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Mechanisms of PTEN loss in cancer: it's all about diversity

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ABSTRACT

PTEN is a phosphatase which metabolises PIP_3, the lipid product of PI 3-Kinase, directly opposing the activation of the oncogenic PI3K/AKT/mTOR signalling network. Accordingly, loss of function of the PTEN tumour suppressor is one of the most common events observed in many types of cancer. Although the mechanisms by which PTEN function is disrupted are diverse, the most frequently observed events are deletion of a single gene copy of *PTEN* and gene silencing, usually observed in tumours with little or no PTEN protein detectable by immunohistochemistry. Accordingly, with the exceptions of glioblastoma and endometrial cancer, mutations of the PTEN coding sequence are uncommon (<10%) in most types of cancer. Here we review the data relating to PTEN loss in seven common tumour types and discuss mechanisms of PTEN regulation, some of which appear to contribute to reduced PTEN protein levels in cancers.

Keywords: Cancer; Tumour Suppressor; PTEN; PI 3-Kinase; Mutation

1. Introduction

PTEN is a ubiquitously expressed tumour suppressor that is commonly inactivated in human sporadic cancers. It was firstly identified in 1997 by two independent research groups while

studying the chromosomal location 10q23, which appeared to show frequent deletion in tumours of the brain, prostate and bladder [1,2]. Shortly thereafter, PTEN mutations were also found in the germline of patients with a group of autosomal dominant syndromes, termed collectively as PTEN hamartoma tumour syndromes (PHTS) that are characterized by the presence of multiple hamartomas, an increased cancer predisposition and neurological symptoms. The role of PTEN as a tumour suppressor has been extensively studied since its initial discovery [3,4] and it was identified as the locus with greatest selection pressure for deletion in an analysis of 746 human cancer genomes [5].

In many cases, PTEN appears to be a haploinsufficient tumour suppressor. In contrast to classical tumour suppressor models that need a complete inactivation to induce cancer and were based upon studies of the Retinoblastoma gene, [6] partial loss of PTEN function can have a dramatic impact on tumorigenesis and cancer progression. Studies using hypomorphic mouse models expressing reduced PTEN levels have shown that even subtle reductions in PTEN expression can significantly increase cancer susceptibility [7–9]. This is also in accordance with evidence that the diverse mechanisms controlling PTEN stability and function can have substantial impacts on cancer development [10–14] and with the observation that loss of one copy of PTEN is far more common than mutation or deletion of both copies (Table 1 and Table S1).

PTEN is a major negative regulator of the signalling pathway defined by class I phosphoinositide 3 kinase (PI3K), AKT and the mechanistic target of rapamycin (mTOR) and which plays a key role controlling a wide range of essential cellular processes including cell proliferation, growth, survival and metabolism [15–17]. The PI3K-AKT-mTOR signalling pathway is evolutionarily conserved within metazoans although the linked functions of the class I PI3Ks and PTEN appear to have evolved earlier as regulators of cell polarity and membrane remodelling [18].

The activation of intracellular class I PI3Ks is caused by diverse cell surface receptors which promote cell growth and proliferation, including many growth factor-activated members of the receptor tyrosine kinases (RTK) cytokine receptors, some integrins and a subset of G-protein coupled receptors which includes several chemokine receptors [19]. These activated receptors directly or indirectly recruit and activate class I PI3K which in turn phosphorylates a small fraction of plasma membrane phosphatidylinositol-4,5-bisphosphate (PIP_2) to generate phosphatidylinositol-3,4,5-trisphosphate (PIP_3), a membrane-associated lipid that acts as a second messenger driving downstream signalling (Figure 1). Increases in local PIP_3

levels facilitate the binding of a large number of proteins that carry selective PIP_3-binding domains which in turn promote the effects of pathway activation on cell metabolism, growth, proliferation, etc [20–22]. The best studied of these PIP_3-binding effector proteins are the AKT protein kinases, which have a large and diverse range of substrates and are important proto-oncogenes in their own right. However, other proteins directly regulated by PIP_3 binding include the BTK/TEC family of tyrosine kinases and several regulators of small GTPase of the ARF and RHO families [19,22].

PTEN's role within the pathway is as a lipid phosphatase, directly opposing the activation of the PI3K signalling by converting the PIP_3 generated by PI3K back to PIP_2. Loss of PTEN results in the lack of regulation of PIP_3 levels which in turn promote the hyper-activation of the pathway thus leading to cellular transformation and tumorigenesis, as observed in studies with PTEN-null tumour cell lines, immortalized fibroblasts and tumours arising in PTEN-deficient mice [3,23,24]. The PI3K/AKT/mTOR signalling axis is one of the most frequently deregulated pathways in cancer with mutations occurring in most of the major components of the network [15,25–27]. Therefore, targeting the pathway has become an attractive strategy for cancer therapy and this has led to the development of numerous compounds designed to counteract activated PI3K signalling, although to date clinical success has been limited to the approval of the PI3K delta inhibitor idelalisib for the treatment of B cell malignancies [15].

Even though its main biological activity relies on its ability to dephosphorylate lipid substrates, PTEN has also been reported to display phosphatase activity against tyrosine, serine and threonine residues towards protein substrates *in vitro* as well as on itself [28] although the biological significance of these functions is still controversial. In addition, PTEN has been proposed to exert some of its biological functions in a catalysis-independent manner through protein-protein interactions [29,30]. Both phosphatase-dependent and independent functions appear related to PTEN's subcellular localization: interestingly, loss of the nuclear pool of PTEN seems to correlate with cancer progression and poor clinical outcome in certain types of tumours thus highlighting the importance of its nuclear localisation [13,31,32]. However, the molecular mechanisms through which PTEN exerts its tumour-suppressor functions in the nucleus and its biological relevance still remain unclear.

