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Oncogenic and Therapeutic Targeting of PTEN Loss in Bone Malignancies

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ABSTRACT

Being a tumor suppressor, PTEN functions as a dual-specificity protein and phospholipid phosphatase and regulates a variety of cellular processes and signal transduction pathways. Loss of PTEN function has been detected frequently in different forms of cancers, such as breast, prostate and lung cancer, gastric and colon cancer, skin cancer, as well as endometrial carcinoma. In this review, we provide a summary of PTEN and its role in bone malignancies including bone metastases, multiple myeloma, and osteosarcoma, etc. We highlight the importance of PTEN loss leading to activation of the oncogenic PI3K/Akt/mTOR pathway in tumorigenesis and progression, which can be attributed to both genetic and non-genetic alterations involving gene mutation, loss of heterozygosity, promoter hypermethylation, and microRNA mediated negative regulation. We also discuss the emerging therapeutic applications targeting PTEN loss for the treatment of these bone malignant diseases. J. Cell. Biochem. 116: 1837–1847, 2015.

KEY WORDS: PTEN; PI3K; Akt; BONE METASTASIS; SARCOMA

The tumor suppressor *PTEN* (phosphatase and tensin homolog deleted from chromosome 10) is a 200 kb gene located on chromosome10q23, a genome region that suffers mutations or loss of heterozygosity in many human cancers [Li et al., 1997]. Loss of PTEN function is a major determinant that affects cancer development across tissues. Functional PTEN loss also occurs in bone metastases, multiple myeloma, osteosarcoma, and other bone malignancies. During the recent years, the role of PTEN loss has been extensively reviewed in many forms of cancers, such as colorectal cancer [Molinari and Frattini, 2013], gastric cancer [Xu et al., 2014], hereditary cancer [Nakanishi et al., 2014], and breast cancer [Davis et al., 2014]. However, no review has been published on this tumor suppressor in bone malignancies. In this review, we summarize the close association between loss of PTEN and these bone tumors. We also discuss the emerging molecular targets for targeted therapy against bone malignancies with PTEN loss.

PI3K/AKT/MTOR PATHWAY

The phosphatidylinositol 3-kinase/Akt/mammalian target of rapamycin (PI3K/Akt/mTOR) signaling pathway is one of the most important intracellular pathways, which is frequently activated in diverse cancers (as reviewed by Chalhoub and Baker [2009]). Signaling through PI3K/Akt/mTOR begins with the activation of receptor tyrosine kinases (RTKs) and/or G protein coupled receptors (GPCRs) in response to growth factors, followed by the activation of phosphatidylinositol 3-kinase (PI3K) [Cantley, 2002]. Class I PI3K leads to phosphorylation of phosphatidylinositol-4,5-bisphosphate (PIP_2) to phosphatidylinositol (3,4,5)-triphosphate (PIP_3). PIP_3 acts as a second messenger by binding to and activate the pleckstrin homology (PH) domain-containing proteins, including the serine/threonine kinase Akt/Protein kinase B (PKB) [Georgescu, 2010].

The activation of Akt regulates several target proteins involved in the control of apoptosis, cell proliferation, and other processes [Luo

Abbreviations: 4E-BP1, eIF4E-binding protein 1; BMP, bone morphogenetic protein; CNA, copy number alteration; EFS, event-free survival; EMT, epithelial-mesenchymal transition; eIF4E, eukaryotic initiation factor 4E; GSK-3, glycogen synthase kinase 3; IKK, I kappa B kinase; JNK, c-Jun N-terminal Kinase; mTOR, mammalian target of rapamycin; MM, multiple myeloma; PI3K, phosphatidylinositol 3-kinase; PIP_2, phosphatidylinositol 4,5-bisphosphate; PIP_3, phosphatidylinositol (3,4,5)-triphosphate; PTEN, phosphatase and tensin homolog deleted from chromosome 10; RCC, renal cell carcinoma; TSC, tuberous sclerosis complex.

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et al., 2003]. Akt promotes cell survival by inhibiting the pro-apoptotic activity of BAD, caspase-9 and the forkhead family, and activating several anti-apoptotic substrates including IκB kinase (IKK) and cAMP response element binding protein (CREB). Akt also stimulates cell cycle progression through its inhibition of glycogen synthase kinase-3 (GSK-3) and decreased proteolytic degradation of cyclin D1 (CCND1) [Radu et al., 2003].

As a target of Akt pathway, mTOR is the catalytic subunit of two structurally distinct complexes (mTORC1 and mTORC2), and its activity is blocked by tuberous sclerosis complex (TSC1/TC2). Following PI3K signaling activation, Akt phosphorylates TSC2 to disrupt TSC complex, which relieves the Rheb GAP activity of TSC2 and allows Rheb to bind ATP. In the presence of ATP, Rheb switches from the inactive GDP state to the active GTP form and subsequently activates mTORC1 by inhibiting FKBP38 [Bai et al., 2007]. Activation of mTOR leads to increased protein synthesis via its two major effectors, eukaryotic translation initiation factor eIF4E-binding protein 1 (4E-BP1) and p70S6 kinase (S6K1) [Engelman et al., 2006]. For a brief schematic of the PI3K/Akt/mTOR pathway, see Figure 1.

FUNCTION AND REGULATION OF PTEN

PTEN functions both as a dual specificity protein phosphatase and an inositol phospholipid phosphatase, and regulates a variety of cellular signaling pathways. The best understood biochemical function of PTEN is its lipid phosphatase activity, through which PTEN can dephosphorylate the lipid second messenger PIP_3 and convert the biologically active lipid PIP_3 to PIP_2, thus antagonizing PI3K activity [Tamura et al., 1999; Di Cristofano and Pandolfi, 2000]. So far, PTEN is the only known lipid phosphatase counteracting the PI3K/Akt pathway and PTEN activity constitutes a major negative regulator of oncogenic PI3K and its downstream Akt signaling. As a protein phosphatase, PTEN dephosphorylates protein substrates on serine/threonine and tyrosine residues, and accounts for some of its biological effects, including inhibition of cell migration and cell cycle arrest.

