Identification of a novel Protein Disulfide-isomerase A3 (PDIA3) transcript variant as a potential biomarker associated with late stage prostate cancer

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Abstract

Background Prostate cancer (PC) is a heterogeneous and unpredictable disease and becomes untreatable when the tumor progress to castrate-resistant (CR) or androgen independent (AI). A major clinical challenge in prostate cancer is the lack of diagnostic and prognostic tests that distinguish between benign and aggressive tumors. Isoforms of gene transcripts are emerging as suitable candidates to represent disease progression. Vitamin D receptor (VDR and PDIA3) transcript isoforms could be the target candidates of study since they have been related with anti-tumoral effects and carcinogenesis in several cancer types.

Methods The current study investigates the role of vitamin D receptor transcript isoforms in prostate cancer progression by using Next Generation Sequencing (NGS), Droplet Digital PCR (ddPCR) and several functional prediction tools.

Results The NGS analysis revealed a novel PDIA3 transcript isoform (PDIA3N) that is higher expressed than the PDIA3 isoform that codifies for the receptor protein, in prostate cells. The expression of PDIA3N was validated by droplet digital PCR (ddPCR) absolute quantification, which confirmed the findings from the NGS analyses. The PDIA3N isoform was present in higher levels than PDIA3, in the metastatic androgen dependent LNCaP cells. Furthermore, analysis of the novel PDIA3 isoform sequence indicate that the variations present in its sequence are altering the original protein function and structure as well as the predicted subcellular localization of the protein.

Conclusions We conclude that, PDIA3N due to the high expression in LNCaP cells and its abnormality in predicted structure, localization and function, is a potential biomarker for prostate cancer disease that needs to be further investigated in prostate cancer samples.
Background

Prostate cancer (PCa) is one of the most common cancer types in men worldwide and is the second leading cause of death among men in the United States (1-3). This disease is very heterogeneous and unpredictable since prostate cancer cells can easily pass from indolent to rapidly progressive and fatal. Furthermore, when the tumor progress to castrate-resistant (CR) or androgen independent (AI) it does not respond to androgen deprivation therapy and it becomes untreatable (4).

A major clinical challenge in prostate cancer is the lack of diagnostic tests that distinguish between benign and aggressive tumors (1, 5). Standard approaches used in clinical decision making are tissue pathology and Gleason score, imaging and prostate specific antigen (PSA) serum levels (1-4). Pathology analysis and Gleason score classification is more reliable but require invasive techniques to extract biopsies from the tumor. Imaging is not used if there is not any suspicion of tumor growth and usually misses small tumors. PSA screening has been used for more than 20 years as the most effective non-invasive method. However, this method lacks the sensitivity to detect early and late stage tumors and lacks specificity since very often gives false positives associated with other uropathies (5). Considering this, there is a need of finding and validating novel biomarkers for the prostate cancer disease progression and aggressiveness.

New gene transcript isoforms are emerging as suitable candidates to represent disease progression since high expression levels of specific isoforms may be associated with specific tumor stages or with the level of the disease progression (6-9). An example is the discovery of an alternative splicing isoform signature associated with overall survival for hepatocellular carcinoma (10). Another example is the isoform switches that are highly predictive for cancer survival and
aggressiveness, such as the one for the DNA excision repair 1 (ERCC1) gene that results in a protein lacking the HHH domain which is associated with lower cancer survival rates (11).

