

Splice variant *PRKC-ζ_{PrC}* is a novel biomarker of human prostate cancer

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BACKGROUND: Previously, using gene-knockdown techniques together with genome expression array analysis, we showed the gene protein Kinase C (PKC)-zeta (*PRKCZ*) to mediate the malignant phenotype of human prostate cancer. However, according to NCBI, the gene has undergone several major iterations. Therefore, to understand the relationship between its structure and biological activities, we have analysed its expressed sequence in prostate cancer cell lines and tissues.

METHODS: Transcriptome-walking and targeted PCR were used to sequence the mRNA transcribed from *PRKCZ*. Hydrophathy analysis was employed to analyse the hypothetical protein sequence subsequently translated and to identify an appropriate epitope to generate a specific monoclonal antibody.

RESULTS: A novel sequence was identified within the 3'-terminal domain of human *PRKCZ* that, in prostate cancer cell lines and tissues, is expressed during transcription and thereafter translated into protein (designated PKC-ζ_{PrC}) independent of conventional PKC-ζ_a. The monoclonal antibody detected expression of this 96 kD protein only within malignant prostatic epithelium.

INTERPRETATION: Transcription and translation of this gene sequence, including previous intronic sequences, generates a novel specific biomarker of human prostate cancer. The presence of catalytic domains characteristic of classic PKC-β and atypical PKC-ι within PKC-ζ_{PrC} provides a potential mechanism for this *PRKCZ* variant to modulate the malignant prostatic phenotype out-with normal cell-regulatory control.

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Previously, we have shown expression of the gene protein kinase C (PKC)-zeta (*PRKCZ*) to be characteristic of human prostate cancer (Cornford *et al*, 1999) where intensity of the protein PKC-ζ_a detected immunohistochemically is predictive of aggressive prostatic malignancy ($P < 0.001$). RNAi gene knockdown, *in vitro* and *in vivo* behavioural studies and gene expression array analysis confirmed *PRKCZ* to be functionally involved in promoting the malignant prostatic phenotype (Yao *et al*, 2010). The gene *PRKCZ* is located on human chromosome 1 (at 1p36.33-p36.2) where it covers 136.21 kb on the direct strand. Containing 104 exons, it potentially encodes 46 structurally distinct splice variants designated in AceView as *a-u* and *va-vy*. During the last decade, the structure of human *PRKCZ* published by NCBI has undergone several major revisions as novel data have accrued. Nevertheless, important structure–function activities of the gene remain incomplete. Presently, the structure of *PRKCZ* splice variant 'a' expressed in prostate cancer comprises 18 exons that are transcribed to a 2295 bp mRNA and translated into 592 amino acids yielding a 67.7 kD protein (Supplementary Table 1). Details of the current structural organisation of *PRKCZ* gene variant 'a' is shown in Figure 1.

PKC isoenzymes are ancient proteins that appeared early during prokaryote evolution (Kruse *et al*, 1996; Manning *et al*, 2002) and are essential for the structural development and functional maintenance of multi-cellular organisms (Suzuki *et al*, 2003). Together, they comprise a complex family of some 14 serine-threonine kinases characterised by fundamental similarities in the structure of certain gene elements and in functional peptide domains (Hanks *et al*, 1988; Kofler *et al*, 2002). Broadly considered to be regulators of cellular homeostasis and behavioural phenotypes (Dempsey *et al*, 2000), these enzymes are increasingly identified to be polyfunctional (Ohno and Nishizuka, 2002). High homology between apparently different members within the overall super family emphasises their common evolutionary origins but can obscure their subtly different functions that are crucial to understanding their roles in cancer cell survival and in designing biologically appropriate therapeutic agents (Ali *et al*, 2009). The genes for all of these enzymes characteristically encode multiple splice variants that are differentially expressed between tissues during morphogenesis (Patel *et al*, 2006; Dobkin-Bekman *et al*, 2010; Makary *et al*, 2011) and especially between malignant tissues and their benign histogenic counterparts (Lee *et al*, 2010; Urtreger *et al*, 2012).

PRKCZ expression profoundly affects cellular behaviour (Le Good and Brindley, 2004; Xin *et al*, 2007), particularly in a diverse range of malignancies (Liu *et al*, 2009; Wu *et al*, 2009) where it modulates different biological mechanisms (Della Peruta

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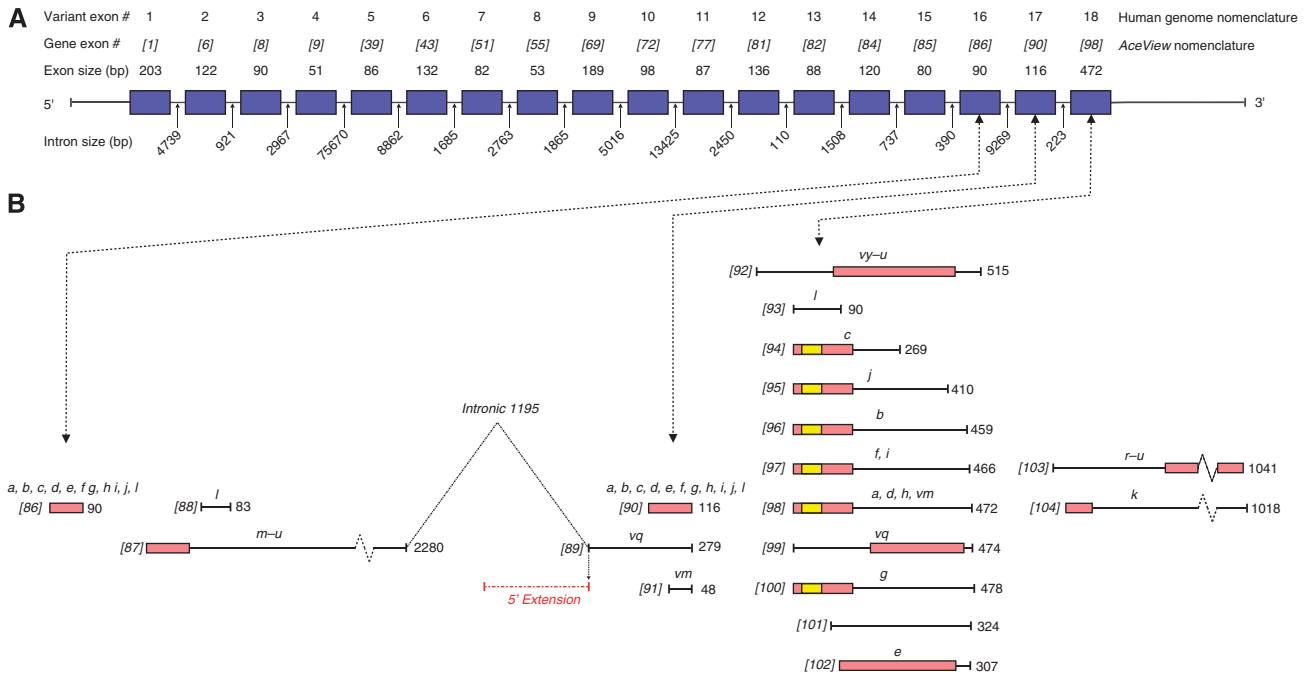


