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Differential expression of Low density lipoprotein Receptor–related Protein 1 (LRP-1) and matrix metalloproteinase-9 (MMP-9) in prostate gland: From normal to malignant lesions

Mónica B. Gilardoni[a,*], María M. Remedi[a], Mabel Oviedo[b], Tristán Dellavedova[c], Juan P. Sarría[c], Laura Racca[c], Mariana Dominguez[c], Claudia G. Pellizas[a], Ana C. Donadio[a]

[a] Centro de Investigaciones en Bioquímica Clínica e Inmunología, CONICET, Departamento de Bioquímica Clínica, FCQ-UNC, Córdoba, Argentina
[b] Histología y Patología, Facultad de Ciencias Médicas, UNC, Córdoba, Argentina
[c] Fundación Urológica Córdoba para la Docencia e Investigación Médica, FUCDIM, Córdoba, Argentina

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ABSTRACT

Background: Metalloproteinases (MMPs) are relevant modulators of inflammation, tumor microenvironment, cancer invasion and metastasis. They can be regulated by the Low density lipoprotein Receptor–related Protein 1 (LRP-1), a receptor reported to mediate the clearance of lipoproteins, extracellular matrix (ECM) macromolecules and proteinases. The aim of this study was to evaluate the expression of LRP-1, MMP-2 and MMP-9 across various grades of prostatic diseases as benign prostatic hyperplasia (BPH), BPH plus prostatitis (BPH + P), high grade prostatic intraepithelial neoplasia (HGPIN) and prostate cancer (PCa).
Methods: LRP-1 was analyzed using immunohistochemistry and MMPs proteolytic activity by zymography in prostate tissues with different prostatic diseases.
Results: LRP-1 was detected in epithelial cells in BPH (16/18), BPH + P (21/21) and HGPIN (6/6), with a staining intensity of 1+, 1+/2+ and 3+, respectively. In PCa, LRP-1 was absent in 19/27 samples while a low expression was observed in 8/27 biopsies. MMP-9 activity was higher and statistically significant in PCa than in BPH ($p \leq 0.01$).
Conclusion: Considering that LRP-1, by mediating the clearance of MMPs, is involved in the regulation of ECM remodeling and cell migration, we conclude that a decreased expression of LRP-1 could be involved with the increasing activity of MMPs shown in cancers.

1. Introduction

Prostate cancer (PCa) is one of the most prevalent cancers in men being the second most common cause of cancer death worldwide [1]. Critical steps in the development of cancer are regulated by the reciprocal interactions between tumor cells and its microenvironment [2]. Recent studies recognize that the tumor microenvironment plays a large role in the pathogenesis and progression of PCa [3].

Tumor progression and metastases are processes involving cellular disengagement from the local milieu, degradation of surrounding extracellular matrix (ECM), tumor cell intravasation and survival in the circulation, and tumor colonization of specific sites. Extracellular proteases such as the matrix metalloproteinases (MMPs), mediate many actions to facilitate these specific steps participating in ECM turnover, cellular migration, the triggering of signaling pathways and the release of growth factors enhancing tumor growth and aggressiveness [4].

Low density lipoprotein Receptor–related Protein 1 (LRP-1) is a member of the low-density lipoprotein receptor gene family. LRP-1 contains binding sites for proteinases such as tissue plasminogen activator (tPA), urokinase plasminogen activator (uPA) and MMPs, for proteinase inhibitors including plasminogen activator inhibitor type 1 (PAI-1) or, for ligands such as lipoprotein lipase and ECM macromolecules [5]. In addition, LRP-1 is involved in the clearance and cellular uptake of proteinases by endocytosis. Since the expression and activation of proteases like serine proteinases and MMPs mediate many of the changes in the tumor microenvironment, LRP-1 may participate in the tumor microen-

* Corresponding author at: Centro de Investigaciones en Bioquímica Clínica e Inmunología, CONICET, Departamento de Bioquímica Clínica, FCQ-UNC, Córdoba, Argentina.
E-mail address: mgilard@fcq.unc.edu.ar (M.B. Gilardoni).

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Table 1
Baseline characteristics of human prostate diseases analyzed.

Characteristics	BPH	BPH + P	PCa
Number of patients	22	34	27
Age,			
Years (range)	53–79	54–88	56–79
Serum PSA,			
ng/mL, mean ±SD	5.28 ± 4.28 (n = 11)	6.33 ± 2.72 (n = 16)	7.60 ± 1.39* (n = 10)
Type of Surgery			
TURP	22/83	8/83	13/83
NB	0/83	26/83	14/83

BPH, Benign prostatic hyperplasia; P, Prostatitis; PCa, Prostate cancer; TURP, transurethral resection of the prostate; NB, needle biopsy; SD, standard deviation.
* $p \leq 0.05$; PCa *vs* BPH, Kruskal-Wallis test.

