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

S Yao¹, SJ Ireland¹, A Bee¹, C Beesley¹, SS Forootan¹, A Dodson¹, T Dickinson¹, P Gerard¹, L-Y Lian², JM Risk², P Smith¹, MI Malki¹, Y Ke¹, CS Cooper³, C Gosden¹ and CS Foster*,¹

¹Division of Pathology, Department of Molecular and Clinical Cancer Medicine, University of Liverpool, 6th Floor, Duncan Building, Daulby Street, Liverpool L69 3GA, UK; ²School of Biological Sciences, University of Liverpool, Liverpool L69 7ZB, UK; ³University of East Anglia, Norwich Research Park, Norwich NR4 7TJ, UK

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. Hydropathy 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–u}.

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-ζ provides a potential mechanism for this PRKCZ variant to modulate the malignant prostate phenotype out-with normal cell-regulatory control.

British Journal of Cancer (2012) 107, 388–399. doi:10.1038/bjc.2012.162 www.bjcancer.com
Published online 29 May 2012
© 2012 Cancer Research UK

Keywords: prostate cancer; PRKCZ; structural variant; novel sequence

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; Utrrreger 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...
et al., 2010; Luna-Ulloa et al., 2011; Valkov et al., 2011; Yu et al., 2011). Therefore, confirming its structure was considered as 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-zeta 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-zeta PRC') that have been shown to be selective for human prostate cancer. This sequence is expressed simultaneously, but independently, from genes expressed in prostate cancer and human prostate cancers. Thus, the PKC-zeta variant selectively expressed in prostate cancer (Yao et al., 2010), were also supplemented with 300 ng ml⁻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-zeta-a in human prostate cancers. The peptide sequence recognised by the rabbit polyclonal antiserum was designed to include the unique epitope 5′-GAGACATGTGTCGTCTT-3′, a 21 nt sequence (Yao et al., 2010) to reveal expression of PKC-zeta-a in human prostate cancers. The peptide sequence recognised by the rabbit polyclonal antiserum was designed to include the unique epitope 5′-GAGACATGTGTCGTCTT-3′, a 21 nt sequence (Yao et al., 2010) to reveal expression of PKC-zeta-a in human prostate cancers. The peptide sequence recognised by the rabbit polyclonal antiserum was designed to include the unique epitope 5′-GAGACATGTGTCGTCTT-3′, a 21 nt sequence (Yao et al., 2010) to reveal expression of PKC-zeta-a in human prostate cancers. The peptide sequence recognised by the rabbit polyclonal antiserum was designed to include the unique epitope 5′-GAGACATGTGTCGTCTT-3′, a 21 nt sequence (Yao et al., 2010) to reveal expression of PKC-zeta-a in human prostate cancers. The peptide sequence recognised by
Protein structure revealed this sequence to correspond to a coding sequence, whereas lower case letters are exonic but untranslated. (B) Back translation of the antigenic peptide to identify its location (bold italics) in the 3' terminal expressed sequence of exon 98 (Figure 2A). Sequence of the antigenic peptide identified by polyclonal antisum sc-216, provided by Santa Cruz. (B) Back translation of the antigenic peptide to identify its location (bold italics) in the 3' terminal expressed sequence of exon 98 PRKCC variant 'a' according to the NCBI database (build 36, 2011). Upper-case letters signify expression sequence, whereas lower case exons are exonic but untranslated. (C) Immunohistochemical detection of PKC-ζ protein in (i) non-neoplastic, (ii) hyperplastic, (iii) prostatic carcinoma (Gleason 3 + 3) and (iv) prostatic carcinoma (Gleason 5 + 5). (D) Western blot of PKC-ζ human prostatic cell lines. (E) Western blot data standardised to β-actin expression. (F) PCR analysis of human prostate cell lines confirming expression of PKC-ζ mRNA. (G) Relative levels of PKC-ζ mRNA standardised to β-actin expression. Immunohistochemical staining

Histological sections of 12 paraffin wax-embedded prostate cancers and 12 non-malignant prostate tissues from specimens of benign prostatic disease without malignancy were obtained from Department of Pathology, University of Liverpool, UK. Conditions (dilution, temperature and pH) for staining with the primary antibody were optimised using known standard positive and negative tissues. Immunohistochemical staining was performed using a fully automated Ventana Benchmark XT-TM immunohistochemistry platform as specified previously (Foster et al., 2009).