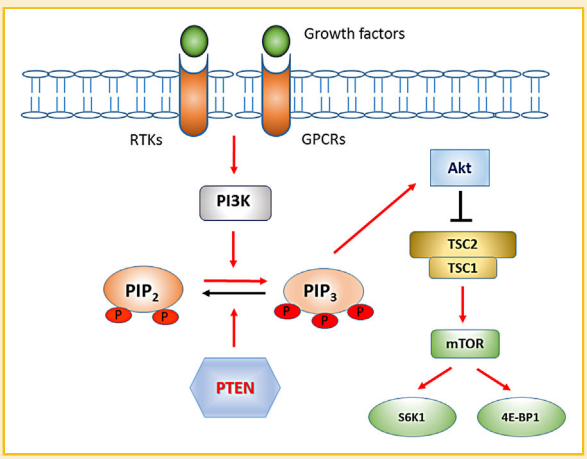

Fig. 1. PTEN and PI3K/Akt/mTOR pathway: The PI3K/Akt/mTOR pathway is activated by ligand (e.g., growth factors) binding to receptors (RTKs and/or GPCRs). PI3K is then recruited to the membrane where it phosphorylates PIP_2 to produce PIP_3, leading to activation of Akt. Disruption of TSC1/TSC2 complex by Akt activates mTOR and results in increased protein synthesis via its effectors, 4E-BP1 and S6K1. PTEN can negatively regulate this pathway by dephosphorylating PIP_3 to convert to PIP_2.

Recently, two novel secretory function of PTEN have been reported. First, a 576-amino acid translational variant of PTEN, termed PTEN-Long, has been identified. PTEN-Long can be secreted from cells and can enter other cells. Importantly, PTEN-Long also has lipid phosphatase activity, thus exerting its antitumor effect in a paracrine mechanism [Hopkins et al., 2013]. Second, PTEN can be secreted in exosomes, and transferred to other cells, where secreted PTEN is internalized by recipient cells with resultant functional activity [Putz et al., 2012].

PTEN loss of function occurs in a wide spectrum of human cancers through both genetic and non-genetic mechanisms, such as mutations, deletions, epigenetic silencing, transcriptional regulation, microRNA-mediated post-transcriptional regulation, as well as protein instability at a frequency that can rival p53 alterations in particular settings [Ali et al., 1999].

GENETIC ALTERATIONS OF *PTEN*

The primary cause of inactivation of PTEN function is gene mutation. Germline mutations cause PTEN hamartoma syndromes (PHTS), including Cowden syndrome (CS), Bannayan–Riley–Ruvalcaba syndrome (BRRS), and Proteus-like syndrome, where exon 5 encoding the PTEN phosphatase domain accounts for approximately 40% of germline mutations [Orloff and Eng, 2008]. Patients with autism spectrum disorders with macrocephaly (ASD-M) also carries germline mutation of *PTEN* gene. Busa et al. analyzed seven children carrying a *PTEN* germline mutation in the absence of family history of CS, and observed that most of the patients did not fulfill usual criteria of BRRS or ASD-M, suggesting a variability of phenotype associated with *PTEN* mutations diagnosed at pediatric age [Busa et al., 2014].

In contrast to germline mutations, *PTEN* somatic mutations including missense and nonsense mutations, deletions, frameshifts and truncations exist in a variety of sporadic human cancers, with the highest numbers found in endometrium, prostate, and central nervous system. For example, 62% (150 out of 242) of endometrical carcinoma patients harbor *PTEN* mutations [Data obtained from The Cancer Genome Atlas (TCGA)] (Fig. 2).

Loss of heterozygosity (LOH) in *PTEN* gene is another genetic mechanism that occurs at a much higher frequency than biallelic inactivation in sporadic tumors, suggesting that PTEN is a haploinsufficient tumor suppressor. In fact, Alimonti et al. demonstrated that even a slight decrease of Pten can alter the steady-state biology of mouse mammary tissues and the expression profiles of genes involved in cancer cell proliferation [Alimonti et al., 2010].

NON-GENETIC REGULATIONS OF *PTEN*

Non-genetic alterations of PTEN also have a profound oncogenic impact. Although Correia et al. comprehensively described the non-genetic regulation of PTEN at multiple levels, as reviewed in [Correia

