Leucine zipper, down regulated in cancer-1 gene expression in prostate cancer

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Received April 8, 2015; Accepted June 12, 2016

DOI: 10.3892/ol.2016.4983

Abstract. Numerous genetic alterations have been implicated in the development of prostate cancer (PCa). DNA and protein microarrays have enabled the identification of genes associated with apoptosis, which is important in PCa development. Despite the molecular mechanisms are not entirely understood, inhibition of apoptosis is a critical pathophysiological factor that contributes to the onset and progression of PCa. Leucine zipper, down-regulated in cancer 1 (LDOC-1) is a known regulator of the nuclear factor (NF)-mediated pathway of apoptosis through the inhibition of NF-κB. The present study investigated the expression of the LDOC-1 gene in LNCaP, PC-3, PNT1A and PNT2 prostate cell lines by reverse transcription-quantitative polymerase chain reaction. In addition LDOC-1 protein expression in normal prostate tissues and PCa was studied by immunohistochemistry. LDOC-1 messenger RNA resulted overexpressed in LNCaP and PC-3 PCa cell lines compared with the two normal prostate cell lines PNT1A and PNT2. The results of immunohistochemistry demonstrated a positive cytoplasmic LDOC-1 staining in all PCa and normal prostate samples, whereas no nuclear staining was observed in any sample. Furthermore, a more intense signal was evidenced in PCa samples. LDOC-1 gene overexpression in PCa suggests an activity of LDOC-1 in PCa cell lines.

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Key words: LDOC-1 gene, prostate cancer, RT-qPCR, protein expression

Introduction

Prostate cancer (PCa) is a slow-growing but often aggressive cancer (1). The frequency of PCa is particularly high in Europe and USA (2,3). Factors such as diet, habit and genetic background have been implicated in the development of PCa (4). The genetic background may contribute to the risk of PCa, as suggested by associations with race, family and specific gene variants (5). Men who have a first-degree relative (brother or father) with PCa have twice the risk of developing PCa, and those with two first-degree relatives affected have a 5-fold greater risk compared with men with no family history (2,3,6). Apoptosis is a programmed cell death process in normal physiological and pathological conditions (7-9). Microarrays studies have enabled the identification of genes that are differentially regulated in cancer (7). Apoptosis inhibition is a critical pathophysiological factor that contributes to the onset and progression of PCa, even if the molecular mechanisms are not completely understood (8,9). The leucine zipper, down-regulated in cancer 1 (LDOC-1) gene, mapping on chromosome Xq27, codes for a nuclear protein of 146 amino acids with a molecular weight of ~17 kDa, and consists of only one exon (Online Mendelian Inheritance in Man 300402) (10). Literature data (10-14) have indicated greater or lesser expression of LDOC-1 in a variety of tumor types. The aim of the present study was to investigate the expression of the LDOC-1 gene in LNCaP and PC3 PCa cell lines compared with normal prostate cell lines PNT1A and PNT2 by reverse transcription-quantitative polymerase chain reaction (RT-qPCR). In addition, immunohistochemical studies for the protein LDOC-1 were performed on histological samples of normal prostate and prostate carcinoma.

Materials and methods

Cell lines. Four cell lines, PNT2, PNT1A, PC-3 and LNCaP, were used in the present study. PNT2 primary culture was obtained post-mortem from the prostate of a 33-year-old man (15,16). This prostate possessed a well differentiated morphology, with the expression of cytokeratin 19, 18 and 8, the latter being a feature of differentiated luminal cells of the

Table I. mRNA expression of LDOC-1 and GAPDH genes in LNCap and PC-3 prostate cancer cell lines compared with PNT1A and PNT2 normal prostate cell lines.