Table 2
Characteristics of human prostate tumors.

	n/total, (%)
Gleason Score	
6 or less	17/27 (63.0)
7	7/27 (25.9)
3 + 4	5/7
4 + 3	2/7
8 or more	3/27 (11.1)
Clinical T stage	
T1	11/27 (40.7)
T2	10/27 (37.0)
T3	5/27 (18.5)
T4	1/27 (3.8)

vironment regulation. In this sense, a low LRP-1 expression has been related with the aggressive phenotype of tumor cells derived from human prostate and breast cancer [6]. According with this, we have demonstrated a decreased LRP-1 expression in prostate samples from highly aggressive tumors in an experimental model in rats [7].

MMPs and especially the gelatinases MMP-2 and MMP-9 are associated with the aggressive biological nature and, accordingly, with the ungovernable clinical course in several types of human neoplasias [8]. In PCa, co-expression of MMP-2 and the tissue inhibitor of metalloproteinase-2 (TIMP-2) was associated with advanced tumor stage and reached near-significance as a predictor of disease recurrence [9]. Besides, an increased MMP-9 expression has been identified in PCa samples carrying the gene rearrangement TMPRSS2:ERG, associated with poor prognosis and an increased relative risk for PCa specific death [10].

The aim of this study was to elucidate the expression and eventual relationship and histopathological significance among LRP-1, MMP-2 and MMP-9 in a spectrum of human prostatic diseases.

2. Patients and methods

2.1. Patients, specimen collection and tissue preparation

A cohort of 83 samples from patients with prostatic diseases that underwent transurethral resection of the prostate (TURP) or prostate needle biopsy (NB) were obtained from Laboratorio Privado de Patología and Fundación Urológica Córdoba para la Docencia e Investigación Médica, FUCDIM (Córdoba, Argentina). Informed consent was obtained from all patients and approved by the institutional research ethics committee. About two-thirds of each sample was processed for histopathology, and the remaining tissue was frozen at −70 °C until use. For histological diagnosis, paraffined sections were cut for hematoxylin and eosin (H&E) and immunohistochemistry staining. H&E slides were characterized as benign prostatic hyperplasia (BPH; n = 22), benign prostatic hyperplasia plus prostatitis (BPH + P; n = 34) and PCa (n = 27). Among the 27 tumor samples, an exhaustive observation allowed us to find high grade prostatic intraepithelial neoplasia (HGPIN; n = 6). Serum samples were obtained at the time of prostate biopsies collection for prostate-specific antigen (PSA) determination. The above patients' characteristics are shown in Table 1. PCa were graded according to the Gleason score and were staged using tumor-node-metastasis (TNM) classification system [7th edition Union Internationale Contre le Cancer (UICC) 2009].

2.2. Immunohistochemistry (IHC)

Prostate sections (4–5 μm) were deparaffinised in three consecutive baths of cooled xylene (5 min each), rehydrated in graded alcohols, and placed in 3% hydrogen peroxide in methanol for 10 min to block endogenous peroxidase activity. Following brief proteolytic digestion with 0.05% trypsin-0.1% $CaCl_2$, pH 7.8 at 37 °C, tissue slides were incubated with the mouse anti-human alpha-LRP-1 subunit monoclonal antibody (ab 20384, Abcam Ltd., Cambridge, UK), 2 μg/ml, overnight at 4 °C. The sections were then processed by the streptavidin-biotin-peroxidase complex method by use of the LV LSAB(+) kit (DAKO, Denmark), following manufacturer's instructions, and the color developed with the substrate chromogen DAB (3,3′-diaminebenzidine, Sigma Chemical Co. St. Louis, MO, USA) at room temperature. Finally, sections were lightly counterstained with Harris' hematoxylin and mounted with xylene-based DPX (Fluka) mounting medium. Liver and prostate normal tissues were used as positive and negative controls for LRP-1 respectively. Negative controls on each sample were obtained by omitting the primary antibody.