Figure 1 (A) Full exonic sequence of NM variant 'a' (NCBI database Build 36, April 2011). Genome exon numbers are in square brackets. Sizes of individual exons (blue boxes) and intervening introns are as shown. (B) Comparative structure of the 3'-terminal region of *PRKCZ* NCBI database Build 36 (November 2011) between bases #124751 and #136201. Horizontal bars indicate the relative size and location of each exon. Preceding numbers (square brackets and italics) denote the genome number assigned to each exon. Letters above each bar identify the splice variants that include the particular exon. Numbers following each bar specify the length (bp) of each exon. Pink rectangles identify the size and location of individual coding regions. Yellow rectangles denote the location of the gene sequence corresponding to the antigenic site identified by polyclonal antiserum sc-216. The upstream (5') extension (253 bp) of exon 89 into the adjacent intronic region is identified in red.

et al, 2010; Luna-Ulloa et al, 2011; Valkov et al, 2011; Yu et al, 2011). Therefore, confirming its structure was considered a prerequisite for understanding the role of this gene in prostate cancer. Thus, the aim of this study was to establish the detailed organisation of the *PRKCZ* gene expressed in prostate cancer and to test the hypothesis that alternative forms of *PRKCZ* might contribute cellular properties distinct from conventional PKC- ζ_{-a} , hence promoting the malignant prostatic phenotype. We now report the retention and transcription of a normally intronic sequence within the 3'-terminal region of *PRKCZ* together with its translation into a novel protein (designated 'PKC- ζ_{-PRC} ') that have been shown to be selective for human prostate cancer. This sequence is expressed simultaneously, but independently, from that of the conventional PKC- ζ_{-a} . Inclusion of catalytic domains characteristically expressed in classic PKC- β and atypical PKC- ι provides evidence that anomalous variants of protein kinase (PK) enzymes may promote the malignant phenotype of prostate cancer cells by mechanisms out-with normal regulatory processes.

MATERIALS AND METHODS

Cell lines

Four human prostate epithelial cell lines PNT-2 (benign), LNCaP, DU145 and PC-3M (highly malignant) were grown as monolayers in RPMI 1640 (Invitrogen, Paisley, UK) supplemented with 10% (v/v) fetal calf serum (Invitrogen), penicillin (100 units ml⁻¹), streptomycin (100 µg ml⁻¹) and L-glutamine (2 mM). Additional cell lines employed as the source of proteins for western blotting included a non-malignant lung epithelial line (Beas-2b) together with malignant cell lines from lung (COR-L88), bladder (HT1197), pancreas (PANC) and breast (MCF-7). The culture media for the

stably-transfected prostatic epithelial cell line si-*PRKC- ζ_{-a}* -PC-3M_{T1-6} in which the gene *PRKC- ζ_{-a}* had been knocked down using si-RNA directed towards the unique 21 nt sequence - 5'-GTGA GAGACATGTGTCGTT-3' - contained within *PRKCZ* exon 1 (Yao et al, 2010), were also supplemented with 500 ng µl⁻¹ Geneticin (Sigma, Gillingham, UK).

Human prostate tissues

Following informed consent from the patients, three human radical prostatectomy specimens containing biopsy-proven prostate cancers were obtained fresh. Following macroscopic inspection and dissection to provide diagnostic specimens, cores of tissue (5 mm diameter) were removed from several sites in the residual material and submitted to frozen section examination to confirm the composition of each specimen with respect to prostatic cancer and non-malignant epithelium. Cores selected for further analysis contained either entirely benign or entirely malignant epithelium on microscopic analysis.

Ethical approval

The studies on human tissues were performed under the National Research Ethics Service Licence 05/Q1505/76.

Rabbit polyclonal antiserum sc-216

Rabbit polyclonal antiserum sc-216, obtained commercially from Santa Cruz Biotechnology (Insight Biotechnology Ltd, Wembley, UK) was identical to that described in the original immunohistochemical and western blotting studies (Cornford et al, 1999; Yao et al, 2010) to reveal expression of PKC- ζ_{-a} in human prostate cancers. The peptide sequence recognised by

PRKCI, potentially homologous with *PRKCZ*, was also transcribed in the cell lines.

Transcriptome-walking

The known structure of the antigenic peptide sequence identified by polyclonal antiserum sc-216 and its corresponding nucleotide-coding sequence were employed as the starting point to investigate the structure of the *PRKCZ* transcripts. mRNAs extracted from PNT-2, LNCaP, DU145 and PC-3M cell lines as well as six primary tissues (one benign and one malignant specimen from each of three patients) were used as templates to generate corresponding cDNAs. Oligonucleotide primer sequences (Supplementary Table 2) were designed to the 3' end of *PRKCZ* (NM_002744), beginning at the antigenic sequence with the aim of walking 5' upstream through the expressed sequence of the gene. Generic primers (*n*-GTTC, where *n* = A-, T-, C- or G-) together with a series of unique target-specific primers (TSP1–9, Supplementary Table 3) were employed to analyse gene expression in a series of consecutive segments. Using cDNA synthesised from the expressed mRNA's, transcriptome-walking was performed using Seegene (Rockville, MD, USA) DNA Speedup Premix Kit involving three-step nested PCR reactions to generate specific products.

Three independent sets of transcriptome-walking experiments were performed to interrogate transcribed sequence upstream of exons 98–81, 81–39 and 39–1. Transcriptome-walking using primers TSP1–TSP3 performed in the 5' ← 3' direction from exon 98 and extending to exon 81 through the expressed message to 750 bp upstream of the epitope sequence identified transcription of a non-coding region corresponding to variant 'vq'. Transcriptome-walking using TSP4–6 primers continued upstream by approximately 800 bp to exon 39. TSP7–TSP9 transcriptome-walking proceeded an additional 450 bp upstream to the 3' end of exon 1. The primers were designed to provide overlapping data and hence confirm that each of the sequences retrieved was an extension of the previous mRNA and not a new variant with a truncated 3' end. The purified PCR product from each first-round amplification (using TSP1, 4 or 7 plus universal upstream primers) was used as the template for the second-round PCR amplification using TSP2, 5 or 8. The PCR products generated were employed as the templates amplified in the third-round PCR using TSP3, 6 or 9 without further purification. All PCR reactions comprised heating at 94 °C for 3 min, then 35 cycles of 94 °C for 30 s, 62 °C for 30 s, 72 °C for 100 s and a final extension step of 72 °C for 7 min. Agarose electrophoresis was performed to identify the amplified products (Supplementary Figure 1A–C). Bands excised from the gels were submitted to nucleotide sequencing (Lark Technologies, Takeley, UK). Derived sequences were subjected to BLAST and ClustalW analysis to compare them with the *PRKCZ* gene sequence within the NCBI database. Following analysis of the initial findings, two additional sets of TSPs were designed to exons 46 and 25 to confirm that the variants encoded by these exons were not expressed in the ranges of interest.

To further characterise the expressed sequence corresponding to variant 'vq', six additional TSPs (Supplementary Table 3) were designed. TSP1–TSP3 were located within the non-coding region of exon 89 ahead of the beginning of exon 90. TSP4–TSP6 were designed within the novel sequence that had been identified 5' of exon 89 (Supplementary Figures 1D and E). Transcriptome-walking with primers TSP1–3 and TSP4–6 continued beyond the published sequence for variant 'vq'. All bands were excised from the gels, submitted to sequence analysis and a consensus sequence compiled. To further elucidate the *PRKCZ* variant(s) expressed in the specimens, a further round of transcriptome-walking was performed, in triplicate, using TSP4–TSP6 designed to exon 81 (Supplementary Table 3). The primers were designed to provide an overlap with the data obtained from TSPs1–3, and hence confirm that the sequence retrieved was an extension of the previous

mRNA and not a new variant with a truncated 3' end. The structure of all products was confirmed by sequence analysis.

