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Abstract:

Loss of function of the PTEN tumour suppressor, resulting in dysregulated activation of the PI3K signalling network, is recognised as one of the most common driving events in prostate cancer development. The observed mechanisms of PTEN loss are diverse, but both homozygous and heterozygous genomic deletions including PTEN are frequent, and often accompanied by loss of detectable protein as assessed by immunohistochemistry. The occurrence of PTEN loss is highest in aggressive metastatic disease and this has driven the development of PTEN as a prognostic biomarker, either alone or in combination with other factors, to distinguish indolent tumours from those likely to progress. Here we discuss these factors and the consequences of PTEN loss, in the context of its role as a lipid phosphatase, as well as current efforts to use available inhibitors of specific components of the PI3K/PTEN/TOR signalling network in prostate cancer treatment.

THE PI3K/PTEN SIGNALLING NETWORK

Elevated and/or uncontrolled activation of the signalling networks activated by growth factors, in particular signalling through the PI3K group of lipid kinases, is a characteristic of most cancers including prostate cancer [1, 2]. The signalling network (Figure 1) is named after a group of lipid kinases, the Class I Phosphoinositide 3-Kinases (here simply termed PI3Ks) which are tightly regulated, with low basal activity and which are activated by diverse cell surface receptors. These activating receptors include those for many growth factors, cytokines and chemokines, as well as by extracellular matrix components acting via integrins [3]. Class I PI3Ks are heterodimeric enzymes, with four human genes encoding catalytic isoforms and five encoding regulatory subunits. Although examples of both catalytic (*PIK3CA* encoding the alpha catalytic subunit of PI3K) and regulatory (*PIK3R1* and *PIK3R2* encoded) subunits have been found to be frequently mutated in several forms of cancer [4, 5], rates of mutation and copy number changes in genes encoding PI3K itself are generally lower in prostate cancer than many other common carcinomas [1, 6]. As part of their activation mechanism, the PI3K catalytic subunits, which are all of approximately 110kDa, directly bind to small GTPases, which appear to be members of the RAS sub-family in the case of the p110α, p110γ and p110δ PI3K isoforms, but members of the RAC GTPase sub-family in the case of p110β [3, 7]. When activated at the plasma membrane, the PI3Ks phosphorylate the relatively abundant phosphoinositide lipid, phosphatidylinositol 4,5-bisphosphate ($PI(4,5)P_2$) on the available 3-position hydroxyl group, to form small amounts of the signalling lipid phosphatidylinositol 3,4,5-trisphosphate (PIP_3). The role of PTEN in the pathway is to catalyse the opposing reaction, metabolising PIP_3 into $PI(4,5)P_2$ as shown in Figure 1A.

Does signalling through the beta isoform of PI3K selectively drive prostate tumorigenesis?

In 2008, genetic analysis of the PI3K isoform dependence of prostate tumour formation in mice lacking Pten function showed that deletion of *Pik3ca* had little effect on tumour formation, yet deletion of *Pik3cb* dramatically reduced tumour burden [8]. Furthermore, similar conclusions were drawn from work with prostate [9] and breast cancer cell lines [10] although a mechanistic basis for this selective link has yet to be identified. This over-reliance on signalling through PI3K beta in cases of PTEN loss may partly explain the low occurrence of mutations and copy number changes in *PIK3CA* in prostate cancer and, although *PIK3CB*, the gene encoding the beta catalytic subunit of PI3K, is very rarely found to be activated by mutation, rates of *PIK3CB* gene amplification are significant in metastatic prostate cancer (approx. 5%) and particularly high in neuroendocrine prostate cancer (21%). This has influenced some efforts to therapeutically target PI3K/PTEN/TOR signalling in the area of prostate cancer, motivated by the potential to selectively target the beta isoform of PI3K and therefore perhaps have lower drug toxicity with similar efficacy compared to inhibitors of all four PI3K isoforms. On the other hand, the links between loss of PTEN, PIP_3 generated by the beta isoform of PI3K and tumorigenesis seem inconsistent [11, 12]. Additionally, more recently data have been presented showing that when one isoform of PI3K is inhibited, feedback loops can lead to increased activation of other expressed isoforms [13, 14]. Regardless, the real value of these ideas and the drug programmes they have motivated should soon be clear, as drugs specifically targeting PI3K beta have entered clinical prostate cancer trials (GSK2636771 and AZD8186 which inhibits both PI3K beta and delta).

Signalling downstream of PI3K/PTEN

The significance in cellular transformation of signalling through PI3K is also highlighted by the very frequent identification in tumours of genetic changes in upstream signalling components which lead to PI3K activation. The clearest examples of such upstream PI3K activating oncogenes are the RAS GTPases and several Receptor Tyrosine Kinases (RTKs), including the Epidermal Growth Factor

Receptor family (EGFR, HER2/ERBB2/NEU etc.) and the PDGF and KIT receptors. However, the list of PI3K network components which appear to be oncogenic drivers extends further to include less central components such as the E3 ubiquitin ligase CBL, which promotes RTK degradation and INPP4B which metabolises the secondary PI3K product lipid $PI(3,4)P_2$ [2]. As with other cancers, integrated genomic studies of primary and metastatic prostate cancers have consistently identified changes leading to the aberrant activation of PI3K signalling in many of these tumours [1, 15-17].

The PI3K product lipid, PIP_3, promotes cell growth, proliferation, cellular polarisation and other changes through a large and diverse group of downstream effector proteins endowed with a lipid binding domain capable of selectively binding to it. It is unclear how broad this PIP_3-binding proteome is in humans, with estimates ranging from a few tens to over 100 [18-20] but the best understood examples by far are the AKT protein kinases, important oncogenes in their own right. Strong genetic experiments in mice, flies and worms have demonstrated the importance of the AKT kinases in mediating the effects of PI3K activation on cell growth and proliferation [21-25]. Additionally, the identification of numerous confirmed substrates for AKT has provided insight into the molecular mechanisms by which these effects are mediated [25, 26]. Downstream of PI3K and AKT, a major regulator of cell growth which has been repeatedly implicated in tumorigenesis is MTOR (mechanistic target of rapamycin). MTOR is a protein kinase subject to multiple complex regulatory inputs in addition to activation downstream of AKT (*e.g*. nutrients and energy) and plays a key role in regulating cell growth and anabolic metabolism, as well as being involved in AKT activation [27].