Immunostaining was assessed semi quantitatively as the percentage of positively stained cells according to the following scale: score 0, absent (no staining present) or <10% of cells with partial membrane staining; score 1+, low intensity or >10% of cells with partial membrane staining; score 2+, moderate intensity and >10% of cells with complete membrane staining; score 3+, strong reaction and >10% of cells with complete membrane and cytoplasm staining. Tumor cells and surrounding stromal cells were assessed separately. The sections were examined, graded and scored by two independent observers, then reviewed together and the average data represent a consensus value of all the observations.

2.3. Gelatin zymography

Prostate specimens (approximately 5 mg), stored at −70 °C, were homogenized for the zymographic studies. The enzymatic activity of MMP-2 and MMP-9 in tissue homogenates of different prostatic disease was assessed by gelatin zymography analysis as previously described [11]. Briefly, tissue samples (40 μg) were analyzed by electrophoresis under no reducing conditions on 7.5% SDS-polyacrylamide gels containing copolymerized gelatin (1.5% w/v; Sigma). After electrophoresis (125 V/1.5 h), the gels were rinsed twice with 2.5% v/v Triton X-100 (Sigma), 45 min each, at room temperature, to remove SDS. After rinsing once in substrate buffer [50 mM Tris – HCl (pH 7.4), 200 mM NaCl, 9 mM $CaCl_2$], gels were incubated at 37 °C for 40 h in the same buffer. The gels were stained for 45 min in 45% v/v methanol-5% v/v glacial acetic acid containing 0.125% w/v Coomasie Brilliant Blue R250 (Sigma) and distained in 25% v/v ethanol–10% v/v glacial acetic acid. MMPs activities were detected as clear bands on the blue background of the stained gel. A capillary blood sample was used as positive control. Prestained SDS-PAGE Standard Molecular Weight (Bio-Rad, Bio-Rad Laboratories Inc., Munich, Germany) was used to determine the molecular weight of the bands. The GELPRO 3.1 software