RT-PCR confirmation of PRKZ variants expressed

A series of strategic RT-PCR reactions together with nucleotide sequencing was performed using the data obtained from transcriptome-walking and applied to the information available within the contemporary NCBI database to confirm the transcription of *PRKCZ*. Primer sequences were designed (Supplementary Table 4; Figure 4A) using Primer 3 software (Whitehead Institute for Biomedical Research, Cambridge, MA, USA; <http://frodo.wi.mit.edu/primer3/>) to span known exon–exon interfaces, hence providing robust data with respect to encoded structures.

Region 1: To confirm the relationship between exon 87 (variant 'm-u') and exon 89 (variant 'vq') and to map the intervening sequence that could not be charted by transcriptome-walking, a common forward primer (Fwd-1_{a/b}) designed to exon 81 was paired with reverse primers located in the novel 5' extension of exon 89 (Rev-1_a) and in the 5' end of exon 89 (Rev-1_b) previously considered to be non-coding.

Region 2: To determine whether the intronic segment between exon 87 and exon 89 was transcribed, a forward primer (Fwd-2) was designed within the 3' end of exon 83 and paired with a reverse primer (Rev-2) in the 5' end of exon 98.

Region 3: The objective of this part of the study was to analyse the structure of the intronic region between exon 98 and exon 104 (the terminal sequence of variant 'k') and hence to obtain structural information on the 13524 bp sequencing gap earlier reported between the two terminal exons of variant 'k'. Separate forward primers (Fwd-3_a and Fwd-3_b) were designed within the 3' end of exon 98 and paired with a common reverse primer (Rev-3_{a/b}) located at the 5' end of exon 104. Alignment of the resulting sequences was made to the consensus sequence of *PRKCZ* within the contemporary NCBI database using BLAST and ClustalW analysis.

Interrogation of NCBI database

To understand the location and distribution of the identified sequences within the *PRKCZ* gene, alignments were made to the contemporary published structure of the human chromosome 1 contig (Gregory *et al*, 2006; Levy *et al*, 2007). Sequences were interrogated with respect to alignment, frequency of sequence recurrence and the presence of any *Alu* sequences. Possible locations of viral insertion sites, especially for viruses HPV and XMRV, were also identified. Nucleotides #16–#370 correspond to the terminal 3' UTR of exon 87 (variant 'm-u'). Nucleotides #90–#386 contain a region that simultaneously corresponds to *Alu* sequences, viral insertion sites and a highly conserved marine metagenome sequence.

Structure and expression of hypothecated protein

Although transcriptome-walking identified transcription of mRNA corresponding to exon 83, including the 5' UTR and continuing into the upstream genomic sequence, western blotting protein extracts from prostate cell lines using polyclonal antiserum sc-216 revealed only a single band at 67.7 kD, corresponding to PKC- ζ_a protein (Figure 2). No additional band was detected at 10.3 kD that corresponded to variant 'vq'. Although sequence analysis of the transcriptome-walking products followed by strategic RT-PCR and subsequent sequencing of those products confirmed expression of the intact mRNA corresponding to exon 83, including its putative coding region, the 5'UTR and the adjacent short 5' intronic segment, there was no evidence that any part of this message was translated into the predicted protein.

Analysis of the exonic structure of *PRKCZ* revealed the coding sequence of the sc-216 epitope contained in exon 90 of *PRKC-ζ_a* to be present also in exon 98 and included in variants 'b', 'c', 'd', 'f', 'g', 'h', 'I', 'j', and 'vm' (Figure 1). To test the hypothesis that the second mRNA sequence detected in the prostate cancer cells was expressed as protein, a monoclonal antibody was generated against the putative peptide obtained by back-translating the identified mRNA sequence into protein followed by western blotting and immunohistochemistry studies.

Generation of monoclonal antibody to the putative peptide sequence

The hypothesized peptide sequence was analysed (<http://gcat.davidson.edu/rakarnik/kyte-doolittle.htm>) to identify hydrophilic domains (Kyte and Doolittle, 1982) as potentially suitable epitopes to generate a monoclonal antibody (Foster, 1982; Bashir *et al.*, 1998), immunise Balb/c mice and perform a classical hybridisation with NS1 mouse myeloma cells (EuroGentech, Seraing, Belgium). A total of 13 hybridomas secreting monoclonal antibodies to the immunising peptide and giving strong signals with clear distinction between benign and malignant prostatic epithelium were cloned by limiting dilution.

Western blotting

Cell lines of different histogenic types were cultured to 80% confluence in 175 cm² flasks. After detachment, cells were extracted by addition of 125 μl of CellLytic M mammalian cell lysis reagent (Sigma-Aldrich, Gillingham, UK) per 10⁶ cells. After lysis and centrifugation at 12 000 × g for 15 min to remove cell debris, each supernatant containing solubilised proteins was collected and quantified using a NanoDrop 1000 instrument.

For polyacrylamide gel electrophoretic separation, aliquots containing 30 μg total cell lysate protein were heated for 2 min at 95 °C and loaded above the separating gel (10% w/v) held in a Mini-Protein 3 Cell apparatus (Bio-Rad, Hercules, CA, USA). Proteins were separated at an initial 80 V until the samples had stacked and then separated at 100 V for 1 h. Proteins were transferred to Hybond ECL nitrocellulose membranes (Amersham Biosciences, Little Chalfont, UK) using a Trans-*alot* electrophoretic transfer cell (Bio-Rad) containing a transfer buffer (pH 8.3) comprising 25 mM Tris-base, 192 mM glycine and 20% methanol (v/v) at 100 V (constant) for 1 h. Each blotted nitrocellulose membrane was placed in 10 ml of 5% (v/v) blocking solution (Geneflow, Fradley, UK) and incubated at room temperature for 1 h on a shaking platform. After draining, each membrane was transferred to a solution of the primary antibody (sc-216: diluted 1:1000 in TBST (20 mM Tris, 500 mM NaCl and 0.1% (w/v) Tween at pH 7.5) containing 5% (w/v) skimmed milk powder or to 10 ml of undiluted hybridoma 5A6 supernatant and incubated overnight at 4 °C. Membranes were washed three times in TBST for 10 min, each time with constant agitation. Thereafter, 20 ml of rabbit polyclonal anti-(mouse immunoglobulin) conjugated with horseradish peroxidase and diluted 1:10 000 in TBST was added and incubated for 30 min with constant agitation. After further washing with TBST (for 10 min, 15 min and 30 min, respectively) membranes were rinsed three times with TBS for 15 min each time before being transferred to fresh ECL-Plus solution (Amersham BioSciences) and incubated for 5 min at room temperature. Membranes were exposed to X-ray film (Hyperfilm, Amersham Biosciences) that was developed and fixed in complete darkness.

RESULTS

Identification of PKC-ζ_a expression in primary prostate cancers and prostate cancer cell lines

Immunohistochemistry using polyclonal antibody sc-216 that detected a 20-mer peptide (Figure 2A) encoded within the 3'-terminal domain of *PRKCZ* (Figure 2B) confirmed strong expression of PKC-ζ_a by

prostate cancer cells but only very weak expression by non-malignant prostatic epithelium (Figure 2C), including PNT-2 cells. Highest expression was found in PC-3M cells, an observation supported by western blotting and PCR (Figure 2D–G).