Quality control of mRNA and RT–PCR controls

For transcriptome-walking and subsequent sequencing studies, cell lines were grown up and extracted in triplicate as separate preparations to provide independent biological replicates. All mRNA preparations were routinely measured using a Nanodrop instrument (LabTech International, Ringmer, UK) to ensure quality. Following each extraction, all preparations were treated with DNase I to destroy any residual contamination by genomic DNA. To exclude the possibility that the amplified PCR product may be derived from genomic contamination, 1 μg total RNA was amplified by PCR using the same primer pairs directly as a control under the same conditions. Since the amount of genomic DNA in total RNA is approximately × 20 than that in the subsequently purified mRNA, failure to amplify specific sequences from total RNA, while generating a reliable product from corresponding mRNA, is a reliable indicator of the latter’s purity. As negative controls, a minus RT first-strand synthesis was performed to ensure that the amplifications were derived from mRNA and not from genomic DNA contamination. Additional negative controls were obtained by PCR amplification of the cDNA and genomic DNA using primers (Supplementary Table 2) to PRKCC variants ‘f’ and ‘n’ that were known from preliminary studies not to be expressed by the prostatic cell lines used in this study. After completion of the PCR reactions, amplified products were separated by electrophoresis in agarose gels containing ethidium bromide (0.5 μg·ml⁻¹). Bands were visualised under UV light, eluted and submitted to sequence analysis.

RT–PCR to confirm expression of PKC-ζ isoform

Total RNA was isolated from the five prostate cancer cell lines, including the si-PRKCC-ζ-a-PC-3M716 knockdown cells, using RNeasy Mini Kits (Qiagen, Crawley, UK), a ready-to-use reagent for the isolation of total RNA from cells and tissues, following the manufacturer’s recommendations. DNase I (Qiagen) was added to the RNA sample to remove any traces of genomic DNA. Total RNA was determined by Nanodrop. All RNA was assessed for quality and purity using a BioAnalyzer (Agilent Technologies, Stockport, UK) before being used in further studies. Only RNA with an RIN > 8.0 was employed in these studies. First strand cDNA was synthesised using Reverse-iT first-strand synthesis kits Superscript III (Invitrogen). PCR was performed using gene-specific primers (Supplementary Table 2) to confirm expression of PRKCC-ζ-a. The employed protocol comprised: initial cycle at 94 °C for 4 min, followed by 35 cycles consisting of denaturation at 94 °C for 20 s, annealing at 57 °C for 30 s and extension at 72 °C for 1 min. These 35 cycles were followed by 10 min of extension at 72 °C.

Assessment of PRKCI expression

As PRKCI (chromosome 1) and PRKCI (chromosome 3) are members of the family of atypical PK genes, and possibly evolutionarily related, PCR reactions using gene-specific primers (Supplementary Table 2) were performed to assess whether polyclonal antisum sc-216 (Figure 2A) was released by Santa Cruz Biotechnology (Santa Cruz, CA, USA). Interrogation of the protein structure revealed this sequence to correspond to a coding sequence within exon 90 at the 5' terminus of the PRKCI gene (Figure 2B). Western blotting had previously confirmed this protein to be strongly expressed in PC-3M cells.
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 antiseraum 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′ 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 part of a new variant with a truncated 3′ end.

To further characterise the expressed sequence corresponding to variant ‘vq’, a further three 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’), exon 89 (variant ‘vq’), and to map the intervening sequence that could not be charted by transcriptome-walking, a common forward primer (Fwd-1) designed to exon 81 was paired with reverse primers located in the novel 5′ extension of exon 89 (Rev-1) and in the 3′ end of exon 89 (Rev-1n) 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, and Fwd-3n) were designed within the 3′ end of exon 98 and paired with a common reverse primer (Rev-3n) located at the 3′ 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 antisera 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.

