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Review Article

Creating a potential diagnostic for prostate cancer risk stratification (InformMDx™) by translating novel scientific discoveries concerning cAMP degrading phosphodiesterase-4D7 (PDE4D7)

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Increased PSA-based screening for prostate cancer has resulted in a growing number of diagnosed cases. However, around half of these are 'indolent', neither metastasizing nor leading to disease specific death. Treating non-progressing tumours with invasive therapies is currently regarded as unnecessary over-treatment with patients being considered for conservative regimens, such as active surveillance (AS). However, this raises both compliance and protocol issues. Great clinical benefit could accrue from a biomarker able to predict long-term patient outcome accurately at the time of biopsy and initial diagnosis. Here we delineate the translation of a laboratory discovery through to the precision development of a clinically validated, novel prognostic biomarker assay (InformMDx™). This centres on determining transcript levels for phosphodiesterase-4D7 (PDE4D7), an enzyme that breaks down cyclic AMP, a signalling molecule intimately connected with proliferation and androgen receptor function. Quantifiable detection of PDE4D7 mRNA transcripts informs on the longitudinal outcome of post-surgical disease progression. The risk of post-surgical progression increases steeply for patients with very low 'PDE4D7 scores', while risk decreases markedly for those patients with very high 'PDE4D7 scores'. Combining clinical risk variables, such as the Gleason or CAPRA (Cancer of the Prostate Risk Assessment) score, with the 'PDE4D7 score' further enhances the prognostic power of this personalized, precision assessment. Thus the 'PDE4D7 score' has the potential to define, more effectively, appropriate medical intervention/AS strategies for individual prostate cancer patients.

Prostate cancer

Prostate cancer is, worldwide, one of the most frequently diagnosed cancers in men above the age of 50 [1]. Its incidence is apparently increasing due to both a growing awareness of its occurrence in the elderly, coupled with new possibilities for its early detection in asymptomatic phases [2]. When confined to the prostate, invasive curative treatment by either surgical removal (radical prostatectomy) or radiotherapy (brachytherapy or external beam) has excellent long-term results, with disease specific mortality <10% in 10 years. Effectiveness is primarily dependent on the biologic aggressiveness of the tumour, expressed as the Gleason grading, and on the local tumour stage. Low grade disease (Gleason score 3 + 3 = 6 or 3 + 4 = 7 without cribriform patterns) [3] has a low growth rate and an extremely low risk of metastasis. Though successful with regard to tumour control, invasive therapies can incur serious side effects such

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as incontinence, erectile dysfunction, radiation proctitis and urethral stenosis. These are evident in some 10–30% of patients [4], incurring a major effect on Quality of Life. Thus, the risk of side effects from invasive therapy has to be balanced against the potential gain in long-term survival.

Increased PSA-based screening has resulted in a growing number of diagnosed low-risk cancers. However, many of these are regarded as 'indolent', are not expected to metastasize and are unlikely to generate tumour-related symptoms during life [5]. In a population-based screening setting, these account for as many as 50% of all diagnosed cancers [6]. Treating such asymptomatic tumours with invasive therapies can be regarded as unnecessary over-treatment. Current, widely accepted guidelines propose actively following such patients up to their eighth decade, only offering invasive therapy where altered clinical signs suggest a change of biological behaviour from low to a higher risk of future symptomatic disease. Clinically refraining from, or delaying, invasive therapy is called active surveillance (AS). It is offered in order to protect from any misinterpretation of the initial low risk tumour classification and detect any altered growth towards a clinically more aggressive tumour status [7]. The criteria for reclassification towards a higher risk follow the clinical experience of decades of tumour treatment where grading of Gleason Score 4 + 3 = 7 and a larger tumour volume are considered justification for invasive therapy to prevent later metastases. However, apart from phenotypic clinical parameters, there is currently no validated prognostic biologic marker that represents an early molecular signature able to signify an adverse course of a prostatic tumour. This is a major deficiency, as tumour reclassification occurs in 40–50% of patients within 5 years of their starting on AS [8]. Currently, it is difficult to predict effectively the clinical and biological behaviour of a tumour at the time of diagnosis. While MRI imaging aids performing targeted biopsies in visible prostatic lesions, there is no clear imaging marker that can identify adverse progression of such lesions [9]. Thus, clinicians and patients rely on histological analysis of repetitive biopsies. This has compliance issues as it would appear that, both patients and physicians, tend to avoid biopsies over time, notwithstanding their utility [10].

Current AS approaches, with a variety of protocols, lack sufficient follow-up to select; currently, an optimal strategy for the safe, long-term surveillance of diagnosed prostate cancers. Thus, it would clearly be of great clinical benefit to have an accurate biomarker able to predict the long-term outcome of cancers at the initial time of diagnosis and biopsy. Such a novel biomarker would, undoubtedly, serve to reduce the number of men that shift towards invasive therapy during the first 3–4 years of follow-up. In such a situation, a stratifying biomarker that identified a very low risk group would be as important as one that indicated adverse progression. Stratification to a very low risk group offers potential for heightened compliance by adjusting follow-up methods to those with much fewer invasive biopsies to perform over time. Furthermore, a biomarker that also highlighted signalling pathways relevant to future tumour behaviour might provide a companion diagnostic for developing novel, targeted therapies.

Cyclic AMP phosphodiesterases

Cyclic 3′-5′ adenosine monophosphate (cAMP) was the first 'second messenger' to be described. It serves as the intracellular manifestation of the binding of various hormones and neurotransmitters ('first messengers') to plasma membrane-located receptors that are able to elicit increased cAMP generation through the activation of adenylyl cyclase via coupling to the GTP-binding protein, G_s [11,12]. Cell type specificity for particular first messengers thus depends upon the types of G_s-coupled receptors that are expressed. The form of the cAMP signal (magnitude and sustainability) is influenced by (i) the efficiency of receptor coupling to G_s, (ii) long- and short-term desensitization processes acting at the receptor, (iii) the particular adenylyl cyclase isoform(s) expressed, (iv) signals acting on adenylyl cyclase through the inhibitory G-protein (G_i) nexus and, of course (v) degradation of cAMP through the action of cyclic nucleotide phosphodiesterases (PDEs) (Figure 1A) [13–15]. cAMP then exerts its action through binding two main effector systems, namely, protein kinase A (PKA) whose activation engenders phosphorylation of target proteins [11,12] and also through cAMP-binding Epac proteins that activate the 'mini' G-proteins, Rap1/2 [16].

As PDEs provide the sole route for intracellular cAMP degradation and signal termination, they are poised to play a pivotal role in regulating intracellular cAMP signalling. Activation of PDE enzymes leads to rapid signal termination and desensitization, while their inhibition will confer either sustained signalling or even initiate signalling through cAMP. Indeed, while manipulation of PDE activity provides a proven therapeutic approach, aberrant PDE activity caused by genetically encoded mutations can underpin the underlying molecular pathology in various diseases.

The PDE superfamily consists of three cAMP specific (PDE4/7/8) members, three cGMP specific (PDE5/6/9) members and five members that degrade both cAMP and cGMP (PDE1/2/3/10/11) [15,17–19]. While their catalytic units show conservation, they are sufficiently different to allow selective inhibitors for each family to be generated. These include PDE5-specific inhibitors, such as Viagra® (erectile dysfunction); PDE3 inhibitors, e.g.