Transcriptome-walking to confirm *PRKCZ* variants expressed in prostate cancers and prostate cancer cell lines

Transcriptome-walking in the 5' ← 3' direction beginning with a primer sequence in *PRKCZ* exon 98 complementary to that encoding the peptide epitope recognised by antibody sc-216, primers TSP1–TSP3 detected strong signals from *PRKCZ* exons 98–81 (Figure 2) in all cell lines and tissue specimens and identified transcription of the regions encoding exons 98, 90, 86, 85, 84, 82 and 81. Primers TSP4–TSP6 identified transcription of exons 77, 72, 69 and 55. Primers TSP7–TSP9 identified transcription of exons 51, 43, 39, 9, 8, 6 and the 3'-terminal sequence of exon 1. Sequence analysis of the amplified products confirmed alignment to variant 'a' *PRKCZ*. In addition, generic primer C-GTTC identified products corresponding *PRKCZ* variant 'vq' (exon 89) together with a contiguous 253 bp extension into the adjacent upstream intron (Figure 1B) in cell lines DU145, PC-3M and in the three primary prostatic carcinomas but not in PNT-2, LNCaP or in the benign tissues (Supplementary Figure 1A–E). Control experiments confirmed each of the extracted mRNAs employed as template for transcriptome-walking and subsequent expression studies to be free from contamination by genomic DNA (Supplementary Figure 1F). Interrogation of the NCBI database revealed this 253 bp sequence to match that defined by GenBank Accession: AA280988. A consensus structure for the upstream intronic extension of exon 89 was generated from the different PCR products (Figure 3). Expression of variant 'vq' and its upstream intronic extension sequence was confirmed by RT-PCR and detected only in the malignant prostatic cell lines and primary tumour tissues but not in PNT-2, LNCaP cells or in benign tissues. Further transcriptome-walking and nucleotide sequence analysis confirmed expression of the entire length of *PRKCZ* variant 'a'.

RT-PCR confirmation of *PRKCZ* variants expressed

Transcription of the *PRKCZ* genome immediately upstream and downstream of exon 89 was analysed by PCR (Figure 4A, Supplementary Table 4). These findings supported the transcriptome-walking data with a sequence identity of 93.2% and confirmed variant of *PRKCZ* (designated *PRKC-ζ_{PrC}*) to be transcribed in prostatic carcinoma PC-3M cells (Figure 4B–E).

Quantitative PCR analysis of mRNA extracted from prostatic epithelial cell lines identified the relative expression of mRNA for *PRKC-ζ* variants 'a' and 'PrC' (Figure 5A). Comparison of the expression levels of these variants in parental PC-3M cells and their corresponding knockdown derivative cells, si-*PRKC-ζ*-PC3-M_{T1-6}, revealed expression of *PRKC-ζ_a* to be significantly reduced, whereas variant *PRKC-ζ_{PrC}* remained unaltered (Figure 5B), suggesting expression of these two variants to be differentially regulated. *PRKC-ι* (the other member of the group of atypical PKC's and having partial homology to *PRKC-ζ*) was not detected in PNT-2 cells or in the non-malignant tissues from one of the two additional prostate specimens (Figure 5C). Quantitative PCR analysis confirmed relatively low levels of *PRKC-ι* in PNT-2, LNCaP and PC-3M cell lines but high levels expressed in DU145 cells (Figure 5D).

Independent confirmation of PKC-ζ_{PrC} expression in fresh prostatic tissues

The 'Check 2' primer set (Supplementary Table 4), used to amplify the novel expressed sequence, identified an ~1 kb band present in mRNA from PC-3M cells and the malignant components of three

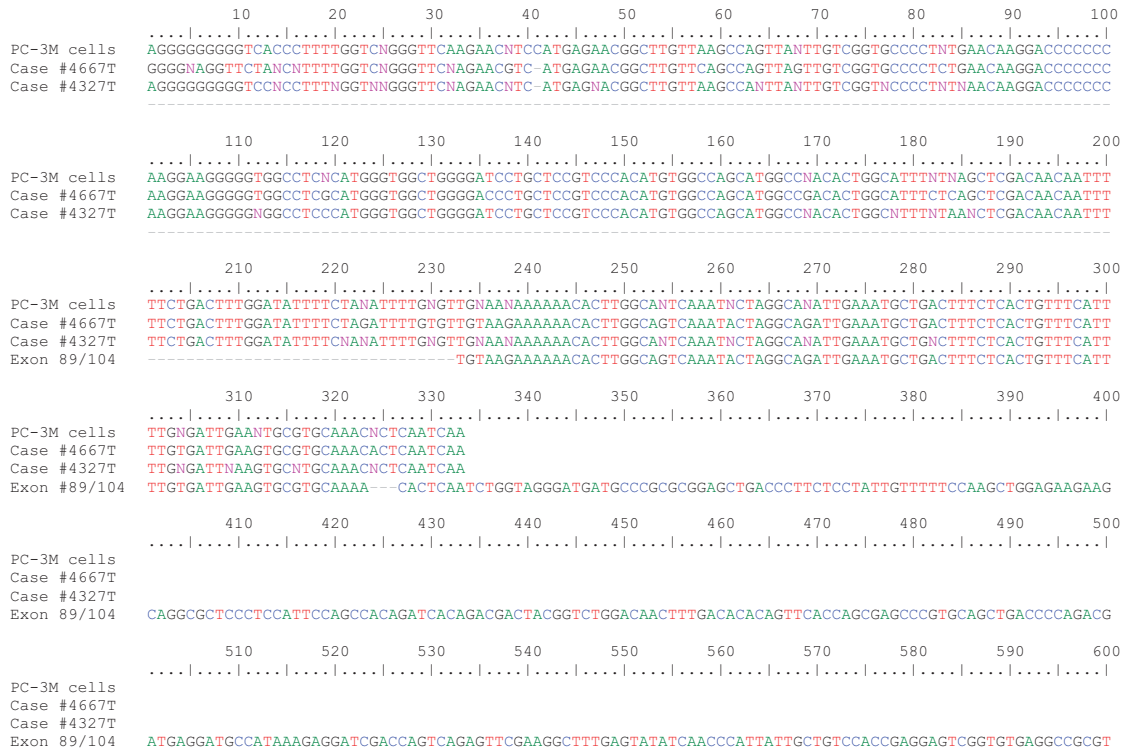


Figure 3 Consensus sequences derived from three replicates for each specimen obtained by genome walking in a 5' ← 3' direction from exon 98 comparing expression sequence data from cell line PC-3M with that from primary carcinomas #4667T and #4327T and their alignment with the published sequence for exon 89 (variant 'vq'). The sequence originating at nucleotide #223 corresponds to the start of the 5' UTR of exon 89 with the obtained sequence 1–222 located within the upstream intronic sequence. Data obtained from the other cell lines and prostate cancer specimens revealed similar matching sequences and alignments (data not shown).

