**ORIGINAL RESEARCH**

**DD3\textsuperscript{PCA3} gene expression in cancer and prostatic hyperplasia**

E Floriano-Sánchez PhD\textsuperscript{1,2}  
N Cárdenas-Rodríguez MSci\textsuperscript{3}  
M Castro-Marín MD\textsuperscript{4}  
P Álvarez -Grave MSci\textsuperscript{5}  
E Lara-Padilla PhD\textsuperscript{6}

\textsuperscript{1}Departamento de Bioquímica y Biología Molecular, Escuela Médico Militar, México, DF,  
\textsuperscript{2}Escuela Superior de Medicina del Instituto Politécnico Nacional. México, DF,  
\textsuperscript{3}Laboratorio de Neuroquímica, Instituto Nacional de Pediatría, México, DF,  
\textsuperscript{4}Servicio de Urología Médica, Hospital Central Militar, México, DF,  
\textsuperscript{5}Departamento de Bioquímica. Escuela Superior de Medicina, IPN, México, DF,  
\textsuperscript{6}Sección de Estudios de Posgrado e Investigación, Escuela Superior de Medicina, IPN,DF.

**Abstract**

**Purpose:** \textit{DD3}\textsuperscript{PCA3} is a novel gene with characteristics that indicate its potentially valuable role in early identification and diagnosis of malignancy and highly upregulated in transformed cells in PCa. The aim of this work was to validate and analyze, by real-time Reverse Transcription-Polymerase Chain Reaction (RT-PCR), the expression of the \textit{DD3}\textsuperscript{PCA3} gene in a mexican population, both in intratumoral tissue with PCa and benign prostatic hyperplasia (BPH).

**Methods:** Human samples from patients with PCa (40 cases) and benign prostatic hyperplasia (40 cases) were analyzed for the mRNA expression of \textit{DD3}\textsuperscript{PCA3} by RT-PCR.

**Results:** The GAPDH gene showed better stability with a Pearson correlation of 0.953 (\textit{P}<0.007) for the determination of housekeeping gene. \textit{DD3}\textsuperscript{PCA3} gene expression was 29.74 times higher in PCa tissue (\textit{P}<0.0001) than in BPH. The gene expression for the PCa and BPH was 1731±280 and 58.23±9.9 fold, respectively.

**Conclusions:** Determination of \textit{DD3}\textsuperscript{PCA3} gene expression by RT-PCR could be a potentially tool for the early detection of PCa in clinical specimens.

In Mexico, prostate cancer (PCa) is the principal cause of urology visits and, in 70% of cases, is found in an advanced stage. Prostate cancer occupies second place in frequency of cancer in men, surpassed only by skin cancer, and is the second principal cause of death in men after lung cancer.\textsuperscript{1} More than 200, 000 men in the United States are diagnosed with PCa each year and 30, 000 die each year from the disease.\textsuperscript{2,3}

The identification of reliable molecular biomarkers for early diagnosis of PCa is desirable.\textsuperscript{4-6} Identification of new markers for diagnosis and new targets for therapy would represent a considerable advance in
the treatment of PCa. Some of the new markers include the following genes: $DD3^{PCA3}$, known as $PCA3$ or $DD3$, hypermethylation of glutathione-S-transferase-1 ($GSTP1$), ERTV17-10, UDP-N-Acetyl-alpha-D-galactosamine transferase, hepsin, $PSMA^{11}$, telomerase reverse transcriptase mRNA ($hTERT)^{12}$, $PSGR$ and $PCGEM1^{13,14}$. The Differential display code 3 ($DD3^{PCA3}$) gene, discovered in 1999 $^9$, is one of the most specific genes for PCa. Its transcription product is a non-coding mRNA located on chromosome $^9$$^{13,15}$ of 25 kb consisting of three introns and four exons with two alternative splicing in exon two. $^9$$^{15}$

Compared with other genetic markers that are associated with prostate tissue, $DD3$ is the most specific marker for malignant disease. It is not expressed in any other normal human tissue, including breast, bladder, testis, gastrointestinal organ and musculoskeletal tissue. $^9$$^{15}$

Moreover, in recent years different methods have been developed for the determination of gene expression: northern blotting and in situ hybridization $^{23}$, RNAse protection assays $^{24,25}$, reverse transcription polymerase chain reaction (RT-PCR) $^{26}$ and cDNA assays. $^2$

The dramatic prostate-specific expression and pronounced upregulation of $DD3$ in PCa $^{28,29}$ suggest unique transcriptional regulation. A quantitative assay for $DD3$ would be a potentially valuable tool for the detection of malignant cells in blood, urine, or other clinical specimens. Therefore, to investigate the potential of $DD3$ in the early diagnosis of PCa we analyzed the genetic expression of this marker in PCa and benign prostatic hyperplasia.

