Original Article
Inhibition of protein kinase C isozymes causes immune profile alteration and possibly decreased tumorigenesis in bladder cancer

Deepika Trehan¹,2, Ranbala Kumari¹,3, Jyoti Sharma¹, Sri Harsha Satuluri¹, Satya Sahay¹, Nitu Kumari Jha¹, Janendra K Batra², Usha Agrawal¹

¹ICMR-National Institute of Pathology, New Delhi, India; ²Jamia Hamdard University, New Delhi, India; ³Amity University, Noida, UP, India

Received March 17, 2023; Accepted June 23, 2023; Epub August 15, 2023; Published August 30, 2023

Abstract: Protein kinase C (PRKC) isozymes activate many signaling pathways and promote tumorigenesis, which can be confirmed by masking the kinase activity. In the present study, the kinase activity of PRKC ε and ζ isozymes was masked by siRNA in bladder cancer, and the consequent gene profile was evaluated. Here, we show that the commonly dysregulated genes affected by both the isozymes were the chemokines (CXCL8 & CXCL10), adhesion molecules (ICAM1, SPP1, MMP3, VEGFA) and mutated isoform of TP53. As these same genes were upregulated in bladder cancer patients, the activity of the kinase in downregulating them is confirmed. These genes are associated with regulating the tumor microenvironment, proliferation and differentiation of cancer cells and poor prognosis. The effect of kinase masking in downregulating these genes in bladder cancer indicates the benefits PRKC inhibitors may have in managing these patients.

Keywords: Protein kinase C, bladder cancer, chemokines, adhesion molecules, TP53, siRNA knockdown

Introduction
Protein kinase C (PRKC) isozymes are known signal transducers that play an important role in normal physiology and numerous diseases, including cardiovascular, neurological, and proliferative dysfunctions. Their significance in oncogenesis is well documented through the regulation of several signalling pathways involved in differentiation, survival, and apoptosis [1]. This family of serine-threonine kinases comprises of three groups of isozymes with unique biochemical properties: classical/conventional or calcium-dependent cPRKCs (α, βI, βII, γ); novel or calcium-independent nPRKCs (δ, ε, η, θ) and atypical PRKCs (ζ & Cι) [2]. Phosphorylation as a result of PRKC action can lead to conformational changes in the target which may modulate biochemical functions and fine-tune tightly regulated biological network [3, 4]. Deregulated kinase signaling has been implicated in the hallmarks of cancers such as differentiation, progression and apoptosis [5]. Although PRKCs exhibit strong functional selectivity in cells due to their distinct intracellular location and differential access to substrates, they exhibit high homology and similar substrate specificity in vitro [6]. Elevated PRKC activity was found to be associated with the increased metastatic or invasive potential of mouse melanoma cells [7], hepatocellular carcinoma [8], human gastric cancer cells [9] and human urinary bladder carcinoma cells [10]. This is well illustrated by members of the novel PRKC family and atypical PRKC family, namely PRKC-ε and PRKC-ζ, which share the similarity in their catalytic domain and exert similar effects, particularly in the context of metastasis and survival [2, 6, 11-13]. Specific PRKC inhibitors have thus been shown to suppress the invasive and/or metastatic potential of cancer cells by reducing cell motility [10, 11], chemotaxis [14] and expression of proteolytic enzymes [15]. Many PRKC inhibitors, such as Imatinib, the first-generation inhibitor, Bcr-Abl, the second-generation, including dasatinib, bosutinib and nilotinib, have been approved for cancer treatment. Other tyrosine kinase inhibitors (TKI’s), such as osimertinib, are being taken up as irreversible inhibitors against various
Protein kinase C knockdown inhibits tumor progression due to the varied role played by PRKCs, studying the effect of PRKC inhibition on signaling pathways may give a deeper understanding of cancer biology. Therefore, we conducted PRKC-ε and PRKC-ζ si-RNA inhibition followed by genome wide analysis using Bladder carcinoma (BC) cell lines as a model. The identification of the underlying mechanism of PRKC inhibition of bladder cancer cells could help to discover targets for designing inhibitors to treat urothelial bladder carcinoma.

This study was designed to conduct si-RNA knockdown of PRKC-ε & ζ isozymes followed by cDNA microarray analysis to identify the common differentially expressed genes (DEGs) after the knockdown. Validation of hub genes has been achieved by analyzing mRNA expression of different grade bladder cancer cells and the expression of bladder tumor tissues from TCGA. This study provides an insight into the regulation of novel genes by PRKC isozymes in bladder cancer.