On the other hand, the significance of the other regulatory mechanisms downstream of PI3K independent of AKT and MTOR remains largely unclear, particularly in cancer. Notably, gradients of PIP_3 have been identified in the plasma membrane of several cell types. Particular attention has been paid to the dynamic enrichment of PIP_3 seen in the leading edge of axonal growth cones, of mammalian neutrophils and fibroblasts and the slime mould *Dictyostelium discoideum* during directed cell migration. More relevant to the prostate, a stable enrichment of PIP_3 has been seen in the baso-lateral membranes of some polarised epithelial cells [28-32].

The significance of the disturbance of such localised and/or AKT-independent PI3K signalling in prostate, or other, cancers is a key remaining question. Many lines of evidence point to such important AKT-independent oncogenic mechanisms, both through the identification of alternative mechanisms [33-35] and also the characterisation of catalytically active mutants of PTEN found in human tumours which retain the ability to suppress AKT activity yet fail in other cell based assays [36, 37]. There are also numerous reports of discordance between the consequences of AKT activation and either activation of PI3K or loss of PTEN (*e.g*. [34, 36, 38, 39]).

Given the significance in oncogenesis of components which activate signalling through the PI3K network, attention has also fallen on the balancing inhibitory components which act to suppress PI3K signalling. The clearest examples of these inhibitory components are the lipid phosphatases which metabolise the growth promoting signals synthesised by the PI3Ks. SHIP and SHIP2 are phosphoinositide 5-phosphatases which convert PIP_3 into the secondary PI3K lipid product $PI(3,4)P_2$. However, the evidence that the SHIPs represent bona fide tumour suppressors is limited, perhaps due to the existence of redundancy between these and other related enzymes, perhaps because $PI(3,4)P_2$ also activates downstream PI3K effectors including AKT [19, 40]. Related to this point, the phosphoinositide 4-phosphatase encoded by *INPP4B*, which converts PI(3,4)P2 to PI3P, has been shown to act as a tumour suppressor in several tissues by removing $PI(3,4)P_2$ and suppressing AKT activity, yet also play conflicting oncogenic roles in some tumours including melanomas, colon cancers and leukaemias possibly by promoting PI3P-dependent activation of SGK3 [41, 42]. These results highlight the contrast with the best studied phosphoinositide phosphatase however, PTEN,

which acts as a PIP_3 3-phosphatase and has consistently been show to act as a significant tumour suppressor in many organ systems, particularly the prostate.

Notably, multiple genetic changes leading to the activation of the broad PI3K pathway within individual prostate tumours are commonly observed. For example, there seems to be a significant association between PTEN loss and PIK3CA mutation or amplification in prostate adenocarcinoma tumour sets (www.cbioportal.org), with both changes being commonly identified in individual tumours. This is in contrast to the frequently observed mutual exclusivity of different mutational events within other specific functional networks that have been observed in previous studies [43, 44].

PTEN: A HAPLOINSUFFICIENT TUMOUR SUPPRESSOR FUNCTIONALLY OPPOSING PI3K

In 1995, Gray *et al*. showed that loss of region q23-24 on chromosome 10 was a frequent occurrence in prostate cancer (62% of tumours studied) [45] contributing to a body of data showing loss of this region in a number of other tumours [46-48]. In 1997, two groups isolated **P**hosphatase and **Ten**sin homolog deleted on Chromosome 10 (*PTEN* - also referred to as *MMAC1* and *TEP1*) as the tumour suppressor gene on Chr10, identifying coding mutations in this gene in samples from prostate cancer patients as well as glioblastoma, breast and kidney cancer derived samples [49, 50]. Steck *et al*. screened chromosome fragments for their ability to suppress tumorigenic phenotypes and were subsequently able to clone *PTEN*, whereas Li *et al*. focussed on identifying *PTEN* mutations in cancer cell lines and tumours [49, 50]. This tumour suppressor status was further confirmed by the identification of *PTEN* mutations in patients suffering from the familial cancer-syndromes Cowden disease and Bannayan-Riley-Ruvalcaba syndrome [51, 52], although notably, prostate cancers have rarely been described in sufferers of these syndromes [53], which are now often considered together under the umbrella term PTEN hamartoma tumour syndrome (PHTS).

Both groups which identified PTEN commented on its homology to the family of protein tyrosine phosphatases [49, 50], and further publications showed that PTEN had weak dual specificity protein tyrosine and serine/threonine phosphatase activity, with a preference for highly acidic substrates [54, 55]. However, it was demonstrated soon after that PTEN dephosphorylates the 3-position on the inositol ring of the PI3K product lipid, phosphatidylinositol 3,4,5-trisphosphate [56] to regenerate phosphatidylinositol 4,5-bisphosphate ($PI(4,5)P_2$). This reaction is illustrated in Figure 1A. Recombinant PTEN expressed in *E. coli* also dephosphorylates the 3-position on the inositol ring of the related substrates, inositol (1,3,4,5) tetrakisphosphate ($Ins(1,3,4,5)P_4$) (which represents the soluble headgroup of PIP_3) and phosphatidylinositol 3-monophosphate and phosphatidylinositol 3,4-bisphosphate, but at reduced rate [56, 57]. Significantly, strong evidence that it is the lipid phosphatase activity that is critical for the tumour suppressor functions of PTEN was provided by the demonstration that a missense mutation, PTEN G129E, identified in two families suffering from Cowden disease, retains protein phosphatase activity but has greatly reduced lipid phosphatase activity towards PIP_3 and was unable to suppress AKT phosphorylation or cell growth in culture [57, 58]. Intense research since these early studies of PTEN has revealed many details regarding how it fulfils its roles in regulating PI3K signalling and blocking tumorigenesis in many tissues (See reviews *e.g*. [59, 60]). However, major questions remain about the downstream mechanisms by which elevated and mislocalised PIP_3 increases the chances of tumour formation (See above in PI3K section) and also whether PTEN has additional unrelated functions, such as dephosphorylating protein substrates or non-catalytic functions. On this point, the consistent finding that heterozygous mice expressing the stable PTEN G129E mutant develop a worse spectrum of tumours than null

allele heterozygous mice, implies that if PTEN fulfils such PIP_3-independent functions, these additional functions have little effect on their own to suppress tumours in most tissues [61, 62].