A, mRNA expression in PNT1A vs. LNCaP cells

	PNTA1		LNCaP	
mRNA expression	M. Cp	RT	M. Cp	RT
Target gene LDOC-1	23.04	1.000	20.66	6.505
Reference gene GAPDH	22.39	-	22.71	-

B, mRNA expression in PNT1A vs. PC-3 cells

	PNTA1		PC-3	
mRNA expression	M. Cp	RT	M. Cp	RT
Target gene LDOC-1	23.04	1.000	20.08	8.819
Reference gene GAPDH	22.39	-	22.57	-

C, mRNA expression in PNT2 vs. LNCaP cells

	PNT2		LNCaP	
mRNA expression	M. Cp	RT	M. Cp	RT
Target gene LDOC-1	23.00	1.000	20.66	9.727
Reference gene GAPDH	21.77	-	22.71	-

D, mRNA expression in PNT2 vs. PC-3 cells

	PNT2		PC-3	
mRNA expression	M. Cp	RT	M. Cp	RT
Target gene LDOC-1	23.00	1.000	20.08	13.190
Reference gene GAPDH	21.77	-	22.57	-

M. Cp, mean crossing point (cycles); RT, ratio normalization; mRNA, messenger RNA; LDOC-1, leucine zipper, down-regulated in cancer 1; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

glandular prostate (15,17). The PNT1A normal human immortalized prostatic cell line has been proved to be a model for analysis of cellular processes such as the proliferation of prostatic epithelium in response to androgens and growth factors, and can be considered a non-tumorigenic cell line based on its molecular and biochemical properties, which are close to those of the normal prostate epithelium (15,16). The PC-3 cell line was originally derived from advanced androgen-independent bone metastasis/metastasized PCa (18). LNCaP is a cell line derived from a needle biopsy of human prostate adenocarcinoma cells of a 50-year-old Caucasian man (19). The PNT1A, PNT2, LNCaP and PC-3 cell lines used in the present study were purchased from the American Type Culture Collection (Manassas, VA, USA) and cultured in RPMI-1640 medium (Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (Thermo Fisher Scientific, Inc.) and 1% penicillin-streptomycin. All cells were maintained at standard cell culture conditions (37˚C and 5% CO_2 in a humidified incubator).

RNA extraction and RT-qPCR. RNA extraction, RT and qPCR for each sample were conducted as previously reported (13). LDOC-1 gene expression level was normalized to glyceraldehyde 3-phosphate dehydrogenase gene expression level, and the target mean crossing point definition was used to indicate the mean normalized cycle threshold (13).

Patient and control prostate tissues. The present study included 15 cases of PCa with different Gleason score (3 prostates removed by prostectomy autopsy and 11 prostatic biopsies) and 12 cases of normal prostatic tissues (3 prostates removed by post-mortem autopsy in normal donors and 9 prostatic biopsies). The samples

Table II. Expression of leucine zipper, down-regulated in cancer 1 protein in normal prostate and prostate carcinoma.

Normal prostate					Prostate carcinoma					
ID	Type	Age, years[a]	Positive cells (%)[b]	Sig. int.	ID	Type	Age, years[c]	Gl. sc.	Positive cells (%)[d]	Sig. int.
N1	Biopsy	68	97	2	PA 1	Prost.	69	4+4	95	3
N2	Biopsy	64	98	1	PA 2	Prost.	61	3+5	98	3
N3	Biopsy	63	95	2	PA 3	Prost.	63	4+4	99	3
N4	Biopsy	65	99	1	PA 4	Biopsy	64	4+4	93	3
N5	Autopsy	79	94	2	PA 5	Biopsy	81	4+3	97	3
N6	Biopsy	74	93	2	PA 6	Biopsy	74	4+4	98	3
N7	Biopsy	72	97	2	PA 7	Biopsy	70	4+4	95	3
N8	Biopsy	67	95	1	PA 8	Biopsy	68	4+4	94	3
N9	Biopsy	56	94	2	PA 9	Biopsy	56	3+3	98	3
N10	Biopsy	69	97	1	PA 10	Biopsy	66	4+3	96	3
N11	Autopsy	71	93	2	PA 11	Biopsy	74	3+3	96	3
N12	Autopsy	88	97	2	PA 12	Biopsy	61	3+3	93	3
					PA 13	Biopsy	67	3+4	94	3
					PA 14	Biopsy	70	4+4	98	3
					PA 15	Biopsy	69	3+5	96	3

[a]Mean=69.66. [b]Mean=95.75. [c]Mean=67.53. [d]Mean=96.00. N, normal prostatic tissues; PA, prostate cancer tissues; ID, identity; Prost., prostates removed by prostectomy; Gl. sc., Gleason score; Autopsy, prostates removed by autopsy; Sig. int., signal intensity.