**Methodology**

**Cell culture and transfection with siRNAs**

Cell transfection for PRKC-ε & ζ was performed in 6-well tissue-culture flasks. The cells (Table 1) were allowed to grow in a medium containing 5% FBS without antibiotics upto 50-75% confluence. Before transfection, the medium was aspirated and 400 μL of transfection medium (opti-MEM) containing 10 mMol siRNA and 30 μL of Lipofectamine 2000 (1:1 ratio) were added followed by incubation at 37°C in a CO₂ incubator upto 48 hrs. Transfection efficiency was checked through qRT-PCR and Western blot analysis. All experiments were performed in triplicate.

**Quantitative real time - PCR**

The cells were harvested for RNA extraction before and after transfection. Total RNA was extracted from all the cell lines using the RNeasy Micro Kit (cat. No.: 74004, Qiagen). cDNA was synthesized at 42°C for 40 min and 85°C for 5 min, and was used as a template for the amplification with pre-denaturation at 94°C for 2 min, 30 cycles of denaturation at 94°C for 30 sec, annealing at 60°C for 30 sec and extension at 72°C for 30 sec, then final extension at 72°C for 2 min (Applied Biosystems™, cat No: 4368814). qRT-PCR was performed using TaqMan polymerase (Applied Biosystems™, cat No.: 4364340) All experiments were performed in quadruplicates.

**Western blot analysis**

The cells were treated with RIPA buffer on ice for 15 mins, followed by 45 sec pulse sonication and 12000 RPM centrifugation for protein extraction. The supernatant was collected and utilized for Western blot development pre and post transfection. The protein was segregated through vertical SDS-PAGE followed by transfer to the nitrocellulose blot. The blot was probed with PRKC-ε & ζ antibodies for the identification of expression in each sample and β-actin as loading control.

**Protein kinase C-ε & ζ activity assessment in cell lines**

The concentration of protein was defined through traditional BCA method. The Enzyme-linked Immunosorbent Assay Kit (MBS2701804 and MBS2019639, My-BioSource’s) was utilized to assess pre and post silenced kinase activity of PRKC-ε & ζ isozymes of each cell lines in triplicates. The ELISA was conducted as per manufacture's protocol and the reading was obtained at 450 nm.

**Microarray experiment**

RNA quality was checked through a Bioanalyzer (Agilent RNA 6000 Pico Kit, cat No.: 5067-1513). The RNA samples which passed the quality (RIN ≥ 7) were processed for cDNA microarray analysis. A total of 12 samples including the cancer cell lines and the knockdown set of (PRKCε and PRKCζ) were processed in duplicate using the Sure Print G3 Human Gene Expression v3 Microarray Kit, 8 × 60K. Data was obtained as text (.txt) files.

**Identification of differentially expressed genes**

Normalization of curated data was conducted in Genespring version 14.9.1. on the isonomic variables and GraphPad Prism version 8 was
used to compare means and calculate paired/unpaired Student’s t-test. For each probe set, the log₂ (treatment/control) ratio was computed, and p values from a one-way analysis of variance were then corrected for false discovery rate (FDR) using the Benjamin-Hochberg method. Probe sets were filtered for single-gene analysis if expression varied by 2-fold in any of the conditions studied and if the p-value for the analysis of variance was 0.05. To find differentially expressed genes, the expression profiles of samples with PRKC expression (PRKC⁺) compared to samples with PRKC knockdown (PRKC⁻) were curated. The upregulated and downregulated sets were separated using data from all statistically significant probe sets in an ANOVA one-way Benjamin-Hochberg analysis.

**Functional enrichment analysis**

The common gene set of upregulated and downregulated genes were processed for GO, KEGG and Hallmark gene sets. For each PRKC isoform, samples from two classes were compared: PRKC expressing versus PRKC-depleted (PRKC⁺ vs PRKC⁻). To identify probable mechanisms and important biological processes connected to overlapping DEGs, functional enrichment analysis and pathway enrichment analysis of genes were carried out with the ClueGO version 2.5.6.

**Construction of PPIs**

A PPI of the common gene set of upregulated and downregulated genes was generated with STRING, version 11 [50] and visualized using Cytoscape (version 8.0; http://cytoscape.org/) [51]. The Cytoscape plugin “MCODE” generated cluster modules and clusters with score > 5 and number of nodes ≥ 10 was considered significant. Hub genes were identified based on centrality values such as closeness, degree, EPC, MCC, and MNC. The hub genes were ranked according to the selected centrality values and the most interacting genes were identified. To discover the important biological processes connected to it, the significant modules were further processed for gene ontology (GO). GO categories and overrepresented pathways were identified using ClueGO version 2.5.6 [52]. Signalling pathways were systematically examined using the Kyoto Encyclopedia of Genes and Genomes (KEGG, http://www.genome.jp/) enrichment database [53]. In choosing GO terms and pathways, the advanced statistical criteria were min#genes = 3, %gene/term = 4. Additionally, a two-sided hypergeometric test was used to do the enrichment/depletion and p value adjustment methods. Kappa score for GO term/pathway network connection was set to 0.4. The cut-off value for significance (p-value) was set to 0.05, with the GO tree interval level set at 3 to 8.

