at a specific level of the pathway sensitize cancers to drugs that inhibit at the same exact level. For example, one could envision *PTEN*-mutant tumors to be more sensitive to PI 3-kinase than Akt- or mTORC-inhibitors. Such linkages can be established in controlled, primary model systems using pharmacological and genetic tools. However, it is also expected that these linkages are perturbed by the context of spontaneous aberrations and feedbacks that arise in a tumor (77). Furthermore, functional readouts for the relevance of phosphatase alterations are needed to prevent false status calls, as recently suggested by comparing different studies on PTEN status in trastuzumab therapy of breast cancer (reviewed in (61)). Yet in spite of this complexity, there is hope for discovering distinct linkages between alterations and therapeutic effects. Patients harboring *TSC* germline mutations that activate mTORC1 clearly benefit from targeting of mTORC1 with rapalogs. Thus it can be envisioned, that the precise matching of drugs with predetermined pathway defects and known resistance routes of a tumor may provide a winning anti-cancer strategy.

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Abbreviations

LOH	Loss of Heterozygosity
PIPs	phosphatidylinositol membrane lipids
PIs	phosphoinositides
PI(3,4,5)P_3	Phosphatidylinositol 3,4,5 trisphosphate

$PI(4,5)P_2$	Phosphatidylinositol 4,5 bisphosphate
PI3Ks	Phosphoinositide 3-kinases
RTKs	receptor tyrosine kinases
PDK1	3-Phosphoinositide-dependent protein kinase 1
INPP4	Inositol polyphosphate 4-phosphatase
INPP4B	Inositol polyphosphate 4-phosphatase type II
PHLPP1	Pleckstrin Homology domain Leucine-rich repeat Protein Phosphatase 1
PHLPP2	Pleckstrin Homology domain Leucine-rich repeat Protein Phosphatase 2
Trp53	Transformation Related Protein 53 gene, p53-gene

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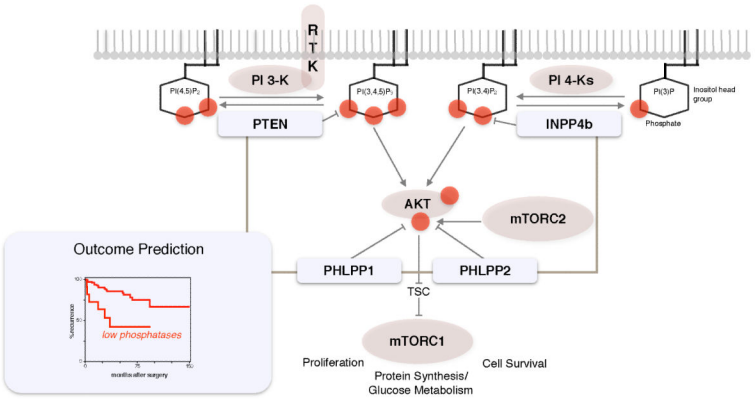

Figure 1.

Core phosphatases of the PI 3-Kinase pathway. PTEN and INPP4b phosphatases inactivate PIP-lipid second messengers to prevent AKT activation. Functionally, they both antagonize class I PI 3-Kinase dependent membrane recruitment of AKT. PHLPP1 and PHLPP2 revert AKT activation by dephosphorylation at Serine 473 to antagonize the phosphorylation that the mTOR Complex 2 carries out on this site. AKT activation signals mTORC1 activation via inhibition of the TSC tumor suppressor complex.

Outcome prediction. Phosphatase status at the DNA, RNA or protein level can be used to predict disease outcome.

Table 1

Clinical trial relevance

Phosphatase status could be used for patient stratification into matching clinical trials. Current Phase II/III trials against pathway targets at the lipid and/or protein level are listed. Phosphatases which are functionally supported by each approach are indicated in the Relevant Phosphatase column.

	Kinase target(s)		Antagonistic phosphatase	Drug	Trial phase	Cancer	Trial ID
Protein kinase inhibitors	AKT			GSK2110183	Phase 2	Solid tumors, hematologic malignancies	NCT01531894
	AKT			MK2206	Phase 2	Relapsed or refractory acute myeloid leukemia	NCT01253447
	mTORC1			Everolimus	Phase 2	Melanoma	NCT01960829
	mTORC1			Sirolimus	Phase 2	Hepatocellular carcinoma	NCT01374750
	mTORC1			Temsirolimus	Phase 1/2	Advanced cancers	NCT00877773
	mTORC1/2			INK128	Phase 1	Advanced non-hematologic malignan-cies	NCT01899053
	mTORC1/2		**PHLPP1/2**	OSI-027	Phase 1	Solid tumor, lymphoma	NCT00698253
	mTORC1/2			AZD8055	Phase 1	Glioblastoma multiforme, other brain tumors	NCT01316809
Lipid kinase inhibitors	PI3K			BAY80-6946	Phase 2	Non-Hodgkin lymphoma	NCT01660451
	PI3K			BKM120	Phase 3	Metastatic breast cancer HR+, HER2-	NCT01633060
	PI3K		**PTEN**	CAL101	Phase 3	Chronic lymphocytic leukemia	NCT01659021
	PI3K			GDC0941	Phase 2	Non-small cell lung cancer	NCT01493843
	PI3K			IPI145	Phase 2	Indolent non-Hodgkin lymphoma	NCT01882803
	PI3K			XL147	Phase 1/2	Breast cancer, breast neoplasms	NCT01042925
Dual specificity inhibitors	PI3K	mTORC1/2		BEZ235	Phase 2	Pancreatic neuroendocrine tumors (pNET)	NCT01628913
	PI3K	mTORC1/2	**PTEN**	BGT226	Phase 1/2	Advanced breast cancer	NCT00600275
	PI3K	mTORC1/2		PF04691502	Phase 2	Endometrial neoplasms	NCT01420081

	Kinase target(s)		Antagonistic phosphatase	Drug	Trial phase	Cancer	Trial ID
	PI3K	mTORC1/2	**PHLPP1/2**	PF05212384	Phase 2	Metastatic colorectal cancer	NCT01925274
	PI3K	mTORC1/2		XL765	Phase 1	Glioblastoma, astrocy-toma	NCT01240460