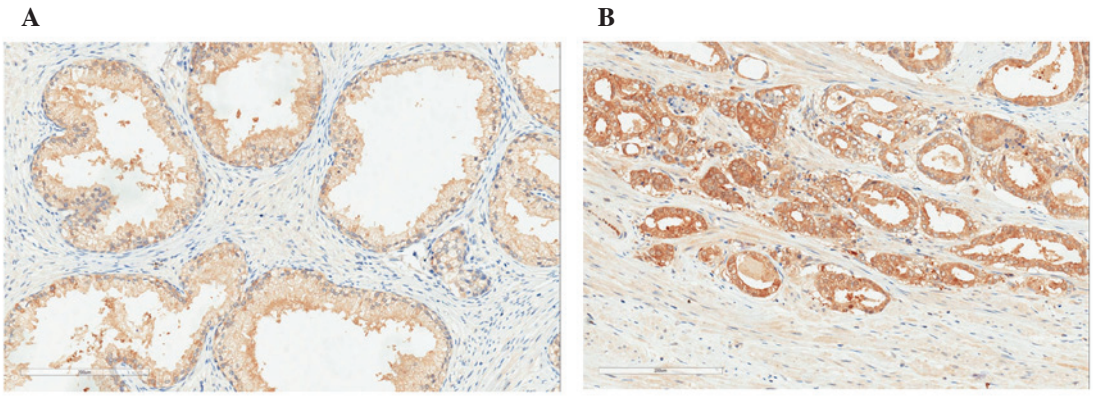

Figure 1. (A) Immunohistochemistry of LDOC-1 protein in normal prostate; hematoxylin counterstain; bar size, 200 μm. (B) Immunohistochemistry of LDOC-1 protein in prostate cancer; Gleason score 3+4; hematoxylin counterstain; bar size, 200 μm. LDOC-1, leucine zipper, down-regulated in cancer 1.

were collected between January 2012 and December 2014. The diagnosis and grading of PCa was made by two different pathologists (A.G. and F.F.) at the Pathology Unit of the Cannizzaro Hospital (Catania, Italy), according to the 2005 International Society of Urological Pathology Consensus Conference (20). All samples were obtained from the Pathology Unit of the Cannizzaro Hospital (Catania, Italy). The protocol was approved by the internal Institutional Review Board of Cannizzaro Hospital, and informed written consent was obtained from each patient with PCa or, if deceased, by his/her relatives.

Immunohistochemical staining. Prostate section tissues (4-μm thick) were obtained from all normal donors and PCa patients. All sections were formalin-fixed and paraffin-embedded following standard methods. A rabbit polyclonal antibody raised against human LDOC-1 protein was used for immunohistochemistry (NBP1-80,323; Novus Biologicals, LLC, Littleton, CO, USA). As indicated by the manufacturer's protocol, this antibody, at a dilution of 1:150, has been demonstrated to reliably recognize LDOC-1 proteins in PCa and normal prostate by immunohistochemistry. In the present study, the slides were deparaffinized, rehydrated, subjected to 3x5 min cycles in a microwave at 360 W in citrate buffer, preincubated in 3% H_2O_2 in citrate buffer and thoroughly washed in Tris-buffered saline (TBS) [50 mm Tris-HCl (pH 7.4) and 150 mm NaCl] containing 0.05% Tween 20 (washing buffer). Slides were then pre-incubated with 3% bovine serum albumin (BSA; Thermo Fisher Scientific, Inc.) in TBS for 30 min, incubated with a 1:150 dilution of anti-LDOC-1 antibody in TBS containing 1% BSA and thoroughly washed in washing buffer

prior to detection with the LSAB 2 kit (contaning anti-mouse, biotinylated, peroxidase-labeled streptavidin and 3,3'-diaminobenzidine-4HCl (Dako North America, Inc., Carpinteria, CA, USA), following the manufacturer's protocol. Upon detection, the sections were counterstained with hematoxylin, dehydrated and mounted in xylene-based DPX mountant (BDH, Ltd., Pool, UK).