**Validation of the hub genes**

The mRNA expression levels of the screened hub genes were validated through qRT-PCR in bladder cancer cell lines and TCGA Bladder cancer datasets (BLCA) consisting of 409 BLCA cases and 19 normal bladder samples. The expression of these genes was analysed with the Tukey HSD (honest significant difference) test. A co-relation analysis was conducted by comparing the TCGA reported PRKC-ε & ζ expression and the hub genes. The protein expression of hub genes in bladder tumor tissue and normal tissues was determined using the Human Protein Atlas (HPA) [54], and the commercially available TMA (NBP3-11846 - Human Bladder Tissue MicroArray Cancer).

**Survival analysis of hub genes**

The survival analysis of hub genes in BLCA was analyised using TCGA database [55]. A Cox proportional hazards model P < 0.05 was considered significant, and associated data analysis was displayed in a survival plot.

**Statistical analysis**

The identification of DEG’s were done using moderate t-test, with a cut-off criteria logFC > 1 or logFC > -1 and p-value < 0.05. The construction of PPI network was obtained using maximum number of interactors = none/query proteins only and confidence score ≥ 0.4. The GO biological attributes were annotated with min#genes = 3 and %gene = 4 in GO term/pathway selection. The GO tree interval level was set as 3, max level as 8 and p-value ≤0.05 as the cut-off criterion of significance. The mean ± SD of the hub genes was assessed using Tukey’s Honest Significant Difference (HSD) test and p values of the pairwise comparisons are indicated as significant (**P**). The correlation analysis was calculated using Spearman correlation.
Protein kinase C knockdown inhibits tumor progression

Results

Kinase Isozymes activity

The activity of each isozyme was observed in terms of concentration (ng/ml) and it was found that the non-invasive cell lines 5637 and J82 had an OD of 0.13 and 0.18 in pre silenced PRKC-ε samples with a significant downregulation to 0.08 post silencing (Figure 1A). Similarly, the invasive cell lines, TCCSUP and T24 had a higher OD of 0.27 and 0.24 in pre-silenced cells, which dropped significantly to 0.09 and 0.08 respectively (Figure 1A). Upon observing the PRKC-ζ concentration in pre silenced cells, it was found to be, almost equivalent in all the cell lines (5637 = 1.15, J82 = 1.15, TCCSUP = 1.24 and T24 = 1.16), which dropped to non-detectable range post silencing among all the cell lines (Figure 1B).

PRKC ε and ζ knockdown

The gene expression of PRKC-ε and PRKC-ζ in bladder carcinoma cell lines 5637 (Grade II carcinoma), J82 (Grade III carcinoma) TCCSUP (Grade IV TCC) & T24 (TCC) was evaluated, before and after transfection with si-RNA by qRT-PCR and western blot analysis. A significant gene downregulation post PRKC-ε and ζ knockdown was observed among the bladder cancer cell line. The survival analysis was assessed through Cox proportional hazards model with significance at $P < 0.005$. The survival analysis was assessed through Cox proportional hazards model with significance at $P < 0.005$.

Am J Cancer Res 2023;13(8):3832-3852
silencing was observed at mRNA (Figure 1C-F) and protein levels (Figure 1G-J) in all bladder cancer cells.

**Differentially regulated genes (DEG’s) in PRKC-ε & ζ knockdown**

Using a cut-off of FDR > 2 and P < 0.05, a total of 1959 and 11537 genes were found to be upregulated and downregulated (Figure 2A) respectively in cells with siRNA mediated PRKC-ε knockdown. Similarly, among siRNA mediated PRKC-ζ knockdown a total of 892 and 1332 genes were found to be upregulated and downregulated respectively (Figure 2B). While 1824 and 757 genes were distinguished under unique upregulated genes (Figure 2C), 11091 and 886 genes (Figure 2D) were identified as unique downregulated for PRKC-ε and ζ knockdown respectively. A total of 134 upregulated genes and 445 down-regulated genes were found to be commonly dysregulated with knockdown of both isozymes bringing the total common dysregulated genes to 579. These sets were marked under group A (PRKC ε & ζ common upregulated genes) and group B (PRKC ε & ζ common downregulated genes).