© 2012 Cancer Research UK
Analysis of the exonic structure of PRKCZ revealed the coding sequence of the sc-216 epitope contained in exon 90 of PRKC-ζa 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 hypothesised 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 hybridoma 5A6. 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 ml of Celllytic M mammalian cell lysis reagent (Sigma-Aldrich, Gillingham, UK) per 10⁶ cells. After lysis and centrifugation at 12000 × g for 15 min to remove cell debris, each supernatant containing solubilised proteins was collected and quantified using a NanoDrop 1000 instrument. 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% v/v) held in a Mini-Protean 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 3% (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 : 10000 in TBST (200 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 : 10000 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-ζa 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-ζa 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, 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 and 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-ζvqPc) 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 PKC-ζ variants ‘a’ and ‘Pc’ (Figure 5A). Comparison of the expression levels of these variants in parental PC-3M cells and their corresponding knockdown derivative cells, si-PKC-ζ-PC3-Mt1–Mt6, revealed expression of PRKC-ζa to be significantly reduced, whereas variant PRKC-ζvqPc remained unaltered (Figure 5B), suggesting expression of these two variants to be differentially regulated. PKCζ variants in all cell lines and tissues examined were expressed at levels similar to PKCζ and having partial homology to PKC-ζvq 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-ζ in PNT-2, LNCaP and PC-3M cell lines but high levels expressed in DU145 cells (Figure 5D).

Independent confirmation of PKC-ζa 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
and respectively (Supplementary Table 4). (B) by genome walking to be expressed in prostate cancer cells. Red and green rectangles identify the positions of forward and reverse PCR primers, Figure 4 PCR amplification of mRNA from PC-3M cells. (C) 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).

| Region | Exon | Primer 1A | Primer 1B | Primer 2 | Primer 3A |
|--------|------|-----------|-----------|----------|-----------|
| 1      | 87   | Primer 1A | Primer 1B | Primer 2 | Primer 3A |
| 2      | 2200 | Primer 1A | Primer 1B | Primer 2 | Primer 3A |
| 3      | 472  | Primer 1A | Primer 1B | Primer 2 | Primer 3A |

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.

© 2012 Cancer Research UK

British Journal of Cancer (2012) 107(2), 388 – 399
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 νp′ (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 hypothesised protein. Protein BLAST confirmed peptide sequence – RDRPLRRDRRGKRQR – (identified in red) to be unique to PRKCZ. Hydrophathy analysis (Kyte and Doolittle, 1982) showed this sequence and to exhibit the putative peptide 985 amino acids long (Figure 7B). Four in-frame segments. The corresponding coordinates on the contig were extended from the 3′-0-terminal region of exon 81 to the 5′-0-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 sequence to be replicated at three additional sites within the gene having 96% identity with 33 gaps. The compiled sequence was 3060 bp long (Supplementary Figure 2) within the human genome contig GRCh37.p2 (Gregory et al, 2006) identified a continuous expressed sequence (Figure 7B) revealed amino acids 21–101 to correspond with the catalytic domain of atypical PRKC–ζ (top) and β-actin (bottom) in malignant and non-malignant prostate cancer cell lines and tissues Amplification cycle number was × 40 for Real-time PCR or × 35 for RT-PCR. –RT (Reverse transcriptase not included) and –cDNA (template not included) represent negative controls. (D) Expression of PRKC–ζ quantified in prostate epithelial cells.

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 catalytic domain of atypical PRKC–ζ, whereas amino acids 624–691 corresponded to the catalytic domain of atypical PRKC–ζ. 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 1592941–1599572. Nucleotide BLAST revealed this expressed sequence to be replicated at three additional sites within the human genome contig.
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) and tissues within the 3' region of human gene PRKCZ. 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-a', 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 PRKCZ-\(s_{PC}\)) 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
prostate cancer cell line PC-3M cells confirmed simultaneous transcription of PRKCZ variant ‘a’ (PRKC\(_z\)-a) together with that of novel sequence PRKC\(_z\)-PrC. Both sequences are translated into distinct proteins (PKC\(_z\)-a and PKC\(_z\)-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\(_z\)-PrC to be expressed only by prostate cancer cells. The novel expressed gene sequence extends between PRKCZ exons 88 and 104 over a distance in excess of 3060 bp including intervening introns and sequences previously
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 (\(\zeta\) and \(\iota\)) 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 insertionional 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-\(\zeta\) 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 promote invasion and metastasis in multiple different malignancies and that the combination of individual variants may explain as yet unidentified differences in their involvement in the spectrum of cellular activities. Variant mRNA transcripts generated by alternative splicing (Brett et al., 2002; Modrek and Lee, 2002), possibly directed by Alu sequences (Krull et al., 2005; Corvelo and Eyras, 2008; Gal-Mark et al., 2008; Romanish et al., 2009) or by ‘read-through’ of intronic sequences (Kaar 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-\(\zeta\) containing a novel PKC-\(\zeta\) catalytic domain has been reported as specific to the brain (Hernandez et al., 2003) and PKC-\(\zeta\)-II, another newly identified member of the PKC-\(\zeta\) family, is a truncated form of the protein actively involved in cell polarity through inhibition of tight junction formation (Peters et al., 2004) or by ‘read-through’ of intronic sequences (Krull et al., 2005). The PKC-\(\zeta\) catalytic domain characteristically expressed in classic PKC-\(\beta\) and atypical PKC-\(\iota\) enzymes provides novel evidence that abnormal 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.
ACKNOWLEDGEMENTS