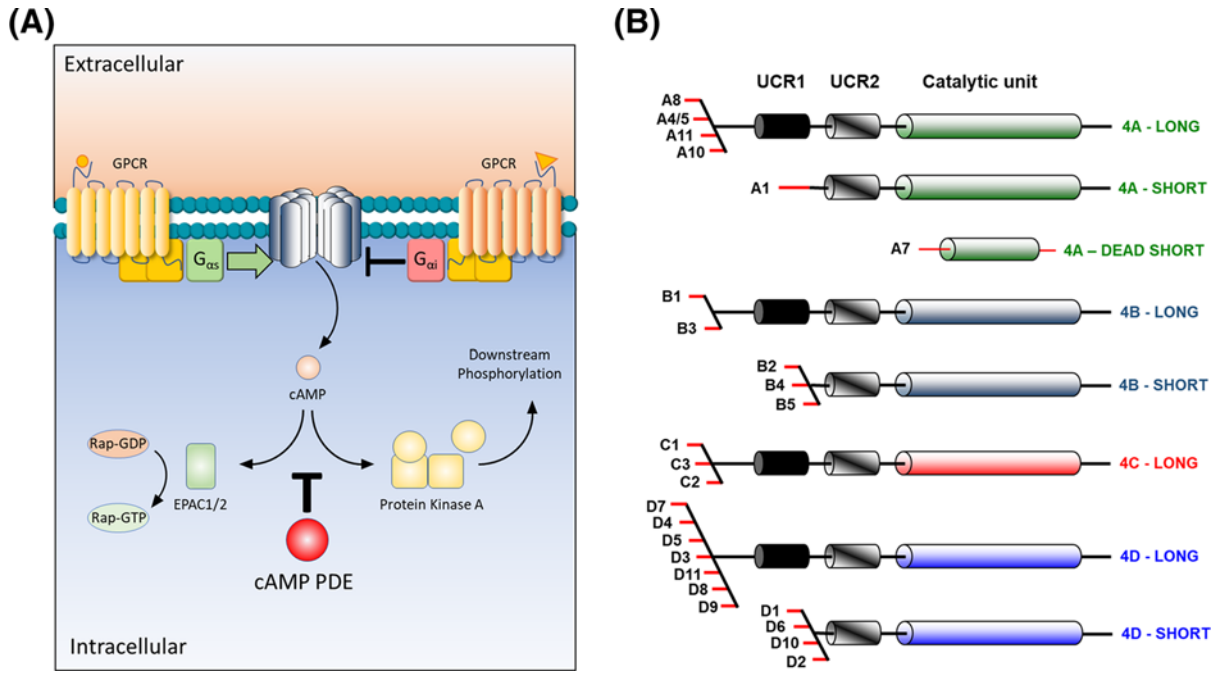

Figure 1. PDE4 isoforms and cAMP signalling cascades

(**A**) A schematic representation of PKA and EPAC effector protein mediated cAMP signalling cascades. The synthesis of cAMP by the various adenylyl cyclase isoforms is influenced by the efficiency of GPCR coupling to stimulatory G_s or inhibitory G_i G-protein α-subunits. The binding of cAMP to the regulatory subunits of PKA, or cAMP-binding domain of EPAC proteins, then leads to the activation of downstream signalling events. The cAMP signal is constrained, or terminated, by the degradation of the cyclic nucleotide through the action of cAMP phosphodiesterases (PDEs) such as PDE4 isoform variants. These isoforms exhibit distinct subcellular localization patterns and regulate discreet pools of cAMP and compartmentalized signalling events. (**B**) A schematic of PDE4 gene family and isoform structure. Four genes (PDE4A, green; PDE4B, gray; PDE4C, red; PDE4D, blue) encode multiple isoform variants each of which are transcribed from independent promoter elements and express unique N-terminal regions. PDE4 isoforms can be separated into groups based upon their conserved domain structure where 'long' form PDE4 enzymes encode both the UCR1 and UCR2 regulatory regions and 'short' isoforms express only UCR2, or a subsection thereof, in addition to the core catalytic domain.

cilostamide (intermittent claudication) and PDE4 inhibitors as anti-inflammatory agents (e.g. roflumilast/Daxas®, COPD; apremilast/Otezla®, psoriasis) and as cognitive enhancers.

There has been much interest in the PDE4 family because of its potential as a therapeutic target [20], that mutations in PDE4D are associated with acrodysostosis [21] and that *PDE4* gene linkages are associated with tumour development including prostate cancer (PDE4D) [22], psychiatric illness (PDE4B) [23] and stroke (PDE4D) [24]. Furthermore, PDE4 members play critical cellular roles in underpinning cAMP signalling compartmentalization within cells [13]. This allows particular PDEs to control specific cellular processes by forming and shaping gradients of cAMP around anchored cAMP effectors (PKA/Epac) to regulate their functioning. Additionally, PDE4 enzymes provide nodes for integrating cross-talk with other signalling pathways via altered functioning elicited by post-translational modification e.g. multisite phosphorylation, ubiquitination and SUMOylation triggered by other signalling pathways [13,25].

Four PDE4 genes (A/B/C/D) encode over 20 distinct isoforms via distinct promoters coupled with alternative mRNA splicing (Figure 1B) [25]. Each isoform is characterized by a unique N-terminal region that, invariably, contains a 'zip-code' allowing isoform targeting to unique intracellular locations/complexes where it regulates local cAMP levels. This allows particular isoforms to control specific intracellular processes [13,26]. Such gradients can be identified using FRET-based, genetically encoded cAMP sensors [27] and the functionality of anchored PDE4 species interrogated by dominant negative approaches and disruption of anchoring [13].

Relatively little, however, is known about the processes and the intronic transcriptional modules underpinning the cell type-specific expression patterns of the various PDE4 isoforms. However, certain isoforms have been shown to

Clinical Science (2019) **133** 269–286
https://doi.org/10.1042/CS20180519

have CRE loci within intronic promoters that provide a feedback regulatory system by up-regulating the expression of such PDE4 isoforms consequent to elevated cAMP levels causing CREB phosphorylation [28,29]. Conversely, a novel cAMP-mediated transcriptional down-regulation of the PDE4A10 isoform occurs through a process involving the transcription factor, ICER (inducible cAMP early repressor) [30]. More studies are required to understand the sophisticated array of processes that allow cell-type specific regulation of PDE4 isoform expression, dynamic reprogramming through various cell signalling processes, alterations in disease and intronic mutations that engender pathological changes.

The catalytic unit of PDE4 isoforms comprises some 315 amino acids, which form three distinct subdomains and is highly conserved between all subfamilies [25]. The catalytic site itself is located at the junction of these subdomains with a deep substrate-binding cleft extending into subdomain-3. The nature of the catalytic site has allowed for the generation of highly selective PDE4 inhibitors, while the similarities between PDE4 subfamilies have compromised the generation of PDE4 subfamily specific inhibitors [20,25]. Attempts to generate PDE4 subfamily specific inhibitors are also complicated by the fact that the structure of full-length PDE4 isoforms in cells can be affected by post-translational modifications and interaction with partner proteins. Thus, 'physiological structures' will undoubtedly differ considerably from those of the purified truncated catalytic unit constructs invariably used in drug development programmes.

In vivo, the functioning and conformation of the catalytic unit is determined by two highly conserved regulatory units located at N-terminal of the catalytic unit (Figure 1B) [20,25]. These are the 60-amino acid UCR1 and the 80-amino acid UCR2 that are found in PDE4 'long' isoforms, where they interact with each other, providing a module that mediates the action of regulatory multisite phosphorylation and drives dimerization [31–33]. In contrast, 'short' PDE4 isoforms lack UCR1 and, as a consequence are monomeric, and demonstrate different regulatory phosphorylation responses [31].

PDE4 isoforms are universally expressed and have undergone evolutionary expansion to provide a library of sophisticated regulatory species serving specific roles in different cell types. As such, alterations in their activity, expression, regulation and targeting in health and disease may lead to marked functional consequences. Here we discuss how following transcript levels of the long PDE4D7 isoform provides a novel prognostic indicator to select the optimal strategy for a long-term surveillance of diagnosed cancers of the prostate.

cAMP and prostate cancer

cAMP-mediated signalling pathways were first implicated as playing a role in prostate cancer when Shima et al. [34] reported a differential expression of adenylyl cyclase in rat prostate lesions. Since that time cAMP signalling has been shown to transactivate the androgen receptor, regulate androgen sensitive transcription and underpin neuroendocrine differentiation in the prostate. Furthermore, the principle cAMP effector proteins, namely protein kinase A (PKA) [35–37] and EPAC1/2 [38], have been directly implicated in aberrant molecular signalling pathways that support the growth of cancerous lesions.

Protein kinase A

PKA is the archetypal effector protein kinase that is directly activated by cAMP and underpins many of the actions linking cAMP signalling to the progression of prostate cancer [39]. PKA is a heterotetrameric enzyme composed of two regulatory subunits (R) and two catalytic kinase subunits (C), where the regulatory subunits, either RIα/RIβ/RIIα/RIIβ, bind directly to the cognate catalytic subunits, either Cα/Cβ/Cγ, which they constitutively inhibit. Binding of cAMP to the R-subunits in this complex releases the activated catalytic unit, which then goes on to phosphorylate various target proteins that are expressed in a cell-type specific manner. The regulation of these signalling events is underpinned by the compartmentalization of cAMP nanodomains by cAMP degrading PDEs coupled with the targeted localization of PKA through interaction with anchor proteins [11–13,26,27]. Changes in cellular PKA distribution, expression and activity have all been shown to contribute to the aberrant proliferation of prostatic tissue [39]. Indeed, the up-regulation of PKA-Cβ2 has been connected with aberrant proliferation in prostate cancer, while altered Cβ3 and Cβ4 expression influences neuroendocrine differentiation, key regulating factors in prostate cancer progression [40]. The up-regulation of PKA RI sub-units has also been associated with aggressive prostate cancer [41].

The more recently discovered EPAC proteins provide a second group of cAMP effector proteins. These bind cAMP to undergo a conformational change that facilitates their role as GEFs for the activation of GTPases Rap1 and Rap2 [16,38]. Evaluation of EPAC proteins in prostate cancer progression is in its infancy. However, EPAC1 can potentiate

cell proliferation and regulate cellular adhesion [16,42,43] and, Rap1 is associated with metastatic potential in the prostate [44] and mediates the synergism between cAMP and EGF signalling in prostate cancer [45].