**Methods**

**Study Subjects**

We obtained 80 samples of prostate tissue through various surgical procedures (transurethral resection, biopsy transrectal and open surgery). Approval was obtained from the local research and ethics committee for use of tissue. Of these samples, 40 patients (50%) had a diagnosis of PCa (Department of Medical Urology, Hospital Central Militar, Mexico). The sample collection was conducted from December 2004 to July 2008 and was considered inclusion, exclusion and elimination criteria. The age of the patients, maximum tumour size, invasion and histological grading were obtained from histopathological study. Stating of PCa was done according to the Gleason score. None of the patients had undergone chemotherapy or radiotherapy before surgery.

Tissues were stored at -83°C (Revco® Legaci ULT2186 3-35 Dupont SVVA Refrigerants) until further processing.

**Extraction and RNA quantification**

Total RNA was extracted with the appropriate kit, using the SV Total RNA Isolation System (Promega, Madison, WI, USA). In brief, 150 mg of the samples were homogenized, after 2µL RNAase inhibitor was added. Verification of the integrity of total RNA was performed by electrophoresis placing 6 µL of total RNA extract from each sample on a 2% agarose gel. The bands were visualized with UV light (EDAS 290 KODAK, New Haven, CT). The quantification of total RNA was realized by SYBR Green with a multidetector Synergy HT-I (BIO-TEK Instruments, Inc. Higland Park, Vermont, U S A).

**Standardizing of real-time RT-PCR technique**

For the development of real-time RT-PCR specific oligonucleotides of the $DD3^{PCA3}$ gene and for the reference genes: subunit ribosomal 18S, glyceraldehyde-3 phosphate dehydrogenase (GAPDH), glucose 6 phosphate dehydrogenase (G6PDH) and β-actin (BACT) were designed. The endogenous genes were validated with BestKeeper software $^{31}$ and the more stable gene was the endogenous control of the total RNA load (housekeeping gene, HKG). The sequences were obtained from GenBankTM (Table 1). The
TABLE 1. Sequence of primers that were used in the RT-PCR, from left to right: gene name, position of amplicon, primer sequence, fragment size, accession (GenBank™) and efficiency.

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Positions</th>
<th>Nucleotide sequence:</th>
<th>Amplicon size (pb)</th>
<th>GenBank™ accession</th>
<th>Primers efficiency</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Forward primer</td>
<td>Reverse primer</td>
<td></td>
<td></td>
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<tr>
<td>BACT</td>
<td>1031-1173</td>
<td>CTGGCACCCAGCACAATG</td>
<td>GGGCCGGACTGCTCATC</td>
<td>143</td>
<td>NM_001101</td>
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<tr>
<td>18S</td>
<td>1601-1713</td>
<td>GTAACCCGTGAAACCCCAT</td>
<td>CCAATACCGTGATAGC</td>
<td>151</td>
<td>X03205</td>
</tr>
<tr>
<td>DD3</td>
<td>98-253</td>
<td>GGGAAAGGACCTGATGATACA</td>
<td>GCTGCAACTGTACTCAAG</td>
<td>156</td>
<td>AF103907</td>
</tr>
<tr>
<td>GAPDH</td>
<td>446-620</td>
<td>GAGCCAAAGGGTCATCATC</td>
<td>CCTCCACGATACCAAG</td>
<td>175</td>
<td>NM_002046</td>
</tr>
<tr>
<td>G6PDH</td>
<td>1777-1935</td>
<td>CCTGGGGAGAGCTGAGAATG</td>
<td>CGGGCTCTCTCGGTACTTG</td>
<td>159</td>
<td>NM_004285</td>
</tr>
</tbody>
</table>

search of sequences was realized in (http://www.ncbi.nlm.nih.gov/BLAST/) to confirm their specificity.