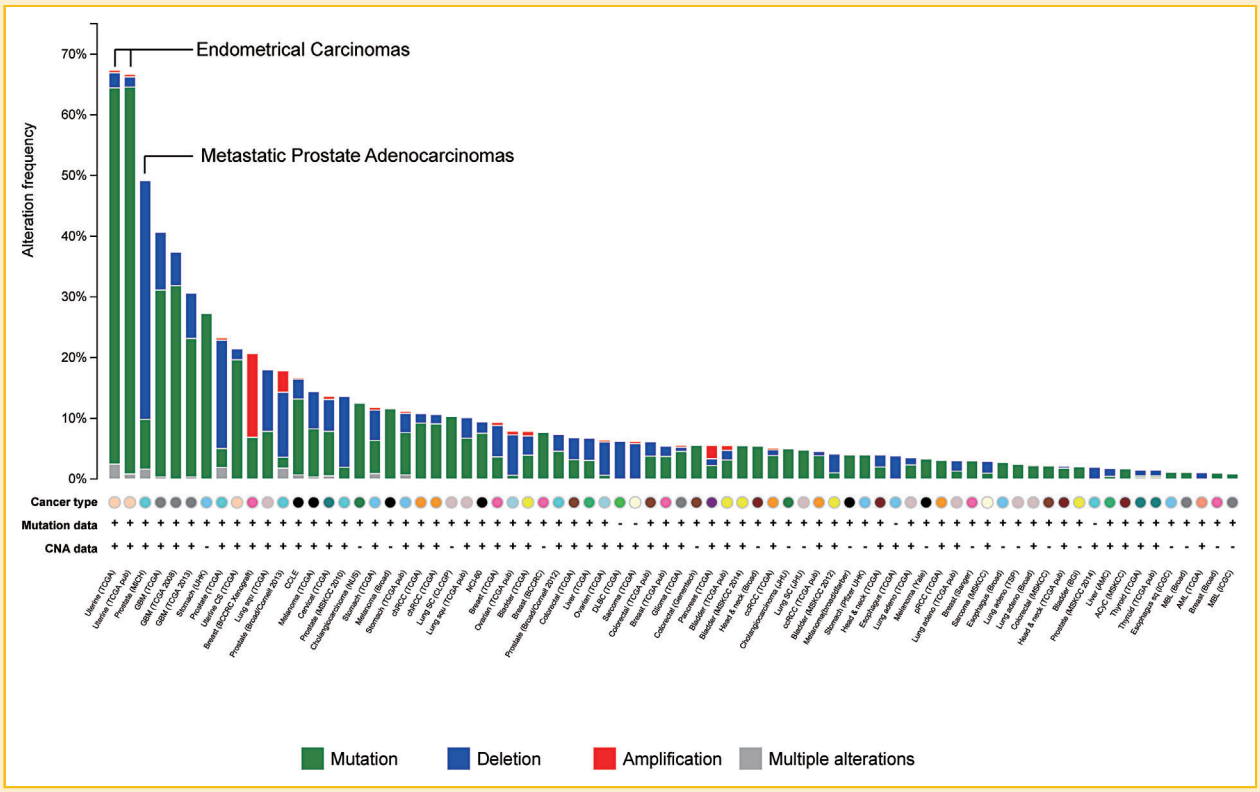

Fig. 2. Cross-cancer alteration summary for PTEN: Genomic sequence data were obtained from The Cancer Genome Atlas (TCGA) via the cBio Portal for Cancer Genomics.

et al., 2014], some new interesting findings have recently been reported. For example, in contrast to the report by Mueller et al. that the methylation of *PTEN* gene occurs commonly in pediatric gliomas and influences their clinical outcome [Mueller et al., 2012], Majchrzak-Celińska et al. found that *PTEN* gene promoter is less frequently methylated in a subset of 95 gliomas of different grades [Majchrzak-Celińska et al., 2015]. This discrepancy may reflect the differences in the molecular aberrations engaged in carcinogenesis in pediatric and adult central nervous system tumor patients. MicroRNAs can negatively regulate gene expression at post-transcriptional level. miR-135b represses the expression of ubiquitin-specific peptidase 13 (USP13) and reduces the stability of PTEN in human colorectal cancers (CRCs). Interestingly, knockdown of USP13 increases the levels of endogenous miR-135b, but not those in CRC cells with PTEN mutation [Xiang et al., 2014], indicating a positive feedback loop between miR-135b and PTEN inactivation in CRCs. Furthermore, miRNA-21 was reported to regulate human telomerase reverse transcriptase (hTERT) expression and control CRC cell growth, and this is mediated via the PTEN/ERK1/2 signaling pathway [Yang et al., 2015]. PTEN phosphorylation is implicated in the regulation of PTEN activity, stability and subcellular localization at post-translational level. Thr366 phosphorylation in PTEN C-terminal region has been associated to decreased PTEN stability in glioblastoma cells, and mutation of this site makes lipid phosphatase activity sufficient for PTEN to inhibit cellular invasion [Tibarewal et al., 2012].

PTEN LOSS IN BONE MALIGNANCIES

The PI3K/Akt/mTOR signaling is frequently activated in a variety of cancers. This pathway regulates cell proliferation, differentiation, cellular metabolism, and cytoskeletal reorganization leading to apoptosis and cancer cell survival. PTEN antagonizes the actions of PI3K signaling by decreasing PIP_3 levels, and is so far the only known lipid phosphatase counteracting this oncogenic pathway. As such, functional loss of PTEN is closely associated with aberrant activation of PI3K/Akt/mTOR signaling in a wide spectrum of human cancers including malignant bone diseases.

VICIOUS CYCLE

During bone metastasis, tumor cells invade into the bone and secrete osteoclast-stimulating factors (e.g., PTHrP, IL-1), which stimulate stromal cells and osteoblasts to express and secrete RANKL. Binding of RANKL to RANK receptor on the surface of osteoclast precursors activates osteoclast differentiation and induces osteolytic bone destruction. In turn, increased bone resorption releases growth factors (e.g., BMP, IGF-1) that attract tumor cells and stimulate their growth, thus propagating a "vicious cycle" between bone resorption and tumor expansion. In prostate cancer, alternatively, bone metastases are prone to be osteoblastic. In these metastases, there is a profound local stimulation of osteoblasts adjacent to the metastatic tumor cells. However, it is becoming more widely recognized that most cases of bone metastasis have both osteoblastic

and osteolytic components and that osteolysis is likely required for advancement of osteoblastic disease [Mundy, 2002; Sturge et al., 2011]. In primary bone tumors, such as osteosarcoma, the osteoblast-derived tumor cells can directly produce RANKL and promote bone destruction. Therefore, RANKL–RANK signaling is not only the master regulator for osteoclastogenesis in bone remodeling, but also a key catalyst for bone malignancies [Dougall, 2007].