**Protein-protein interaction (PPI) network analysis**

The 579 common dysregulated genes generated a PPI network of 353 nodes of enrichment p-value: < 1.0e-16 in Cytoscape (Figure 2E). The remaining nodes were in small clusters or single, not interacting with any other genes. The big network of 353 nodes was analyzed for modules with a score of > 5 and the number of nodes ≥ 10 in MCODE. A total of 3 modules were constructed, out of which only one module with MCODE score of 16.316 (20 nodes, 155 edges) was identified to be statistically significant (Figure 2F).

**HUB gene identification**

Out of 19, a set of 15 hub genes were identified as the most interacting genes based on centrality values (Table 2; Figure 2G). The genes included CSF3, LCN2, VEGFA, SPP1, CXCL8, CD86, CXCL2, TP53, CXCL10, MMP1, CX3CL1, MMP3, IL6, CCL3 and ICAM1.

**HUB gene basal expression through qRT-PCR**

To verify the identified hub genes dependability of PRKC, qRT-PCR was conducted on control and knockdown samples of four different bladder cancer cell lines. A total of 12 genes, including CSF3, VEGFA, SPP1, CXCL2, TP53, CXCL10, CX3CL1, MMP3, IL6, CCL3, ICAM-1 and CXCL8 showed statistically significant downregulation in all the four grades of bladder tumor. Total 3 genes, LCN2, CD86, and MMP1 were found to be upregulated in all the tested cell lines. The differences in expression levels of each hub gene between control and knockdown samples are shown as bar plots in Figure 3A.

**mRNA expression analysis of hub genes from TCGA**

The mRNA expression of the identified hub genes was explored using TCGA database in tumor and normal cases, and the levels of LCN2, VEGFA, SPP1, CD86, TP53, CXCL10, CXCL8, MMP1, CCL3, MMP3, ICAM1 were found to be significantly upregulated in TCGA BLCA database. CSF3, CXCL2, L6 and CX3CL1 showed significant downregulation (Figure 3B) in comparison to the normal bladder cases.

**Co-relation analysis of hub genes with PRKC-ε & ζ expression**

The expression of PRKC-ε was co-related with the expression of hub genes in TCGA data. The results of the Spearman correlation test indicated that an increased expression of PRKC-ε is statistically significant and in a negative correlation with the expression of CSF3, LCN2, SPP1, CXCL8, CD86, CXCL2, CXCL10, TP53, MMP1, MMP3, CCL3 and ICAM1 genes (Figure 3C). Similarly, PRKC-ζ expression shows the statistical significance and inverse correlation with CSF3, SPP1, CX3CL1, CXCL8, VEGFA, CD86, CXCL2, CXCL10, TP53, MMP1, MMP3, IL6, CCL3, LCN2 and ICAM1 (Figure 3D). The genes which are inversely regulated by both the isozymes are CSF3, SPP1, CXCL8, CD86, CXCL2, CXCL10, MMP1, MMP3, CCL3 and ICAM1.

**Regulatory functions identification**

The 15 hub genes show significant enrichment under biological regulatory functions (GO category) for positive regulation of leukocyte migration (CCL3, CX3CL1, CXCL10, CXCL8, ICAM1, IL6, VEGFA), cellular response to lipopolysaccharide (CCL3, CD86, CSF3, CX3CL1, CXCL10, CXCL2, CXCL8, ICAM1, LCN2), regulation of neuroblast formation (CX3CL1, TP53, VEGFA) (Figure 3E). Upon KEGG pathway analysis IL-17 signalling (CSF3, CXCL1, CXCL10, CXCL2, CXCL8, IL6,
Protein kinase C knockdown inhibits tumor progression

Figure 2. Hub genes were identified using analytical methods. (A) PRKC-ε and (B) PRKC-ζ, Volcanic plot illustrating the set of Differentially expressed gene sets as a result of knockdown. The differentiation is computed upon $p$-value ≤ 0.05 and fold change > 2. Red colour depicts the set of significantly upregulated genes and blue shows the set of significantly downregulated genes. (C) A total of 134 common upregulated genes were identified in PRKC-ε & ζ knockdown, (D) A total of 445 common downregulated genes were identified in PRKC-ε & ζ knockdown. (E) PPI network with 353 nodes and 102 edges, $p$-value: < 0.001. (Red color shows upregulated genes and blue are downregulated genes). (F) Module with MCODE Score: 16.316 and interaction of 19 genes. (G) Venn diagram based upon five intersecting parameters identifies 15 genes. These are the set of significant hub genes in the module of 19 genes. Areas with different colours correspond to different parameters.
Protein kinase C knockdown inhibits tumor progression

LCN2, MMP1, MMP3), TNF signalling pathway (CXCL1, CXCL10, CXCL2, IL6, MMP3), bladder cancer (CXCL8, TP53, VEGFA, MMP1), and Toll like receptor signalling (CCL3, CD86, CXCL10, CXCL8, IL6, SSPI) pathways were enriched (Figure 3F).