The synthesized primers (Invitrogen, Carlsbad, CA) were as follows: DD3PCA3 gene; endogenous genes candidates: BACT, 18S, and GAPDH, G6PDH gene, which were optimized at 57 °C (Table 1). Conditions for the RT-PCR were optimized with a thermal cycler (gradient Pxn 2 Thermal Cycler Hybaid, Franklin, MA) using the SuperScript™III Platinum® SYBR® One Step qRT-PCR Kit with ROX (Invitrogen, Carlsbad, CA).

The sequence corresponds to the records in the GenBank™ under the following number of accesions: DD3PCA3, AF103907; BACT, NM_001101; GAPDH, NM_002046; subunit 18S, X03205 and G6PDH, NM_004285. The amplification products by real time RT-PCR were displayed by electrophoresis on a 2% agarose gel and studied with the electrophoresis EDAS 290 analysis system. The results of the amplifications such as temperature, primer concentrations, dNTPs and volumes were transferred to the amplification protocol in real time with the Rotor Gene 6.0 detection system (Corbett Life Science, Sydney City, Australia).

The reaction of the real-time RT-PCR was realized with approximately 10 ng of total RNA. The real-time RT-PCR profile consisted of an initial temperature of 52°C for 10 min for the synthesis of cDNA and at 94°C for 5 min, followed by 40 cycles in the stage of PCR, each cycle at 95 °C for 20 s, 57 °C for 20 s, 72 °C for 20 s. The denaturation stage had an interval temperature of 60-98°C.

Quantitative determination of DD3PCA3 mRNA

Data from the CP of endogenous candidates genes (GAPDH, G6PDH, BACT and 18S) and the DD3PCA3 gene, were exported from Rotor-Gene 6.0 software (Corbett Life Science, Sydney City, Australia), to calculate efficiencies with the REST® statistical model[30,31] and data were plotted constructing a linear regression which compares the logarithmic concentration (total RNA) against CP (CP is defined as the number of cycles in which the fluorescence intensity increases above the baseline fluorescence of the sample).

To correlate the candidate endogenous genes and determine the more stable gene, the BestKeeper software was used, exporting the CP of Rotor-Gene 6.0 software at Excel tool to show the melting temperature (Tm) characteristic of each amplified product. Determination of HKG was realized using BestKeeper statistical model analized CP values by Pearson correlation (Table 2).[32]

Statistical Analysis

Data from the absolute quantification of all samples were normalized with the HKG and were analyzed by
Student's $t$ test to compare both groups (PCa and BPH), considering statistical significance at $P < 0.05$. The results were represented as mean± SEM using the PRISMA software (GraphPad Prism 3.02 Software, San Diego, CA, USA).

Results

Determination of sample degradation

The integrity and the specificity of amplified products of each sample, was verified. The results indicated that degradation was not observed in the sample. (Figure 1).

Housekeeping gene determination

The GAPDH gene showed better stability than the BACT, G6PDH and 18S with a Pearson correlation of 0.953 ($P < 0.007$) (Table 2 and figure 2).

Quantitative determination of housekeeping gene

Standard curve of GAPDH was produced by Real-time RT-PCR SYBR Green of serial dilutions (0.001 to 10 ng) of the endogenous GAPDH (Figure 3).

Quantitative determination of $DD3^{PCA3}$ mRNA

In the PCa samples, the $DD3^{PCA3}$ gene is over-expressed 29.74-fold than in BPH ($P < 0.0001$). The DD3 gene expression in BPH was 58.3± 9.9 and in PCa was 1731 ± 280 (Figure 4).

Discussion

The current study showed that $DD3$ was expressed more in PCa than in BPH. A close association between $DD3$ and PCa has been reported in previous studies.

Floriano-Sánchez et al. $DD3^{PCA3}$ in cancer and prostatic hyperplasia
In conclusion, this study suggests that a quantitative assay by RT-PCR for DD3 would be a potentially valuable tool for the detection of PCa in clinical specimens. The combination of DD3 and PSA could be used as molecular markers in the early diagnosis of PCa. Although further studies are needed, gene therapies based on identification of transcription factors that interact with DD3 promoter represent an area for preclinical investigation.

Acknowledgments

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References


Correspondence to:
M. en C. Noemí Cárdenas Rodríguez
Instituto Nacional de Pediatría Av. Imán 1 Insurgentes Cuicuilco México, D. F., 04530 México.
Email: noemicr2001@yahoo.com.mx