The convergence of androgen and cAMP signalling

Androgen signalling is central to the initial proliferative drive and the outgrowth of prostate tumours, and is therefore targeted in the mainstay prostate cancer treatments [46]. However, cAMP signalling is closely tied to androgen receptor activity, not only modulating androgen synthesis by mediating the downstream signalling events associated with the action of luteinizing hormone, but also by impacting on androgen receptor signalling itself. Furthermore, cAMP plays a role in regulating ligand-independent activation of the androgen receptor that occurs in response to the activation of NF-B and Rho signalling cascades [47–49].

As well as playing a role in regulating androgen receptor activity, cAMP can also modulate the co-operative binding of transcription factors at androgen response elements within gene promoters. This was uncovered when PSA, the archetypal androgen responsive gene and prostate cancer biomarker, was identified as increasing upon treatment of prostate cancer cells with forskolin, a direct activator of adenylyl cyclase [50]. PKA phosphorylation of the transcription factor CREB confers interaction with its protein partners, p300 and CBP. These are KAT3 family of proteins, which act as lysine (K) acetyl transferases (KAT) able to modify histones and non-histone proteins, thereby regulating chromatin accessibility and transcription. In particular, they exert a synergistic action on androgen sensitive signalling [51,52].

Neuroendocrine (NE) cells are found in the lumen of the healthy prostate and secrete a variety of signalling molecules including chromogranin A, vasoactive intestinal peptide and vascular endothelial growth factor [53–55]. The NE differentiation of epithelial cells within prostatic tumours is well-documented and thought to result in the elevated incidence of these cells in biopsies from advanced cases of prostate cancer. An increased level of such trans-differentiated cells can occur after prolonged androgen deprivation therapy and is associated with elevated serum levels of both chromogranin A and neurone-specific enolase: markers of NE cell types. Indeed, prostatic tumours can nucleate around colonies of neuroendocrine-like cells during androgen withdrawal therapy with the associated secretory neuroendocrine products stimulating the proliferation of prostatic epithelial cells [56,57]. This reflects the formation of cAMP-regulated, paracrine and autocrine signalling loops associated with the progression of prostate cancer [58–60]. *In vitro*, NE differentiation can be stimulated in androgen-sensitive cells by either androgen withdrawal or, importantly, by increased cAMP signalling [61]. This highlights further the notion that increased cAMP signalling in prostate cancer cells leads to disease advancement and a poor prognosis.

PDE expression in prostate cancer: the importance of PDE4D7

PDEs provide the sole means for the degradation of cAMP. As such they are poised to provide a key regulatory role. Thus, aberrant changes in their expression, activity and intracellular location may all contribute to the underlying molecular pathology of particular disease states. Indeed, it has recently been shown that mutations in PDE genes are enriched in prostate cancer patients leading to elevated cAMP signalling and a potential predisposition to prostate cancer [62]. However, varied expression profiles in different cell types coupled with complex arrays of isoform variants within each PDE family makes understanding the links between aberrant changes in PDE expression and functionality during disease progression challenging. Several studies have endeavoured to describe the complement of PDEs in prostate, all of which identified significant levels of PDE4 expression alongside other PDEs. In particular, Uckert et al. [63] not only identified transcripts for PDE1A, PDE1B, PDE2A, PDE4A, PDE4B, PDE4C, PDE4D, PDE5A, PDE7A, PDE8A, PDE9A and PDE10A within prostate tissues, but also showed that PDE4, PDE5 and PDE11 enzymes were each localized to distinct prostate compartments [64].

Using sequence information on currently identified PDE isoforms, we profiled PDE superfamily member expression in 19 prostate cancer cell lines and xenografts [65]. Our studies identified PDE3B, PDE4B, PDE4D, PDE7A, PDE8A, PDE8B and PDE9A isoforms as being abundantly expressed at the mRNA level in cancerous prostate cells [65], while PDE1, PDE3A, PDE5A, PDE10A and PDE11A mRNA are present at lower levels (unpublished data), highlighting the complexity of cyclic nucleotide signalling in the prostate epithelium. Importantly, by separating the prostate cancer cell samples into androgen sensitive and androgen insensitive, castration resistant prostate cancer (CRPC), cellular phenotypes, we discovered that the expression of PDE4D isoforms was down-regulated in CRPC samples. In particular, we found that the most abundant PDE4 isoform in many of the androgen sensitive samples, PDE4D7, exhibited a significant degree of down-regulation in the CRPC cell models, presenting a scenario where the down-regulation of PDE4D7 could directly contribute to the exacerbation of disease driving cAMP signalling

Clinical Science (2019) **133** 269–286
https://doi.org/10.1042/CS20180519

changes. Moreover, these observations suggested that measurement of PDE4D7 had potential to inform on prostate cancer disease progression where low levels of PDE4D7 expression may be connected with a more aggressive phenotype.

The intracellular function and regulation of PDE4D7

While changes in the expression level of mRNA transcripts can be utilized as a disease biomarker, understanding the biological outcome or function of these changes builds confidence in how the target is integrated with the underlying disease.

PDE4D7 is characterized by its unique 80 amino acid N-terminal region [66]. It is a so-called 'long' PDE4 isoform by virtue of it having both the UCR1 and UCR2 regulatory domains [25]. These, and the catalytic unit, can be subject to multisite phosphorylation by various protein kinases, which allows PDE4D7 to integrate inputs from other signalling pathways as well as for feedback activation by cAMP signalling through PKA phosphorylation of a conserved serine (Ser129) within UCR1 [13]. As with other PDE4 isoforms [13], the unique N-terminal region of PDE4D7 is involved in intracellular targeting to distinct intracellular locales, which are critical for the particular functionality of this isoform [65]. An insight into functionality was gained using the exemplar androgen-sensitive (VCaP) and CRPC (PC3) cell lines, where the differences in mRNA abundance are translated at protein level with the level of cAMP PDE4D7 activity being significantly reduced in the CRPC cancer cell line.

A major fraction of PDE4D7 is localized to the plasma membrane where it has a major influence on sub-plasma membrane cAMP dynamics detected using a targeted, genetically encoded FRET-based cAMP sensor. Thus PDE4D7 can exert effects on a discrete cAMP 'pool' / compartment in cells, indicating that it will have a defined functionality, in this case in modulating cellular proliferation. This was exemplified by (i) a dominant negative strategy, where displacement of PDE4D7 from its functionally important membrane site is achieved by overexpression of a catalytically inactive PDE4D7 mutant, and (ii) by siRNA-mediated knockdown. Both strategies elicited enhanced androgen-sensitive cellular proliferation. Conversely, overexpression of exogenous PDE4D7, in CRPC PC3 cells, suppressed their proliferation by increasing PDE4D7 levels,.

The PDE4D7 promoter has been identified with initial studies identifying multiple CREB binding sites (CRE loci), suggesting that the cAMP/PKA signalling pathway may regulate PDE4D7 transcriptional expression [65]. Otherwise, little more is known, save that susceptibility markers for ischaemic stroke map to the region of Chr5q12 where PDE4D7, and the overlapping androgen-sensitive PART1 exons, locate [24,65–68].

Functional studies give insight into how down-regulation of PDE4D7 may impact on the progression of prostate cancer. It must also be noted, however, that pan-PDE4 and PDE4D-selective inhibitors have been reported to suppress the growth of some prostate cancer cell models [69]. These reports suggest the importance of isoform-specific signalling events where the inhibition of additional isoforms, alongside PDE4D7, may affect the initial cellular response to modulation of PDE4 activity. Recently, a large-scale genome wide investigation [22] also indicated that genetic aberration of the PDE4D locus is a common feature of prostate cancer where early loss of heterozygosity or homozygous deletion of the PDE4D/IL6ST locus in aggressive disease imparts a cancer-suppressive role. In this context, the activity of PDE4D7 may allow for the modulation of cAMP signalling events in early prostate cancer that is lost during its down-regulation, thereby allowing for a more aggressive prostate cancer phenotype. Such mechanistic insights into the purported role of PDE4D7 in the progression of prostate cancer provide functional underpinning for the potential use of using PDE4D7 transcript levels as a biomarker for prostate cancer progression.