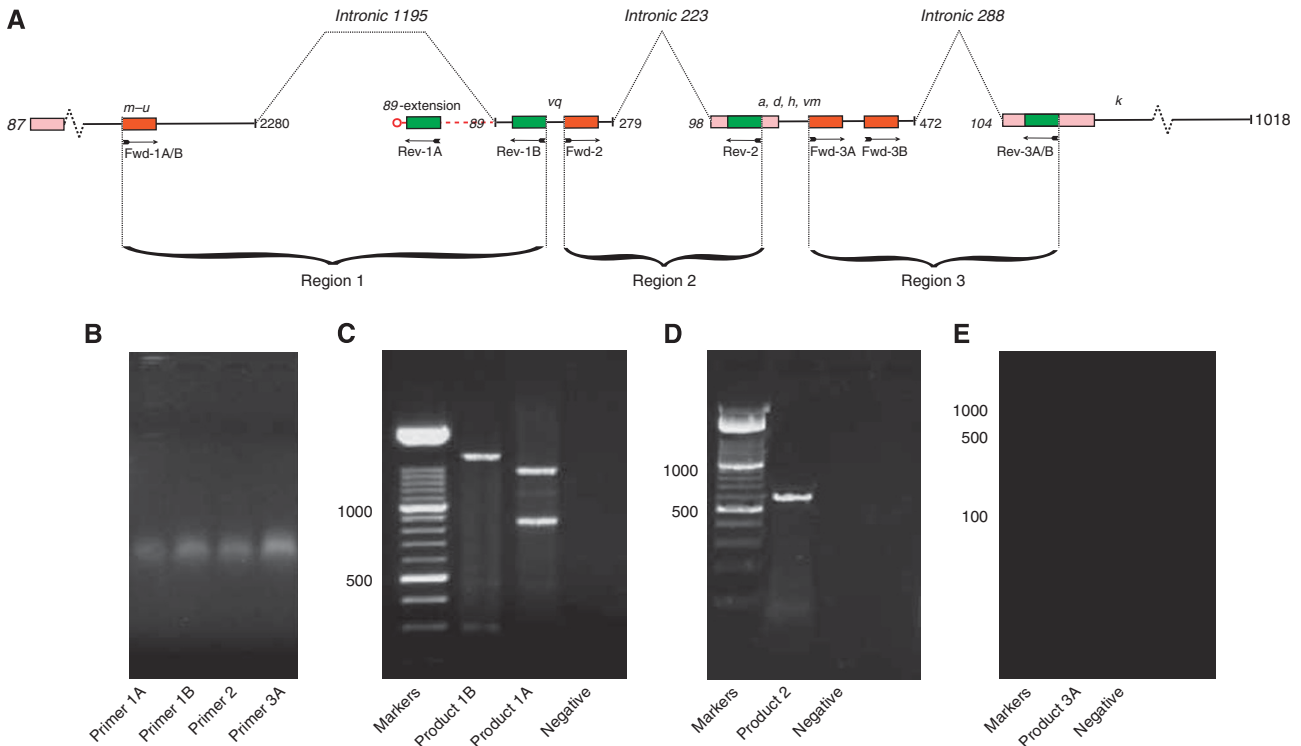


Figure 4 PCR amplification of mRNA from PC-3M cells. **(A)** Schematic diagram of the three contiguous regions, including intronic sequences, identified by genome walking to be expressed in prostate cancer cells. Red and green rectangles identify the positions of forward and reverse PCR primers, respectively (Supplementary Table 4). **(B)** Control gel confirming absence of contaminating genomic DNA. **(C)** Region 1: Three products at ~700, ~1400 and ~1600 bp obtained using primer pairs 1A/B spanning exon 87 to the 5' extension of exon 89 within the intervening intronic sequence. **(D)** Region 2: Single product at ~600 bp spanning exons 89–98 including intervening intronic 223 bp. **(E)** Region 3: Single product of ~594 bp from primer pair 3A spanning exons 98–104, including intronic 288 bp. A smaller product at ~554 bp (data not shown) was derived from primer pair 3B.

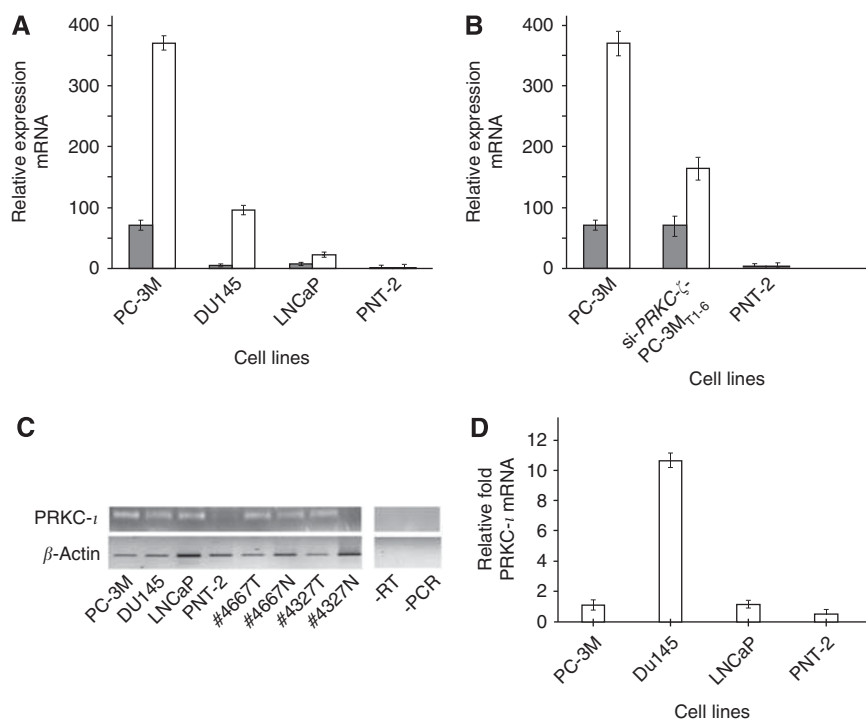


Figure 5 Expression of genes *PRKC-ζ* and *PRKC-ι* in prostate epithelial cells. **(A)** Real-time PCR analysis of *PRKC-ζ* variants 'a' (white bars) and 'PrC' (dark bars) in prostate cell lines using the $\Delta\Delta CT$ method. **(B)** Expression of variants 'a' (white bars) and 'PrC' (dark bars) in the parental PC-3M cells and their knockdown derivative si-*PRKC-ζ*-PC-3M_{T1-6} cells relative to that in PNT-2 cells **(C)** 1% (w/v) agarose gel electrophoresis of *PRKC-ι* (top) and β -actin (bottom) in malignant and non-malignant prostate cancer cell lines and tissues Amplification cycle number was $\times 40$ for Real-time PCR or $\times 35$ for RT-PCR. -RT (Reverse transcriptase not included) and -cDNA (template not included) represent negative controls. **(D)** Expression of *PRKC-ι* quantified in prostate epithelial cells.

fresh radical prostatectomy specimens but not in the non-malignant tissues (Figure 6A and B). The amplified sequence extended from the 3'-terminal region of variant *m-u* (exon 87) through the intervening exonised intronic region to the 5' extension of variant '*vq*' (exon 89). Sequencing analysis confirmed identity between this region expressed in the PC-3M cells and that detected in the primary malignant tissues (Figure 6C).

Generation of monoclonal antibody to the putative peptide sequence

Translation of the novel transcribed sequence (Figure 7A) with run-through of stop codons (Williams *et al*, 2004) identified a putative peptide 985 amino acids long (Figure 7B). Four in-frame candidates were considered as potential antigenic sites to raise monoclonal antibodies to this hypothesized protein. Protein BLAST confirmed peptide sequence – RDRPLRRDRRGKRQR – (identified in red) to be unique to *PRKCZ*. Hydropathy analysis (Kyte and Doolittle, 1982) showed this sequence and to exhibit the lowest hydrophobicity (Figure 7C). Conformation modelling revealed the sequence to be optimally expressed by the intact folded protein. This peptide was employed as the immunogen to generate monoclonal antibodies. Clone 5A6 was selected for further analysis because of its relative strength, lack of non-specific background staining and its discrimination between benign and malignant prostatic epithelium.