Prognostic value of hub genes for overall survival

Finally, we investigated the possible predictive value of hub genes by comparing mRNA expression and survival analysis from TCGA using several independent datasets with varying probe IDs for bladder cancer patients. In all, LCN2, VEGFA, TP53, CXCL10, CX3CL1 and MMP3 exhibited better prognosis with < 1 hazard ratio (HR) (Figure 3G; Table 3). Simultaneously, a significant Cox p value was also observed in TP53, CXCL10, ICMA1 and CCL3 (Table 3). Genes among upregulated expression in bladder cancer from TCGA database with significant survival rate and HR ratio < 1 are TP53 and CXCL10.

Protein expression of hub genes

In order to validate the hub genes at the protein expression levels, immunohistochemical data of patients were analysed with Bladder tumor tissue data and Normal bladder tissue obtained from the Human Protein Atlas (HPA). The protein expressions of SPP1, VEGFA, TP53, MMP3 and ICAM1 shows > 75% quantity and strong intensity in urothelial bladder cancer and has moderate to no intensity in Bladder normal tissue respectively. CXCL8 shows weak intensity in both urothelial carcinoma and in urothelial cells. The protein expression of CXCL10 in the urothelial bladder cancer is not available through Human Protein Atlas, therefore the expression in tumor was identified through commercially available TMA and the expression was identified to be 75-25% in Bladder tumor tissue and < 25% in Bladder normal tissue (Figure 3H).

The evaluation of the TCGA database expression and experimental data (siRNA knockdown)
Protein kinase C knockdown inhibits tumor progression
Protein kinase C knockdown inhibits tumor progression
Protein kinase C knockdown inhibits tumor progression
Protein kinase C knockdown inhibits tumor progression
Protein kinase C knockdown inhibits tumor progression
Protein kinase C knockdown inhibits tumor progression
Protein kinase C knockdown inhibits tumor progression
Protein kinase C knockdown inhibits tumor progression

**CXCL8**
- HPA057179
  - Patient id: 3465
  - Tumor cells
  - Staining: not detected
  - Intensity: Weak
  - Quality: <25%
  - Location: cytoplasmic/membrane

**SPP1**
- HPA027541
  - Patient id: 3543
  - Tumor cells
  - Staining: Low
  - Intensity: weak
  - Quality: <25%
  - Location: cytoplasmic/membrane

**VEGFA**
- CAB05429
  - Patient id: 3265
  - Tumor cells
  - Staining: High
  - Intensity: Strong
  - Quality: 75-25%
  - Location: cytoplasmic/membrane

**CXCL10**
- HPA045942
  - Patient id: 2470
  - Tumor cells
  - Staining: not detected
  - Intensity: Negative
  - Location: none

**TP53**
- CAB039239
  - Patient id: 3112
  - Tumor cells
  - Staining: High
  - Intensity: Strong
  - Quality: >75%
  - Location: cytoplasmic/membrane

**MMP3**
- HPA007875
  - Patient id: 1774
  - Tumor cells
  - Staining: High
  - Intensity: Strong
  - Quality: >75%
  - Location: cytoplasmic/membrane

**ICAM1**
- HPA004877
  - Patient id: 1984
  - Tumor cells
  - Staining: High
  - Intensity: Strong
  - Quality: >75%
  - Location: cytoplasmic/membrane

**NBP3-11846**
- Human Bladder
- Tissue MicroArray (Cancer)
Protein kinase C knockdown inhibits tumor progression

**Figure 3.** Hub gene dysregulation and biological variance. A. mRNA expression of 15 hub genes in response to PRKC-ε & ζ silencing among different grade of bladder cancer cell (5637, J82, TCCSUP and T24); non-transfected cells are taken as controls. B. Expression of hub genes in Urothelial Bladder cancer vs normal Bladder tissue from TCGA data. Statistical significance has been indicated in the figures. ***P < 0.001. C. D. Correlation analysis of hub genes with Protein kinase C-ε & ζ with a Spearman correlation scatter plot with regression line (red line) depicting a statistically significant co-relationship of hub genes with the kinase. The negative value of Spearman indicates an inverse co-relationship between the kinase and the hub genes. E, F. The image represents the functional enrichment analysis under GO KEGG pathway. Gene annotations are in terms of the percentage of gene/term for represented Module. G. Cox multivariate proportional hazard model curves for overall survival of the hub genes obtained from bladder cancer survival database. The two line shows high (red) and low (blue) percentage of the genes in respect to the time interval (months), n = 201. H. Immunohistochemical analysis of the hub genes. The protein expression of identified hub genes (CXCL8, SPP1, VEGFA, TP53, MMP3 and ICAM1) was assessed in Bladder cancer tissues and normal urothelial bladder tissue derived from Human Protein Atlas database and the expression of CXCL10 was assessed through Tissue microarray (NBP3-11846 - Human Bladder Tissue MicroArray Cancer).