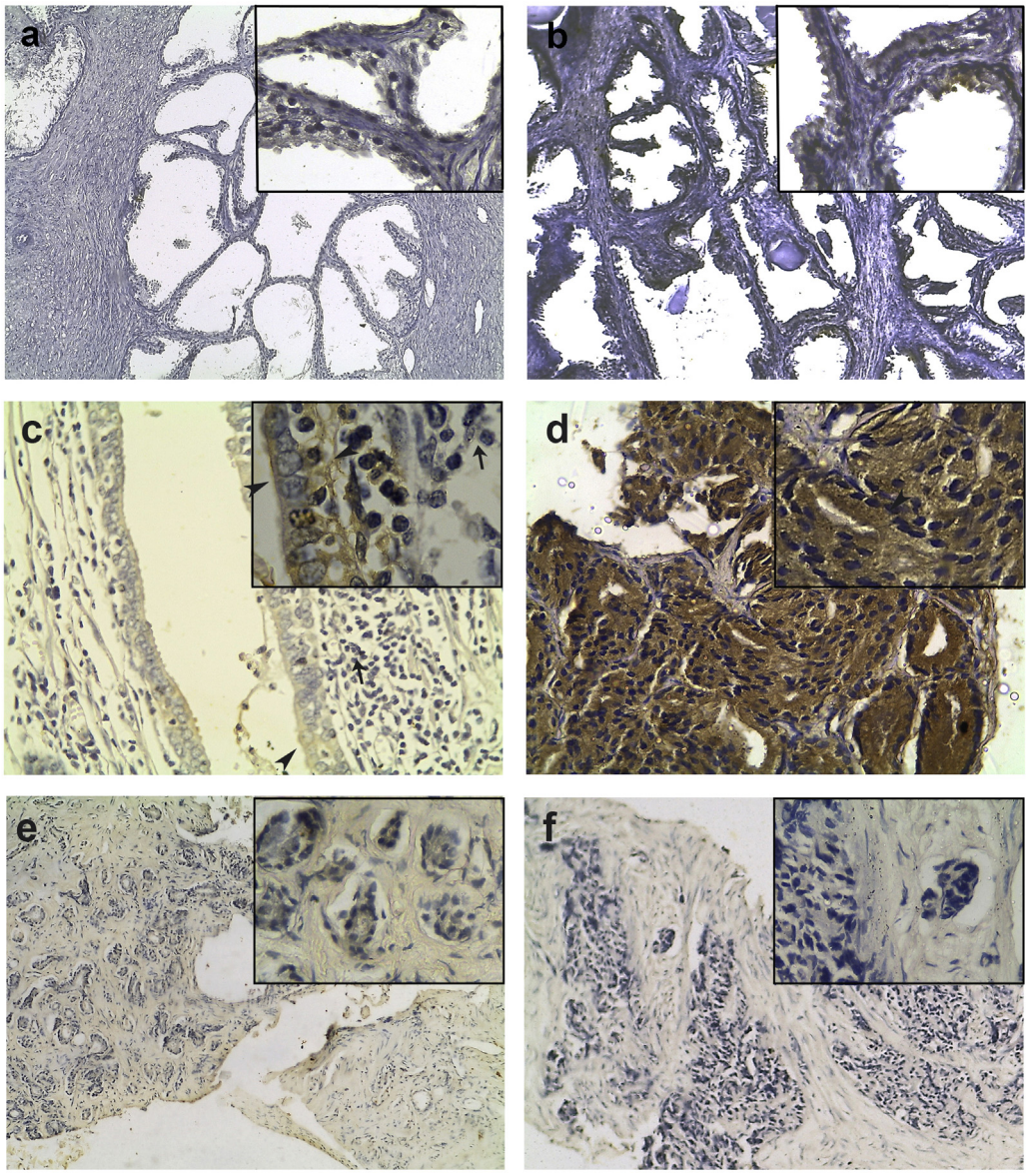

Fig. 1. Immunohistochemical staining of LRP-1 in normal prostate, BPH, BPH + P, HGPIN and PCa. (a) Normal prostatic tissue: Absence of LRP-1 staining (×100; insert: ×400); (b) BPH: Low membranous LRP-1 expression (1+) in epithelial cells (×100; insert: ×400); (c) BPH + P: Moderate membranous LRP-1 immunostaining (2+) in apical, lateral and basal membranes (►) with mixed inflammatory infiltrate of neutrophils and mononuclear cells (→)(×400; insert: ×1000); (d) HGPIN: High immunostaining (3+) in cell membranes and cytoplasm (►) (×400; insert: X600); (e) Well differentiated PCa (Gleason 6- T2): Low LRP-1 immunostaining (1+) in tumoral epithelial cells (×100; insert: ×400); (f) Undifferentiated PCa (Gleason 8- T4): Absence of LRP-1 staining (×100; insert: ×400).

(Media Cybernetics Inc., Rockville, MD, USA) was used for the analysis of the bands.

2.4. Statistical analysis

Differences between means were determined by the Kruskal-Wallis test and Dunn's Multiple comparisons test. P value ≤ 0.05 were considered statistically significant.

3. Results

3.1. Histopathological characterization and PSA values in the studied population

The baseline characteristics of the 83 cases analyzed are shown in Table 1. PSA values were significantly higher in PCa than BPH patients. No differences could be observed between PCa and BPH + P patients, although a trend to reach higher values in PCa samples was observed. PCa were graded according to the Gleason score of 6 or less, 7 and 8 or more and were staged using TNM classification system (Table 2).

3.2. Immunohistochemical expression of LRP-1

LRP-1 immunostaining was evaluated in BPH (n = 18), BPH + P (n = 21), HGPIN (n = 6) and PCa (n = 27) to delineate its expression profile. LRP-1 was not expressed in normal prostate glandular cells (Fig. 1a), although was positive in 16 out of 18 BPH samples (1+) (Fig. 1b). LRP-1 was upregulated in BPH + P samples, with a moderate (2+) immunoreactivity in 11 out of 21 samples (Fig. 1c) and a low signal (1+) in the remaining cases (n = 10). LRP-1 immunostaining was exclusively membranous in BPH samples and membranous and cytoplasmic with high scores (3+) in HGPIN lesions (n = 6) (Fig. 1d). Eight of twenty-seven Gleason 6 or less score PCa cases