Expression of protein identified by 5A6 in epithelial cell lines and prostatic tissues

Western blotting using hybridoma supernatant 5A6 revealed a single strong band at 96 kD in the malignant prostatic epithelial cell lines PC-3M, DU145 and LNCaP but not in the benign PNT-2 cells (Figure 7D). The 96 kD immunoreactive band was not

detected in proteins extracted from non-malignant lung cell line (Beas-2b) or from four malignant cell lines including lung (COR-L88), bladder (HT1197), pancreas (PANC) and breast (MCF-7). The '*PrC*' and '*a*' variants were differentially identified as distinct bands by monoclonal antibody 5A6 and conventional polyclonal antiserum sc-216, respectively (Figure 7E). Tissue sections from benign and malignant prostatic tissues stained using monoclonal antibody 5A6 revealed strong staining of malignant prostatic epithelial cells in contrast to an absence of staining non-malignant prostatic epithelium (Figure 7F).

Interrogation of putative protein structure

NCBI protein Blast of the putative protein from the novel transcribed sequence (Figure 7B) revealed amino acids 21–101 to correspond with the hypothetical protein encoded by LOC 100507445, amino acids 607–694 corresponded to the catalytic domain of atypical *PRKC-ι*, whereas amino acids 624–691 corresponded to the catalytic domain of *PRKC-β*. Although no other structural similarities or motifs were identified, potentially, this novel protein could exhibit kinase activity that is not under normal regulatory control.

Interrogation of human genome

Alignment of all amplified sequences to human genome contig GRCh37.p2 (Gregory *et al*, 2006) identified a continuous expressed sequence 3060 bp long (Supplementary Figure 2) within the *PRKCZ* gene having 96% identity with 33 gaps. The compiled sequence extended from the 3'-terminal region of exon 81 to the 5' end of exon 94, providing novel data completing intervening sequencing gaps and confirming transcription of the included intronic segments. The corresponding coordinates on the contig were identified as 1592941–1595972. Nucleotide BLAST revealed this expressed sequence to be replicated at three additional sites within

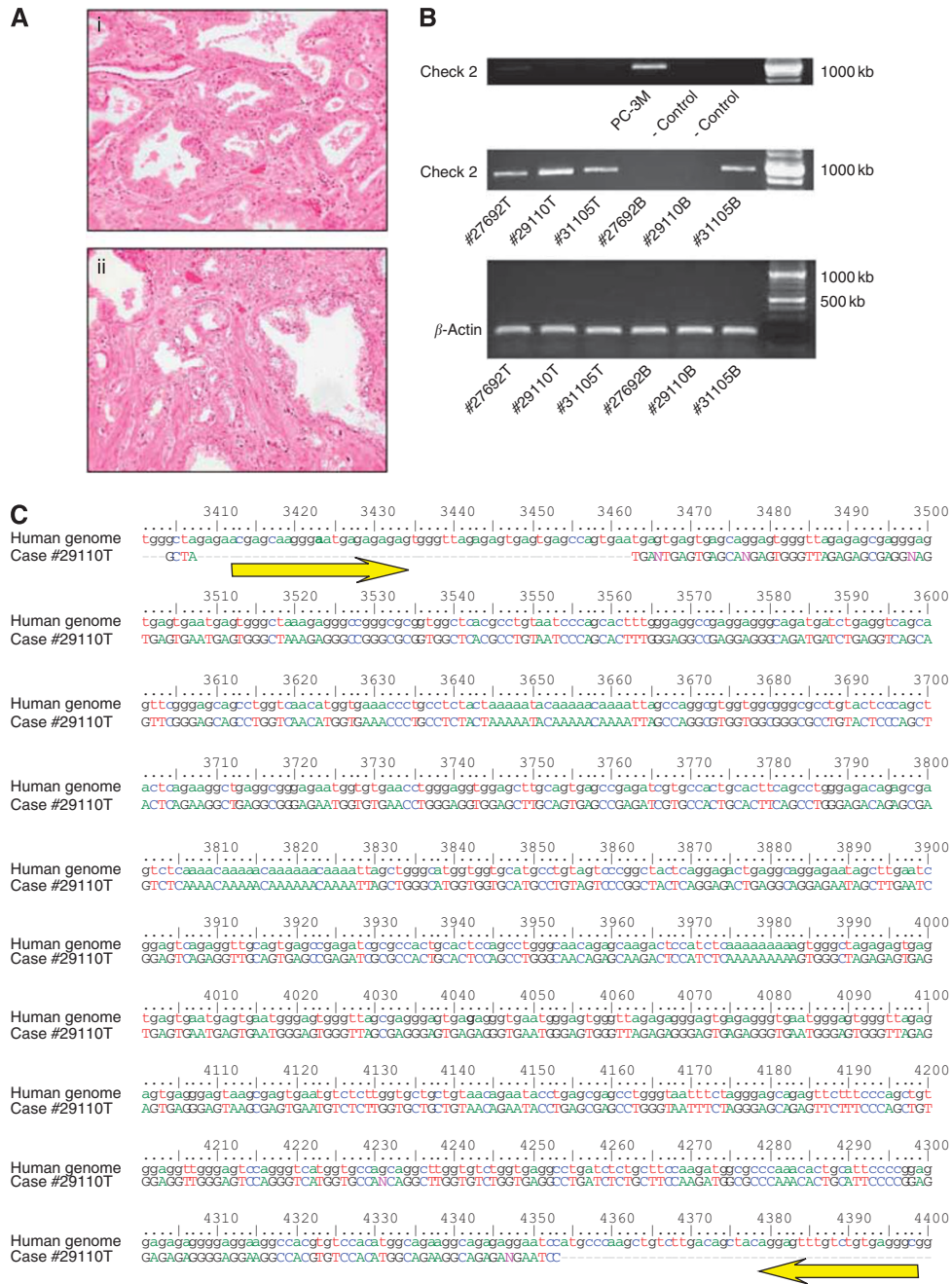


Figure 6 Confirmation of novel sequence expression in prostatic carcinomas. **(A)** Tissue morphology of primary prostatic tissues (i) Primary prostatic carcinoma (#29110T), (ii) predominantly benign prostatic tissue (#31105T) but containing small clusters of carcinoma cells infiltrating between the benign glands. **(B)** Amplification of novel sequence using Check 2 primer set (Supplementary Table 4) in PC-3M cells (top) and in three primary prostatic tissues (middle). Abbreviations: B = predominantly benign tissue; T = tumour tissue. Contamination of benign tissue by prostate cancer cells revealed as a weak band in the benign lane (#31105B). **(C)** Nucleotide sequence of the products from the gels illustrated in panel B aligned with the consensus sequence. Yellow arrows indicate location of the Check 2 primer pair.

the human genome: on the negative strand of chromosome 1p in the open reading frame C1orf86 (NM_001146310, coordinates 2135481–2103093), in locus LOC100506504 (XM_003120338) adjacent to C1orf86 on the negative strand of 1p36 and on the positive strand of chromosome Xq23 (NT 011651.17) with 98% identity. Interrogation of expressed sequence from 1 kb above (5') the start of the compiled sequence (position 1590926) to the end (3') of the sequence (position 1594986) revealed the presence of a single *Alu* block 285 bp long beginning at nucleotide #90 of the compiled sequence and located within the 3'-terminal domain of exon 87 (variant 'm-u', Figure 4). This region also contained a

potential insertion site for viruses HPV and XMRV, although neither was identified in these PC-3M cells (data not included).