The *PTEN* gene is located on chromosome 10q23 and its 9 exons encode a predominant protein product of 403 amino acids and 48 kDa that shares sequence homology with the tyrosine phosphatase superfamily as well as with tensin and auxilin. Therefore it was named

Phosphate and Tensin Homolog deleted from chromosome ten (*PTEN*) when first discovered [1]. The protein sequence is highly conserved within vertebrates with only one amino acid difference between the human and murine orthologs. The first analysis of a crystal structure of human PTEN revealed the existence of 2 tightly associated domains: a catalytic N-terminal phosphatase domain (amino acids 6-185) and a C2 domain required for membrane binding (amino acids 186-351) [33]. The protein also includes an extreme N-terminal PtdIns(4,5)P_2 binding sequence (amino acids 6-15) that enables the interaction with substrate-containing membrane surfaces and cytoplasmic and nuclear localization signals (amino acids 19-25) that helps dictate its subcellular localization [34–36]. Furthermore, the C-terminal portion of PTEN has a less-structured C-terminal tail (amino acids 352-403) that contributes to the post-translational regulation of the PTEN protein, containing two clusters of phosphorylation sites and a PDZ binding sequence [33].

In addition to the most abundant 403 amino acid form of PTEN, many cells contain lower levels of N-terminally extended isoforms of the enzyme [37,38]. The first of these to be discovered, PTEN-L, has an additional 173 amino acid N-terminal region translated from an alternate upstream start codon which notably includes a signal peptide. This leads to the secretion of PTEN-L and suggested the hypothesis that active PTEN-L protein may be shared between cells to suppress PI3K/AKT signalling [39]. However, the functions of these longer forms of PTEN remains somewhat mysterious.

2. Changes in PTEN activity in health and disease

Given the biological importance of its functions and the profound pathological effects that arise as a consequence of subtle disruptions on its expression and activity, it is unsurprising that PTEN levels are tightly regulated through multiple physiological mechanisms [40,41]. These mechanisms of PTEN regulation act at transcriptional, post-transcriptional and post-translational levels. Significantly, these physiological mechanisms appear to be subverted in some cancers to suppress PTEN expression and activity. In particular, strong evidence has established the role of promoter methylation and miRNAs suppressing PTEN expression in certain tumour types and these effects are discussed in more detail below (Figure 2). Moreover, PTEN regulation by the transcriptional product of its pseudogene (PTENP1) has gained great interest recently, thus adding a further degree of complexity to the already intricate regulation of PTEN expression [42]. Notably, PTENP1 contains similar 3'UTR miRNA binding sites to PTEN, and has been proposed to act as a miRNA sponge with significance in a number of cancers [12].

Genetic alterations in cancer

The *PTEN* gene has been found to display point mutations in several tumour types predominantly in glioblastoma, endometrial and prostate cancer and to a lesser extent in tumours of the breast, lung and colon [43,44]. Somatic inactivating PTEN mutations tend to be fairly evenly distributed across its 9 exons, which is a common feature of tumour suppressor genes. However, a great number of mutations are found in the codons encoding arginine residues 130, 173 and 233 (cancer.sanger.ac.uk/cosmic). These hotspots are currently unexplained, although the occurrence of both nonsense and missense mutations at these codons and the apparent lack of any distinct functional consequences of these mutations imply they may be driven by higher mutation rate rather than stronger selection.

Multiple genetic alterations have been identified in the *PTEN* coding sequence including missense, nonsense and frameshift mutations; splice site variants, deletions and insertions. The majority of tumour-associated missense mutations result in a completely abolished or at least greatly reduced phosphatase activity of the encoded enzyme [45–47]. Moreover, the vast majority of possible frameshift mutations or truncations occur in regions encoding for either the phosphatase or C2 domains (exons 1-8) and invariably result in a complete loss of catalytic activity since both domains are required to form an active catalytic unit [33,45,48]. The frequency of mutations within the region that encodes for the C-terminal tail of the PTEN protein (exon 9) is lower than in other regions required for its catalytic activity and these C-terminal mutations are more likely only to affect protein stability and its post-translational regulation instead of causing a complete loss of function.

Epigenetic and transcriptional regulation

There are a number of well-established transcription factors that have been shown to directly bind to the *PTEN* promoter and positively regulate its expression, including tumour protein 53 (p53), peroxisome proliferator-activated receptor γ (PPARγ) and early growth response protein 1 (EGR1) [49–51]. Interestingly, several lines of evidence point towards an interplay between PTEN and p53 in which they regulate each other at the transcriptional as well as at the protein level. p53 binds to the *PTEN* promoter at its responsive element (RE) site thus activating its expression while PTEN indirectly increases p53 expression through the regulation of MDM2 transcription, which is a key regulator of p53. Additionally, p53 negatively regulates PTEN protein stability through protein-protein interactions and this physical association between the two proteins also prevents p53 from binding to other RE sites in the genome, therefore modulating its target gene transcription [52].

The *PTEN* promoter has been described as a potential target for a number of transcriptional repressors. The zinc finger-like transcription factors Snail1 and SLUG have been found to compete with p53 for the *PTEN* promoter-binding region [53,54]. Other transcription factors such as polycomb complex protein BMI1, c-Jun and nuclear factor kappa B (NFκB) have been reported to bind to the *PTEN* promoter and negatively regulate its gene transcription in different biological settings [55–57]. Moreover, several studies have revealed a potential dual role of NOTCH signalling in the modulation of PTEN expression by acting on some of the transcription factors that are known to bind to the *PTEN* promoter, although the role of NOTCH on PTEN modulation depends on the cellular context and it is still unclear. Interestingly, NOTCH1 has been shown to negatively regulate PTEN expression by activating the inhibitor hairy and enhancer of split1 (HES1). Conversely, NOTCH1 inhibits the suppressor c-repeat binding factor1 (CBF1, also known as RBPJ) thus upregulating PTEN expression [58,59].