We are extremely grateful to Mrs Jill Gosney for her invaluable assistance in preparing this manuscript. This work was supported by the North West Cancer Research Fund (UK) (grant: CR 760); Prostate Research Campaign (UK) (project: #73910) and the National Cancer Research Institute (MRC-UK) (grant: MRC99X). Funding bodies had no involvement in the design and conduct of the study; in collection management, analysis and interpretation of the data or in preparation, review and approval of this publication.

Supplementary Information accompanies the paper on the British Journal of Cancer website (http://www.nature.com/bjc).

REFERENCES

Agell L, Hernandez S, de Muga S, Lorente JA, Juanpere N, Esqueva R, Serrano S, Gelabert A, Lloreta J (2008) KLF6 and TP53 mutations are a rare event in prostate cancer: distinguishing between Taq polymerase artifacts and true mutations. Mod Pathol 21: 1470–1478
Ahman AK, Jonsson BA, Damber JE, Bergh A, Emanuelsson M, Gronberg H (2000) Low frequency allelic imbalance at the prostate cancer susceptibility loci HPC1 and 1p36 in Swedish men with hereditary prostate cancer. Genes Chromosomes Cancer 29: 292–296
Al Olama AA, Kote-Jarai Z, Giles GG, Guy M, Morrison J, Severi G, Leongamornlert DA, Tymrakiewicz M, Jhavar S, Saunders EJ, Hopper JL, Huddart RA, Khoo VS, Parker CC, Woodhouse CJ, Thompson A, Christmas T, Ogden C, Cooper C, Donovan JL, Hamdy FC, Neal DE, Eeles RA, Easton DF, UK Prostate Cancer Study Collaborators/British Association of Urological Surgeons’ Section of Oncology, UK Prostate testing for cancer and treatment study (ProtecT) Study Collaborators (2009) Multiple loci on 6q24 associated with prostate cancer susceptibility. Nat Genet 41: 1058–1060
Ali AS, Ali S, El-Rayes BF, Philip PA, Sarkar FH (2009) Exploitation of protein kinase C A as a useful target for cancer therapy. Cancer Treat Rev 35: 1–8
Andrea JE, Walsh MP (1995) Identification of a brain-specific protein kinase C zeta pseudogene (ps PKC zeta) transcript. Biochem J 310: 835–843
Badisch M, Eroles R, Leblanc G, Foukles WD, Giles G, Edwards S, Goldgar D, Hopper JL, Bishop DT, Moller H, Clark J, Attard G, Foster CS, Proctor CS, Southey MC, Lophatananon A, Liu JF, Kolonel LN, Le Marchand L, Walfors T, Tammeda TL, Auvine V, Sørensen J, Campbell PJ, Scott PD, Ford D, Goodfellow P, Foulkes WD, Eeles RA, Easton DF, UK Prostate Cancer Study Collaborators/British Association of Urological Surgeons’ Section of Oncology, UK Prostate testing for cancer and treatment study (ProtecT) Study Collaborators (2009) Multiple loci on 6q24 associated with prostate cancer susceptibility. Nat Genet 41: 1058–1060