PTEN IN BONE METASTASIS

Numerous studies have implicated the loss of PTEN in prostate cancer progression including bone metastasis. In the initial identification of this gene, homozygous genetic inactivation of PTEN was observed in the LNCaP and PC-3 human prostate cancer cell lines, both of which have bone metastatic properties [Steck et al., 1997]. *PTEN* deletions are associated with 15–30% of localized cancers [Li et al., 1997; McCall et al., 2008], whereas 41% of patients with metastatic (including bone) prostate adenocarcinoma display *PTEN* gene deletions, and 10% of patients carry *PTEN* mutations [Data obtained from TCGA] (Fig. 2). Both genetic and epigenetic alterations of the *PTEN* gene in bone malignancies are generalized in Table 1.

In contrast to a variety of observations of PTEN loss in prostate cancer, little is known about this tumor suppressor in breast cancer or cancer induced bone metastasis. In a clinicopathological study involving 46 primary tumors and 52 metastases (including six bone metastatic samples), PTEN loss was found in 14 (30.4%) of the primary tumors and 13 (25%) of the metastases, and there were no significant differences in the proportion of tumors with PTEN loss between primary tumors and metastases [Gonzalez-Angulo et al., 2011]. Patients with human HER2-overexpressing tumors experience a shorter time to relapse and shorter overall survival than patients with tumors of normal HER2 levels. Loss of PTEN function was found to significantly correlate with shorter survival in patients with HER2-overexpressing metastatic breast cancer who have received trastuzumab-based chemotherapy [Esteva et al., 2010]. Razis et al. reported that the efficacy of trastuzumab therapy in patients with HER2-positive metastatic breast cancer is highly dependent on the activation of the PI3K/Akt pathway, induced by activating *PTEN* loss or *PIK3CA* mutations [Razis et al., 2011].

Although expressed at low level in renal cell carcinoma (RCC), the PTEN expression is further reduced in tumor cells that have metastasized to bone than those of non-metastatic. Calcium treatment increased tumor cell migration and proliferation exclusively in bone metastatic RCC cells and this treatment completely abolished PTEN expression [Joeckel et al., 2014]. Oligodendrogliomas (OGDs) rarely metastasize outside the central nervous system. However, a G to A transversion mutation in *PTEN* gene at codon 234 of exon 2 has been recently identified in one anaplastic OGD patient with bone metastasis [Li et al., 2014].

TABLE 1. Genetic and Epigenetic Status of *PTEN* in Human Bone Malignancies

Disease	Event	Frequency	Reference
Osteosarcoma	Del	44%	Chen et al. [2014] Freeman et al. [2008]
Chondrosarcoma	LOH	67%	Raskind et al. [1996]
	Mut	2.50%	Lin et al. [2002]
Ewing sarcoma	Del	14%	Lynn et al. [2013]
Multiple myeloma	Hypermeth	12%	Piras et al. [2014]
	Mut	NA	Ge and Rudikoff [2000]
Metastatic breast cancer*	Mut	4%	[TCGA]
	Amp or Del	6%	[TCGA]
Metastatic prostate cancer*	Mut	10%	[TCGA]
	Del	41%	[TCGA]
Bone metastatic ODG	Mut	NA	Li et al. [2014]

Abbreviations: Del, homozygous deletions; LOH, loss of heterozygosity; Mut, mutation; Hypermeth, hypermethylation; AMP, amplification; ODG, oligodendrogliomas.
*Metastasis including but not exclusive to bone.

PTEN IN MULTIPLE MYELOMA

Multiple myeloma (MM) is a B-cell neoplasm characterized by end-organ damage including osteolytic bone lesions. Deletion mutations of the *PTEN* gene have been identified in human MM cells, leading to activation of PI3K/Akt [Ge and Rudikoff, 2000; Hyun et al., 2000]. Choi et al. reported that *PTEN*-null myeloma cells are stringently dependent on the PI3K/Akt activation for cell survival [Choi et al., 2002]. *PTEN* gene is also hypermethylated in 12% (7 of 58) MM patients. Notably, *PTEN* deregulation seems independent or weakly associated with its epigenetic status, suggesting a lack of functional consequence to *PTEN* methylation [Piras et al., 2014].

In another study involving 55 MM patients, a decreased PTEN expression was shown to be accompanied by an increased *FAK* mRNA, a key molecule in the integrin signaling pathway that has been associated with several kinases including PI3K. The opposing expression of PTEN and FAK in patients seems to be correlated with disease progression and extramedullary infiltration, as PTEN protein was higher and total FAK protein was significantly lower in six controls than in 12 patients with stage III MM, whereas phosphorylated FAK protein was detected in 11 patients with MM, but not detected in six controls [Wang et al., 2012].

Aberrant expression of miRNAs also occurs in human MM. miR-221/222, which negatively regulates *PTEN* expression, was found to be upregulated in >50% of all 38 MM patients [Di Martino et al., 2013], whereas miR-21 is expressed in eight human MM cell lines, and its overexpression increases proliferation of tumor cells [Leone et al., 2013].

PTEN IN OSTEOSARCOMA

Osteosarcoma is the most common malignant bone tumor in children and young adolescents. Pediatric osteosarcoma is characterized by multiple somatic chromosomal lesions, including structural variations (SVs) and copy number alterations (CNAs). Deletion mutations of the *PTEN* gene was first identified in canine osteosarcoma cell lines [Levine et al., 2002]. Beyond *TP53, RB1, ATRX*, and *DLG2* genes that have shown recurrent somatic alterations in 29–53% of human osteosarcomas, *PTEN* was also identified to be recurrently mutated in about 44% of tumors [Freeman et al., 2008; Chen et al., 2014].