In terms of cell biology, PTEN is a relatively small 50kDa enzyme, which associates transiently with the plasma membrane to metabolise its lipid substrate [29, 59, 63]. Additional functions and sites of action for PTEN, such as in the nucleus and on endomembranes, have been proposed but these remain controversial and detailed mechanisms remain to be determined [59, 60, 64, 65]. PTEN binds to many other proteins, several through its extreme C-terminal PDZ binding sequence and it is this binding mode which seems responsible for the localisation of PTEN to adherens junctions in epithelial cells and to neuronal synapses [29, 66-69]. The apical junctional localisation of PTEN in several epithelial tissues has been described, including in the prostate although in most cases this localisation appears as an enrichment, rather than a bold unique localisation [67, 68, 70, 71] (See below in PTEN IHC section).

It seems significant and in contrast to some other tumour suppressors, that the potential impact of even small changes in PTEN expression appears large, given the evidence from clinical data and murine models that PTEN is a haploinsufficient tumour suppressor, with function being strongly dose dependent. Mice with only a single functional *Pten* copy throughout the body develop tumours in a diverse range of organs [72, 73]. Furthermore, elegant studies using a hypomorphic allele, which generates approximately a half dose of normal PTEN protein relative to a wild-type allele, have found that heterozygous mice carrying either a single copy of wild-type *PTEN*, or one hypomorphic allele show prostate cancer initiation and progression which correlates closely with reducing PTEN dose [74] and even animals carrying one wild-type and one hypomorphic copy show increased rates of tumour formation in some organs, particularly the breast [75]. Conversely, over-expression of PTEN in mice leads to a tumour resistant state [76, 77].

PROSTATE CANCER AND PTEN

Prostate cancer is the second most common cancer in the UK (CRUK, 2013), with ~50,000 new cases diagnosed in 2013, and ~10,000 deaths in 2012, making it the second most common cause of death from cancer in men. Age is the most significant risk factor for prostate cancer, with >50% of the cases diagnosed between 2011-2013 in men >70 years of age, although family history is also a risk factor [78]. Treatment of prostate cancer is challenging, because histologically similar tumours give rise to diverse clinical outcomes. At this time, there are no reliable prognostic markers to discriminate indolent tumours from those likely to cause aggressive metastatic disease, and this complicates treatment decisions. Genetically, prostate tumours show inter and intra-tumoural heterogeneity, suggesting multiple genetic changes and environmental factors are involved. Along with surgery and radiotherapy, androgen deprivation therapy is a very common treatment for metastatic prostate cancer, but almost all such cancers eventually become resistant to this treatment and their disease will progress, at this stage being termed castrate-resistant prostate cancer (CRPC).

Around half of primary prostate cancers carry driving gene fusions between androgen regulated promoters and members of the E26 transformation specific (ETS) family of transcription factors, most commonly the *TMPRSS2-ERG* fusion. However, primary tumours are otherwise genetically very diverse [79]. Metastatic tumours show more recurrent changes, with four dominant events each

being present in around half of these tumours: 1) amplifications of the Androgen Receptor (*AR*) gene 2) ETS family gene fusions, again most commonly the *TMPRSS2-ERG* fusion 3) disruption of the *TP53* gene and 4) as will be discussed here, disruption of *PTEN* [17]. The significance to pathology of these changes in PTEN has been confirmed in animal studies, with full genetic deletion of Pten specifically from the developed prostate gland causing rapid high grade Prostate Intraepithelial Neoplasia (PIN) and in some studies an invasive prostate carcinoma within 3-6 months of age [74, 80].

PTEN MUTATIONS IN PROSTATE CANCER

Recent detailed molecular analyses using exome and genome sequencing, array comparative genomic hybridisation (aCGH), *in situ* hybridisation and immunohistochemistry have confirmed early observations that PTEN function is frequently lost in prostate cancer. These studies found copy number alterations, structural rearrangements and/or point mutations to the *PTEN* gene in between 16% and 41% of tumour samples [1, 16, 17, 79, 81-83]. The difference in mutation frequency is likely to reflect, at least in part, the stage of cancers analysed – studies looking at CRPC showed higher levels of mutation, with homozygous loss of *PTEN* being the most common alteration [16, 17]. *PTEN* appears more frequently subject to deletion than point mutation in prostate cancer, and in all studies point mutations (nonsense, missense or insertion/deletion changes) were in the minority. For example detailed genetic analyses of >300 primary prostate tumours, found 2.4% had missense or nonsense mutations, compared to 15% with copy number alterations [79]. Similarly, consultation of the TCGA Research Network showed an overall alteration rate of 20.6% for PTEN from 9 studies, with around three quarters of these changes representing PTEN deletions. These data are summarised in Figures 2A and 2B. In other studies, point mutations were found only combined with heterozygous gene loss [16, 17]. One reason why copy number alteration may be more common is that loss of PTEN appears can occur through a series of "shuffling" events dubbed chromoplexy, where multiple distant loci undergo concurrent rearrangement [15, 81, 84]. This is in keeping with the emerging theme that relative to other cancer types, prostate cancer is driven more by large scale genomic structural rearrangements than by point mutations [84, 85]. Baca *et al.* proposed that PTEN mutations may be a "gating" point for the development of aggressive prostate cancer, based on the subclonal nature of PTEN loss in their tumour set [81]. However, data such as the detection of shared PTEN alterations in multifocal PCa [86], support a role for PTEN loss in prostate cancer initiation, at least in some cases (see below).

DETECTING PTEN CHANGES IN PROSTATE CANCER AND ASSOCIATION WITH DISEASE SEVERITY

In a clinical setting, fluorescence *in situ* hybridisation (FISH) and immunohistochemistry (IHC) are commonly used to assess patient samples for changes in the abundance of PTEN mRNA and protein respectively. Substantial effort has been expended to optimise IHC methods in particular in light of evidence that PTEN expression is regulated post-transcriptionally [87].