Translation of the tissue marker PDE4D7 into a clinical application

Verification of the results generated during the discovery phase

Biomarker validation presents a typical 'chicken and egg' situation. Ideally, the first step is to develop a robust, sensitive, efficient and reproducible assay for the biomarker of interest that will work with high integrity using various tissue materials, such as cDNA and total RNA isolated from either fresh, frozen or formalin-fixed tissue sections. Translating this, from the initial research observation, takes a considerable investment in terms of both time and money under conditions when it is not known whether the proposed marker will have value in a clinically relevant patient cohort. Rather than immediately recruiting patients to assess, an alternative approach is, firstly, to analyse publically available data sets. These can be interrogated regarding the expression of the proposed biomarker in order to try and uncover potentially useful correlates. Such a pure '*in silico*' approach has a potential disadvantage in that, often, the clinical annotation associated with publically available data sets is limited. Furthermore, in many instances,

it is unclear as to how the respective patient samples were collected. Often this is done on an 'availability and access' basis, which makes it difficult to determine how much bias comes with the data. In order to control this more effectively, material collected prospectively from a consecutively managed patient cohort is required. This demands a major resource commitment, especially in situations where the clinical value of the proposed biomarker remains to be proven.

In developing PDE4D7 expression as a prognostic biomarker for prostate cancer, we adopted a hybrid development path, taking the initial laboratory observations through to clinical utility using a combination of primary research and re-analysis of existing public gene expression datasets [65,70–73]. Firstly we developed an effective bio-assay for PDE4D7 that, in its early form, was sufficient for validation and assessment of clinical utility. Secondly, we conducted an *in silico* analysis of PDE4D7 expression across publicly available prostate cancer data sets. The first clinical validation analysis used data from eight different publically available databases represented a total of 1405 patients across a multitude of different clinically relevant prostate disease states [73]. These data sets represented tumour states in terms of their tissue representation where normal adjacent prostate (NAP), benign lesions (BL) and hyperplasia (BPH) of the prostate were detailed alongside primary prostate cancer (Primary PCa) with various levels of post-surgical disease progression (none, progression to biochemical relapse, progression to metastases), as well as metastatic prostate cancer and hormone-refractory CRPC (castration resistant prostate cancer). Five DNA Exon array datasets (1006 samples in total) were interrogated with probe sets specific for PDE4D7 and the RNAseq data available via the TCGA consortium (193 samples in total) were analysed for PDE4D7 expression using isoform specific sequencing reads. Such DNA microarray and RNAseq data were then normalized using standard methodologies (e.g. RMA or FPMK). In parallel to the *in silico* analysis, two of the data sets (206 samples in total) were investigated using an early development of our RT-qPCR assay for PDE4D7 using an initial set of reference genes for normalization [73].

Data generated during these studies provided multiple important insights. Crucially, we confirmed, in a clinical setting [73], our initial, laboratory-based hypothesis derived from observations on prostate cancer cell lines and xenografts [65]. Namely that PDE4D7 was significantly down-regulated in tumours presenting aggressive characteristics associated with reduced androgen dependency (Figure 2A). This was a critical, convincing verification given that we obtained similar results across independent data sets. Thus, we showed that PDE4D7 measurement informs on the progression of prostate cancer regardless of the bias of sample collection, unknown clinical patient characteristics and other potentially confounding factors. Furthermore, our early stage RT-qPCR assay delivered reproducible data across two independent sample sets where we analysed PDE4D7 expression in normal adjacent tissue (NAP) versus primary cancer tissue (tumour; Figure 2B). Such data allowed comparable conclusions to those obtained from our analyses of DNA Exon microarray data sets (Figure 2B). Finally, we showed that, across multiple datasets, reduction in normalized expression of PDE4D7 mRNA is associated with adverse pathology outcome in terms of the post-operative Gleason score (Figure 2C). This allowed us to further propose [73] that PDE4D7 down-regulation in prostate cancer is associated with longitudinal biological outcome after primary intervention (Figure 2D).

An additional outcome of our validation studies was the demonstration that, despite the complexity of transcription and splicing associated with PDE4 isoform regulation, it is possible to both detect and quantify individual PDE4 transcripts from DNA Exon microarray, next-generation RNAseq and RT-qPCR approaches. Having developed the technical tools necessary to quantify individual PDE4D transcripts, and leverage the statistical power of the different sources of available '–omics' data, we thought it prudent to determine whether tumorigenesis of the prostate leads to additional changes in PDE4D transcript expression profile or whether changes were unique to PDE4D7. In particular, we wanted to build on our original cell line and xenograft study [65] and understand which PDE4D isoforms are expressed in prostate cancer tissue and whether there are selective, disease-associated changes in PDE4D isoform expression concerning the known short (PDE4D1, PDE4D2, PDE4D6) forms and the long (PDE4D3, PDE4D4, PDE4D5, PDE4D7, PDE4D8 and PDE4D9) forms and if this might provide a multifactorial signature for PDE4D expression in prostate cancer. In line with the PDE4D isoform expression pattern that we observed in the initial cell line and xenograft studies [65], across the clinical data sets we found evidence for consistent transcription of PDE4D1 and PDE4D2 short forms as well as clear evidence of consistent transcription of PDE4D5, PDE4D7 and PDE4D9 [72,73]. Inconsistent expression patterns were noted for the other long forms of PDE4D [72]. Consequently, we focused our attention on the analysis of the PDE4D1 and PDE4D2 short isoforms and the PDE4D5, PDE4D7 and PDE4D9 long forms.

TMPRSS2-ERG-positive tumours and PDE4D7 expression

The prostate cancer-specific TMPRSS2-ERG gene fusion event occurs in approximately 50% of prostate cancer patients and has become recognized as a molecular hallmark of prostatic tumourigenesis [74]. This occurs between the

Clinical Science (2019) **133** 269–286
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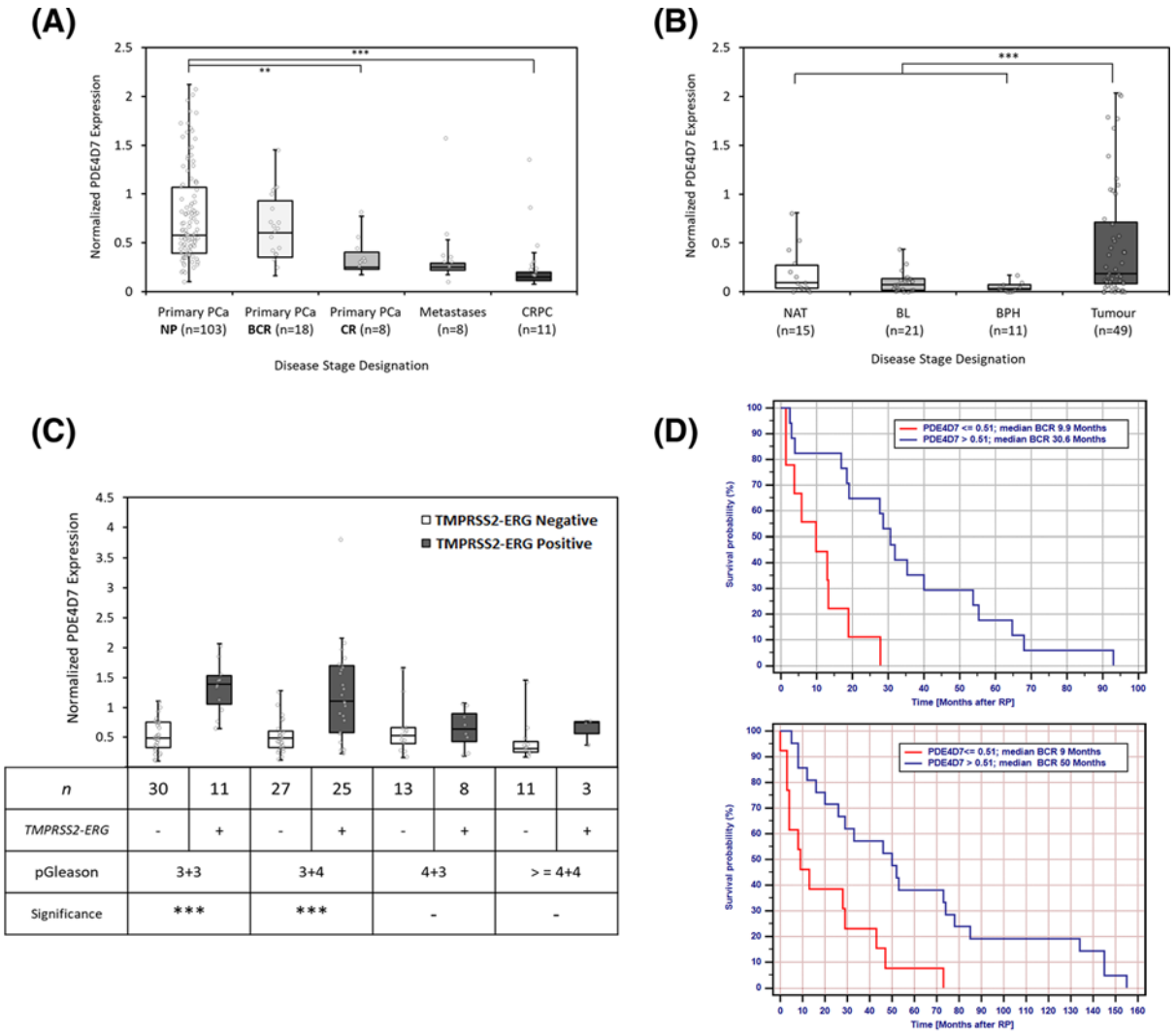