**Table 3.** Hub genes expression in study and TCGA data and association with survival

<table>
<thead>
<tr>
<th>S.No</th>
<th>ID</th>
<th>Cox p-value</th>
<th>HR (Hazard Ratio)</th>
<th>Dataset</th>
<th>Probe ID</th>
<th>Cohort</th>
<th>mRNA expression (TCGA)</th>
<th>Experimental data (siRNA knockdown) expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>CSF3</td>
<td>0.34</td>
<td>1.58</td>
<td>GSE13507</td>
<td>ILMN_1706852</td>
<td>CNUH</td>
<td>downregulated</td>
<td>downregulated</td>
</tr>
<tr>
<td>2</td>
<td>LCN2</td>
<td>0.42</td>
<td>0.91</td>
<td>GSE13507</td>
<td>ILMN_1692223</td>
<td>CNUH</td>
<td>upregulated</td>
<td>upregulated</td>
</tr>
<tr>
<td>3</td>
<td>VEGFA</td>
<td>0.80</td>
<td>0.94</td>
<td>GSE5287</td>
<td>210512_s_at</td>
<td>Aarhus(1995-2004)</td>
<td>upregulated</td>
<td>downregulated</td>
</tr>
<tr>
<td>4</td>
<td>SPP1</td>
<td>0.18</td>
<td>1.11</td>
<td>GSE13507</td>
<td>ILMN_1651354</td>
<td>CNUH</td>
<td>upregulated</td>
<td>downregulated</td>
</tr>
<tr>
<td>5</td>
<td>CD86</td>
<td>0.64</td>
<td>0.15</td>
<td>GSE13507</td>
<td>ILMN_1714602</td>
<td>CNUH</td>
<td>upregulated</td>
<td>upregulated</td>
</tr>
<tr>
<td>6</td>
<td>CXCL2</td>
<td>0.61</td>
<td>1.07</td>
<td>GSE13507</td>
<td>ILMN_1682636</td>
<td>CNUH</td>
<td>downregulated</td>
<td>downregulated</td>
</tr>
<tr>
<td>7</td>
<td>TP53</td>
<td>0.00</td>
<td>0.43</td>
<td>GSE5287</td>
<td>201746_s_at</td>
<td>Aarhus(1995-2004)</td>
<td>upregulated</td>
<td>downregulated</td>
</tr>
<tr>
<td>8</td>
<td>CXCL10</td>
<td>0.09</td>
<td>0.96</td>
<td>GSE13507</td>
<td>ILMN_1791759</td>
<td>CNUH</td>
<td>upregulated</td>
<td>upregulated</td>
</tr>
<tr>
<td>9</td>
<td>MMP1</td>
<td>0.27</td>
<td>1.07</td>
<td>GSE13507</td>
<td>ILMN_17264648</td>
<td>CNUH</td>
<td>upregulated</td>
<td>upregulated</td>
</tr>
<tr>
<td>10</td>
<td>CX3CL1</td>
<td>0.38</td>
<td>0.92</td>
<td>GSE13507</td>
<td>ILMN_1654072</td>
<td>CNUH</td>
<td>downregulated</td>
<td>downregulated</td>
</tr>
<tr>
<td>11</td>
<td>MMP3</td>
<td>0.57</td>
<td>0.92</td>
<td>GSE13507</td>
<td>ILMN_1784459</td>
<td>CNUH</td>
<td>upregulated</td>
<td>downregulated</td>
</tr>
<tr>
<td>12</td>
<td>IL6</td>
<td>0.16</td>
<td>1.18</td>
<td>GSE13507</td>
<td>ILMN_1699651</td>
<td>CNUH</td>
<td>downregulated</td>
<td>downregulated</td>
</tr>
<tr>
<td>13</td>
<td>CCL3</td>
<td>0.05</td>
<td>1.42</td>
<td>GSE13507</td>
<td>ILMN_1724449</td>
<td>CNUH</td>
<td>downregulated</td>
<td>downregulated</td>
</tr>
<tr>
<td>14</td>
<td>ICAM1</td>
<td>0.07</td>
<td>1.36</td>
<td>GSE13507</td>
<td>ILMN_1812226</td>
<td>CNUH</td>
<td>upregulated</td>
<td>downregulated</td>
</tr>
<tr>
<td>15</td>
<td>CXCL8</td>
<td>0.47</td>
<td>1.22</td>
<td>GSE5287</td>
<td>202638_s_at</td>
<td>Aarhus(1995-2004)</td>
<td>upregulated</td>
<td>downregulated</td>
</tr>
</tbody>
</table>