Vitamin D and its metabolites have been suggested as potential candidates for the prevention and therapy of several cancer forms, including prostate cancer (12, 13). Numerous reports demonstrate that Vitamin D has an antitumor effect since it stimulates differentiation, increases apoptosis and inhibits proliferation, invasiveness and metastasis of cancer cells (13-15). Two receptors are involved in the activation of the Vitamin D signaling pathway and its mediated effects: The classic nuclear vitamin D receptor (VDR) and the protein disulfide isomerase family A, member 3 (PDIA3) receptor (16). VDR is a receptor localized in the cytosol that gets activated upon binding with vitamin D which initiates its heterodimerization with the RXR receptor. This complex migrates to the nucleus where it modulates gene transcription after binding with the vitamin D response element (VDRE) in the genome (17, 18). Thus, VDR is responsible of the “genomic or long-term actions” which are the activation of gene transcription by chromatin remodeling and the regulation of vitamin D biosynthesis (18). The PDIA3 receptor is a chaperone localized mainly in the endoplasmic reticulum and it is responsible for “non-genomic or rapid actions” of vitamin D in the plasma membrane (16). These actions include the regulation of intracellular, extranuclear pathways and signaling cascades, such as the activation of the protein kinase C (PKC) pathway and the calcium transport (16). The role of PDIA3 in cancer regulation remain controversial since some studies suggest that it is responsible of the activation of proapoptotic pathways (19, 20) and other studies that it is associated with cancer proliferation, inhibition of the apoptosis and poor prognosis (21, 22). VDR was found to be lower expressed in
prostate tissue while PDIA3 was higher expressed at protein and transcript level in both normal and cancer prostate tissue (23).

The current study investigates the role of vitamin D receptor isoforms in prostate cancer progression. Furthermore, we searched for novel vitamin D receptor transcript isoforms, related to prostate cancer progression. Therefore, next generation sequence (NGS) was performed on prostate cancer RNA from stages of progression. The NGS analysis identified a new PDIA3 transcript isoform in prostate cells that is higher expressed than the actual isoform that codifies for the PDIA3 receptor protein. We did an absolute quantification by droplet digital PCR (ddPCR), absolute quantification, which confirmed that this PDIA3N isoform was present in higher levels, compared to the normal PDIA3 isoform, in the metastatic and androgen dependent stage of the disease. These results also show that there is change in expression level of PDIA3 isoforms between normal and metastatic cancer cells that could be an indicator of normal and aggressive stage of prostate cancer.

The novel PDIA3 isoform has a second and different translation initiation site (TIS) than the one observed in the normal PDIA3 isoform. This novel TIS lead to a different protein sequence in the N-terminus and it is an indication that this isoform is delivered to a different subcellular compartment in the cell to accomplish another function. Furthermore, this novel PDIA3 isoform shows a truncated N-terminus sequence that changes the active thioredoxin sites of the protein. Moreover, most of the amino acid substitutions as well as deletions in PDIA3N sequence, are predicted to be pathogenic or damaging in the PDIA3N sequence compared to the PDIA3 sequence.

Altogether, these data suggest that this novel transcript isoform of PDIA3 novel transcript isoform is a potential biomarker for the aggressiveness of the prostate
cancer disease, that truly needs to be further investigated in patient samples in correlation with the Gleason score.

Methods

Reagents

RPMI-1640 medium, Dulbecco’s Modified Eagle’s Medium (DMEM), Minimum Essential Medium Eagle (EMEM), Stemline Keratinocyte Medium II, Stemline Keratinocyte Growth Supplement, Fetal Bovine Serum (FBS), Penicillin-streptomycin (PEST), sodium pyruvate (SP) and Trypsin-EDTA solution were purchased from Sigma Aldrich (St. Louis, MO, USA).

RNA extraction was performed with the RNeasy Mini Kit from QIAGEN including DNA digestion with DNase set (Hilden, DE) and reverse transcription PCR (RT-PCR) with the High Capacity cDNA Reverse Transcription Kit from Applied Biosystems (Foster City, CA, USA). Both RNA and DNA samples were eluted in nuclease free water from VWR (Radnor, PA, USA).

For the droplet digital polymerase chain reaction (ddPCR): ddPCR\textsuperscript{TM} Supermix for Probes (No dUTP), ddPCR\textsuperscript{TM} Droplet Generation Oil for Probes, Droplet Reader Oil and two different Prime PCR Assays were all purchased from Bio-Rad Laboratories (Hercules, CA, USA). Prime PCR Assay for PDIA3-Normal (PDIA3) consist in a forward primer (5´-GTGTGGCGCTGCTTCTTG- 3´), a reverse primer (5´-AAGAACTCGACGAGCATGAG-3´) and a FAM probe or internal primer FAM (5´-GCCTCGCCGCTGCCTCCGAC-3´). Prime PCR Assay for PDIA3-Novel (PDIA3N) consist in a forward primer (5´-G GCAGTGATTGTGATGTTAGCCTCCA-3´), a reverse primer (5´-CAAGTCTCTGCA GTGTCCA-3´) and a HEX probe or internal primer HEX (5´-ACACACACACCTGGTGTCCTCCAGA-3´). Positive target controls for the ddPCR
experiment consisted in DNA fragments or oligos with the same sequence as the target (PDIA3 and PDIA3N respectively).