Figure 2. PDE4D7 expression in prostate cancer

(**A**) Expression of PDE4D7 Splice Variant in Prostate Tissues. Annotations: 'Primary PCa NP' – primary prostate cancer, no post–surgical progression; 'primary PCa BCR' – primary prostate cancer, post-surgical progression to biochemical recurrence (BCR); 'primary PCa CR' – primary prostate cancer, post-surgical progression to clinical recurrence (CR); Metastases – metastatic prostate cancer tissue; CRPC – castration resistant prostate cancer tissue. (**B**) Expression of PDE4D7 Splice Variant in Normal, Benign versus Cancerous Prostate Tissues. Annotations: NAT – normal adjacent tissue; BL – benign prostate lesions; BPH – benign prostate hyperplasia lesions; Tumour – prostate cancer with various Gleason grades. (**C**) Correlation of PDE4D7 Expression to Pathology Gleason Score. Annotations: BCR – post-surgical progression to biochemical recurrence (-/+ = no/yes). TMPRS2-ERG – status of the gene fusion between the TMPRSS2 and the ERG genes (-/+ = no/yes). pGleason – pathology Gleason score after surgery. (**D**) Kaplan–Meier survival curves in men with biochemical recurrence after primary treatment. Using a cut-off of <0.51 (corresponding to the Youden-Index) for normalized PDE4D7 expression, two patient cohorts can be separated with a median time to biochemical recurrence (BCR) after primary treatment of 9.9 and 9 months versus a median time to BCR of 30.6 and 50 months, respectively (HR = 0.29; $P = 6.0E-04$; HR = 0.36; $P = 1.6E-03$, respectively). Significant P-values of group comparison are indicated (**$P<0.01$; ***$P<0.001$). Figures and legends are adapted from reference [73].

5′ untranslated region of TMPRSS2 (transmembrane protease, serine 2), an androgen-regulated gene and the ETS transcription family member, ERG. It leads to a deletion of an interstitial chromosomal region containing 16 genes on chromosome 21, by either complete deletion or by chromosomal translocation of this interstitial area to another region of chromosome 21. This event was identified by an outlier approach as it does not occur in all prostate tumours [74]. Subsequently, it was found that other ETS transcription factor family members can be found fused to the

TMPRSS2 gene in prostate cancer, namely ETV1, ETV4 and ETV5, although at typically much lower prevalence compared with the TMPRRS2-ERG rearrangement [75]. Interestingly, we identified a distinct expression pattern for the individual prostate cancer relevant PDE4D transcripts in five patient cohorts where we had access to information on their TMPRRS2-ERG status [72]. Importantly, in contrast with PDE4D5, PDE4D9, PDE4D1 and PDE4D2, transcript levels of PDE4D7 were found at a significantly higher level in TMPRRS2-ERG positive cancers compared with fusion gene negative prostate cancers (Figure 3A). Further analysis revealed that when PDE4D transcript levels were compared between primary TMPRSS2-ERG positive tumours and adjacent normal tissue (NAP), we not only observed PDE4D7 up-regulation but also found that the other evaluated PDE4D transcripts were down-regulated [72,73]. Such observations uncovered a context-dependent pattern of events where PDE4D7 exhibits a unique up-regulation of expression in TMPRSS2-ERG positive tumours versus NAP [72,73]. However, in contrast with this, the striking down-regulation of PDE4D7 is clearly correlated with a more aggressive tumour phenotype (Figure 3B) [72,73].

The basis and functional significance of PDE4D7 up-regulation in the presence of the TMPRRS2-ERG gene fusion remains to be determined. However, the effect is clearly specific for the fusion of the ERG transcription factor as we did not find the same PDE4D7 up-regulation where TMPRSS2 was fused to ETS transcription factors other than ERG, namely either ETV1 or ETV4 [72]. Presumably, PDE4D7 up-regulation is due either directly or indirectly to the increased expression of the ERG gene, which is driven by androgen-stimulated transcription of the TMPRSS2 gene [74,76].

Very recently, differences in the genomic evolution of TMPRRS2-ERG gene fusion positive tumours compared with tumours without such a gene rearrangement have been uncovered from whole genome sequencing (WGS) of a total of 112 primary and local/distant metastatic prostate cancers [22]. This indicated that the earliest homozygous deletions appeared on Chr5 in the region 55–59 Mb in fusion positive tumour tissues while, in ERG negative cancers, the losses emerged in the region 60–100 Mb on the same chromosome. Intriguingly, it is the region between 59 and 60 Mb on Chr5 that encompasses the exons and the promoters of both PDE4D7 and PDE4D5. How this might affect the differential expression of PDE4D transcripts remains to be ascertained. However, it is striking that the breakpoint in genomic losses on Chr5, in either the presence or absence of the TMPRRS2-ERG gene fusion, occurs within the putative promoter and transcriptional start regions of long PDE4D isoforms whose expression is altered in prostate cancer.

Early insights into prostate cancer-regulated transcription of PDE4D isoforms

In order to assess potential involvement of transcriptional regulation of PDE4D transcripts by either the ERG transcription factor or by the AR we interrogated a ChiP-seq dataset generated from a prostate cancer cell line treated with the synthetic androgen R1881 [77]. With ChiP-seq, proteins binding to genomic DNA are cross-linked, followed by precipitation of the protein of interest, namely ERG and AR in this case, using selective antibodies. The cross-linked DNA is then sequenced allowing the identification of DNA sites to which the protein of interest has bound. This approach identified a multitude of putative binding sites for the AR, as well as for the ERG transcription factor, within the PDE4D gene locus. Some of these were found to overlap partially with the first, isoform-specific exon of each of the long isoforms PDE4D5, PDE4D7 and PDE4D9 [72]. Subsequent analysis confirmed that there was very strong enrichment for both putative AR and ERG binding sites compared with randomly selected genomic regions of the same size as the PDE4D gene (approximately 1.5 Mb) across all chromosomes [72]. Coupled with the fact that PDE4D7 exons overlap with those of the androgen regulated gene PART1, these results support the view that both androgen, as well as the ETS transcription factor ERG, are involved in the regulation of the PDE4D gene locus.

DNA methylation plays a key role in transcriptional regulation and has been evaluated in the context of prostate cancer based diagnosis [78,79]. The methylation of promoter regions of particular genes leads to their transcriptional repression [80]. In cancer CpG-rich regions of gene promoters can undergo aberrant hypermethylation, leading to the silencing of gene expression [81]. Evaluating various DNA methylation data sets generated from prostate cancer tissues, we noted [72] that hypermethylated patterns were consistently identified for five PDE4D transcripts, namely PDE4D1, PDE4D2, PDE4D4, PDE4D5 and PDE4D8 and that, in some cases, these regions included the transcriptional start sites (TSS) of such PDE4D isoforms. To elucidate the impact of the hyper-methylated regions of PDE4D gene we compared PDE4D transcript levels recorded in DNA microarray datasets, with patient matched methylation data [72]. From this information we calculated Spearman's correlation coefficient and found that PDE4D5 showed the strongest negative correlation ($r = -0.57$) between the extent of TSS methylation and transcript expression. Thus it may be that differential methylation is relevant for the regulation of the expression of some, but clearly not all,