Microscopic evaluation. Slides were observed under a microscope, and cells were visually scored at x10, 20 and 40 magnification. To evaluate the percentage of positive tumor cells and the signal intensity, ≤500 cells were analyzed for each PCa and normal prostate tissue. LDOC-1-positive cells were evaluated independently in a blinded fashion by two observers (A.G. and M.S.). No significant difference was observed between the two observers. The signal intensity was evaluated in arbitrary units from 1 (light) to 3 (dark).

Statistical analysis. Results are presented as the mean ± standard error. Data were analyzed bu Student's t-test or one-way analysis of variance, followed by Duncan's multiple range test. Statistical analyses were performed using Statview version 5.01 for Windows (SAS Institute, Cary, NC, USA). $P<0.05$ was considered to indicate a statistically significant difference.

Results

RT-qPCR. The LDOC-1 gene was overexpressed in LNCaP and PC-3 PCa cell lines compared with the two PNT1A and PNT2 normal prostate cell lines (Table I). Particularly, a marked increase in messenger RNA (mRNA) expression of LDOC-1 in LNCaP and PC-3 PCa cell lines was observed in comparison with the PNT2 cell line (Table I).

Immunohistochemical. The 12 normal tissues sections exhibited cytoplasmic signal for the protein LDOC-1 (mean percentage of positive cells, 95.75±2.01%), with a signal intensity of 1 or 2 (Table II and Fig. 1A). LDOC-1-positive cytoplasmic signal was present in all 15 PCa samples (mean number of positive cells, 96.00±1.96%), with a signal intensity of 3 in all PCa sections (Table II and Fig. 1B). No nuclear staining was observed in any of the samples. A more intense signal of LDOC-1 protein was evidenced in samples of PCa samples.

Discussion

RT-qPCR analysis demonstrated that mRNA LDOC-1 was overexpressed in LNCaP and PC-3 PCa cell lines compared with normal prostate cell lines. These data were confirmed by an increased expression of the protein LDOC-1 in the tissues of PCa patients compared with normal prostate tissues. Gene conversions on the X chromosome (LDOC-1 maps in Xq27) have been linked with genetic diseases and disorders (21). These variations lead to haplotypes with an increased susceptibility to PCa; therefore it cannot be excluded that any rearrangements may favor an overexpression of LDOC-1 in PCa. LDOC-1 is a known regulator of the nuclear factor (NF)-κB-mediated pathway of apoptosis through the inhibition of NF-κB (12). The expression of Wiskott-Aldrich syndrome protein family, member 3 induces the translocation of LDOC-1 from the nucleus to the cytoplasm, resulting in the inhibition of LDOC1-induced apoptosis (22,23). In addition, the transcription factor myeloid zinc finger gene 1 was demonstrated to interact with LDOC-1 and to enhance the activity of LDOC-1, thus favoring apoptosis (22,23).

Recently, Buchholtz *et al* (24) observed a low expression of LDOC-1 in ovarian cancer cells. This does not exclude the fact that, in certain tumors, there may be a higher expression of the gene LDOC-1, where the mechanism of pro-apoptotic activity may be triggered as a last resort to block the tumor progression. Furthermore, it cannot be excluded that, in PCa, there may be less mechanisms of methylation, thus leading to gene overexpression for various reasons that remain to be clarified.

In conclusion, the present results could be useful for better understanding the potential role LDOC-1 in the search of novel anticancer mechanisms in PCa.

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