Epigenetic silencing of the *PTEN* promoter has been identified as an alternative method for gene inactivation [60]. Aberrant hypermethylation of CpG islands on the *PTEN* promoter have been found in multiple human cancers including breast, colorectal, multiple myeloma and gastric carcinoma [61–64]. Notably, although consideration in these studies is required to exclude signals from the PTEN pseudogene *PTENP1* [65], promoter methylation at *PTEN* tends to be seen in tumour types in which loss or mutation of both the *PTEN* gene copies is rare. Furthermore, *PTEN* transcription can be modulated by histone acetylation. The transcription factor SAL-like protein 4 (SALL4) has been reported to bind to the *PTEN* promoter and downregulate its transcription by recruiting a strong epigenetic repressor, the nucleosome remodelling and deacetylase complex (NuRD), that contains a chromatin remodelling ATPase and also displays histone deacetylase activity [66].

Post-transcriptional regulation of PTEN by non-coding RNAs

PTEN expression is susceptible to post-transcriptional regulation through a variety of microRNAs (miRNAs), and some have been associated with PTEN repression in human cancers, although the difficulty of dissecting direct effects in cancer from experiments carried out in cultured cells must be taken into consideration. For instance, miRNA21 is one of the most frequently upregulated oncogenic miRNA (oncomir) in cancer [67] and it has been reported to modulate PTEN levels in multiple tumour types [68,69]. miRNA124 constitutes another example of PTEN downregulation and plays an important role in the development of ovarian cancer as well as in cisplatin resistance [70]. Additionally, the

genomic cluster miR-17-92 miRNA encodes for a total 15 miRNAs that together target PTEN, and its upregulation has been associated with lymphoproliferative disease and autoimmunity [71]. A plethora of different miRNAs have been linked to PTEN regulation in human cancers including miR19a in leukaemia, miR22 in prostate and miR26a in high-grade glioma. Interestingly, the contribution of miRNAs to tumorigenesis through the regulation of PTEN levels tends to be cancer-type dependent. Direct inhibition of miRNA function to increase PTEN expression has been revealed as a promising tool in oncology. Furthermore, miRNA regulation might have indirect effects on PTEN expression when targeting transcription factors involved in the expression of the *PTEN* gene. However, the broader biological effects of most of the newly discovered miRNAs are still unknown so further studies will need to be conducted in order to gain knowledge on their functions and therefore uncover their therapeutic potential.

Post-translational modification: Phosphorylation

Phosphorylation is a key regulatory mechanism controlling PTEN phosphatase activity, stability and subcellular localization. PTEN is phosphorylated upon a cluster of serine and threonine residues (Ser380, Thr382, Thr383 and Ser385) located on its C-terminal tail, apparently in many cell types to a high stoichiometry by the kinase CK2 [72]. Other nearby sites that appear to be phosphorylated at lower stoichiometry include Ser370, Thr366 and Ser362. Phosphorylation of Ser370 is mediated by CK2, and this event promotes a subsequent phosphorylation of Thr366 and probably Ser362 by glycogen synthase kinase 3 (GSK3) [73,74]. These C-terminal phosphorylation events promote the maintenance of a closed but more stable conformation in which the C-tail binds to the C2 and phosphatase domains, blocking the active site perhaps by acting as a pseudosubstrate [72,75–77]. In agreement with this, Thr366 appears to be subject to slow autodephosphorylation [76]. As a result of this conformational change, closed PTEN shows reduced plasma membrane localization and decreased lipid phosphatase activity compared to the open conformation state [78–80]. The increase of PTEN stability upon C-tail phosphorylation occurs, at least in part, as a consequence of the closed conformation being less accessible to ubiquitin ligases, probably largely due to its reduced membrane localisation, therefore making the protein less prone to proteasome-mediated degradation [72,81]. Several studies have also shown that C-tail phosphorylation reduces the ability of PTEN to bind to PDZ-domain containing proteins, including membrane-associated guanylate kinase inverted protein 2 (MAGI-2). MAGI-2 has been shown to act as a scaffold protein that facilitates the assembly of PTEN to

a multiprotein signalling complex, enhancing PTEN stability and therefore increasing its ability to supress Akt activation [82,83].

Post-translational modification: Ubiquitination

Ubiquitination of PTEN by E3 Ubiquitin (Ub) ligases affects its catalytic activity as well as its stability and subcellular localization. It has been shown that the addition of multiple ubiquitin chains can target PTEN for proteasome-dependent degradation whereas adding a single ubiquitin unit can increase PTEN stability and promote its nuclear localization [13]. The HECT-domain protein neural precursor cell expressed, developmentally downregulated 4-1 (NEDD4-1) was the first identified and remains the most intensively studied PTEN Ub ligase. Wang and colleagues have shown that NEDD4-1 binds to PTEN, catalyses its polyubiquitination and induces PTEN degradation [14]. Moreover, the levels of NEDD4-1 inversely correlated with PTEN levels in tumour biopsies while overexpression of NEDD4-1 promoted cellular transformation *in vitro* thus pointing towards an oncogenic function of NEDD4-1. However, the effects of NEDD4-1 on PTEN must be considered at least partially redundant, since later analysis in two different strains of NEDD4-1 deficient mice found no apparent changes in the regulation of PTEN levels and its subcellular distribution [84]. Further studies on the mechanisms of PTEN ubiquitination mediated by NEDD4-1 have implicated several ubiquitin ligase adaptors and activator proteins. For instance, Ndfip1 and Ndfip2 have been proposed to act as potent activators of several E3 ubiquitin ligases, including NEDD4-1, through binding to the WW domains characteristic of HECT E3 ligases. They also function as adaptor proteins by promoting the interaction between the ligase and its substrate, namely NEDD4-1 and PTEN [85]. In line with this, the adaptor protein Numb has been recently found to be a binding partner of NEDD4-1 and to play an active role in the modulation of PTEN ubiquitination [86].

PTEN has several sites of ubiquitination and it seems likely that several Ub-ligases are involved in PTEN mono- and polyubiquitination perhaps with some redundancy between related ligases [14,87]. WWP2, a member of the NEDD4-like protein family, has been reported to tag PTEN for degradation through an ubiquitination-dependent pathway [88]. Interestingly, *in vitro* ubiquitin-transferase assays have found that purified WWP2 appears to be more active than NEDD4-1 in ubiquitinating unphosphorylated PTEN, suggesting that both enzymes might show distinct ubiquitination preferences for PTEN depending on the phosphorylation profile of the phosphatase [89]. Additionally, the RING domain E3 ligase X-linked inhibitor of apoptosis protein (XIAP) and the chaperone-assisted E3 ligase C-terminus

of Hsc70-interacting protein (CHIP) have both been reported to induce ubiquitination and regulate the proteasome turnover of PTEN [90,91].