DISCUSSION

This study has identified a previously unreported transcript (designated *PRKC-ζ_{PrC}*) expressed in prostate cancer cell lines and tissues within the 3' region of human gene *PRKCZ*. Transcriptome-walking together with targeted PCR analysis and subsequent oligonucleotide sequencing studies performed in

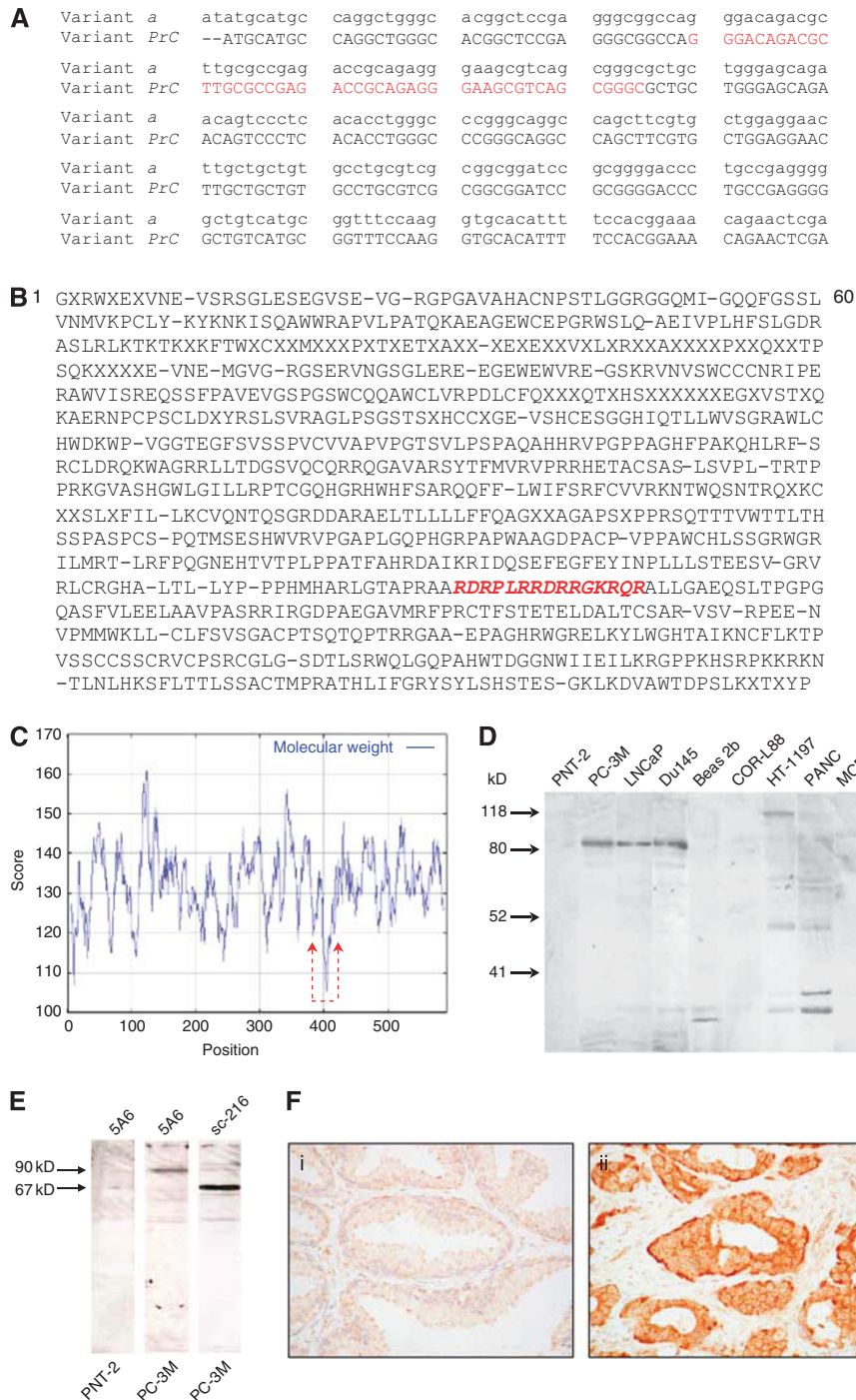


Figure 7 Analysis of novel peptide epitope expression. **(A)** Transcript sequence obtained from the 3'-UTR of exon 98 amplified as part of Region 2 (Figure 4). The sequence encoding the epitope peptide (below) is shown in red. **(B)** Translation of DNA consensus sequence to a hypothetical peptide. The subsequent immunising epitope sequence used as immunogen selected by hydropathy analysis is indicated in red. **(C)** Kyte and Doolittle hydropathy analysis of hypothecated peptide indicating (arrows) the hydrophilic immunogenic region. **(D)** Western blot of human epithelial cell lines using monoclonal antibody 5A6. Cell lines: PNT-2: non-malignant prostate; PC-3M, LNCaP, Du145: malignant prostate; . Beas 2b, COR-L88: malignant lung; HT1197: malignant bladder; PANC: malignant pancreas; MCF-7: malignant breast). **(E)** Western blot comparing the relative motilities of the bands identified in benign (PNT-2) and malignant (PC-3M) prostatic epithelial cell lines using monoclonal antibody 5A6 (variant 'PrC') and polyclonal antiserum sc-2116 (variant 'a'). **(F)** Immunohistochemistry of human prostate tissues using monoclonal antibody 5A6: (i) benign epithelium (ii) malignant epithelium.

prostate cancer cell line PC-3M cells confirmed simultaneous transcription of *PRKCZ* variant 'a' (*PRKC-ζ_a*) together with that of novel sequence *PRKC-ζ_{PrC}*. Both sequences are translated into distinct proteins (*PKC-ζ_a* and *PKC-ζ_{PrC}*, respectively) in prostate cancer cell lines and in primary prostatic carcinomas but not in benign prostatic epithelia or in non-prostatic epithelial cell lines.

Western blotting and immunohistochemistry using monoclonal antibody 5A6 raised to the hypothecated peptide encoded by the novel sequence revealed protein *PKC-ζ_{PrC}* to be expressed only by prostate cancer cells. The novel expressed gene sequence extends between *PRKC* exons 88 and 104 over a distance in excess of 3060 bp including intervening introns and sequences previously

considered as non-coding. Interrogation of contig GRCh37.p2 (Gregory *et al*, 2006) revealed a block of *Alu* sequences (*Alu-Sx*, *Alu-Sb*, *Alu-Sq*, *Alu-Sb1*, *Alu-Sc*, *Alu-Sp*, *Alu-Sj*, *Alu-Sb2*) 285 bp long close to the origin of the novel sequence. Translation of the expressed sequence with run-through of the contained stop codons (Williams *et al*, 2004) identified a potential protein 985 amino acids long that contained a single region corresponding to the catalytic domains of classic *PRKC-β* and atypical *PRKC-ι* enzymes but no other functional homologies or motifs. PCR analysis of mRNA extracted from fresh primary prostatic tissues supported the proposition that *PRKC-ζ_{PRC}* is expressed only in malignant prostatic epithelium. The finding that *PRKC-ζ_a* and *PRKC-ζ_{PRC}* are expressed simultaneously together with the observation that RNAi directed at *PRKC-ζ_a* failed to reduce expression of *PRKC-ζ_{PRC}* supports a mechanism whereby these alternative sites, possibly pseudogenes (Vanin, 1985; Andrea and Walsh, 1995), potentially allow simultaneous expression of the two distinct variants, possibly from identical genomic sequences that are located separately within the genome. There is documented support for the proposal that alternative *PKC-ζ* transcripts can be transcribed simultaneously from a common *PRKCZ* gene sequence in the absence of gene duplication or existence of a structurally identical pseudogene (Marshall *et al*, 2000). Although there is no evidence that *PRKCZ* is oncogenic, anomalous expression of *PRKCZ* variant *PKC-ζ_{PRC}* exhibiting alternative (that is, *PKC-β* or *PKC-ι*) kinase activity may promote the aggressive phenotype of prostate cancer.