Fluorescence *in situ* hybridisation

Estimates of *PTEN* gene loss in prostate cancer by FISH are quite variable, and likely reflect differences in sample cohorts, intra-tumoural heterogeneity, methodology and analyses. For example 2 studies using the same FISH probes but different sample sets found loss of *PTEN* in 68% and 44% of primary prostate carcinomas [88, 89]. More recent 4 colour FISH protocols have been developed – now incorporating probes for regions flanking the PTEN locus as well as for the centromere of ch10 and for *PTEN* [90-92]. This provides information about the size of Ch10 deletions and allows the identification of false positive results due to sectioning artefacts. In an initial study of 330 samples, almost all from patients who had undergone radical prostatectomies, 40% showed *PTEN* loss by FISH [92]. This number was in good agreement with Choucair *et al*. who showed that 41% of primary tumours had heterozygous loss of *PTEN* and that this was associated with a reduced time to recurrence and decreased AR signalling [93]. Studies of more advanced

disease samples have found higher rates of *PTEN* loss, for example 77% of CRPC samples studied by Sircar *et al*., supporting the hypothesis that *PTEN* loss is associated with these more aggressive tumours [90]. In support of this, a later study, from 612 patient tumour samples from radical prostatectomies, ~20% had lost one or both copies of *PTEN*, this was very strongly correlated with increased Gleason score and poorer outcome [91]. Consistent with this picture, a recent meta-analysis and review using data from 7 previous published studies, most discussed here, found a strong correlation of PTEN genomic deletion with both higher Gleason score and increased capsular penetration [94].

Immunohistochemistry

Although some initial analyses of PTEN by immunohistochemistry (IHC) suffered from problems with poor methods and antibodies limiting sensitivity and PTEN specificity, optimisation of method and materials have supported the generation of strong data, much strengthened by controls demonstrating a good correlation of PTEN loss by IHC with reduced PTEN signals at the DNA and mRNA levels [95-98]. By IHC analyses both in published studies and online resources such as the Human Protein Atlas (www.proteinatlas.org), staining for the PTEN protein shows a cytoplasmic and nuclear pattern in the luminal and basal epithelial cells of the prostate, as well as being expressed in other cell types [82]. Reported rates of loss of PTEN staining in prostate cancers have been observed in a range of 15-30% in unselected primary tumours, either from biopsy samples or from radical prostatectomies [82, 99-102]. One common feature to come from this work has been frequently observed intra-tumour heterogeneity, with regions positive and negative for PTEN staining [82, 103, 104] indicating that PTEN loss is often not an early founding event in prostate cancer formation. The lack of evidence for disturbed PTEN function in high grade PIN also argues that PTEN loss may be a later event in prostate cancer development [105].

A very important feature which has been observed is a correlation of PTEN loss as judged by IHC with higher grade tumours as judged by Gleason score [82, 99, 103, 104] and with stage, with higher rates of PTEN loss have being consistently observed in metastatic and in castration resistant cancers [1, 82, 101, 103, 106]. Accordingly, loss of PTEN assessed by IHC has also been independently associated with a variety of poor outcomes, including PSA-measured biochemical recurrence [100], poor clinical outcome [107], and tumours which scored as Gleason grade 6 at tumour biopsy but which were upgraded to Gleason 7 or higher when the prostate gland was removed and analysed [71]. The confirmation of the association of PTEN with lethal progression in a recently published prospective study implies that the association of PTEN loss with aggressive disease is not simply because these tumours have a higher mutational burden and more disrupted genome, but further support the hypothesis that loss of PTEN is not usually an initiating even in prostate cancer, but rather is most frequently a later event which worsens patient outcome [108].

Is PTEN protein expression often reduced in tumours retaining normal PTEN mRNA?

Where reported together, assessment of the *PTEN* gene by FISH and protein by IHC from tumour biopsies have showed good correlation in many but not all cases and this finding has been used to provide confidence in the presented data. For example, Han *et al*. reported that all but 1 of the 21 tumours that had *PTEN* deletion by FISH showed reduced PTEN staining by IHC [109]. More recently, similar results were reported by Lotan *et al*. and Murphy *et al*. [71, 110]. However, a strong correlation between gene and protein analysis should not always be expected and a clear possibility, with some support, is that PTEN protein levels are suppressed in prostate tumours retaining wild-type copies of the *PTEN* gene, due to changes at one or more steps in the gene expression pipeline. Accordingly, it is common for samples to show a positive FISH result and a negative/reduced PTEN IHC, and much less common to have IHC positivity reported in cells carrying *PTEN* gene loss as assessed by FISH [99, 109, 111, 112].

PTEN function is known to be regulated at the transcriptional, post-transcriptional and post-translational levels through diverse mechanisms both physiologically and in disease [87]. In many cases, the significance of these regulatory mechanisms to the loss of PTEN function during carcinogenesis is still emerging. Examples of reduced PTEN transcription in prostate cancer by changes in transcription factor networks have been described [113, 114] and in other cancers by promoter methylation [115, 116], although there is little evidence for this in prostate cancer. However, the most recent attention in this area has fallen on changes in the micro-RNA networks which are able to suppress PTEN expression. Good evidence for a role of miRNA in regulating PTEN in prostate cancer exists [117-120] and a number of miRNAs, representing candidate oncogenic miRNAs, or oncomiRs, were shown to bind the 3' UTR of *PTEN* [117, 119, 121]. An added layer of complexity in the post-transcriptional regulation of PTEN has come with its use as an example in work establishing a novel mechanism controlling gene expression. Several mRNAs, including the PTEN pseudogene *PTENP1*, contain similar miRNA binding sites to PTEN and these have been referred to as competing endogenous RNA [122]. Depletion of *PTENP1* or other competing transcripts is proposed to free-up more miRNA, allowing it to bind to the PTEN mRNA and experimentally led to a reduction in PTEN protein levels [120, 121]. Similarly and in support of this model, *PTENP1* has been found to undergo genomic copy number loss in a small percentage of sporadic colon cancers [120]. A more recent study also identified a number of long non-coding RNAs from clinical data as candidate tumour suppressors in prostate cancer, which were shown to be able to act as such miRNA-competing 'sponges' and the knock down of which reduced PTEN expression and increased proliferation in the DU145 prostate cell line [118]. These studies add to the great complexity found in mRNA gene expression data and leave the challenge of dissecting the relative significance of individual changes observed in tumour miRNA signatures and telling oncogenic drivers from passengers.