In contrast to the growing number of E3 ubiquitin ligases that have been linked to PTEN so far, the regulation of PTEN deubiquitination remains poorly understood. Ubiquitin C-terminal hydrolase 13 (USP13) has been found to reverse PTEN ubiquitination thus leading to its stabilization in breast cancer cells [92]. Other deubiquitination enzymes that have been associated with PTEN include OUT-domain containing protein 3 (OTUD3) and herpesvirus-associated ubiquitin-specific protease 7 (HAUSP, also known as USP7) [93,94].

Other ubiquitin-like modifications: sumoylation and ISGylation

PTEN is post-translationally modified by the addition of other small ubiquitin-like proteins including small ubiquitin-related modifier (SUMO) and interferon-stimulated gene 15 (ISG15). Lys266 and Lys254, both located in the C2 domain, have been identified as important SUMO-1 acceptors in PTEN [95,96]. More recently, PTEN has been reported to be one of the first validated substrates of ISGylation in cancer biology. Similar to ubiquitin, ISG15 conjugation to protein substrates requires a cascade that includes an E1-activating protein (UBE1L), an E2-conjugating protein (UBCH8) and an E3-ligase, typically HERC5. Although the E3-ligase(s) involved in the last step of ISG15 conjugation of PTEN remains unidentified, the functional consequences of PTEN ISGylation are related to its destabilization in *in vitro* studies, thus suggesting that ISG15 conjugation might control PTEN protein levels. Conversely, the deubiquitinating enzyme ubiquitin specific peptidase 18 (USP18) has been reported to reverse PTEN ISGylation. Additionally, USP18 and PTEN immunostaining were positively correlated in human lung cancer samples, suggesting the role of USP18 as a potential therapeutic target in cancer [97].

Post-translational modification: Oxidation

Like other members of the PTP family, PTEN contains a reactive catalytic site cysteine nucleophile that is susceptible to oxidation. Indeed, several studies have shown that the catalytic activity of PTEN is subjected to physiological regulation by reactive oxygen species (ROS). The reversible oxidation of the Cys124 site by ROS can promote the formation of a disulphide bond with Cys71 that in turn supresses PTEN phosphatase activity [98]. Moreover, it has been shown that endogenous ROS generated in stimulated cells promote the transient oxidation and therefore inactivation of a fraction of the PTEN protein pool and this correlates with a ROS-dependent activation of downstream Akt signalling [99,100]. PTEN reversible oxidation seems to be regulated by thioredoxin-interacting protein (Txnip), which

acts as a negative regulator of the thioredoxin-NADPH-dependent reduction of disulphide bonds in proteins. Studies on a total Txnip knockout mouse model have shown an accumulation of oxidized PTEN and increased Akt signalling in oxidative tissues [101]. Similarly, the peroxidase peroxiredoxin 1 (Prdx1) and more recently the apoptosis-inducing factor (AIF) have been reported to protect PTEN from H_2O_2-mediated oxidation through direct interaction, therefore promoting PTEN tumour suppressive functions [102,103]. Interestingly, analysis of the PTEN interactome using *in vitro* affinity capture have shown that the redox status of PTEN can also modify its protein-protein interactions suggesting that ROS might also play a role in the regulation of PTEN phosphatase-independent functions [104].

Post-translational modification: Acetylation

PTEN lipid phosphatase activity is subjected to modulation through acetylation. Okumura and colleagues provided the first evidence of PTEN acetylation when reporting that the histone acetyltransferase PCAF (p300/CBP-associated factor) acetylates PTEN at Lys125 and Lys128, both located within the catalytic pocket. Acetylation on these residues inhibits PTEN lipid phosphatase activity and therefore prevents PTEN from downregulating the Akt signalling pathway. Intriguingly, PTEN acetylation by PCAF only seems to occur in the presence of growth factors, thus indicating that PCAF might be a regulator of PTEN in response to mitogenic stimulation [105]. Similarly, the histone acetylatransferase CBP targets PTEN on Lys402, which is located within its extreme C-terminal PDZ binding sequence. Accordingly, acetylation of Lys402 does not directly affect PTEN catalytic activity, but enhances instead its interaction with some PDZ proteins, including MAGI-2. The deacetylase sirtuin 1 (SIRT1) has been found to be responsible for the deacetylation of PTEN in this context [106]. In SIRT1 knockout cells, PTEN was hyperacetylated and excluded from the nucleus and this correlates with increased Akt activity, suggesting that acetylation might be involved in the modulation of PTEN subcellular localization and activity [107].

TUMOUR TYPE-SPECIFIC PATTERNS OF PTEN LOSS

Stark changes in PTEN activity are seen in tumours that display changes in the *PTEN* gene, mutating or deleting the gene in many cases leading to complete loss of activity or of expression. PTEN shows different patterns of loss in different tumour types and these are discussed in individual sections below and illustrated in Table 1 and Table S1. It should be noted that despite work to compare antibodies and advise best practice [108], sources of uncertainty within datasets analysing PTEN loss remain the use of poor antibodies in

immunohistochemistry (IHC) as well as difficulties in standardizing IHC data. A few monoclonal antibodies have been well validated using cells and tissues genetically deleted for PTEN. The first of these was the 6H2.1 mouse monoclonal validated in the Charis Eng laboratory and subsequently used very widely [109]. Other validated antibodies have emerged more recently, with the 138G6 rabbit monoclonal antibody probably now having its selectivity supported by the greatest body of negative data from diverse genetically engineered PTEN null tissues [110,111] .