In contrast to some human malignancies, prostate cancer may be associated with multiple gene loci each independently conferring a low but cumulative risk (Al Olama *et al*, 2009; Eeles *et al*, 2009). Genetic susceptibility to prostate cancer is complex, possibly caused by interaction of low-penetrance genes that include rare putative autosomal dominants (0.003–0.06 allele frequency). Linkage analysis has identified susceptibility with loci on chromosome 1p36 (Gibbs *et al*, 1999; Xu *et al*, 2001), particularly the CAPB (carcinoma prostate brain) locus (Badzioch *et al*, 2000). However, the effect of CAPB appears to be independent of the HPC1 locus (1q24–25) that has been confirmed in families with aggressive prostate cancer occurring at median age. Furthermore, very low levels of allelic imbalance found at HPC1 and 1p36 make it unlikely that these loci contain classic tumour suppressor genes (Ahman *et al*, 2000). Nevertheless, human chromosome 1p36 is recognised to be a fragile region, as evidenced by the *Alu* blocks identified in this study. The frequent structural changes occurring in this region, particularly deletions (Battaglia and Shaffer, 2008), are associated with many different mesenchymal and epithelial malignancies. Decreased expression of *TERE1* that maps at 1p36.11–36.33 occurs in some 61% of aggressive prostate cancers, particularly metastases (McGarvey *et al*, 2003). *TERE1* is closely linked to *DISP3* (1p36.22) that encodes multi-span transmembrane proteins implicated in the processing of lipid-enhanced secreted proteins (Katoh and Katoh, 2005), a process we have reported to be functionally implicated in aggressive prostate cancer (Foorootan *et al*, 2009). Within the 1p36 locus, *p73* encodes a protein with a high level of homology to *p53* that is infrequently sporadically uninhibited. Although germ-line mutations within *p73* do not predispose to prostate cancer (Peters *et al*, 2001) and expression of *p73* is not modulated in prostate cancer PC-3M cells when *PRKC-ζ* (at 1p36.33) is knocked down following RNAi (Yao *et al*, 2010), it is possible that *p73* may substitute for some *p53* activities when that gene is mutated in ~3–42% of prostate cancers (Agell *et al*, 2008), thus promoting the aggressive phenotype. Similarly, expression of *TNFR2* (also encoded at 1p36.2) is deleted or mutated in a range of human malignancies but not modulated in PC-3M cells following *PRKC-ζ* knockdown.

Structural aberrations (deletions, tandem duplications, translocations, insertions and inversions) within the genome of malignant cells are frequent (Beerenwinkel *et al*, 2007; Bignell *et al*, 2010).

PKCs comprise a large family of enzymes that mediate multiple functions including maintenance of internal homeostasis, response to external stimuli and modulation of cellular phenotype. This family of enzymes, defined by conserved sequences within their catalytic domains (Hanks *et al*, 1988) is a large, heterogeneous and continually evolving group with origins extending to the most primitive unicellular organisms (Mellor and Parker, 1998; Manning *et al*, 2002). Within the PKC super family, atypical PKCs (λ/ι and ζ) are structurally and functionally divergent from other members (Schmitz and Heinisch, 2003). These are powerful regulatory enzymes that control the polarity and migration of individual cells as well as the development of multi-cellular structures, (Suzuki *et al*, 2003) frequently mediating opposing effects on homeostatic regulators and mechanisms such as ion channels (Muscella *et al*, 2005). The spectrum of putative *PK* genes has been augmented following identification of individual *PK* gene duplication, pseudogenes and genes encoding truncated enzymes (Standaert *et al*, 2001), emphasising that the family of *PKC* genes and corresponding proteins is not only continually evolving but is constantly diversifying with respect to the functions of individual members. Genetically, the presence of *Alu* sequences in an open-reading frame, such as those identified in this study, produce genetic instability, usually altering gene expression or causing disruption by insertional mutation (Batzer and Deininger, 2002) resulting in homologous recombination leading to generation of a new genetic variant. Genomic analyses have revealed structural alterations in the subtelomeric region of human chromosome 1p to be associated with several different malignancies and to harbour a novel tumour suppressor (Bagchi and Mills, 2008). This region (chromosome 1p36) is not only under intense evolutionary pressure (van der Drift *et al*, 1999) but is an important region of phylogenetic divergence separating *Homo sapiens* from members of the *Pongidae* (Conte *et al*, 1999) and other ape species (Weise *et al*, 2005). Therefore, it is not surprising that this relatively unstable region of the human genome is also involved in promoting the malignant phenotype as well as contributing to evolutionary divergence, possibly employing similar mechanisms (Krull *et al*, 2007).

The specific mechanism(s) by which *PKC-ζ* promotes invasion and metastasis of human prostate cancer are not yet elucidated, although the gene is recognised to be pleiotropic. Discovery of gene splice variants has revealed that one gene may potentially produce multiple different isoforms emphasising the complexity of their involvement in the spectrum of cellular activities. Variant RNA transcripts generated by alternative splicing (Brett *et al*, 2002; Modrek and Lee, 2002), possibly directed by *Alu* sequences (Krull *et al*, 2005; Corvelo and Eyra, 2008; Gal-Mark *et al*, 2008; Romanish *et al*, 2009) or by 'read-through' of intronic sequences (Kaer *et al*, 2011) may be translated into proteins containing (or omitting) alternative motifs, binding sites and subcellular localisation signals. With respect to PK, isoform PKM- ζ containing a novel *PKC-ζ* catalytic domain has been reported as specific to the brain (Hernandez *et al*, 2003) and *PKC-ζ*II, another newly identified member of the *PKC-ζ* family, is a truncated form of the protein actively involved in cell polarity through inhibition of tight junction formation (Parkinson *et al*, 2004). Retention of intronic sequences and subsequent generation of novel products exhibiting biological activities out-with the recognised function of specific genes is a powerful driver of phenotypic diversity within malignant cells (De Rosa *et al*, 2007) as well as in normal cellular evolution (Calvanese *et al*, 2008; Gontijo *et al*, 2011). Inclusion of catalytic domains characteristically expressed in classic *PKC-β* and atypical *PKC-ι* enzymes provides novel evidence that anomalous variants of PK enzymes are powerful agents potentially able to independently modulate the malignant prostatic phenotype out-with normal regulatory mechanisms. Our current studies emphasise the importance of independently characterising all gene sequences transcribed in each individual cancer as expression of particular gene variants may significantly modify the malignant phenotype,

particularly the biological behaviour of individual malignancies, with profound implications for their appropriate management and clinical outcome.

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