In cancer cells, EGFR aberrations impact a variety of cell signaling pathways, especially the PI3K/Akt and JAK/STAT pathways [Hynes and Lane, 2005]. In fact, Akt activation or functional loss of PTEN has been found to comprise an important cause of resistance to the anti-EGFR agent, gefitinib [She et al., 2003]. Interestingly, the

prevalence of EGFR expression and genomic gains at the *EGFR* locus in osteosarcoma tumors correlates with deletions at the *PTEN* locus [Freeman et al., 2008]. Such an imbalance suggests that growth-promoting pathways (notably, the PI3K/AKT/mTOR) influenced by EGFR, is constitutively upregulated in osteosarcoma.

Based on a global microarray analyses of 19 human osteosarcoma cell lines, 177 miRNAs were identified that were differentially expressed in tumor cell lines relative to normal bone. Among those, *PTEN* expression correlates inversely with oncogenic miR-92a and members of the miR-17 and miR-130/301 families [Namlos et al., 2012]. miR-221 was shown to be significantly upregulated in osteosarcoma cell lines than in osteoblasts, and this upregulation induces cell survival and cisplatin resistance, and also reduces cell apoptosis [Zhao et al., 2013]. Recently, miR-128 and miR-17 have been reported to promote tumor cells proliferation, migration, and invasion by repressing the *PTEN* gene [Gao et al., 2014; Shen et al., 2014].

PTEN IN CHONDROSARCOMA AND EWING SARCOMA

Chondrosarcoma is the second most common primary malignant neoplasm of bone in adults. Although 67% (12 of 18) chondrosarcoma had LOH on chromosome 10q [Raskind et al., 1996], Lin et al. found only one mutation resulting in a truncated PTEN protein among 40 chondrosarcoma tumors and cell lines examined, suggesting that *PTEN* mutation is an uncommon event in chondrosarcomagenesis [Lin et al., 2002]. The high frequency of LOH on chromosome 10q may suggest the presence of additional tumor suppressor genes at these loci.

Both deletions and LOH in chromosome 10q, 11p, and 17p were identified in Ewing sarcomas. In particular, *PTEN* deletion occurs in 14% of the tumors [Lynn et al., 2013]. The expression of PTEN at protein level is reduced in approximately 25% (4/15) of Ewing sarcomas. Clinical trials of IGF-1-targeted inhibitors have demonstrated robust but limited patient responses, PTEN loss can decrease the sensitivity of Ewing sarcoma cells to IGF1R inhibitors NVP-AEW541 and OSI-906, but increase autophagic response to mTOR inhibition [Patel et al., 2014]. PTEN loss also occurs in a subset of Ewing sarcoma cell lines and this loss strongly correlates with high baseline PI3K pathway activity [Niemeyer et al., 2015].

PTEN LOSS FACILITATES TUMOR GROWTH IN BONE MICROENVIRONMENT

The close association between PTEN loss and bone tumors indicates that PTEN has a significant impact in bone microenvironment; loss of which may facilitate tumor expansion in bones. In bone malignancies, such as bone metastasis, the interaction between tumor cells and the bone microenvironment is critical to the development of bone lesion. The osteoblastic nature of prostate cancer bone metastasis suggests that the interaction of tumor cells with bone involves cells of osteoblastic lineage [Logothetis and Lin, 2005]. In fact, prostate cancer cells could be specifically attracted by bone-specific factors and migrate preferentially to bone [Keller et al., 2001]. Importantly, PTEN can specifically inhibit prostate cancer cell migration to bone-conditioned medium, but not lung-conditioned medium in vitro, and this is mediated through activation of small GTPase Rac1 [Wu et al., 2007].

Chemokines are known to regulate the migration and chemotaxis of cells and play a key role in the regulation of metastasis. In particular, the chemokine CXCL12 and its receptor CXCR4 have been strongly linked to bone metastasis and are markers for poor prognosis [Sun et al., 2003; Mochizuki et al., 2004]. PTEN is essential to its negative regulation on CXCR4-mediated chemotaxis [Gao et al., 2005]. Recent studies have shown that PTEN loss can activate PI3K/Akt, which induces CXCL12/CXCR4 expression and promotes prostate cancer cell proliferation and invasion, leading to enhanced tumor growth in the bone [Conley-LaComb et al., 2013]. Furthermore, MT1-MMP has been shown to facilitate prostate cancer cell invasion and promote bone metastasis. Tumor cells harboring biallelic *PTEN* deletion display higher levels of MT1-MMP at the cell surface than $PTEN^{+/+}$ and $PTEN^{+/-}$ cancer cells and exhibit enhanced migration and invasion activities [Kim et al., 2010].

On the other hand, PTEN also suppresses osteoclast differentiation and activity thereby interfering with "vicious cycle" in bone tumors. For example, RANKL activates Akt, which is essential for cell survival in osteoclasts. However, this process can be antagonized by overexpression of PTEN [Sugatani et al., 2003]. PI3K/Akt/GSK3β/NFATc1 signaling axis plays an important role in RANKL-induced osteoclastogenesis [Moon et al., 2012]. PTEN can therefore inhibit osteoclastogenesis by negatively regulating PI3K/Akt pathway. In a recent report, myeloid-specific *Pten*-null mice display increased osteoclastogenesis compared to wild-type mice. Pten loss did not affect the generation or survival of osteoclast precursor cells, but greatly enhanced RANKL-induced expression of NFATc1, the master transcription factor of osteoclastogenesis, resulting in markedly increased terminal differentiation of osteoclast differentiation [Bluml et al., 2015].

In general, these data suggest that PTEN may exert a dual anti-tumor roles, such that: (1) PTEN directly acts on tumor cell behavior (proliferation, migration, apoptosis, etc); (2) PTEN inhibits osteoclast activity and blocks osteoclast-mediated bone destruction, leading to interruption of the "vicious cycle." Loss of PTEN can therefore facilitate tumor growth and expansion in bones (Fig. 3).