PTEN loss in Glioma

The frequent mutations in PTEN that are found in glioblastoma provided key data in the discovery of the tumour suppressor [1,2] and have provided motivation for the intense studies of the role of PTEN loss in the progression of these particularly deadly tumours. Almost all glioblastomas display loss of function of the PTEN tumour suppressor. Data from The Cancer Genome Atlas identified deletions including the *PTEN* locus in 143/170 (85%) of glioblastomas, making this the most frequent genetic change identified. Additionally, approximately half of the remaining 15% of tumours which did not display evident genetic changes in *PTEN* displayed reduced expression of the *PTEN* mRNA relative to control samples. This is in accordance with the recognised diversity of mechanisms by which PTEN function is lost in cancers. Genetic loss of *PTEN* is not strongly associated with any specific subtype of GBM, occurring in the majority of all 4 sub-types defined by Verhaak *et al*, although occurring at highest rates in the 'classical' subtype (37/37 tumours) [112]. In contrast to the major carcinomas, mutations within the *PTEN* coding sequence are common in glioblastoma, occurring in around 30% of these cancers and often accompanied by deletion of the second allele [113,114] spread throughout the coding sequence and disrupting both regulatory as well as catalytic aspects of PTEN function [34,35,43,115,116]. Accordingly, loss of detectable PTEN protein by IHC is also observed in many, probably most, of these cancers [117–120]. Notably, genetic disruption of *PTEN* is much less common in lower grade gliomas than in glioblastoma, although methylation of the *PTEN* promoter is a common hallmark of low-grade gliomas including grade II astrocytomas, oligodendrogliomas and oligoastrocytomas [121].

PTEN loss in breast cancer

Breast cancer is the most commonly diagnosed malignancy as well as the leading cause of cancer death among women worldwide [122]. Breast cancer is a heterogeneous disease that can be classified into different subtypes, each of them displaying different clinical and

pathological features and showing differential therapeutic responses. The main molecular classification of breast carcinomas is based on the expression of immunohistochemistry markers namely estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2), identifying tumours which respond differently to anti-estrogen therapy.

Multiple studies have confirmed the initial observation that PTEN loss is common in breast cancer [123,124] although the frequency and clinical relevance of PTEN alterations in this particular tumour type has not been fully elucidated. Immunohistochemistry analysis of patient-derived samples has revealed that the expression of the PTEN protein is lost or reduced in 40% of primary breast carcinomas as assessed by IHC (Table 1). The prognostic value of PTEN loss in breast cancer has been intensively studied. For instance, a recent meta-analysis pooling results from 27 studies and including a total of 10,231 cases found an association between PTEN loss and a more aggressive behaviour of the disease as assessed by the analysis of several clinicopathological parameters including tumour size, lymph node metastasis and cell differentiation. Moreover, the pooled results showed that PTEN loss was associated with negative ER and PR expression while positively correlating with triple negative phenotypes [125].

Multiple mechanisms can lead to reduced PTEN expression in breast cancer although up to which extent each of them contributes to PTEN inactivation is still unclear. Hemizygous deletion of the *PTEN* gene locus is a fairly common event in breast carcinomas, particularly in aggressive disease [123,126,127]. Contrary to the high rate of LOH, sequence variants in the *PTEN* coding region have rarely been documented in sporadic breast carcinomas [128]. The reported PTEN mutation frequencies across the literature is <5% in concordance with the mutation frequency reported at the COSMIC database, which is of 4.43% (318/7176 samples). This discrepancy between homozygous mutation and loss of protein expression suggests that other non-genetic mechanisms contribute to PTEN inactivation.

Accumulating evidence has suggested that epigenetic silencing of the *PTEN* promoter is a major mechanism leading to PTEN inactivation in breast cancer. However, the reported rates of promoter hypermethylation are diverse (Table S1). For instance, a study conducted by Garcia *et al* found that the *PTEN* promoter was hypermethylated in 48% of the analysed samples and that *PTEN* promoter hypermethylation correlated with other prognostic factors such as ERBB2 overexpression, larger size and higher histologic grade [129]. Conversely, a recent systematic literature review by Lu and collaborators revealed that aberrant

methylation of the promoter is a major contributor to PTEN loss during the early stages of breast cancer but it does not seem to play a role during the progression of the disease since the presence of promoter hypermethylation showed no correlation with tumour grade, ER, PR or HER2 status [130].

PTEN loss in endometrial cancer

Endometrial carcinoma is the most common malignancy of the female genital tract and it is the fifth most common cancer affecting women worldwide. Based on differences in histology and clinical outcomes, endometrial carcinomas have been traditionally classified into two major types. Type 1, also called endometrioid endometrial carcinoma (EECA), represents the majority of cases (70-80%), occurs in pre- and peri-menopausal woman and it is related to estrogen exposure. Clinically, this phenotype is mainly low grade and overall carries a good prognosis. In contrast, the type 2 subtype typically features high-grade serous carcinomas or clear cell tumours to a lesser extent. It is often described as being estrogen-independent and it features a lower degree of cell differentiation. Compared to type 1 subtypes, type 2 tumours have poorer prognosis and they account for a disproportionately high number of deaths (around 40%) considering their relatively low incidence that accounts for 10-20% of all endometrial cancers. Each phenotype shows distinctive molecular alterations.

PTEN represents the most frequently mutated gene in endometrial cancer with the endometrioid subtype showing the highest percentage of PTEN coding sequence variants of all tumour types [131,132]. Conversely, mutations on the *PTEN* gene appear to be highly uncommon in type 2 serous carcinomas [133–135]. In terms of its clinical significance, PTEN loss of function in the endometrium has been postulated to be an early event in carcinogenesis and to also correlate with good prognosis given that a higher frequency of PTEN mutations is often found in pre-malignant lesions or type I tumours compared to more advanced or even metastatic disease [132]. In both of these characterisrics, the pattern of PTEN loss in EECA contrasts with several other major cancer types in which loss correlates with disease severity and does not appear to be an initiating event.