Ahman AK, Jonsson BA, Damber JE, Bergh A, Emanuelsson M, Gronberg H (2000) Low frequency allelic imbalance at the prostate cancer susceptibility loci HPC1 and 1p36 in Swedish men with hereditary prostate cancer. Genes Chromosomes Cancer 29: 292–296
Al Olama AA, Kote-Jarai Z, Giles GG, Guy M, Morrison J, Severi G, Leongamornlert DA, Tymrakiewicz M, Jhavar S, Saunders EJ, Hopper JL, Huddart RA, Khoo VS, Parker CC, Woodhouse CJ, Thompson A, Christmas T, Ogden C, Cooper C, Donovan JL, Hamdy FC, Neal DE, Eeles RA, Easton DF, UK Prostate Cancer Study Collaborators/British Association of Urological Surgeons’ Section of Oncology, UK Prostate testing for cancer and treatment study (ProtecT) Study Collaborators (2009) Multiple loci on 6q24 associated with prostate cancer susceptibility. Nat Genet 41: 1058–1060
Ali AS, Ali S, El-Rayes BF, Philip PA, Sarkar FH (2009) Exploitation of protein kinase C A as a useful target for cancer therapy. Cancer Treat Rev 35: 1–8
Andrea JE, Walsh MP (1995) Identification of a brain-specific protein kinase C zeta pseudogene (ps PKC zeta) transcript. Biochem J 310: 835–843
Badisch M, Eroles R, Leblanc G, Foukles WD, Giles G, Edwards S, Goldgar D, Hopper JL, Bishop DT, Moller H, Clark J, Attard G, Foster CS, Proctor CS, Southey MC, Lophatananon A, Liu JF, Kolonel LN, Le Marchand L, Walfors T, Tammeda TL, Auvine V, Sørensen J, Campbell PJ, Scott PD, Ford D, Goodfellow P, Foulkes WD, Eeles RA, Easton DF, UK Prostate Cancer Study Collaborators/British Association of Urological Surgeons’ Section of Oncology, UK Prostate testing for cancer and treatment study (ProtecT) Study Collaborators (2009) Multiple loci on 6q24 associated with prostate cancer susceptibility. Nat Genet 41: 1058–1060
Al Olama AA, Kote-Jarai Z, Giles GG, Guy M, Morrison J, Severi G, Leongamornlert DA, Tymrakiewicz M, Jhavar S, Saunders EJ, Hopper JL, Huddart RA, Khoo VS, Parker CC, Woodhouse CJ, Thompson A, Christmas T, Ogden C, Cooper C, Donovan JL, Hamdy FC, Neal DE, Eeles RA, Easton DF, UK Prostate Cancer Study Collaborators/British Association of Urological Surgeons’ Section of Oncology, UK Prostate testing for cancer and treatment study (ProtecT) Study Collaborators (2009) Multiple loci on 6q24 associated with prostate cancer susceptibility. Nat Genet 41: 1058–1060