Cell culture

Cells cultured for further experiments were ranging in the severity of the prostate cancer disease. PNT2 (Control), normal prostatic epithelial cells well differentiated; P4E6 (Early stage), immortalized human prostate cell line derived from a biopsy of a well-differentiated early stage prostate cancer. DU145, prostate derived from metastatic site in the brain (Prostate adenocarcinoma, grade II, AR negative). PC3, prostate cell line derived from bone metastasis (Prostate adenocarcinoma, grade IV, AR negative). LNCaP from a lymph node metastasis (carcinoma metastatic, AR positive).

PNT2, P4E6 and LNCaP were obtained from Sigma Aldrich. PNT2 and LNCaP cell lines were cultured in RPMI-1640 complete medium supplemented with 10% FBS and 1% PEST. The cell lines DU145 and PC3 were obtained from ATCC (Manassas, VA, USA). P4E6 cells were cultured in Stemline Keratinocyte Medium II with Stemline Keratinocyte Growth Supplement, 2mM Glutamine and 2% of Foetal Bovine Serum (FBS). Addition of 2% serum was found to increase cell viability. DU145 cells were cultured in EMEM supplemented with 10% FBS and 1% PEST. PC3 cells were cultured in DMEM containing 10% FBS, 5% of pyruvate sodium and 1% PEST. All the cells were cultured in T25 flasks and culture adherent cells were detached with Trypsin-EDTA solution and maintained at 37°C in 5% CO2.

RNA extraction and RT-PCR

Total RNA was extracted from one million cells for each cell line: PNT2, P4E,
DU145, PC3 and LNCaP by following the protocol for the RNeasy Mini Kit from Qiagen including genomic DNA digestion with DNase. Samples were eluted in RNase-Free Water and stored at -80°C. 20 µl of each cell line sample that fulfilled the quality standards (A260/280 = 1.9-2.0, A260/230 = 1.5-1.8, RNA integrity number ≥ 8) was sent to The National Genomic Infrastructure (NGI) in The Science Life Lab in Stockholm, Sweden.

From the PNT2, DU145, PC3 and LNCaP samples, RNA (up to 2 µg) was reversed transcribed to cDNA according to the protocol for the High Capacity cDNA Reverse Transcription Kit from Applied Biosystems. The obtained cDNA samples were used as a reaction template in the ddPCR absolute quantification experiment.

**NGS**

Five RNA samples (PNT2, P4E6, DU145, PC3 and LNCaP) were sequenced with Illumina HiSeq 2500 High Output v4, 2x125bp in one lane giving >37.6M read pairs/sample. The library used was TruSeq Stranded Total RNA Illumina RiboZero and was also performed in the NGI. Data delivered from the NGS included demultiplexing, quality control and raw data.

The Software TopHat/2.0.4 was used for mapping reads to the Human genome assembly, build GRCh37 (hg19). The output of the mapping was BED files, which comprises the splice junctions reported by the TopHat algorithm; and “accepted hits” BAM files, which contains a list of the reads aligned to the reference genome. Both files were sorted and indexed with Samtools. Quantification of normalized expression values were obtained as FPKM (Fragments per Kilobase of Exon per Million Fragments Mapped) values generated by cufflinks/2.1.1. FPKMs values were obtained from genes and their different transcript isoforms.
Droplet digital™ PCR (ddPCR)