It seems important that in these and other studies, functional loss of PTEN would not be detected if only FISH and/or sequencing are performed, without IHC. Therefore strong well validated IHC seems likely to provide the best assessment of PTEN function. PTEN IHC appears reliable and is a promising way to identify patients incorrectly presumed as low risk who may be prone to disease progression, a conclusion recently strengthened by a large prospective study [108]. The fact that PTEN is regulated at many levels beyond transcription may also means that studies that have used DNA analyses to look at PTEN loss in prostate tumours may have underestimated the frequency at which PTEN is lost in prostate cancer.

In Figure 3 and Table I, we present a summary diagram showing mean percentage frequencies of reported PTEN loss assessed by different technologies in a large number of independent studies which contain data addressing the frequencies of PTEN loss and reduction in prostate cancer cohorts. This diagram is clearly a simplification, as side-by-side it presents data from many studies using somewhat different methodologies and of patient groups, ignoring many specific features of the studies included. However, the overall pattern of data is compelling, illustrating the higher rates of loss observed in metastatic/CRPC patients. Also, the occurrence of undetectable PTEN expression by IHC appears consistently higher than the apparent rates of homozygous loss by FISH and other genetic assessments, giving further support to the proposal (see above) that PTEN protein levels in prostate cancer are often reduced in tumour cells retaining healthy levels of wild-type PTEN mRNA [111]. This is in notable contrast to some other cancers where loss is usually at the level of mRNA, *e.g*. breast cancer [123].

As a final note in consideration of methods to detect genetic changes in *PTEN*, progress is being made in detecting tumour specific gene copy number changes and mutations in cell-free DNA in blood and urine, which is a challenge given the large background of un-mutated DNA from other tissues [124, 125]. The possibility for prostate cancer patients of tumour characterisation and

prognosis from non-invasive liquid biopsy is appealing, yet the detection sensitivities for free variant DNA must be improved before these techniques can be applied to most patients.

PTEN AS A COMPONENT OF PCA BIOMARKER SIGNATURES

A challenge in the field has been to find prognostic markers, in the face of inter and intra-tumoural heterogeneity (especially in patients presenting with lower Gleason grade tumours ~7). A major specific goal is to predict patients who will experience rapid disease progression, distinguishing them from patients with disease which will remain indolent. Furthermore, this would be useful if it could be performed on initial biopsy samples, and not be impacted by the treatment regime of the patients. In addition to the studies discussed above which have selected PTEN to test its utility as a prognostic biomarker, other studies have undertaken to identify markers associated with disease severity taking a global unbiased approach and in some cases have identified PTEN loss as a component of aggressive disease signatures (*e.g*. [1, 126, 127]). Of these global studies, the majority look for signatures of aggressive disease – one notable exception was the work of Irshad *et al*., which identified an "indolence signature" [128]. It will take time for the identification of these signatures to have an impact on therapeutic decisions for a large number of patients. PTEN has been suggested as a biomarker in several further studies - for example changes in expression of a combination of PTEN, SMAD4, SPP1 and Cyclin D1 and was found to have prognostic value superior to Gleason scores alone [129]. Unfortunately, this promising initial finding did not extend to a different cohort [130]. Liu *et al*. found that copy number changes in *PTEN* and *MYC* provided additional prognostic information over and above standard analyses, and both were over-represented in lethal prostate cancer. These findings were also extended to two other cohorts, which had lower death rates during the studies [127].

DRUGS TO TREAT PROSTATE CANCERS LACKING PTEN

One notable feature of the PI3K/PTEN signalling pathway is its reliance on protein and lipid kinases, which are enzyme groups with a good track record of druggability. Therefore, interest in the signalling network has been motivated not only by its causal role driving tumorigenesis, but by the success of programmes to develop drugs to inhibit these different lipid and protein kinases, many of which have been, or are being, tested in prostate cancer patients. The strategies and progress of drug programmes targeting the PI3K-pathway and components downstream including TOR has been reviewed elsewhere [131-133], so this will not be covered in great detail here. To summarise briefly, rapamycin/sirolimus and other related allosteric inhibitors of the MTOR complex 1 (TORC1) protein kinase complex have been approved as immunosuppressants and to treat renal cell carcinoma for some years. Several different class I PI3Ks inhibitors have progressed to phase III clinical trials, with "pan PI3K inhibitors" which inhibit all 4 PI3K isoforms (e.g. BKM120) being the furthest progressed. One PI3K inhibitor has been approved, Idelalisib, a PI3K delta selective inhibitor, developed to treat chronic lymphocytic leukaemia due to the specific biology of this disease [134].

Additionally, inhibitors of the AKT protein kinases are in phase II trials and active site inhibitors of the MTOR kinase and even of the upstream kinase PDK1 are also in clinical and pre-clinical development respectively. Several clinical trials of inhibitors of TOR (specifically MTOR kinase complex 1) and PI3K in prostate cancer patients have achieved very poor response rates as monotherapy (*e.g*. [135]) and efforts are now testing combination therapies which include PI3K-MTOR inhibitors, a strategy which has recently yielded some success in other solid tumours [134, 136]. A key point here is that many of these drugs targeting the PI3K pathway do appear to successfully and relatively selectively inhibit their target *in vivo*. This provides hope that successful applications for them in the treatment of prostate cancer may be identified, but also highlights the difficulties of achieving lasting tumour regression through the inhibition of the complex and dynamic

signalling networks which promote mitogenesis and growth [137]. It is noteworthy that in most of these trials, a small minority of patients did respond to treatment, yet no rationale and predictive biomarkers have yet emerged which would allow them to be selected in advance. Some successes in other cancers (*e.g*. data indicating that TSC1 mutations predict response to rapamycin/everolimus in bladder cancer [138]) provides hope for this work which seems likely to be crucial for the successful targeting of PI3K/PTEN signalling in prostate cancer.