expression (Table 3) indicates that genes CXCL8, CXCL10, SPP1, VEGFA, TP53, MMP3 and ICAM1 are hub genes which have statistically significant downregulated mRNA expression in different grades of bladder cancer cells due to kinase inhibition. Upon TCGA database analysis their expression was found to be up-regulated in BLCA. Upon correlation analysis, these genes show statistical significance with both the isozymes, and have significant role in bladder cancer.

**Discussion**

Protein kinase C (PRKC) transfers phosphoryl groups onto target proteins, thereby altering their activity and regulating signalling pathways. They are reported as tumor promoters and contribute to oncogenesis [17, 18]. Despite the distinct intracellular location and differential access to substrates, PRKC isozymes show impressive functional selectivity in cells with high homology and similar substrate specificity in vitro [19]. The effect of PRKC-α inhibition in epithelial cancers has been studied [20]; however, the overall profile of genes and signalling pathways involved in the suppression of carcinogenic characteristics with the ε & ζ isozymes has not been reported yet. Therefore, in this study, we evaluated the effect of the knockdown of PRKC ε & ζ isozymes in combination on bladder cancer tumorigenesis. Our analysis shows a statistically significant downregulatory effect on the expression of 15 hub genes with a strong association of differentially regulated pathways relevant for controlling the immune response, inflammation, proliferation, differentiation, apoptosis, and angiogenesis. The identified genes were further validated in control (non-transfected) and knockdown samples via
Protein kinase C knockdown inhibits tumor progression

Figure 4. Masking of kinase activity revealed potential genes associated with PRKC ε & ζ tumorigenesis in bladder cancer cells.
Protein kinase C knockdown inhibits tumor progression

Upon evaluating the mRNA expression of hub genes in PRKC-ε & ζ knockdown samples, it was found that the hub genes were regulated by both the kinases in all the four grades of bladder cancer cells (5637, J82, TCCSUP, T24). Among these only 7 genes TP53, CXCL8, CXCL10, MMP3, SPP1, VEGFA and ICAM1 were identified to be directly associated with bladder cancer progression and tumorigenesis (Figure 4). Further analysis revealed that these genes were primarily enriched in hallmark gene sets such as inflammatory response (M5932), TNF-α signalling via NFK-β interaction (M5890), Epithelial Mesenchymal Transition (M5930), cytokine-cytokine receptor interaction (hsa04060), and IL-17 signalling (hsa04657) and display statistical significance for survival in bladder cancer.

The initiation of an appropriate microenvironment of tumor mediated immune response requires chemokine-receptors expressed on tumor cells [21]. These receptors enhance the chemokines activity, which may lead to invasiveness and evoke a cascade of changes leading to epithelial to mesenchymal transition [22, 23]. In this study, two inflammatory chemokines CXCL10 and CXCL8 were downregulated by both PRKC-ε & ζ isozymes. The increased levels of these cytokines are associated with leukocyte homing to inflamed tissues, which exacerbates inflammation and significantly damages tissue [24]. These chemokines are secreted by different cell types, including lymphocytes, neutrophils, macrophages, and many tumor cells [25, 26]. Many advanced human malignancies, such as ovarian carcinoma, multiple myeloma and B-cell lymphoma, have also been linked to elevated expression of CXCL10 [27-29]. It is also correlated with poor survival in patients with ovarian cancer and colon cancer [30, 31]. Similarly, the binding of CXCL8 to the CXCR1 and CXCR2 cell-surface G protein-coupled receptors allows inflammation which induces Protein Kinase C (PRKC), Akt/PKB, and MAPK signalling [32]. CXCL8 enhances the neutrophil chemoattractant protein, which further increases the infiltrating monocytes and lymphocytes in the tumor microenvironment (TME) [33]. This property of CXCL8 allows it to play a key role in tumor progression by enhancing the proliferation, invasion, and angiogenesis of tumors [34]. Many studies have projected CXCL8 as a urinary biomarker in bladder cancer [35].