Droplet digital polymerase chain reaction (ddPCR; QX200, Bio-Rad, Hercules, CA, USA) was performed according to the manufacturer’s instructions. A total of 178 cDNA samples were analyzed to quantify the amount of PDIA3-Normal and PDIA3-Novel target. Each sample was partitioned into 8000-15000 droplets, there every droplet can be considered as one PCR reaction, with target and background DNA randomly, but uniformly, distributed among the droplets. The reactions were performed in 25 µl reaction volumes that consisted of up to 10 µl and 330ng of template cDNA, 12.5 µl of ddPCR supermix for probes (No dUTP), 1.25 µl of PDIA3-Normal Prime PCR Assay, 1.25 µl of PDIA3-Novel Prime PCR Assay and DNase free water (variable volume). The non-template controls (NTC) contained 8 µl of purified water instead of cDNA template and each positive control contained a complementary single DNA sequence or oligo to the PDIA3-Normal isoform and to the PDIA3-Novel isoform respectively. 20 µL of each reaction mix was loaded into the DG8 Cartridge and 70 µL of droplet generation oil onto QX200 Droplet Generation system for droplet generation. Total amount of droplets of each reaction was transferred to a 96 well plate to perform the PCR amplification. In order to determine the optimal annealing temperature for the PCR, thermal gradient experiments were performed with one sample (Additional file 1). After evaluating which temperature that showed the best separation between positive droplets with the target and negative droplets, the PCR temperature conditions were set to 95 °C for 10 min (1 cycle), 94 °C for 30 s, 58.1 °C for 60 s (40 cycles), 98 °C for 10 min and infinite hold at 4 °C. After that the PCR 96-well plate was read by the QX200 Droplet Reader and the type of experiment was set to absolute quantification. The
Poisson-corrected determination of template concentration (copies/ µl) and the ratio between both isoforms (PDIA3-Novel/PDIA3-Normal) was calculated using QuantaSoft™ Analysis Pro Software (v1.0.596, Bio-Rad).

**Statistical analysis**

Differences between sample groups and template concentrations were assessed by non-parametric Kruskal-Wallis multiple comparisons test (p<0.05) in GraphPad Prism version 7.00 for Windows (GraphPad Software, La Jolla California USA). Groups pairwise comparisons were assessed by Mann-Whitney U test (p>0.05). These tests were selected assuming that the data did not follow a normal (Gaussian) distribution (Kolmogorov-Smirnov test, P value <0.0001).

**Functional analysis**

Protein sequences were retrieved from UniprotKB (P30101) for PDIA3 and from UniParc (UPI000066D935) for PDIA3N (24). Both protein sequences were analysed by the I-TASSER (Iterative Threading ASSEmbly Refinement server) for generating the protein structure model (25). Differences in the protein sequences were highlighted with UCSF Chimera 1.0 (26). A prediction of the damage or pathogenicity of the 56 variations (amino acid substitutions and deletions) that PDIA3N present in comparison with PDIA3 was carried out with PolyPhen-2 (27) and PROVEAN (Protein Variation Effect Analyzer) v1.1 (28) software tools. The threshold score used to determine that the variant was pathogenic in PolyPhen-2 (HumDiv and HumVar algorithms) was 0.403 and in PROVEAN was -2.5. In PolyPhen-2 an amino acid substitution was considered “Possibly Damaging” if the score was higher than 0.403 and “Probably Damaging if it was higher than 0.975. In PROVEAN an amino acid
substitution/deletion was considered “Deleterious” in PROVEAN if the score was under -2.5. A prediction of the subcellular localization of PDIA3 and PDIA3N was performed by DeepLoc-1.0 (29).

Results

**NGS analysis reveals the PDIA3N, a novel transcript isoform of PDIA3, that is higher expressed in the metastatic cell line LNCaP**

NGS results analysis was focused on Vitamin D receptor transcript isoforms of VDR, RXRA and PDIA3. VDR and RXRA transcript isoforms showed low expression levels (0-7.4 FPKMs) for the five samples analyzed. The analysis of the results for the PDIA3 detected a novel transcript isoform (ENST00000538521.1, GRCh37.p13 Ensembl 2018) (30) in prostate cells (PDIA3 Novel, Figure 1A). The PDIA3N isoform has 14 exons and is 1628 bp shorter than the actual PDIA3 transcript isoform (ENST00000300289, GRCh38.p12 Ensembl 2018) (30) and contains a different nucleotide sequence fragment of 178bp (Figure1A). Moreover, an alternative translation initiation sequence (TIS) is present in this sequence fragment (Figure 1A). The novel PDIA3 transcript isoform was annotated in the previous reference genome GRCh37 (hg19) and it was deprecated when the new reference genome GRCh38 (hg38) was released. This previous annotation of the novel isoform was performed by the Ensembl gene build and it was first registered as genomic DNA sequence by the European Nucleotide Archive in 2007 and as a protein coding in kidney and colon cancer in 2009 and 2010 respectively (UPI000066D935, UniParc) (24).