THERAPEUTIC TARGETING PTEN LOSS IN BONE MALIGNANCIES

Loss of PTEN function frequently occurs in a variety of cancers, leading to constitutive activation of oncogenic PI3K/Akt signaling and upregulation of its downstream mTOR pathway. In case of bone, both tumor cell behaviors (e.g., proliferation, migration, and invasion) and the interaction between tumor cells and bone microenvironment (i.e., "vicious cycle") are critically involved in bone tumor development and progression. As such, restoration of normal PTEN function and inhibition of PI3K/Akt/mTOR pathway targeting tumor cell property (direct effect) and the "vicious cycle" (indirect effect) are two major therapeutic strategies for PTEN loss associated bone malignancies (Fig. 3).

RESTORATION OF NORMAL *PTEN* ACTIVITY

Gene therapy. Gene therapy provides a useful method for the direct delivery of a functional *PTEN* gene into tissues to restore PTEN

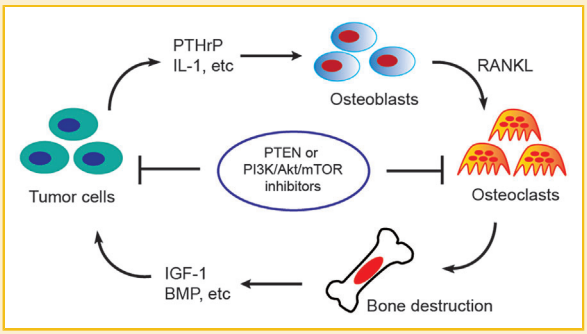

Figure 3. Effects of PTEN or PI3K/Akt/mTOR inhibitors in "vicious cycle": During bone malignancies, tumor cells produce growth factors and cytokines (e.g., PTHrP, IL-1) and stimulate osteoblasts or stromal cells to produce RANKL, which can induce osteoclast differentiation and lead to bone destruction. In turn, increased bone resorption releases growth factors (e.g., BMP, IGF-1) that attract tumor cells and stimulate their growth, thus propagating a "vicious cycle" between bone resorption and tumor expansion. PTEN or PI3K/Akt/mTOR inhibitors may directly target tumor cells behaviors (direct effect) or inhibit osteoclasts (indirect effect), thus interrupting the "vicious cycle."

expression. In MM tumorigenesis, PTEN loss induced Akt activation is a potential mediator of tumor expansion. Restoration of PTEN expression in MM tumor cells using gene therapy suppressed Akt activity, increased apoptosis and abolished in vivo tumor growth, concomitant with cell cycle arrest at the G2/M phase [Choi et al., 2002; Wang et al., 2010]. Overexpression of PTEN using gene transfer could also suppress PC-3 human prostate cancer cell growth primarily by blocking cell cycle progression and completely inhibit the formation of bone metastases [Davies et al., 2002]. Lentiviral vector mediated PTEN overexpression in Ewing sarcoma cells resulted in a significant increase of apoptosis and anchorage-independent growth [Patel et al., 2014]. Since PTEN can be secreted once produced in cells and enter the other cells [Hopkins et al., 2013], the antitumor effect of PTEN can be augmented following gene therapy mediated PTEN overexpression in tumor cells. Hence, restoration of a fully functional wild-type PTEN using gene therapy represents a potential approach against bone tumors associated with PTEN loss.

Demethylating agents. Since promoter hypermethylation and resulting silencing of *PTEN* occurs in many cancers, DNA demethylating agents act as a suitable therapeutic approach in cancer therapy. However, among all bone malignant diseases, *PTEN* gene methylation has only been reported in MM patients (12%), and surprisingly, this methylation has no functional consequences and seems independent of PTEN deregulation [Piras et al., 2014]. Interestingly, methylation inhibitor 5-azacytidine (5-Zac) can significantly increase PTEN expression through decreased binding of the CG sites to SP1 and MYC, both of which are main transcription factors in the *PTEN* promoter. 5-Zac treatment can also induce apoptosis in human osteosarcoma MG63 cells [Song et al., 2014]. As such, although there is no proof of *PTEN* promoter methylation in the majority of bone malignancies, it still warrants investigation for the effectiveness of demethylating agents to treat bone tumors with PTEN loss.

miRNA inhibitors. In light of miRNAs that contribute to tumorigenesis by negatively regulating *PTEN* expression, miRNAs inhibitors may function as another suitable approach for cancer therapy. miR-221 is one of the most commonly and highly upregulated miRNA in cancer, and can directly target *PTEN* gene by binding to its 3′-UTR leading to inhibition of PTEN and activation of Akt. Knockdown of miR-221 was reported to downregulate Akt signaling, inhibit osteosarcoma cell growth and induce apoptosis, and this was associated with miR-221 regulated several downstream genes of Akt, such as *Bcl-2* and *CCND1* [Zhao et al., 2013]. Likewise, miR-17 also targets 3′-UTR of *PTEN* gene leading to inhibition of PTEN expression and activation of Akt. miR-17 was shown to be elevated in osteosarcoma tissues than in matched normal tissues, whereas its inhibitor suppressed proliferation, colony formation, migration, and invasion in MG63 osteosarcoma cells [Gao et al., 2014].

In MM cells highly expressing miR-221/222, enforced expression of miR-221/222 inhibitors triggered antiproliferative effects, and exerted strong antitumor activity in xenografted mice [Di Martino et al., 2013]. miR-21 inhibitors also suppress tumor growth and abrogate the supporting activity of human bone marrow stromal cells on MM cells [Leone et al., 2013]. In both of these studies, overexpression of miRNA inhibitors leads to PTEN upregulation with functional Akt impairment in MM cells, supporting a framework for clinical development of miRNA inhibitors-based therapeutic strategy.