Another crucial protein which acts as a ligand for the leukocyte adhesion protein LFA-1 (integrin alpha-L/beta-2) and has a critical role in cell adhesion, leukocyte trans endothelial migration to areas of inflammation, and lymphocyte activation [33] is ICAM-1. The affinity and clustering of LFA-1 rise when leukocytes are triggered by any of several exogenous stimuli, such as chemokines [36]. It is observed in prostate tumor cells, ICAM-1 and TGFβ2 signalling caused perforin to be downregulated via MMP's interactions which caused invasion [37]. Similarly, in our data analysis, it was found that MMP3 displays interaction with the ICAM1 via Epithelial Mesenchymal Transition (map04520) in bladder cancer.

Another hallmark of tumor progression includes mutation of the tumor suppressor gene TP53, which results in an increased risk of bladder cancer development [38]. Mutations in p53 influence cytokine signalling via NF-kB mediated transcriptional activation of CXC chemokines. The enhancement in pro-inflammatory signal results in the activation of transcription, apoptosis, and altered cell cycle events [39]. Previous research revealed that TP53 function was inactivated in 76% of muscle invasive bladder cancer (MIBC) and 50% of MIBC samples had TP53 mutations [40]. The mutation in “TP53” called as driver mutation in bladder cancer affect response to cancer chemotherapy and drug susceptibility, hence lead to poorer prognosis [41]. These mutations are reported for neoplastic processes, tumor vascularization and metastatic spread of tumor via regulating vascular endothelial growth factor (VEGF-A), which is reported to be the most significant angiogenic regulator [42]. Its overexpression in pre-chemotherapy samples was found to be a strong predictor of recurrence and death in locally advanced urothelial cancer undergoing cystectomy and chemotherapy [43]. Also, the higher levels of VEGF in tumor tissue led to progression in bladder transitional cell carcinoma (TCC), and increased urinary levels act as a marker for recurrence in patients with superficial lesions [44, 45]. This proangiogenic factor is regulated by MMPs [46], which are responsible for SPP1 upregulation and stimulate the JAK1/STAT1 signalling pathway. This, in res-
Protein kinase C knockdown inhibits tumor progression

Response, increases proliferation and invasion and prevents apoptosis [47]. Recent studies revealed that SPP1 expression is correlated with the development of tumors in the breast, lung, prostate, liver, stomach, colon, cervix, and ovary and that the plasma concentration of SPP1 in patients with metastatic disease is significantly higher than that of normal serum [48, 49]. The kinase inhibition downregulated the expression of all the 7 genes, and may be beneficial to target tumorigenesis.

The current analysis has uncovered the PRKC interaction and modulation of oncogenes and tumor suppressor genes. Protein Kinase C signalling has come out to be vital for the regulation of events that are important for tumor progression via cytokines interaction, adhesion molecules such as ICAM, VEGFA, SPP1 and MMP3 along with mutated oncogene TP53 for regulation of cell death. All the identified hub genes modulate the cell cycle control, DNA damage, angiogenesis, immune response, and apoptosis. The current study shows that the functionality of PRKC isoforms, ε & ζ, drive the tumorigenic phenotypes in bladder cancer. Further studies may establish the role of PRKC inhibitors, based on cell selectivity and toxicity in bladder cancer treatment by in vivo studies.

Acknowledgements

The work is supported by ad-hoc research grant to Usha Agrawal (UA), Indian Council of Medical Research (ICMR) (Grant No. 5/13/37/2014/ NCD-III).

Disclosure of conflict of interest

None.

Abbreviations

PRKC, Protein kinase C; TKI’s, tyrosine kinase inhibitors; BC, Bladder carcinoma; DEGs, Differentially expressed genes; TCC, Transitional cell carcinoma; MIBC, muscle invasive bladder cancer; MMP, Matrix metalloproteins; VEGFA, Vascular endothelial growth factors; ICAM, Intracellular adhesion molecule; SPP1, Secreted Phosphoprotein 1; CXCL8, C-X-C Motif Chemokine Ligand 8; SDS-PAGE, Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis; RIN, RNA integrity number; FDR, false discovery rate; GO, gene ontology; KEGG, Kyoto Encyclopaedia of Genes and Genomes; BLCA, Bladder cancer datasets; Tukey HSD, honest significant difference.

Address correspondence to: Usha Agrawal, ICMR-National Institute of Pathology, New Delhi, India. ORCID: 0000-0001-7539-4102; E-mail: ushakaggarwal.nip@gov.in

References


Protein kinase C knockdown inhibits tumor progression


Protein kinase C knockdown inhibits tumor progression