The methylation of the *PTEN* promoter is a relatively frequent event in endometrial carcinomas and it appears to be associated with advanced stage and with microsatellite instability (MSI) phenotype [136]. Several onco-miRNAs have been identified *in vitro* and validated in clinical samples as potential regulators of PTEN in EECA, including miR-200a, miR-200b, miR-141 and miR-429 [137–139]. Finally, a post-translational mechanism for PTEN

loss has been studied in endometrial cancer involving the E3 ligase WWP2. Expression levels of WWP2 were significantly elevated in tumour samples with little or no PTEN staining as assessed by immunohistochemistry despite showing normal or high levels of the PTEN mRNA, implicating WWP2 in the regulation of PTEN protein levels at a post-translational level in endometrial malignancies [140].

PTEN loss in ovarian cancer

Based on invasiveness, epithelial ovarian cancer has been classified as either low or high grade and four histological subtypes of the disease are commonly used: serous, endometrioid, mucinous and clear cell [141]. The mutational landscape is rather specific to each of the main types of cancers that arise in the ovary [142]. PTEN mutations are rare in ovarian cancer and found largely in the endometrioid subtypes studies [143]. More frequent is loss of one or occasionally both copies of *PTEN,* most commonly observed in endometrioid, clear-cell and high-grade serous ovarian carcinomas [144,145]. Multiple groups have also reported frequent reductions in PTEN levels in IHC analysis of ovarian cancer in accordance with the levels of PTEN copy number loss and with stroma sometimes accounting for any detectable PTEN protein in tumour biopsies [141,146,147]. Interestingly, both at a morphological and at a mutational level there is a clear difference between samples harbouring TP53 mutations (serous) and those harbouring PTEN mutations (endometrioid), leading to the idea that these two genes might be involved in the lineage differentiation of these carcinoma types [142]. Furthermore, loss of PTEN heterozygosity was identified in the transition from endometriosis to endometrioid ovarian cancer [148].

These combined genetic and immunohistochemical analyses imply, as with many carcinomas, that many tumours retaining at least one wild-type copy of the *PTEN* gene yet display no detectable PTEN protein. Promoter hypermethylation, does not appear to be an important mechanism reducing PTEN expression in clear-cell and endometrioid ovarian carcinomas [146,149]. However, miRNA activity may play an important role in silencing PTEN in ovarian cancers. In 2007 and 2008, two independent research groups showed that miRNA expression patterns in cancerous ovarian tissue were dramatically different from those found in the healthy ovary following a series of microarray and Northern Blotting experiments [150,151]. A plethora of miRNA's have been discovered to interact with PTEN and contribute to tumorigenesis, however in the context of ovarian cancer, mir214 comes across as a crucial player. Being involved in several physiological processes including *in utero* development, cell fate and musculo-skeletal formation, mir214 is known to reduce apoptotic

cell death and thus has the ability of increasing cellular proliferation when deregulated [152,153]. In ovarian cancer, mir214 seems to contribute to cisplatin resistance by targeting the 3' UTR of PTEN and downregulating its expression leading in turn to enhanced Akt signalling and cell survival. Mir214 was found in circulating exosomes from ovarian tumours suggesting a correlation with malignancy but also revealing its potential as a diagnostic marker [154,155]. Other miRNA's such as mir21, mir93, mir130a, mir374a and mir106 have also been shown to not only bind to and downregulate *PTEN* transcripts, but also to increase cisplatin resistance in cell lines while knockdown of these miRNAs leads to drug resistant cells becoming sensitive to treatment, including paclitaxel [156–158]. In addition, mir130a was also shown to bind to the multidrug resistance gene (MDR1) in SKOV3/DDP (cisplatin resistant) and SKOV3 cell lines. By doing so, MDR1 gets upregulated and PTEN downregulated, leading to cellular proliferation and enhanced cisplatin resistance [156,159].

Mir19a was shown to be upregulated and leading to ovarian cancer cell proliferation by means of Akt signalling as a result of the miRNA binding to *PTEN* transcripts [160]. Mir205 binds not only to PTEN mRNA but also to SMAD4 transcripts suppressing their activity and promoting cell proliferation and invasion [161] while has-miR-222 was found in ovarian tumour effusions suggesting a role in metastasis via PTEN and PAK 1 downregulation, the former being another predicted target of has-miR-222, found highly expressed in effusions alongside PTEN and involved in cell motility and morphology [162]. MiRNA's have also been shown to play a role in a key process involved in metastasis and tumour spreading: the epithelial to mesenchymal transition (EMT). To this end, mir216a, mir18b and mir175p among others have been shown to bind to PTEN's 3'UTR and to facilitate EMT [163].

PTEN loss in lung cancer

Lung cancer is the highest cause of cancer mortality in the UK and worldwide with patients' 5 year survival rate of around 15%. Based on histology, two major subtypes of the disease have been defined and become widely used: small-cell (SCLC) and non-small-cell lung cancers (NSCLCs) with the latter being further divided into three subtypes also based on histological features: adenocarcinoma (ADC, most common), squamous cell carcinoma (SCC) and large cell carcinoma (LCC) [164].

Immunohistochemical analysis of PTEN expression in NSCLC shows loss of the tumour suppressor in around 30-50% of NSCLC (Table 1). Notably, evidence has identified methylation of the *PTEN* promoter but found no correlation with PTEN protein levels [165]. Loss of PTEN function also appears to be far less common in adenocarcinoma, where it is a

very rare event, compared to other forms of NSCLC in which both mutation of the *PTEN* gene and loss of PTEN protein are commonly observed [166–168].