INHIBITION OF PI3K/AKT/MTOR PATHWAY

Substantial progress in uncovering PI3K/Akt/mTOR signaling and their roles in tumorigenesis has enabled the development of novel targeted therapy for anticancer treatment. PI3K/Akt/mTOR small molecule inhibitors in both preclinical studies and clinical trials have been reviewed [Dillon and Miller, 2014; Polivka and Janku, 2014]. Here we generalize most advances of these inhibitors and their preclinical applications in bone malignancies (Table 2).

PI3K inhibitors. PI3K has become of key interest in cancer therapy not only because it is activated in cancers with PTEN loss, but also because of its high mutation frequency and/or gain in function of its catalytic subunits in cancer cells [Janku, 2013]. PI3K inhibitor PF-04691502 can significantly suppress breast cancer induced bone metastasis in a mouse model. This anti-metastatic effect is distinct from drug mediated antiproliferative action, but correlates with abrogation of cytoplasmic p27 accumulation; the latter associates with Akt activation [Wander et al., 2013]. In another study, PI3K inhibitor LY294002 prevented phosphorylation of Akt and induced G0/G1 cell cycle arrest and apoptosis in osteosarcoma cancer stem-like cells. The apoptosis induction by LY294002 is accompanied by activation of caspase-9, caspase-3, and PARP, which are involved in the mitochondrial apoptosis pathway [Gong et al., 2012]. Recently, combination of ZD55-TRAIL (an oncolytic adenovirus expressing TRAIL) with LY294002 in RPMI-8226 MM cells was reported to inhibit the virus-mediated activation of Akt/mTOR and promoted cell death [Tong et al., 2014].

BYL719, a new PI3K inhibitor, has been reported to inhibit osteosarcoma cell proliferation and migration, and also reduces tumor growth in vivo. Combination of BYL719 and conventional chemotherapeutic drug ifosfamide displayed a synergistic antitumor

TABLE 2. Preclinical studies of PI3K/Akt/mTOR inhibitors in bone malignancies

Target	Drug	Disease model	Effect	Reference
PI3K	PF-04691502	Bone metastasis (BC)	Inhibited tumor cell motility and invasion Reduced bone metastasis in vivo	Wander et al. [2013]
PI3K	BYL719	Osteosarcoma	Inhibited tumor cell proliferation and migration Inhibited tumor growth in vivo Reduced osteoclast differentiation	Gobin et al. [2015]
PI3K	LY294002	Osteosarcoma	Induced apoptosis in cancer stem-like cells	Gong et al. [2012]
PI3K	LY294002	Multiple myeloma	Induced cell death	Tong et al. [2014]
Akt	Perifosine	Multiple myeloma	Inhibited tumor cell DNA synthesis Induced tumor cell apoptosis Inhibited osteoclast differentiation	Huston et al. [2008]
Akt	Perifosine	Osteosarcoma	Induced tumor cell apoptosis	Yao et al. [2013]
Akt	MK-2206	Osteosarcoma	Inhibited tumor cell proliferation	Kuijjer et al. [2014]
Akt	MK-2206	Osteosarcoma	Increased event-free survival in vivo	Gorlick et al. [2012]
Akt	AKTi-1/2	Bone metastasis (BC)	Reduced bone metastasis in vivo	Denoyer et al. [2014]
mTOR	Rapamycin	Osteosarcoma	Inhibited tumor cell proliferation Induced tumor cell apoptosis	Gazitt et al. [2009]
mTOR	Rapamycin	Bone metastasis (BC)	Reduced bone metastasis in vivo	Hussein et al. [2012]
mTOR	Rapamycin	Bone metastasis (NB)	Induced OPG production Increased bone thickness Reduced osteoclast differentiation	Hartwich et al. [2013]
mTOR	Everolimus Everolimus + ZOL	Osteosarcoma	Inhibited tumor cell proliferation Reduced tumor growth in vivo	Moriceau et al. [2010]
mTOR	Everolimus + Bendamustine	Multiple myeloma	Inhibited tumor cell proliferation Increased cytotoxicity and apoptosis	Lu et al. [2013]
mTOR	Everolimus + ZOL	Bone metastasis (LC)	Increased tumor cell apoptosis Reduced bone metastasis in vivo	Yu et al. [2014]
mTOR	Everolimus + Docetaxel + ZOL	Bone metastasis (PC)	Reduced tumor growth in vivo	Morgan et al. [2008]
mTOR	MLN0128	Multiple sarcomas	Inhibited tumor cell proliferation Reduced tumor growth in vivo	Slotkin et al. [2014]

Abbreviations: BC, breast cancer; LC, lung cancer; NB, neuroblastoma; OPG, osteoprotegerin; PC, prostate cancer; ZOL, zoledronic acid.

effect on in vivo tumor development. Importantly, BYL719 also inhibited osteoclast differentiation and interfered with the "vicious cycle," and this inhibition was due to the direct effect on both osteoclast precursors and mature osteoclasts [Gobin et al., 2015].

Akt inhibitors. Akt inhibitor perifosine is a novel oral inhibitor of Akt activation and is already in clinical trials in patients with metastatic colorectal cancer or MM [Alexander, 2011]. Besides perifosine induced direct MM cell cytotoxicity, this Akt inhibitor was found to completely inhibit osteoclast formation, suggesting that perifosine exert a dual antitumor effect in MM [Huston et al., 2008]. In osteosarcoma, perifosine can induce tumor cell apoptosis and growth inhibition, and this correlates with perifosine-mediated blockade of Akt signaling and activation of Caspase-3, JNK, and P53 [Yao et al., 2013].