Supplementary Information accompanies the paper on the British Journal of Cancer website (http://www.nature.com/bjc)
Marshall BS, Price G, Powell CT (2000) Rat protein kinase c zeta gene contains alternative promoters for generation of dual transcripts with 5′-end heterogeneity. DNA Cell Biol 19: 707–719
McGarvey TW, Nguyen T, Puthiyaveetil R, Tomaszewski JE, Malkowicz SB (2003) TERE1, a novel gene affecting growth regulation in prostate carcinoma. Prostate 54: 144–155
Mellor H, Parker PJ (1996) The extended protein kinase C superfamily. Biochem J 332: 281–292
Modrek B, Lee CI (2002) A genomic view of alternative splicing. Nat Genet 30: 13–19
Muscella A, Storelli C, Marsiglione S (2005) Atypical PKC-zeta and PKC- iota mediate opposing effects on MCF-7 Na + /K + ATPase activity. J Cell Physiol 205: 278–285
Ohno S, Nishizuka Y (2002) Protein kinase C isoforms and their specific functions. Prologue. J Biochem 132: 509–511
Parkinson SJ, Le Good JA, Whelan RD, Whitehead P, Parker PJ (2004) Identification of PKCζatell: an endogenous inhibitor of cell polarity. EMBO J 23: 77–88
Patel NA, Song SS, Cooper DB (2006) PKCζdelta alternatively spliced isoforms modulate cellular apoptosis in retinoic acid-induced differentiation of human NT2 cells and mouse embryonic stem cells. Gene Expr 13: 73–84
Peters MA, Janer M, Kolb S, Jarvik GP, Ostrander EA, Stanford JL (2001) Germline mutations in the p73 gene do not predispose to familial prostate-brain cancer. Prostate 48: 292–296
Romanishin MT, Nakamura Y, Uchida K, Yang W, Mager DL (2009) A novel protein isoform of the multicopy human NAIP gene derives from intragenic ALU SINE promoters. PLoS One 4: e6761
Schmitz HP, Heinisch JJ (2003) Evolution, biochemistry and genetics of protein kinase C in fungi. Curr Genet 43: 245–254
Standaert ML, Urtreger AJ, Kazanietz MG, Balde Kier ´ ED (2012) Contribution of individual PKC isoforms to breast cancer progression. Int Union Biochem Mol Biol Life 64: 18–26
Suzuki A, Akimoto K, Ohno S (2003) Protein kinase C lamda/iota (PKClamda/iota): a PKC isoform essential for the development of multicellular organisms. J Biochem 133: 9–16
Urtreger AJ, Kazanietz MG, Balde Kier JoF Ed (2012) Contribution of individual PKC isoforms to breast cancer progression. Int Union Biochem Mol Biol Life 64: 18–26
Vanlova K, Sörbye SW, Kılvaer TK, Donnen E, Bremnes RM, Busund LT (2011) The prognostic impact of TGF-β1, fascin, NF-xB and PCKc expression in soft tissue sarcomas. PLoS One 6: e17507
van der Drift P, Chan A, Zehetner G, Westerveld A, Versteeck R (1999) Multiple MSP pseudogenes in a local repeat cluster on 1p36.2: An expanding genomic graveyard? Genomics 62: 74–81
Vanin EF (1985) Processed pseudogenes: characteristics and evolution. Annu Rev Genet 19: 253–272
Weise A, Starke H, Mrašek K, Clausen U, Liehr T (2005) New insights into intragenic Alu SINE promoters. Cytogenet Genome Res 108: 217–222
Williams I, Richardson J, Starkey A, Stansfield I (2004) Genome-wide production of stop codon readthrough during translation in the yeast Saccharomyces cerevisiae. Nucleic Acids Res 32: 6605–6616
Wu J, Zhang B, Wu M, Li H, Niu R, Ying R, Zhang N (2009) Screening of a PKC zeta-specific kinase inhibitor PKCζ1257.3 which inhibits EGF-induced breast cancer cell chemotaxis. Invest New Drugs 28: 268–275
Xin M, Gao F, May WS, Flagg T, Deng X (2007) Protein kinase Czeta abrogates the proapoptotic function of Bax through phosphorylation. J Biol Chem 282: 21268–21277
Xu J, Zheng SL, Chang B, Smith JR, Carpent JD, Stine OC, Isaacs WD, Wiley KE, Henning L, Ewing C, Bujnovszky P, Bleeker ER, Walsh PC, Trent JM, Meyers DA, Isaacs WB (1999) Linkage of protein cancer susceptibility loci to chromosome 1. Hum Genet 105: 338–345
Yao S, Bee A, Brewer D, Dodson A, Beesley C, Ambroise L, Fisher G, Moller H, Dickinson T, Gerard P, Lian L-Y, Risk J, Lane B, Ke Y, Reuter VE, Berney DM, Gosden CM, Scardino P, Cuzick Y, Djamgoz MBA, Cooper CS, Foster CS (2010) PRKCζ, a protein expression promotes the aggressive phenotype of human prostate cancer cells and is a novel target for therapeutic intervention. Genes Cancer 1: 444–464
Yu LR, Lv JQ, Jin LY, Ding SD, Ma XY, Wang JJ, Zhu XQ (2011) Over-expression of protein kinase C isoforms (ζ, δ, θ and ε) in squamous cervical cancer. Neoplasma 58: 491–498

This work is published under the standard license to publish agreement. After 12 months the work will become freely available and the license terms will switch to a Creative Commons Attribution-NonCommercial-Share Alike 3.0 Unported License.