NGS analysis showed that PDIA3 Novel is higher expressed in prostate cells than the actual PDIA3 isoform with normalized values of expression 8.5-14.5 and 44.6-69.3
FPKM values respectively (Figure 1B). The expression of PDIA3N was correlated with the tumor stage, being more expressed in the metastatic cell lines (DU145, PC3 and LNCaP) than in the control cell line (PNT2) and the earlier prostate cancer stage one (P4E6). The highest value of expression (69.3 FPKM value) was obtained for the metastatic androgen dependent cell line LNCaP. Since the number of samples was only five (one for each cell line) these results from NGS needed to be further validated for statistical significance.

**ddPCR absolute quantification confirms that PDIA3N was significantly higher expressed in LNCaP cells compared with PDIA3**

A total of 178 cDNA prostate cell samples from PNT2, DU145, PC3 and LNCaP were analyzed by ddPCR amplification experiment to quantify the amount of target PDIA3 Normal and Novel transcript. Figure 2.A shows two examples of amplitude plots for the PDIA3 and PDIA3N for one of the LNCaP samples. Amplitude limit was set manually to 2000 for PDIA3 and 4000 for PDIA3N since it was the most appropriate value to represent the separation of positive and negative droplets for all the samples analyzed. PDIA3N amplification plot shows more positive droplets (26) compared to the number of positive droplets (blue) in the PDIA3 amplification plot (Figure 2.A). This difference was observed across all the amplitude plots for the LNCaP samples. Figure 2.B. shows the ratio of quantification between the number of copies/µl for PDIA3N and PDIA3 for PNT2, DU145 and LNCaP prostate cell samples. Kruskal-Wallis multiple comparisons test confirmed that the difference among the cell line groups was significant (****, P<0.0001). The median ratio PDIA3 N/PDIA3 was significantly higher in the metastatic cell line LNCaP compared with the normal or epithelial cell line PNT2 (Mann-Whitney U test, ****: P <0.0001) (Figure 2.B).
Figure 3 shows in detail the target concentrations (copies/µl) after the ddPCR analysis of both targets (PDIA3 and PDIA3N) in PNT2 and LNCaP cells. First observation is that PDIA3 concentration was higher in LNCaP cells than in PNT2 cells (***, P<0.001). Second observation is a significantly higher PDIA3N concentration in LNCaP cells than in PNT2 cells (****, P<0.0001). PDIA3N concentration was also significantly higher when compared with PDIA3 concentration in LNCaP cells (#, P<0.0001).

**Functional prediction analysis shows that PDIA3N is potentially pathogenic and is localized in the cytoplasm**

Figure 4 shows a representation of the generated model for both PDIA3 and PDIA3N proteins. The protein sequence for PDIA3N has 485 amino acids and the first fragment in the N-terminus of the sequence differs in 56 amino acids (36 amino acid substitutions and 20 amino acid deletions) from the PDIA3 sequence (Figure 4A). The model generated by I-TASSER for PDIA3N had higher confidence (C-Score) than the one generated for PDIA3 (Figure 4B). At the secondary structure level the PDIA3N lacks an α-helix and one of the thioredoxin active sites is truncated to CGH from WCGH in PDIA3 (Figure 4A, 4B). The total 56 variations that PDIA3N shows with respect to PDIA3 were analyzed with Polyphen-2 (27) and PROVEAN (28). From these 56 variations, 22 were confirmed as variants affecting the structure and function of the protein, by the two predictors PolyPhen-2 and PROVEAN (Table 1). The rest of variants were either benign or neutral (Polyphen-2 Score < 0.432, PROVEAN Score >2.5) or not confirmed by both methods as damaging variants. The prediction of subcellular localization by DeepLoc-1.0 shows that PDIA3N is a soluble protein present in the cytoplasm of the cell while PDIA3 is present in the endoplasmic
Discussion

Previous studies pinpoint PDIA3 as a plausible candidate for studying cancer prognosis and as a target for cancer treatment (19-22, 31). The role of PDIA3 in cancer treatment remains controversial and the implication of different PDIA3 transcript isoforms has not yet been studied in connection with prostate cancer progression.