In a pediatric preclinical testing program, in vivo administration of Akt inhibitor MK-2206 induced significant differences in event-free survival (EFS) distribution compared to control in 12 of 29 (41%) of the evaluable solid tumor xenografts and in 2 of 8 (25%) of the evaluable acute lymphoblastic leukemia (ALL) xenografts. Notably, significant differences in EFS distribution were most frequently noted in the osteosarcoma panel (6 of 6) [Gorlick et al., 2012]. In another study, MK-2206 inhibited proliferation of U2OS and HOS osteosarcoma cells, but not of 143B, which harbors a KRAS oncogenic transformation [Kuijjer et al., 2014].

Activation of both Akt and ERK has been shown to facilitate migration, invasion and survival in bone metastatic 4T1BM2 breast cancer cells. Preemptive treatment of these tumor cells with Akt inhibitor VIII (AKTi-1/2) has shown capacity to suppress experimental metastasis to bone in vivo [Denoyer et al., 2014].

mTOR inhibitors. The mTOR inhibitors rapamycin and its derivatives are also powerful antitumor compounds. mTOR pathway is able to promote osteoclastogenesis and inhibit osteoclast apoptosis, and is associated with bone metastatic cancer [Bertoldo et al., 2014]. Rapamycin is the first-generation mTOR inhibitor that strongly inhibits osteosarcoma cell growth and decreases Akt/mTOR activity [Gazitt et al., 2009]. Rapamycin is able to decrease breast cancer cells induced osteolytic bone metastasis in a mouse model, and this is associated with blockade of abnormal production of osteoclasts by late osteoclast precursors [Hussein et al., 2012]. In neuroblastoma bone metastasis, rapamycin treatment was shown to induce OPG production and inhibit osteoclast formation in vitro. Notably, in a xenograft model, increased OPG expression correlated with a delay to pathologic fracture suggesting a potential role for mTOR inhibitors in the treatment of neuroblastoma bone metastases [Hartwich et al., 2013].

Recent studies reported that mTOR inhibitor everolimus, when combined with bendamustine (a bifunctional alkylating agent) shows synergistic antitumor effect in MM. Combination treatment with these two agents inhibits proliferation and promotes cytotoxicity and apoptosis in MM cell via its downregulation of downstream target proteins of the mTOR pathway, S6K1, and 4E-BP1 [Lu et al., 2013]. The combination of everolimus and bisphosphonate zoledronic acid also synergistically promotes apoptosis and significantly reduces lung cancer induced bone metastasis [Yu et al., 2014]. A second-generation mTOR inhibitor MLN0128 has been shown to have anti-proliferative effect in vitro and growth inhibitory effect in vivo in multiple sarcoma subtypes, including Ewing sarcoma and osteosarcoma [Slotkin et al., 2014].

Other inhibitors. In osteosarcoma, caffeine-assisted chemotherapy has induced an enhancement of the 5-year survival rate to ~90% [Tsuchiya et al., 1998]. Recent studies demonstrated that caffeine can inhibit proliferation and increase apoptosis in human MG63 osteosarcoma cells and HT1080 fibrosarcoma cells, and this correlates with caffeine induced activation of PTEN and inactivation of Akt. Interestingly, caffeine induced PTEN activation is mediated through an intracellular cAMP signaling-dependent mechanism [Miwa et al., 2011]. In addition, caffeine also induces apoptosis of HOS osteosarcoma cells, and this effect may involve inhibition of multiple pathways, such as Akt/mTOR, ERK, and NF-κB [Miwa et al., 2012].

Cyclooxygenase-2 (COX-2) inhibitors can exert antitumor activity via COX-2-dependent and independent pathways. COX-2 inhibitors are known to inhibit the PI3K/Akt pathway [Arico et al., 2002]. Celecoxib, a COX-2 inhibitor, induces apoptosis in human osteosarcoma cell line MG63 via downregulation of PI3K/Akt, a mechanism that is COX-2-independent but a survivin and Bcl-2-related [Liu et al., 2008]. Notably, Celecoxib was also shown to reduce cytosolic and nuclear β-catenin and inhibit survival in osteosarcoma MG63 cells [Xia et al., 2010]. Since aberrant activation of Wnt pathway also plays an important role in bone malignancies such as osteosarcoma [Cai et al., 2014], COX-2 inhibitors may exert a dual inhibitory effect on both oncogenic PI3K/Akt and Wnt pathways.

SUMMARY

Functional loss of PTEN has been detected in a variety of bone malignant diseases. PTEN loss leads to aberrant activation of oncogenic PI3K/Akt/mTOR pathway which is instrumental in promoting tumor development and progression. Restoration of PTEN function and inhibition of PI3K/Akt/mTOR pathway represent two major therapeutic strategies for molecular cancer therapy in PTEN loss associated bone malignancies.

So far, our knowledge concerning how PTEN regulates malignant bone diseases is still limited. Although PTEN acts as a cytoplasmic negative regulator for PIP_3 and its downstream PI3K/Akt signaling cascade, PTEN may have phosphatase-independent functions and this needs to be investigated in bone tumors. The absence of nuclear PTEN is associated with more aggressive cancers, suggesting PTEN plays a tumor suppressor role in the nucleus, independently of its ability to regulate PIP_3. It is therefore worthwhile to explore the function of nuclear localization of PTEN in bone tumors. Many regulatory mechanisms to modulate PTEN activity have been identified with in vitro studies. Yet, these exciting findings must be extended to in vivo models to understand how they are relevant to bone cancer. Although anticancer drugs targeting PI3K/Akt/mTOR pathway are already in clinical trials in a variety of cancers, the application of these drugs to treat patients with bone malignancies is still in its infancy, and the development of multiple inhibitors for different components of this oncogenic pathway may allow for tailored therapy in personalized medicine.

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