While several clusters of miRNAs have been identified and implicated in lung cancer pathogenesis, probably the most widely researched is mir21, located on chromosome 17 and also found to be involved in tumorigenesis of other cancer types including ovarian. Other groups have further researched this oncomir, with Xu and colleagues observing that mir21 did bind to PTEN directly and that mir21 knockdown decreased migration and invasion of NSCLC cells while its downregulation increased apoptosis *in vitro* inducing cell cycle arrest in G2/M phase [169]. Lang and their team also proved mir21 oncogenicity *in vivo* using geftinib resistant subcutaneous tumour xenografts in mice and restoring their drug sensitivity by anti-mir21 therapy [170]. Other miRNAs recently discovered to be interacting with PTEN include mir-93-5p, also found in NSCLC. Its knockdown led to cell migration and proliferation being severely inhibited and its overexpression was followed by metastasis into lymph nodes in mice. Additionally, mir19 overexpression was shown to induce EMT-like alterations *in vitro* in A549 and HCC827 cells [171] and mir205 was found to be inversely correlated with PTEN expression and positively correlated with enhanced cell migration and chemoresistance to standard therapy [172]. Literature reports myriads of miRNAs that have been recently discovered to be interacting with PTEN, binding to its 3'UTR and downregulating it in NSCLC. Among these: mir93 [173], mir543 [174], mir494-3p [175], mir92a [176], mir26b [177], mir181 [178,179], mir29b and mir222 are the most commonly associated with lung cancer. One very interesting miRNA that seems to be going against the grain and being downregulated in NSCLC is mir130. A series of lung cancer patient survival analyses revealed that life expectancy seems to be increased with upregulated miRNA130, making this miRNA a very attractive therapy target. It has been demonstrated that it binds to PTEN mRNA and it stabilizes it, being positively correlated with higher PTEN protein levels [180].

Considering the many ongoing studies that are looking into PTEN regulation by means of miRNAs and knowing the importance of functional PTEN in cancer, the development of drugs targeting the oncomirs seems to be a very interesting potential new horizon in cancer therapy development.

PTEN loss in prostate cancer

In 1995, Gray *et al* showed that the region q23-24 on chromosome 10 was frequently lost in prostate cancer (62% of tumours studied) [181] and subsequently, the use of such deleted

prostate cancer samples contributed to the cloning of PTEN in 1997 [1,2]. The frequency of PTEN mutations in prostate cancer has been intensively studied using a number of techniques, and some common themes emerge. In prostate cancer PTEN is most frequently altered by copy number alteration, rather than point mutation [182]. Missense or nonsense mutations are usually found in <5% of primary prostate cancers, and slightly more commonly in metastatic cancers (Table 1). On the other hand, when present, point mutations are often found in combination with deletions in the second *PTEN* allele, contributing to homozygous functional loss [182,183]. The other most notable finding is that the frequency of PTEN deletion is higher in metastatic disease than in primary prostate tumours, seemingly in the ranges 30-45% and 10-20% respectively [184].

This is consistent with the conclusion that prostate cancer is driven by genomic rearrangement more strongly than most other types of cancer [182,185], supported by large-scale sequencing data as well as more focused FISH analyses. An additional possibility is that deletion of 10q23 also inactivates other genes than *PTEN* that may play a role in the development of prostate cancer [186]. This latter study identified six genes in a minimally deleted region on 10q23 in prostate cancer datasets that were lost along with *PTEN*. Two particularly intriguing genes are KLLN (a p53 target, which encodes the killin protein) and Multiple Inositol-Polyphosphate Phosphatase 1 (MINPP1), a histidine phosphatase involved in inositol-3-phosphate metabolism. Another study also reported that genes lost in the 10q23 region, including Fas cell surface death receptor (FAS) and 3'-Phosphoadenosine 5'-Phosphosulfate Synthase 2 (PAPSS2), as well as PTEN, were implicated in prostate-specific antigen (PSA) biochemical recurrence [187]. FAS and PTEN may cooperate in causing programmed cell death and PAPSS2 may have a role in androgen synthesis. PAPSS2 has been shown to be poorly expressed in prostate tumours [187].

In a number of cases, PTEN status according to both FISH and IHC has been reported for prostate tumours, and in many cases there is good concordance between the techniques [188]. However, PTEN function is known to be regulated at the transcriptional, post-transcriptional and translational levels, and the question remains to what extent dysregulation of these processes could contribute to prostate cancer development. A preliminary comparison of the frequency of PTEN loss using sequencing, array CGH, FISH or IHC showed that IHC consistently reported higher frequencies of PTEN loss than FISH or other genetic assessments consistent with the hypothesis that PTEN may be regulated at this level in prostate cancer.

Therefore the question of which mechanisms could be involved is pertinent. There is relatively poor evidence for the role of promoter methylation in prostate cancer [183], compared to other tumours. Conversely, there is increasing evidence for miRNA suppression of PTEN levels having a role in progression of prostate cancer [188–192]. Care must be taken when interpreting these results, given that expression of the candidate oncogenic miRNAs would have to be high in order to reduce PTEN mRNA levels and translation, and most experiments have been performed using tissue culture systems rather than *in vivo* models (for example [193]). Nevertheless, a number of miRNAs have been implicated in regulation of PTEN in prostate cancer including mir4534[194], mir153[195] and mir410-3p[196], and a number of miRNA levels are altered in tumour tissue compared to non-tumour human prostate samples (eg [197,198]).

PTEN loss in Colorectal cancer (CRC)

Colorectal cancer, also known as bowel cancer, is the third most common cancer worldwide with approximately 1.3 million new cases diagnosed each year. Biologically, CRC can be divided into right sided and left sided and rectal tumours, which have different embryonic origins and tumours from the two regions are thought of as distinct genetic entities [199–201]. Crucially, the response of patients to therapy can vary according to tumour location. Loss of function of the PTEN tumour suppressor is one of the most frequently observed events that drive sporadic colorectal cancer. As with several other major carcinomas, the predominant genetic change is deletion of a single PTEN gene copy. Importantly, these deletions are often associated with loss of PTEN protein expression by IHC, which is observed in approximately half of colorectal cancers [167,202–205].

Despite the high level of *PTEN* gene deletions, the rate of mutation/focal deletion of PTEN in CRC is generally reported as relatively low. For example, a Cancer Genome Atlas study found a 4% focal deletion rate and a single PTEN mutation in 165 samples [206]. Similarly, from a larger dataset including 1867 patients, a 2% PTEN mutation rate was detected [207]. In this report, the frequency of mutation varied depending on the origin of the tumour, and was higher in right sided tumours (5%), a finding supported by others [208]. However, higher PTEN mutation frequencies of between 9%-20% have also been reported [204,209–211]. The stage of tumours analysed may account for some of these differences, given that PTEN mutations tend to increase with CRC disease severity.