By performing NGS we could detect a novel PDIA3 transcript isoform in prostate cells. This novel isoform (PDIA3N) was previously reported by the Ensembl gene build (ENST00000538521.1, GRCh37.p13 Ensembl 2018) and is not included in the new genome assembly, GRCh38 (hg19)(30). The information reported by Ensembl GRCh37 revealed that the PDIA3N could be associated with the progression of kidney and colon cancer. However, there are not further studies evaluating the existence of this isoform and/or the level of expression in prostate cancer.

Furthermore, the NGS analysis showed that this isoform was higher expressed than the normal isoform in prostate cells and that the level of expression was especially high in LNCaP cells. Since the number of samples was not enough to assess statistical significance, these results were further validated by ddPCR. The results from ddPCR confirmed that PDIA3N is expressed in prostate cells and that the concentration is significantly higher in the metastatic androgen dependent LNCaP cells in comparison to the PNT2 control prostate cells. In the metastatic androgen independent cell lines DU145 and PC3, we could not detect any differences between the PDIA3N and PDIA3 isoform concentrations compared to PNT2 was not significant. These results suggest that the overexpression of PDIA3N and PDIA3 may be
somehow related with cells in advanced stages that have androgen dependent growth.

Several studies associate deregulation of PDIA3 with multiple pathologies including cancer and neurodegenerative disease (31-33). Aberrant expression of PDIA3 is shown to be correlated with poor prognosis in several cancer types as well as to the increase of cell proliferation mediated by vitamin D binding and subsequent activation of the epidermal growth factor receptor (EGFR)(31).

Until now, there have been only one study evaluating the expression of PDIA3 in prostate cancer tissue (19). In this study, they showed that tumor samples with a higher Gleason score (GS 8-10) had significant increased levels of the PDIA3 transcript abundance levels compared to GS 6 tumors. However, the difference in PDIA3 expression between GS 8-10 and benign tumor tissue was not significant (19). Furthermore, the study did not focused on assessing potential new PDIA3 isoforms playing a role in the expression between benign and tumor tissues (19). The novel PDIA3 isoform detected in this study was significantly higher expressed in comparison with the benign prostate cells.

The protein predicted sequence reveals that, PDIA3N is shorter in length and contains a different N-terminus sequence than the normal isoform due to an alternative 5´-proximal TIS. It has been shown that several TIS can be recognized by the ribosomes in the coding sequence (CDS) at the same time. Thus, the 40S ribosomal subunit, during the scanning of an open-reading sequence, may recognize a second TIS and start the protein translation from that point. This mechanism is called “leaky scanning” and is the responsible of the translation of functional different isoforms (34-36). Most of these isoforms are N-truncated proteins that contain secretory signals in order to be delivered to different cell compartments
We have confirmed by prediction analysis that PDIA3N has a different subcellular location since it is mainly located in the cytoplasm while PDIA3 is in the endoplasmic reticulum. Considering all this information and our results, we suggest that PDIA3N is an N-truncated protein isoform of PDIA3 produced by alternative translation or “leaky scanning”.

One of the predicted functional differences, compared to the normal PDIA3, (See results section, Figure 4B) is the lack of one α-helix in its secondary structure and the truncation in one active site of the thioredoxin (See results section, Figure 4A). These differences are likely to affect, first of all the conformation of the protein and secondly the activity as a thiol oxidoreductase.

The analyses of the sequence variations in this novel isoform PDIA3N present in comparison with PDIA3 a confirmation that a big proportion of the variations are damaging or harmful for the function and/or structure of the protein.