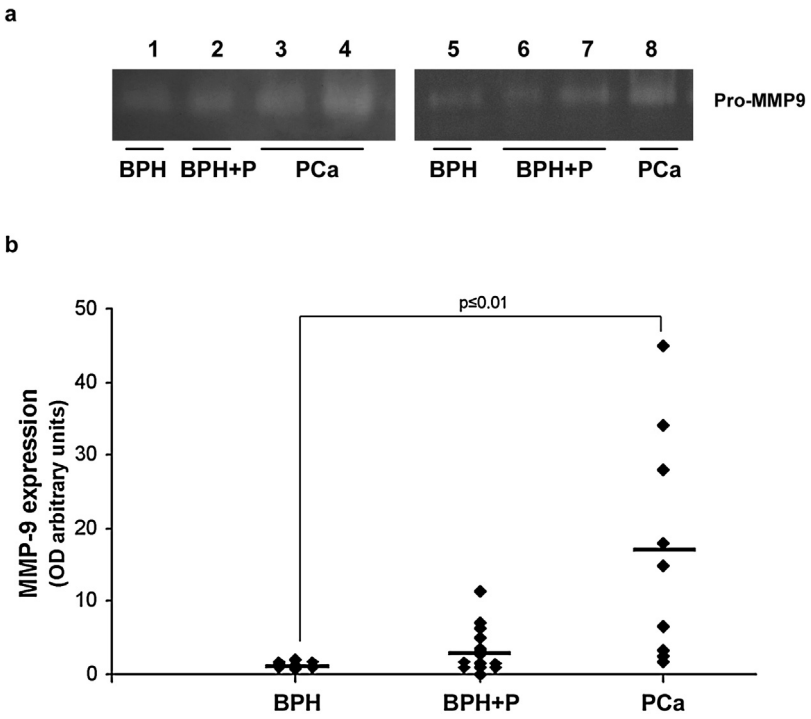

Fig. 2. MMP-9 activity in prostate samples. (a) Representative zymograms showing MMP-9 gelatinolytic activity in prostate samples with BPH, BPH + P and PCa. Areas of protease activity are indicated by clear bands in the gel for proMMP-9. (b) Data from densitometric analysis of proMMP-9 in BPH, BPH + P and PCa are depicted. The filled line in the graphic indicates the mean of each group of data. A significant difference in proMMP-9 activity between PCa and BPH samples was observed ($p \leq 0.01$; Dunn's Multiple comparisons test).

Table 3
Histopathologic patterns and LRP-1 staining grade association.

	Absent	Low	Moderate	High
BPH (n = 18)	2/18	16/18	0/18	0/18
BPH + P (n = 21)	0/21	10/21	11/21	0/21
HGPIN (n = 6)	0/6	0/6	0/6	6/6
PCa (n = 27)	9/27[a]; 10/27[b,c]	8/27[a]	0/27	0/27

BPH, Benign prostatic hyperplasia; P, Prostatitis; HGPIN, high grade prostatic intraepithelial neoplasia; PCa, Prostate cancer.
Ratio of patients with score indicated vs total number of patients studied.
[a] Gleason 6 or less.
[b] Gleason 7.
[c] Gleason 8 or more.

had 1+ immunoreactivity. Conversely, the expression was absent in nineteen Gleason 7 and 8 or more PCa cases. Neoplastic glands corresponding to T2 and T4 TNM scale were shown (Fig. 1e and f respectively). LRP-1 was unreactive in all over the stromal tumoral tissue. The association of the histopathological features and LRP-1 staining grade is shown in Table 3.

3.3. MMP-2 and MMP-9 activities in prostatic tissues

A higher and significant proMMP-9 expression was observed in samples of PCa (n = 9) patients in relation to BPH (n = 9) samples ($p \leq 0.01$; Dunn's Multiple comparisons test). No significant differences were detected between PCa and BPH + P (n = 13) biopsies (Fig. 2). Related with MMP-2 expression, a slight proMMP-2 activity was detected in few random cases. In turn, MMP-2 active protein was only detected in tumor samples (data not shown).

4. Discussion

MMPs play a critical role in the classic hallmarks of cancer [12]. They may originate from malignant cells as well as from their surrounding stroma and the autocrine and paracrine cross-talk between tumor and stromal cells may influence their expression.

It is known that MMPs mediate many of the changes in the microenvironment during tumor progression [4]. *Via* clearing of MMPs and other proteins like serine proteinases and proteinase-inhibitor complexes, LRP-1 contributes to the homeostasis of secreted proteins and the integrity of the ECM. In addition, LRP-1 is involved in regulating the abundance of many other proteins and receptors associated to the plasma membrane as well as in multiple signaling pathways that regulate migration, invasion, proliferation and cell survival [13,14]. Considering that LRP-1 modulates cell behavior, either as a cargo protein that mediates endocytosis of a wide range of ligands or as a signal transducer, we analyzed the presence of this protease clearance factor in the studied prostate pathologies.