Clinical Science (2019) **133** 269–286
https://doi.org/10.1042/CS20180519

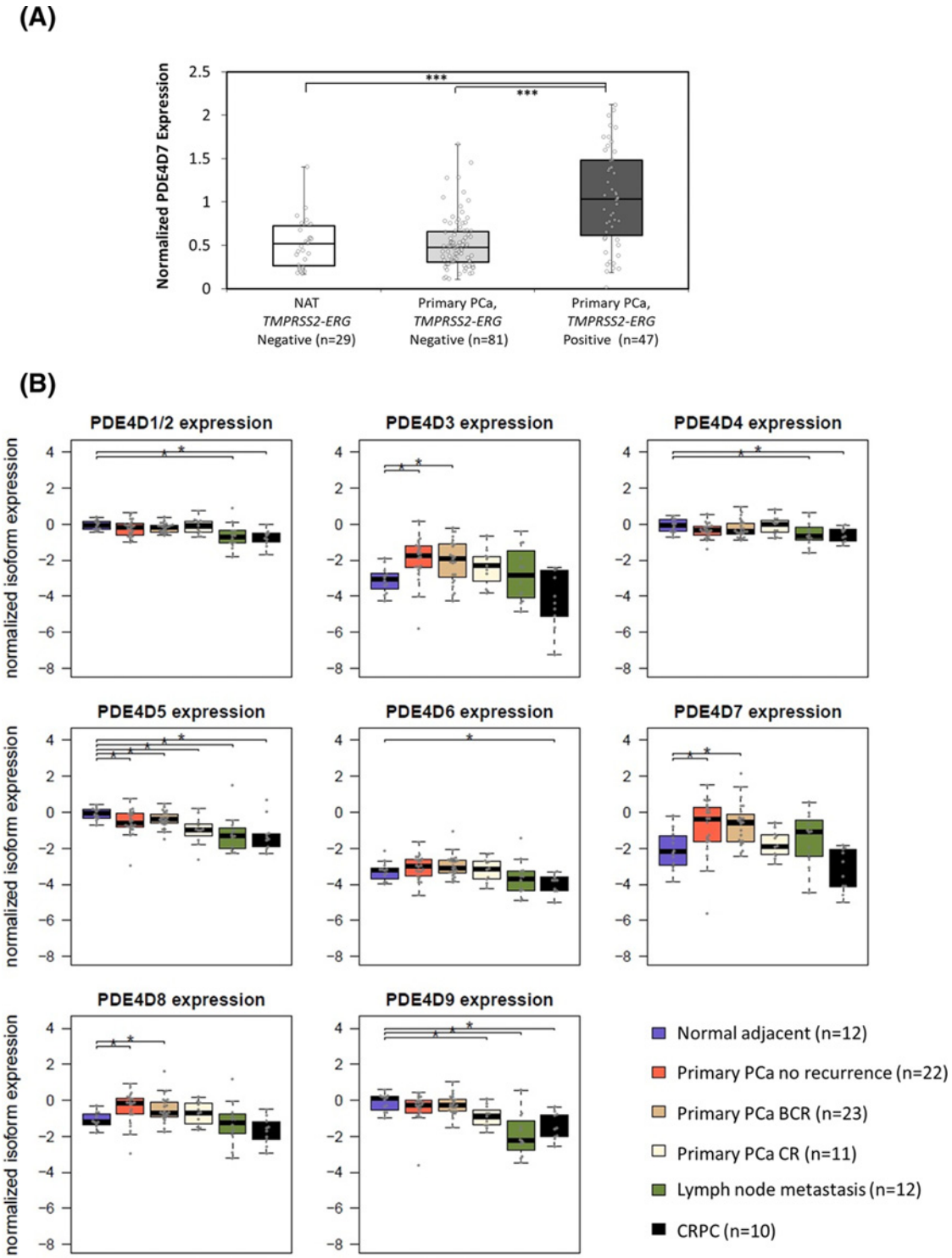


Figure 3. PDE4D7 expression and TMPRSS2-ERG gene fusion status

(**A**) Correlation of PDE4D7 Expression in Normal and Cancerous Human Prostate Tissues to TMPRSS2-ERG Gene Fusion Status. Samples were divided into three different groups: (1) normal adjacent tissue without TMPRSS2-ERG fusion events (NAT TMPRSS2-ERG negative); (2) prostate tumour tissue without TMPRSS2-ERG fusion events (Primary PCa, TMPRSS2-ERG negative) and (3) prostate tumour tissue with TMPRSS2-ERG fusion events (Primary PCa, TMPRSS2-ERG positive). (**B**) PDE4D isoform expression in prostatic tissues. Annotations: CR – clinical recurrence, BCR – biochemical recurrence, CPRC – castration resistant prostate cancer. Significant *P*-values of group comparison for difference of mean PDE4D7 expression are indicated (*$P<0.05$). Figures and legends are adapted from references [72,73].

PDE4D isoforms expressed in prostate tissue. Certainly, it would appear that PDE4D7 expression is not regulated by methylation and, in this regard, PDE4D7 and PDE4D5 show opposing expression behaviour in prostatic tissue.

The data generated by this hybrid approach to the validation of PDE4D7 as a prognostic biomarker of prostate cancer reinforced the strong link between PDE4D7 expression and the aggressiveness of prostatic cancer and proved to be sufficiently convincing to allow us to progress to the next stage of clinical validation.

Validation within a clinical setting

The translation of a laboratory-based proposal for a disease-relevant biomarker into a clinical setting requires the development of a robust and accessible assay for the gene of interest as well as its rigorous evaluation in a multitude of different patient cohorts and settings. Therefore, a suitable assay technology that enables the sensitive and reproducible measurement of the relevant biomarker needs to be developed. Despite enormous progress in the development of gene expression platform technologies, such as DNA microarrays and next-generation RNA sequencing, the technology chosen for data validation and the development of a specific, reproducible, assay remains quantitative PCR (qPCR). This is because it provides a robust and affordable means for assessing virtually any defined transcript. Furthermore, this assay not only is able to work on freshly isolated nucleic acids, but also can function effectively using formalin-fixed, paraffin embedded tissue, which is routinely generated in the clinic. Additionally, this approach allows the straightforward selection of appropriate reference genes for use in the normalization of the biomarker expression across various sample preparations. However, while multiple reference genes might increase robustness, this will also increase the assay complexity.

In order to streamline the assays for commercial use, the qPCR assay has to be developed from a multistage protocol into a one-step RT-qPCR assay where the RNA starting material is reverse-transcribed and assayed in a single reaction step. Similarly, data quantification and assay processing require automation, aligning the procedure to translate raw data into C_q values that represent the normalized expression value of the biomarker transcript which is then evaluated using a pre-defined 'quality' metric in order to determine which data elements are robust, and which outliers should be discarded from downstream statistical analysis. Most importantly, a concept of how to interpret the data in order to provide a result of clinical value needs to be defined and executed.

Undertaking further optimization and testing of the PDE4D7 RT-qPCR assay, we were able to generate a robust and sensitive assay with very high target specificity and a high sensitivity of target detection (the limit of detection is close to 1 copy of RNA in FFPE, formalin-fixed, paraffin embedded tissue). In its final form this sensitive assay had a high level of assay efficiency (close to 100%), assay linearity over a range of target concentrations ($R^2 > 0.98$) as well as high levels of assay reproducibility (technical replicates SD < 0.3 C_q).

We then set out to identify an appropriate set of reference genes. A range of ten candidate reference genes that are commonly used in qPCR studies were used to evaluated PDE4D7 transcript levels in human prostate FFPE samples as well as a number of cell lines. From these studies we chose PUM1, TBP, TUBA1B and HPRT1 as the most stable reference genes for evaluating PDE4 transcripts in prostatic tissue [71]. In order to ensure robust data quality we pre-defined a C_q threshold to either accept or refuse a sample for downstream data analysis. The selected threshold was based on the analysis of qPCR data on FFPE tissue extracted RNA of known concentrations from which we deduced a C_q threshold value as the limit of detection of a single copy of RNA in the sample [71].

Regarding normalization of PDE4D7 C_q values, we used a modified ΔC_q approach by subtracting the C_q mean of the four selected reference genes from the PDE4D7 C_q value of each tumour sample. Thus, we generated a normalized PDE4D7 expression value with a normal distribution between -8 and $+4$ [71]. Subsequently, we translated the normalized expression values by linear transformation into a +1 to +5 distribution. This provides a 'PDE4D7 score' which relates to PDE4D7 abundance and is categorized into four groups: (1) all PDE4D7 scores between 1 and <2; (2) all PDE4D7 scores between 2 and <3; (3) all PDE4D7 scores 3 and <4; (4) all PDE4D7 scores between 4 and 5 [71].

Based on the correlation between PDE4D7 expression and pathological features of the disease, our defined aim was to identify prognostic associations between the expression of PDE4D7 in a patient prostate tissue, collected by either biopsy or surgery, and clinically useful information relevant to the outcome of individual patients. Clinically relevant endpoints, or surrogate endpoints that are significantly correlated to the development of metastases, cancer specific or overall mortality have, typically, been evaluated as prognostic cancer biomarkers [82]. The most relevant rational for using a surrogate endpoint relates to situations where either data on established clinical endpoints are not available or when the number of events in the data cohort is too limited for statistical data analysis. For the development of the PDE4D7 prognostic biomarker, we evaluated either BCR (biochemical relapse) progression-free survival or the start of post-surgical secondary treatment as surrogate endpoints for metastases and prostate cancer death. Using these

Clinical Science (2019) **133** 269–286
https://doi.org/10.1042/CS20180519

particular endpoints, we identified a relevant number of events in our clinical cohorts (e.g. >30% for BCR), which is particularly relevant for multivariable data analysis [83].