The higher percentage of colorectal tumours showing undetectable PTEN expression by IHC in tumours retaining a wild-type copy of *PTEN* suggest that additional mechanisms regulate

PTEN expression in CRC. It has been suggested that loss of expression of PTEN in CRC is frequently via mixed genetic and epigenetic mechanisms, and that this is similar to its loss in breast and endometrial cancer [212]. miRNA based mechanisms are a focus of research for how PTEN expression is lost in CRC. Mir-21, mir32, mir92a, mir200a and mir494 are all upregulated in CRC samples, and this was also correlated with reduced PTEN protein levels [213–218]. Again, care must be taken with interpreting some of these data since the different miRNAs can have pleiotropic effects; eg miRNA-21 has also been reported to regulate the APC/beta-catenin and Notch pathways and it is unclear if this occurs via the PI3K/PTEN pathway [219,220]. Intriguingly, loss of PTEN has also been shown to elevate levels of miR-135b, accelerating development of CRC in a mouse model [221].

Conclusion

The spectrum of changes and processes which contribute to the loss of PTEN function in different types of tumours is diverse and as discussed reveals some specific patterns. In many cases, the source and consequence of these patterns are unclear. It seems likely that some may be driven by mutational processes, such as tumour cells which are deficient in mismatch repair, identifiable by microsatellite instability. However, other patterns of PTEN loss seem likely to be driven by selection, although very little is known about how different functional classes of PTEN mutation bestow different selective advantages on tumours. For example, knock-in mice expressing the lipid phosphatase inactive point mutants, PTEN C124S or PTEN G129E display a more severe tumour phenotype than mice carrying deletion mutants [222,223]. It is notable that these PTEN mutant proteins are both stable and inactive, suggesting a dominant negative mechanism of action. However, how this may act and whether such dominant negative effects have greater impact on cancer development in specific lineages is currently unclear. It must be hoped that as with the many studies of miRNA dysregulation, this evolving understanding that complex non-genetic factors contribute to the loss of PTEN function and tumour formation may pave new avenues for successful cancer therapy.

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Table 1.

Tumour Type	% Frequency of PTEN loss by:			
	Mutation	Deletion	Loss of Protein (IHC)	Promoter methylation
Breast	3	27	40	35
Colon	7	12	40	17
Lung	8	34	56	38
Prostate (Primary)	3	26	29	<5
Prostate (Metastatic)	13	51	54	<5
Glioblastoma	30	78	65	6
Endometrial	41	48	45	19
Ovarian (High Grade Serous)	1	30	34	10
Ovarian (Endometrioid)	16	48	44	10

Table 1. Estimated frequencies of PTEN loss by different mechanisms in the listed tumour types is shown as a percentage. Mutation refers to sequence variants within the coding region of the PTEN gene. Deletion refers to deletion of either one or both copies of the PTEN gene as assessed by loss of heterozygosity, reduced copy number in array CGH analysis or FISH. Loss of protein refers to low or undetectable PTEN protein in immunohistochemical (IHC) studies. Numerical values were assigned as follows. Independent publications reporting values for frequencies of PTEN loss from clinical samples were studied and any reports with very low sample numbers or perceived methodological flaws were discarded (almost all for the use of antibodies in IHC with poor and/or unvalidated specificity). Of the remaining reports, for each tumour type, those reporting the highest and lowest values were discarded and a mean value calculated from the remaining data. References, including details of more specific pathologies are provided in Table S1. It should be noted that for most tumour types this analysis does not reflect a comprehensive analysis which includes all available published data.

Figure Legends

Figure 1. Overview of the class I PI3 kinase signalling cascade. The binding of diverse ligands to their cognate cell surface receptors activates PI3K. The relevant ligand/receptor pairs include many growth factors and cytokines, as well as chemokines and neurotransmitters and their receptors. The diagram uses a receptor tyrosine kinase as an example, although many G-protein coupled receptors and integrins also activate PI3K. Activated PI 3-kinase phosphorylates the membrane phosphoinositide $PI(4,5)P_2$ converting a small fraction of this lipid to $PI(3,4,5)P_3$ which then continues the signalling cascade by regulating a large group of PIP_3-binding proteins including AKT. AKT influences cellular behaviour by phosphorylating its substrates including FOXO transcription factors, TSC1 and TSC2, TBC1D4 and GSK3. PTEN acts as an antagonist to PI3K de-phosphorylating PIP3 and converting it back to PIP2.

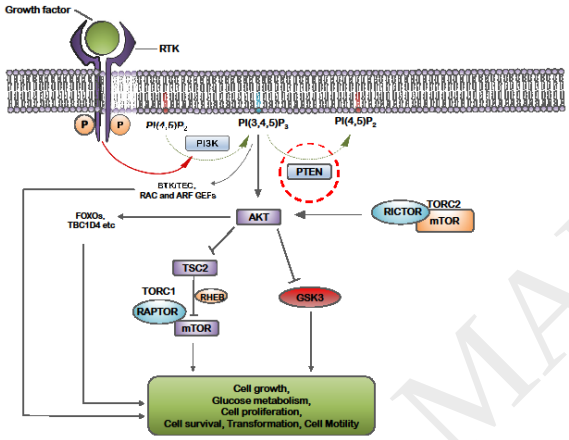

Figure 2. Mechanisms of PTEN regulation and potential loss of function are represented. a) Genetic deletion and mutation. b) promoter hypermethylation. c) micro RNA (miRNA) binding to the 3' UTR of the PTEN gene blocking translation. d) inactivating point mutations which directly block the function of the PTEN enzyme. Reported effects include those on catalytic activity, regulation and protein stability. e) post-translational modifications, including those indicated: ubiqutination, oxidation, acetylation, phosphorylation, SUMOylation and ISGylation.