Isoforms of gene transcripts have been suggested as plausible biomarkers for different diseases, such as cancer. The expression pattern of different isoforms could change depending on the stage or severity of the disease and, also the relation between the expression of two gene isoforms could be correlated with a specific disease pattern (7, 11, 37). In this study we confirm PDIA3 as a plausible biomarker to test in prostate cancer human samples. Furthermore, the detected novel isoform of PDIA3 (PDIA3N) is even higher expressed than the normal PDIA3 transcript in the metastatic androgen dependent cell line LNCaP. The relation of expression between PDIA3N and PDIA3 (Ratio PDIA3 Novel/PDIA3 Normal) could also be a predictor of the disease aggressiveness since changes, from almost two times in control samples to nine times higher expression of PDIA3N, are seen in the metastatic androgen dependent samples. Functionality analysis of the variations in
the PDIA3N sequence also confirm the abnormality of the protein with respect to PDIA3.

Considering that VDR is lower expressed in prostate cancer samples and the increased expression of PDIA3, according to the cancer stage, we propose that PDIA3 could be inhibiting the competitively antitumorigenic actions of vitamin D in cancer cells. Thus, Vitamin D would to a greater extend bind the PDIA3 receptor instead of with VDR receptor due to the abundance of the PDIA3 receptor in pathogenic conditions. The role of PDIA3N remains unclear but what is evident is that it also plays a relevant role in pathogenesis due to its abundant expression in the metastatic androgen-dependent stage, compared to PDIA3, and due to its predicted aberrant structure and function. One explanation that supports our results is that PDIA3N is novel and not studied before is that the seen contradictory effects could have been wrongly attributed to the PDIA3 normal isoform/receptor, when they instead are caused by the PDIA3N isoform. Thus, there could be different PDIA3 isoforms playing a contrary role in cancer progression. The switch in expression between these isoforms could indicate a different stage or form of the disease. PDIA3N could be the pathogenic form of PDIA3 which inhibit cell apoptosis and stimulates the pathways leading to cancer cell proliferation. This aberrant isoform/protein would only be overexpressed under aggressive stages of cancer. We conclude that, PDIA3N due to the high expression in LNCaP cells and its abnormality in predicted structure, localization and function, is a potential biomarker for prostate cancer disease that needs to be further investigated in prostate cancer samples. Further studies could be focused in studying expression patterns of PDIA3N in different prostate cancer human samples, assessing if there is a dual role of PDIA3 and PDIA3N in cancer progression and evaluating vitamin D
ligand affinities with the PDIA3N. Functional analysis and comparison on protein level of PDIA3N could be possible with overexpression of PDIA3N in LnCap cells.

Abbreviations

AD androgen dependent
AI androgen independent
AR androgen receptor
CDS coding sequence
CR castrate resistant
ddPCR droplet digital PCR
EGFR epidermal growth factor receptor
ERCC1 DNA excision repair protein 1
GS Gleason score
NGS next generation sequencing
PCa prostate cancer
VDR vitamin D receptor
PDIA3 protein disulfide isomerase A3
PDIA3N novel protein disulfide isomerase A3
PSA prostate specific antigen
TIS Translation initiation site
RXR retinoid X receptor
RXRA retinoid X receptor alpha
PKC protein kinase C

Declarations
**Ethics approval and consent to participate**

Not applicable

**Consent for publication**

Not applicable

**Availability of data and materials**

The analysis supporting the conclusions of this article are included within the article and its additional files. NGS and ddPCR Raw data are available from the corresponding author on reasonable request.

**Competing interests**

The authors declare that they have no competing interests.

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**Authors' contributions**

DLA conceived of the study, MADC carried out the design and MADC, PK and GH the experiments. MADC, FS, DL and SK participated in the analysis of results. MADC wrote the manuscript. DLA, SK, FS, JH and DL reviewed the manuscript. All authors have read and approved the final manuscript.

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Tables

**Table 1.** PDIA3 Sequence variations, present in PDIA3N, that are predicted as harmful or damaging for the protein structure and function by Polyphen-2 and PROVEAN.