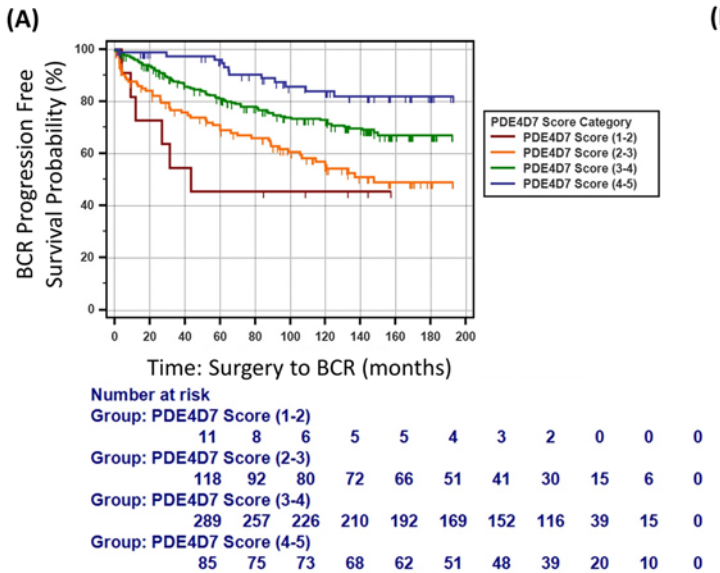
In our evaluation, we selected standard methods of multivariable analysis such as Cox regression and Kaplan–Meier survival analysis in order to investigate the added and independent value of the continuous and/or the categorical 'PDE4D7 score' compared with established prognostic clinical variables such as PSA and Gleason score [71]. We thus built risk models where we combined the 'PDE4D7 score' with either pre- or post-surgical clinical predictors of post-surgical progression using logistic regression. The resulting models were subsequently tested on multiple independent patient cohorts in Kaplan–Meier survival and ROC curve analysis in order to predict post-treatment progression free survival [71].

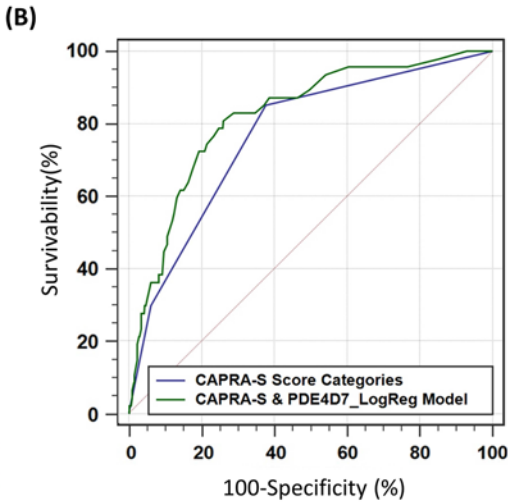
Using such a strategy, we set out to test the prognostic value of the PDE4D7 score on a biopsy from retrospectively collected, resected prostate tissue in a consecutively managed patient cohort from a single surgery centre in a post-surgical setting [71]. The patient population comprised some 500 individuals where longitudinal follow-up, of both pathology and biological outcomes, was undertaken. These clinical data were available for all patients and collected during a follow-up of a median 120 months after treatment. The 'PDE4D7 score' was determined as described above and then tested in both uni- and multivariable analyses using the available post-surgical co-variates (i.e. pathology Gleason score, pT stage, surgical margin status, seminal vesicle invasion status and lymph node invasion status) in order to adjust for the multivariable setting. In this instance, biochemical progression-free survival after primary intervention was set as the evaluated clinical endpoint. The univariable analysis of these clinical samples [71] showing the inverse association between PDE4D7 expression (in terms of 'PDE4D7 score') and post-surgical biological relapse (HR = 0.53 per unit change; 95% CI 0.41–0.67; $P<0.0001$), robustly confirmed our previous data [65,71–73]. In multivariable analysis with such clinical variables, the 'PDE4D7 score' remained as an independent and effective means for predicting clinical outcome (HR = 0.56 per unit change; 95% CI 0.43–0.73; $P<0.0001$). Furthermore, we obtained [71] a very similar outcome when we evaluated the 'PDE4D7 score' in multivariable analysis (HR = 0.54 95% CI 0.42–0.69; $P<0.0001$) with the validated and clinically used risk model CAPRA-S [84,85]. The CAPRA-S score, which is based on pre-operative PSA and pathologic parameters determined at the time of surgery, was developed to provide clinicians with information aimed to help predict disease recurrence, including BCR, systemic progression, and PCSM and has been validated in US and other populations [84,85]. This takes account of preoperative PSA, pathologic Gleason score, the presence or absence of seminal vesicle invasion (pT3b), positive surgical margins, extra- capsular extension (pT3a)/lymph node positivity within a multivariable Cox proportional hazards statistical model.

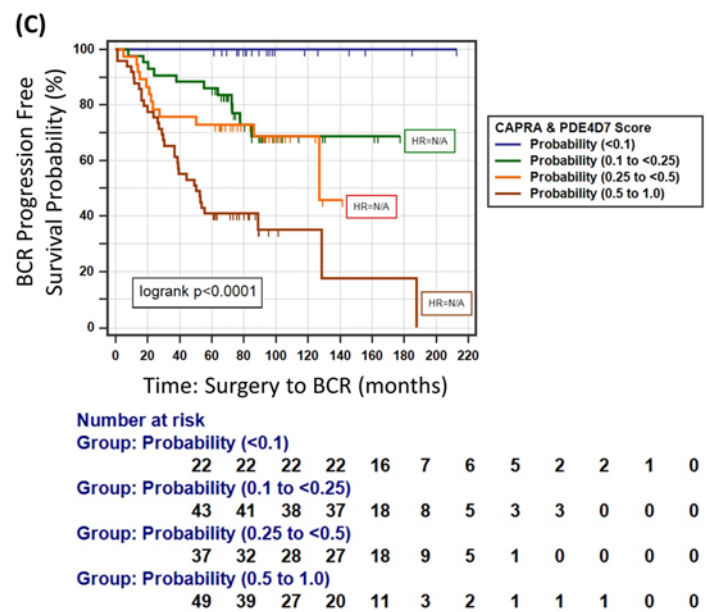
Interestingly, when assessing the hazard ratio (HR) compared with the continuous 'PDE4D7 score' we uncovered [71] a linear increase in risk with decreasing 'PDE4D7 score' for score values lying between 2 and 5. However, at PDE4D7 scores <2, then the risk of post-surgical progression increases steeply [71]. This is also evident in the Kaplan–Meier survival curves where patients that are grouped within the lowest 'PDE4D7 scores' category exhibit the highest risk of disease recurrence (Figure 4A). Using logistic regression analysis we then combined the CAPRA-S score with the continuous 'PDE4D7 score'. Testing this model using ROC curve analysis, we noticed a 4–6% significant improvement in AUC compared with the CAPRA-S alone for both 2- and 5-year predictions of post-treatment progression to BCR (Figure 4B). Thus we evaluated a combined CAPRA-S & 'PDE4D7 score' Cox regression combination model in Kaplan–Meier survival analysis and compared this with the CAPRA-S score categories alone. Undertaking this, we confirmed the added value in risk prediction when using a model the combined 'PDE4D7 & CAPRA-S' score, compared with using the clinical metric of CAPRA-S score alone [71].

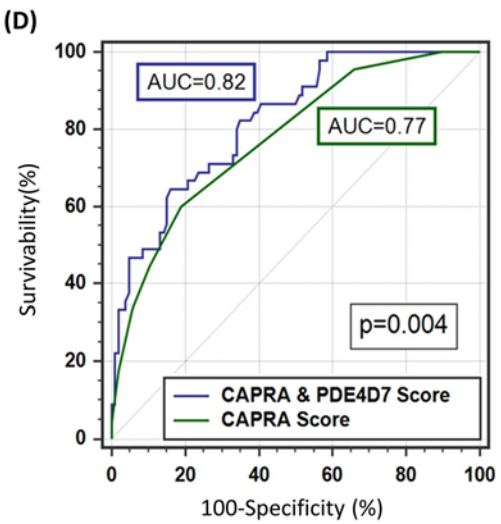
Subsequent to the diagnosis of prostate cancer, an accurate risk assessment needs to be undertaken before stratification to a defined primary treatment. With this in mind, we set out to see if we could translate the prognostic use of the 'PDE4D7 score' in a pre-surgery situation testing tumour tissue obtained from diagnostic needle biopsy samples [70]. In this, needle biopsies were performed on 168 patients, from a single diagnostic clinical centre, who had undergone surgery as a primary treatment. The minimum follow-up period for each patient was 60 months after this intervention. The clinical co-variates used to adjust the 'PDE4D7 score' in the multivariable analysis were age at surgery, pre-operative PSA, PSA density, biopsy Gleason score, percentage of tumour positive biopsy cores, percentage of tumour in the biopsy and clinical cT stage. In this we evaluated the utility of the 'PDE4D7 score' and the combined 'PDE4D7 & CAPRA' scores compared to the pre-surgical CAPRA score in Cox regression analysis for biochemical relapse [70].