As described earlier in the article (Heading: "Does signalling through the beta isoform of PI3K selectively drive prostate tumorigenesis?"), one specific feature of efforts to therapeutically target PI3K/PTEN/TOR signalling that has received particular attention in the area of prostate cancer has been the potential to selectively target the beta isoform of PI3K and perhaps minimise drug toxicity. There are arguments for and against this strategy [139], but ongoing clinical trials of the PI3K inhibitors with some selectivity for the beta isoform (GSK2636771 and AZD8186 which inhibits both PI3K beta and delta) should begin to answer these questions.

REGULATORY SYSTEMS INTERACTING WITH PI3K/PTEN

At the molecular level, several proteins which directly and robustly interact with PTEN are found to be altered themselves in prostate cancer. Amplification of the recognised PTEN inhibitor and RAC1 activator, PREX2, has been observed as well as (uncommon) deletion of the PTEN scaffold PAR3 ([140, 141] and www.cbioportal.org). Additionally, potential crosstalk with other regulatory systems with established roles in oncogenesis is provided in reports that PTEN can directly interact with other major tumour suppressors and oncogenes, such as p53, beta-catenin and SRC [142-144]. On the other hand, the significance of these regulatory influences on, or by, PTEN remains poorly understood.

At a higher level, signalling through the PI3K network influences major cellular processes such as cell cycle entry and protein synthesis providing outputs that are integrated with those from other semi-independent functional networks. As discussed, examples of such pathways are also frequently dysregulated in prostate cancer, *e.g*. programmes of transcriptional regulation by the Androgen Receptor, TP53 and by ETS family transcription factors. The consequences of such pathway integration and cross-talk generally appear strongly context dependent and hard to study and predict. However, with strong relevance to prostate cancer development, multiple mechanisms of bi-directional negative feedback have been observed between AR and PI3K/PTEN signalling, the disruption of which is required for the strong activation of both PI3K and AR pathways observed in some aggressive tumours [14, 145-147].

CONCLUDING REMARKS

The role of PTEN as a suppressor of prostate cancer and the association of its loss with aggressive disease are well established and increasing numbers of drugs are available to target different points in the PTEN/PI3K/TOR signalling network. However, these agents have so far had limited success as monotherapies and progress has been slow to develop combination therapies and to develop predictive biomarkers to match drugs with patients who will respond. The first significant impact of our understanding of signalling via PTEN/PI3K in terms of clinical impact may be the use of PTEN functional assessment as a prognostic biomarker**.**

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	primary tumours					metastatic/CRPC					p-value
Method	**%**	**n**	**het/%**	**hom/%**	**Ref**	**%**	**n**	**het/%**	**hom/%**	**Ref**	
FISH	**17**	251	**10**	**7**	[109]	**53.7**	41	**29.3**	**24.4**	[109]	0.1721 (het)
	23	57			[106]	**52**	57			[106]	0.0001 (hom)
	40	330	**24.8**	**15.2**	[92]	**62**	32	**38**	**34**	[92]	
	40.7	118	**18.6**	**22.1**	[148]	**44.9**	49	**12.2**	**32.7**	[148]	
	43.9	107	**39.3**	**4.6**	[88]	**90**	10	**60**	**30**	[88]	
	14.9	643	**2.8**	**12.1**	[99]*	**40.5**	37	**18.9**	**21.6**	[149]*	
	17.4	322			[150]	**63.6**	55	**27.3**	**36.3**	[151]	
	18.3	612	**9**	**9.3**	[91]	**76.8**	56	**33.9**	**42.9**	[90]	
	19.7	2131	**8.1**	**12.1**	[152]						
	21.8	339	**9.4**	**12.4**	[153]						
	22.4	3756	**8**	**14.4**	[154]						
	30.9	97	**17.5**	**13.4**	[110]						
	32.6	187	**5.3**	**27.3**	[112]						
	41.7	134	**31.3**	**10.4**	[155]						
	41.9	43	**41.9**	**0**	[93]						
	68.6	35	**62.9**	**5.7**	[89]						
IHC	**15**	282			[101]	**45**	122			[101]	0.0056
	38	308			[82]	**48**	50			[82]	
	55.2	58			[156]	**68.8**	15			[156]	
	11.5	174			[71]	**60.5**	38			[149]*	
	17.6	675			[99]*	**78.9**	19			[157]	
	20.2	109			[103]						
	21.5	65			[158]						
	25	1044			[108]						
	27.2	103			[100]						
	31	357			[159]	**40**	357			[159]	
	33	107			[110]*						
	33.1	118			[160]						
	35.1	316			[153]						
	36	194			[104]						
	61	57			[161]						
Other genomic	**16**	32			[162]	**33.3**	6			[162]	0.0003
	21	181			[1]	**42**	37			[1]	
	23	112			[163]	**59.5**	42			[163]	
	11.8	51			[83]	**33.3**	81			[164]	
	16	57			[81]	**40**	150			[17]	
	16.7	60			[165]	**44.4**	18			[166]	
	17	333			[79]	**47**	50			[16]	
	17.5	40			[167]						
	23	126			[110]*						
	24	112			[168]						
	28	55			[169]						
	35	40			[170]						
	38.4	125			[127]						