Our findings remarkably showed a loss of LRP-1 expression in PCa, results that were previously described in an experimental prostate tumor model [7]. Although several studies have implicated LRP-1 in tumorigenesis, its precise role and potential underlying mechanisms remain controversial. For example, related with LRP-1 performance as a clearance receptor, the invalidation of LRP-1 expression in human thyroid carcinoma cells resulted in an increased level of MMP-2 and uPA in the extracellular compartment, though an inhibition in cell migration [15]. In turn, other authors reported an inverse relationship between LRP-1 expression and prostate and breast cancer cell invasion *in vitro* [6], results that are also consistent with those of Webb and coworkers [16] who observed that LRP-1 inhibits HT 1080 cell migration and invasion by suppressing cell signaling downstream of uPA receptor. Likewise, Amos et al. [17] reported that reduced LRP-1 level in glioblastoma cells led to increased uPA secretion, events that worked in concert to drive tumor invasion *in vitro* and in a xenograft glioblastoma mouse model. In the same way, in hepatocellular carcinoma (HCC) cells, the inhibition of LRP-1 coupled to an increased expression and activity of MMP-9 enhanced tumor cell migration and inva-

sion *in vitro* and *in vivo*. Furthermore, in HCC patients a low level of LRP-1 in tumor samples predicted an unfavorable prognosis, while low-LRP-1/high-MMP-9 patients had the worst prognosis [18]. Nevertheless, the activity of LRP-1 as a regulator of cancer progression *in vivo* remains incompletely understood. For instance, the xenotransplantation of LRP-1 silenced CL16 cells resulted in tumor development and tumor cell dissemination to the lungs although the metastases failed to enlarge [19].

In addition of LRP-1 analysis, we also studied MMP-2 and MMP-9 expression. MMPs deregulation is an important pathogenic factor in a wide range of pathologies, including chronic inflammatory diseases and cancer. In the present work, we described a high and significant expression of proMMP-9 in PCa samples. Accordingly, similar findings have also been reported [20] as well as a significant increase in plasma MMP-9 level and activity in patients with PCa, associated with clinical variables like PSA and Gleason score [21]. In turn, MMP-9 was found overexpressed in prostate tumor samples compared with corresponding non tumor tissues, while their negative regulators were under expressed relating these events with tumor prognosis [22]. Accordingly, Tian et al. [10] also described an increased MMP-9 expression in PCa samples associated with poor prognosis and increased risk for PCa related death. Interestingly, inflammatory cells could also participate as relevant sources of MMPs [23,24]. This could contribute to explain the trend to higher MMP-9 values described in BPH + P compared with BPH samples.

In reference to our results, it is known that LRP-1 binds and internalizes MMP-9 [25]. However, the anticancer effect of LRP-1 related to its ability of endocytosing metalloproteinases may be counterbalanced by its capability to endocytose their inhibitors or by its protumor signaling properties. LRP-1 down-regulation was described in aggressive malignant cells [14]. To add more difficulty, LRP-1 expression may itself be controlled by metalloproteinases, which shed LRP-1 ectodomain (ECD) [26]. Similarly, the LRP-1 proteolysis mediated by membrane-type 1 matrix metalloproteinase (MT1-MMP) has been described as a mechanism to balance the functional activity of LRP-1 [27]. Interestingly, MT1-MMP is associated with advanced PCa and, more recently, implicated in the oxidative stress and induction of a more invasive phenotype in prostate cancer cells [28]. On the other hand, LRP-1 shedding, that results from the proteolytic cleavage of the LRP-1 ectodomain in the extracellular portion of the alpha-chain, could also have important implications by impairing clearance of proteinase activities [29]. Likewise, LRP-1 shedding from human lung fibroblasts impairs endocytosis of MMP-2 and −9 [30]. Moreover, the inhibition of LRP-1 shedding decreased MMP-2 and −9 activities in cultures of human endometrial explants [31] and fibrosarcoma cells [32]. Paradoxically, LRP-1 acting as a membrane receptor that transduces intracellular signals induces MMP-9 expression [33–35]. The dual nature of LRP-1, both scavenger and signaling receptor, and the variable functions of its numerous ligands adds to the complexity of deciphering the role of LRP-1 in biological processes. In our work, the overexpression of LRP-1 in HGPIN and a decreased expression in PCa, contrasting with an increased MMP-9 level in malignant lesions could be related with signaling events and matrix proteolysis related with PCa progression.

5. Conclusion

We demonstrated that LRP-1 is absent in normal prostate and its expression is increased in non- malignant and premalignant lesions. In PCa the expression of LRP-1 is almost undetectable whereas MMP-9 shows a significantly higher level of activity. These findings could supply an eventual role for LRP-1 in extracellular matrix proteolytic enzymes recycling, which might be related with prostate cancer progression.

Conflicts of interest

The authors declare no conflicts of interest.

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Dr. Claudia G. Pellizas and Dr. Ana C. Donadio are established researchers at CONICET.

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