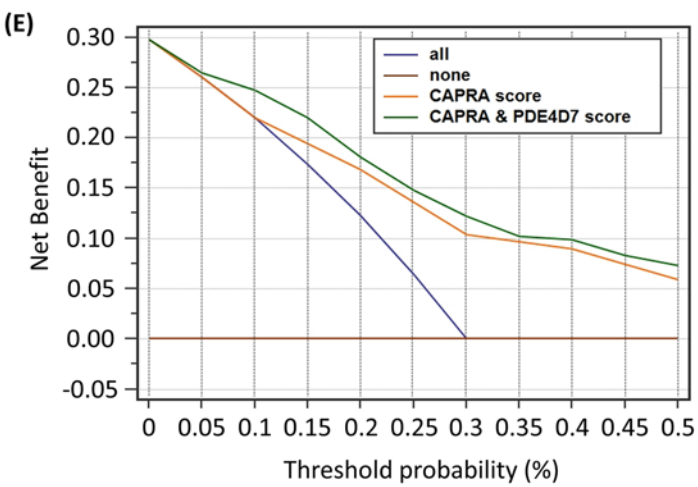
Evaluating this patient cohort we found [70] that the 'PDE4D7 score' was inversely associated with BCR in multivariable analysis when adjusting for clinical variables (HR = 0.43; 95% CI 0.29–0.63; $P<0.0001$) as well as for the clinical CAPRA score (HR = 0.53; 95% CI 0.38–0.74; $P=0.0001$). Kaplan–Meier analysis demonstrated that, as before, in a post-surgical setting, the 'PDE4D7 score' categories were significantly associated with BCR progression











Clinical Science (2019) **133** 269–286
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Figure 4. The 'PDE4D7 score' as a prognostic biomarker in prostate cancer

(**A**) Kaplan–Meier analysis of the time to PSA relapse (BCR – biochemical recurrence) after prostatectomy of the PDE4D7 score categories in a surgery study cohort (n=503; logrank P<1.0E-04). PDE4D7 score categories are defined as: PDE4D7 (1–2): PDE4D7 scores (1 to <2); PDE4D7 (2–3): PDE4D7 scores (2 to <3); PDE4D7 (3–4): PDE4D7 scores (3 to <4); PDE4D7 (4–5): PDE4D7 scores (4–5). The highest PDE4D7 score category (4–5) was used as the reference category. (**B**) ROC cure analysis of 2-year biochemical recurrence (BCR) for the incremental value of the PDE4D7 score added to the CAPRA-S score categories by logistic regression analysis in the study cohort (n=469). The PDE4D7 score was modelled as a continuous variable in the logistic regression. For the prediction of the 2-year PSA relapse after surgery, the incremental value of PDE4D7 was 6% yielding an AUC of 0.82 in comparison with 0.76 for the CAPRA-S score category model alone (P=0.0004). (**C**) Kaplan–Meier analysis of the BCR-free survival in the patient diagnostic biopsy (n=151) cohort of a CAPRA & PDE4D7 score combination model. The CAPRA & PDE4D7 combination model was created by logistic regression on the independent surgery cohort with complete 5-year follow-up (n=449). The CAPRA & PDE4D7 score categories were defined according to the probability to experience PSA failure after surgery based on the logit(p) function of the logistic regression model as indicated. (**D**) ROC curve analysis of 5-year biochemical recurrence (BCR) in the diagnostic biopsy cohort (n=151) of the CAPRA score versus the CAPRA & PDE4D7 logistic regression model which was developed on the independent surgery patient cohort with complete 5-year follow-up (n=449). The incremental value of PDE4D7 to the CAPRA score yielded a 5% increase in AUC (P=0.004). (**E**) Decision curve analysis in the diagnostic biopsy (n=151) patient cohort of the net benefit of four different treatment decision strategies for men at risk of disease recurrence within 5 years after surgery. Treatment strategies were tested for their net benefit across indicated threshold probabilities (0.05 step size) to trigger prostate surgery based on the probability of future disease recurrence. Figures and legends are adapted from references [70,71].

free survival (logrank P<0.0001) and secondary treatment free survival (logrank P=0.01). We then employed [70] a combination logistic regression model, which was developed on the previous cohort. This consisted of the combined 'CAPRA & PDE4D7' score, demonstrating that patients within the highest combined 'CAPRA & PDE4D7' combined score category have virtually no risk of biochemical progression or transfer to any secondary treatment after surgery (Figure 4C). This logistic regression model was also evaluated using ROC curve analysis in order to predict 5-year BCR after surgery. This revealed an increase in AUC of 5% over the CAPRA score alone (AUC = 0.82 vs. 0.77, respectively; P=0.004; Figure 4D). Decision curve analysis of the combined 'CAPRA & PDE4D7' score model confirmed the superior net benefit of using this combined score, compared with either score alone, across all decision thresholds in order to decide on whether to undertake intervention (e.g. surgery) based on the risk threshold of an individual patient to experience post-surgical disease progression (Figure 4E).

Conclusions

Disease severity and prognostic outcome vary considerably between incidences of prostate cancer. Indeed, many prostate cancers present as a slowly progressive or even as a non-progressive indolent disease that are unlikely to elicit either significant morbidity or death. There has thus been considerable concern that unnecessary harm is done to patients as a result of over-diagnosis and over-treatment with radiotherapy and radical prostatectomy. Indeed, there has been a move to AS as a management strategy to monitor disease progression in order to avoid or delay potential harm to low-risk patient groups [86,87]. However, it is becoming increasingly evident that such a close surveillance regime comes with substantial challenges concerning both its clinical implementation and as regarding patient compliance [10,88,89]. In this context, the identification of accurate and reliable prognostic biomarkers is key in further personalizing treatment strategy and better defining the risk of disease progression.

Here we present a summary of the scientific and clinical research on the cyclic AMP degrading phosphodiesterase PDE4D7 long isoform in the development of a novel, clinically relevant and personalized prognostic biomarker assay for prostate cancer (InformMDx™). This progressed from initial *in vitro* observations and characterization using cell lines and xenograft material, through to clinical validation on patient cohorts with longitudinal pathology and biological outcomes. Specifically, we have shown that PDE4D7 transcript abundance is inversely associated with increasingly aggressive states of prostate cancer. Thus, for patients with low 'PDE4D7 scores', the risk of post-surgical disease progression increases steeply, while risk decreases markedly for those patients with high 'PDE4D7 scores'. The predictive nature of this personalized assessment can be further enhanced through combining the 'PDE4D7 score' with the CAPRA clinical-biased scoring system [71]. Thus the 'PDE4D7 score' provides a robust readout that enables more accurate patient risk stratification, yielding a clinically effective prognostic test poised to have significant clinical impact for pre-surgical risk stratification. In particular, the PDE4D7 stratification biomarker has potential utility in selecting men with very minimal (or virtually no) risk of progressive disease for inclusion to AS, thus reducing the burden that both AS and inappropriate therapeutic intervention place on both the patient and the healthcare system.

In light of the significant literature that connects cAMP signalling and the molecular hallmarks of advancing prostate cancer [36,37,39,40,51,53,58], the correlation between decreased PDE4D7 expression and the progression of prostate cancer suggests that PDE4D7 activity might well influence the underlying cellular pathology. These clinical correlates prompt a need to uncover the precise consequences of how altered PDE4D7 expression in various states of the disease has an impact on cellular pathology and, indeed, whether PDE4D7 might provide a potential therapeutic target. Thus, employing the 'PDE4D7 score' to stratify patients might have use not only for directing individuals to particular therapy options that are currently available, but also to stimulate the search for novel treatment options and even novel therapeutics for patients having low PDE4D7 levels associated with recurring and progressive forms of prostate cancer. In this regard, during the last decade the treatment landscape of metastatic and/or CRPC has changed substantially and a number of additional therapeutic options for systemic therapies are available today [90,91]. However, aside from examples in the adoptive immune cell therapy space [92], newly developed therapeutics have continued to centre around modulators of AR activity [93,94]. As time-dependent resistance to various of these novel therapies is now emerging [95], therapeutics aimed at novel molecular targets are urgently required. Indeed, it may be that positive modulation of PDE4D7 activity, or signalling events associated with it, might present an opportunity to develop novel therapeutic options for use alongside PDE4D7 as a target matched, clinically validated, companion biomarker.

Competing Interests

D.J.P.H.,M.D.H., and R.H. are co-inventors on patents related to the presented work.

Abbreviations

AS, active surveillance; CRPC, castration resistant prostate cancer; NP, neuroendocrine; PDE, phosphodiesterase; PDE4D7, phosphodiesterase-4D7; PKA, protein kinase A; qPCR, quantitative PCR; TSS, transcriptional start sites.

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Clinical Science (2019) **133** 269–286
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