Table 1

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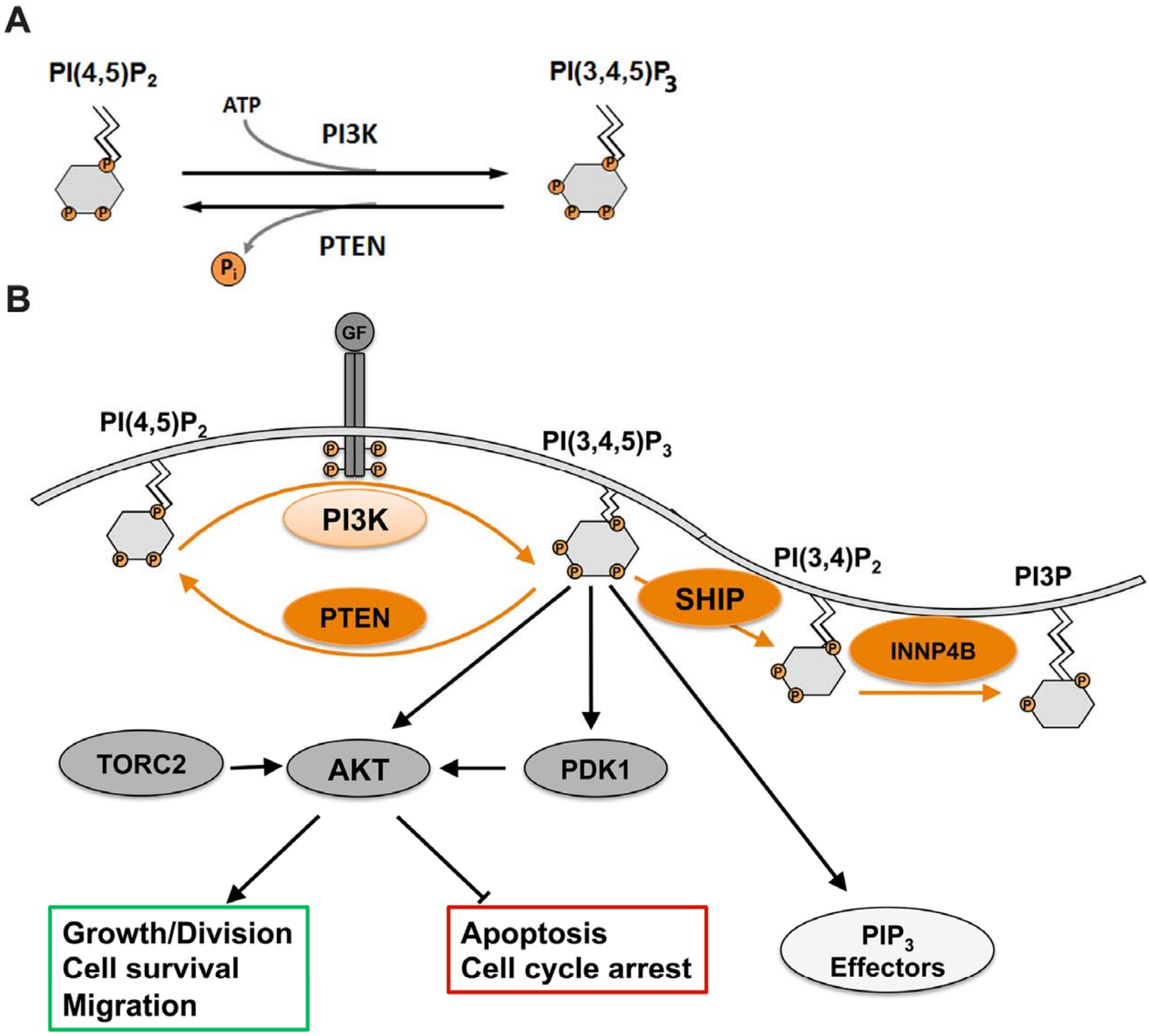
Figure Legends

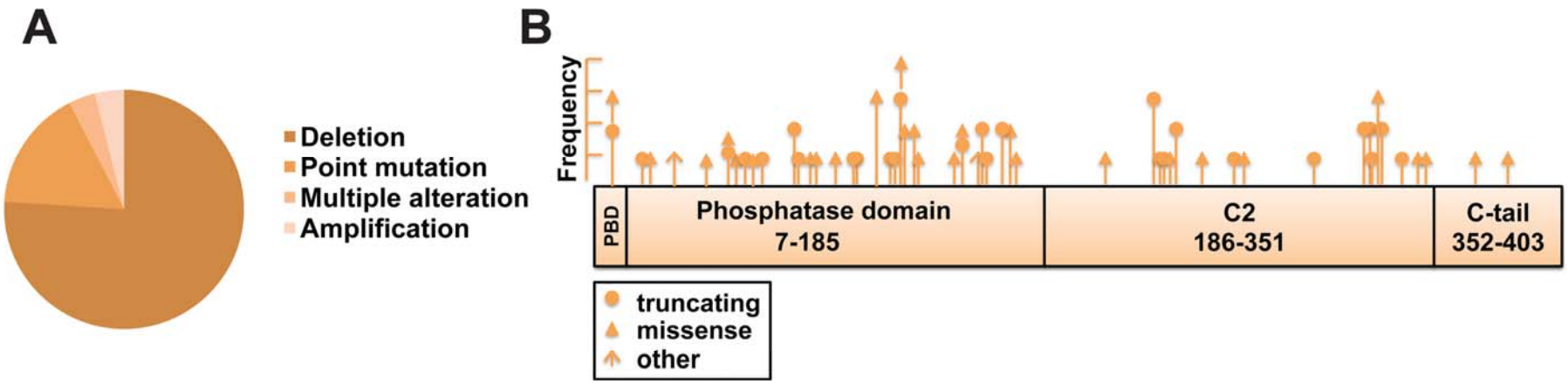
Figure 1 PI3K/PTEN pathway signalling
A. The reactions catalyzed by the phosphoinositide lipid kinase PI3K that is antagonized by the lipid phosphatase PTEN.
B. A hypothetical model for the working of the PI3K signalling network is represented. Lipid kinases are represented by light orange ovals, lipid phosphatases dark orange, protein kinases are shaded grey. Phosphoinositide lipids are also labeled. Orange reaction arrows indicate conversion of lipid species, and black arrows represent functional interactions. PIP_3 effectors are also included representing the large PIP_3-binding proteome.