| Mutation | Polyphen-2 HumDiv Prediction-Score | Polyphen-2 HumVar Prediction-Score | PROVEAN Prediction-Score |
|----------|-----------------------------------|-----------------------------------|--------------------------|
| L15S     | Probably damaging-0.999           | Probably damaging-0.998           | Deleterious-(-2.841)     |
| L16P     | Probably damaging-0.994           | Probably damaging-0.840           | Deleterious-(-2.534)     |
| S25W     | Probably damaging-0.999           | Probably damaging-0.995           | Deleterious-(-3.646)     |
| D26L     | Probably damaging-1.0             | Probably damaging-1.0             | Deleterious-(-8.089)     |
| V27L     | Probably damaging-0.999           | Probably damaging-0.998           | Deleterious-(-2.596)     |
| L28P     | Probably damaging-0.999           | Probably damaging-0.980           | Deleterious-(-5.534)     |
| E29R     | Possibly damaging-0.660           | Possibly damaging-0.518           | Deleterious-(-3.547)     |
|     | Possibly damaging-0.660 | Possibly damaging-0.480 | Deleterious-(-3.609) |
|-----|------------------------|-------------------------|----------------------|
| T31L|                        |                         |                      |
| D32E| Possibly damaging-0.975 | Possibly damaging-0.927 | Deleterious-(-3.203) |
| N34L| Possibly damaging-0.956 | Possibly damaging-0.882 | Deleterious-(-4.518) |
| F35I| Probably damaging-1.0  | Probably damaging-1.0  | Deleterious-(-5.266) |
|     |                        |                         |                      |
| S37del|                      |                         | Deleterious-(-4.275) |
| R38del|                      |                         | Deleterious-(-4.842) |
|     |                        |                         |                      |
| I39del|                      |                         | Deleterious-(-3.643) |
| L49del|                      |                         | Deleterious-(-3.824) |
| V50del|                      |                         | Deleterious-(-6.236) |
|     |                        |                         |                      |
| E51del|                      |                         | Deleterious-(-8.939) |
| F52del|                      |                         | Deleterious-(-12.045)|
|     |                        |                         |                      |
| F53del|                      |                         | Deleterious-(-12.045)|
|     |                        |                         |                      |
| A54del|                      |                         | Deleterious-(-12.826)|
| P55del|                      |                         | Deleterious-(-15.312)|
| W56del|                      |                         | Deleterious-(-18.703)|

**Figures**
Panel figure showing NGS results from prostate cell line RNA samples. (A) Differences in nucleotide composition of NGS results. FPKM values are shown for the prostate cell lines PNT2, P4E6, DU145, PC3 and LNCaP.

Exons: 13, Coding exons: 13, Transcript length: 3727 bps, Translation length: 505 residues

Gene Expression (FPKM)

Prostate cell line

Exons: 14, Coding exons: 13 Transcript length: 2099 bps Translation length: 485 residues
Figure 2

Panel figure showing amplification results from ddPCR experiment with prostate cell line samples. (A) Amplification of PDIA3 gene. (B) Ratio of PDIA3Novel/Normal for PNT2, DU145, PC3, and LNCaP cell lines. The results were assessed by Mann-Whitney U test (*: P < 0.05, **: P < 0.01, ***: P < 0.001, ****: P < 0.0001).
Figure 3

Graph showing the differences in PDIA3 and PDIA3N target concentration (copies/µl).
**Figure 4**

Predicted Functional analysis of PDIA3 normal isoform and PDIA3 novel isoform with I-TASSER.

(A) Protein sequence for PDIA3, Lenght: 505 aa

MRLRLALFPGVALLLAAARLAAADVLLELTDONFESRISDTGSAILMLVEFFAPWCGHCKRLAPEYEAAATRLKIGVPLAKVDC3TANTNC5KYGVSPGTLMKIFRGEAGAYDGPRTADGVYHLKQAGPASVPLRTEEEFKKFSIKDASIVGFFDDSSEAHSEFLKAASNLRDNYRFAHTNVESLVNEYDNGEGIILFRPSHLYNKFEDKTVAYTEQKMTSGKIKKFIQENIFGIC...

C-score = 0.27

(B) Protein sequence for PDIA3N, Lenght: 485 aa

MTLWLPFLKFAVDFSPSLAAYTHTWILLPPFLEILICGHCKRLAPEYEAAATRLKIGVPLAKVDC3TANTNC5KYGVSPGTLMKIFRGEAGAYDGPRTADGVYHLKQAGPASVPLRTEEEFKKFSIKDASIVGFFDDSSEAHSEFLKAASNLRDNYRFAHTNVESLVNEYDNGEGIILFRPSHLYNKFEDKTVAYTEK...

C-score = 1.79

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**Supplementary Files**

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