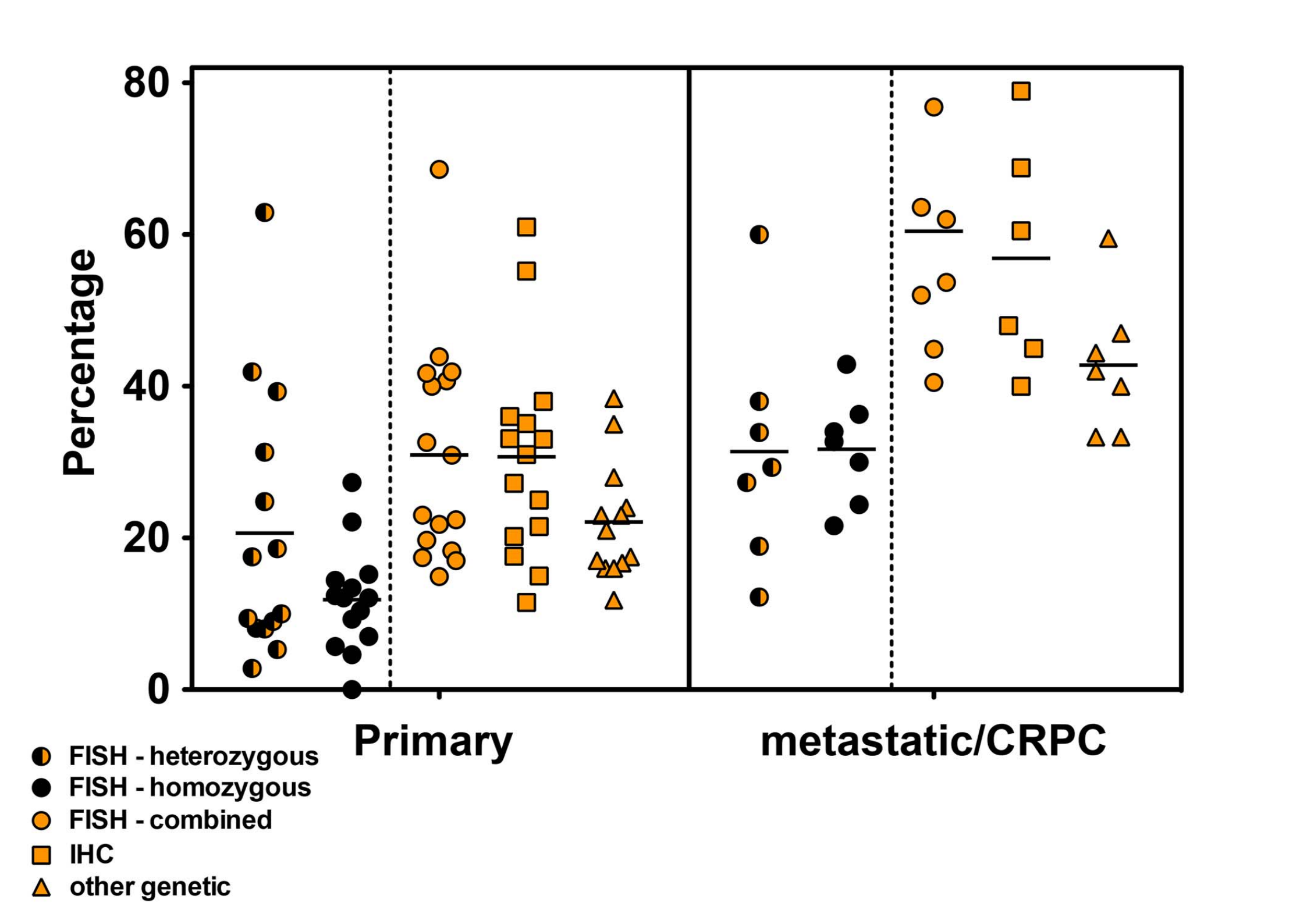
Figure 2 Classes and locations of PTEN alterations in prostate cancer.
A. PTEN is primarily altered by deletion in prostate cancer. The data used to generate this figure are derived from the TCGA Research Network (http://cancergenome.nih.gov/.). 9 prostate cancer studies were selected, and the total number of PTEN mutations was pooled (279 mutations, from 1355 samples, giving a PTEN alteration rate of 20.6%). These 279 alterations were classified into deletion, mutation, multiple alteration and amplification.
B. The site of PTEN mutations mapped onto the PTEN domain structure.
The data used to generate this figure are derived from the TCGA Research Network (http://cancergenome.nih.gov) and COSMIC (cancer.sanger.ac.uk [171]). Mutations are mapped onto the primary structure of PTEN, and classified according to whether they are truncating (including nonsense and frameshift mutations), missense or other (*e.g*. in frame deletion). In several cases, multiple mutations are present at a single amino acid in different patient samples, and this is depicted by the height of the bars.

Figure 3 A comparison of PTEN alteration frequencies in primary tumours and CRPC/metastatic prostate cancer. The percentages of samples displaying loss of PTEN detected by Immunohistochemistry (IHC), Fluorescent *in situ* Hybridisation (FISH), or other genetic methods (*e.g*. Sequencing, array CGH) in individual studies of primary and CRPC/metastatic prostate cancers is shown, along with the mean percentage from these aggregated studies. Where possible, data for loss of *PTEN* detected by FISH have been separated to display heterozygous and homozygous loss, along with the combined number for each.

Table 1 Detailed analysis for Figure 3. The percentage of samples with altered PTEN and total sample number (n) are shown for each study, grouped by technique used and by primary or CRPC/metastatic disease (for primary disease, PIN was not included, and tumours were not sorted according to stage in our analyses). Where the same study reported PTEN alterations in primary and CRPC/metastatic disease, the alteration frequency and sample size appears in adjacent columns. Where the same study used and compared different methods *e.g.* FISH and IHC, this is indicated by a * by the reference.
The scoring criteria were as follows:
FISH analysis – counted as loss initially, then sorted into heterozygous or homozygous loss.
IHC – scored according to the paper criteria. Where possible, reduced/weak was included as loss, along with absent, but this was not always possible.
Other genomic – including sequencing, PCR, microsatellite analyses, array CGH.
A Student's t-test was used to compare the primary and CRPC/metastatic data, and the p-values are shown.


A
PI(4,5)P2
ATP
PI3K
PI(3,4,5)P3
PTEN
Pi
B
GF
PI(4,5)P2
PI(3,4,5)P3
PI3K
PTEN
PI(3,4)P2
PI3P
SHIP
INNP4B
TORC2
AKT
PDK1
Growth/Division
Cell survival
Migration
Apoptosis
Cell cycle arrest
PIP3
Effectors


A
Deletion
Point mutation
Multiple alteration
Amplification
B
Frequency
PBD
Phosphatase domain
7-185
C2
186-351
C-tail
352-403
truncating
missense
other


80
60
40
20
0
Percentage
Primary
metastatic/CRPC
FISH - heterozygous
FISH - homozygous
FISH - combined
IHC
other genetic