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CHANGES IN PROLACTIN RECEPTOR LOCATION IN PROSTATE TUMORS

Gastón López Fontana[1,2], Lara Rey[3], Flavia Santiano[3], Rodrigo López Fontana[2], José Daniel López Laur[2], Leila Zyla[3], Paula Valdemoros[4], Martín E. Guerrero-Giménez[3], Juan Manuel Fernández-Muñoz[3], Silvina Gómez[3], Flavia Bruna[3], Juan Manuel Guglielmi[2], Rubén Carón[3] and Constanza López Fontana[3].

[1]*Facultad de Ciencias Médicas. Universidad Nacional de Cuyo. Mendoza. Argentina.*
[2]*Clínica Andina de Urología. Mendoza. Argentina.*
[3]*Instituto de Medicina y Biología Experimental de Cuyo (IMBECU). Centro Científico y Tecnológico Mendoza. Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET). Mendoza. Argentina.*
[4]*Laboratorio de Anatomía Patológica Vega Valdemoros. Mendoza. Argentina.*

Summary.- *INTRODUCTION: Prolactin (PRL) binds its receptor (PRLR) and stimulates cell proliferation, differentiation and survival in prostate cancer (PCa) cell lines via STAT5a, MAPK and AKT.*

OBJECTIVE: To evaluate the expression of PRL and PRLR in normal and tumor prostate tissues with different Gleason patterns.

METHODS: Samples of normal, benign prostatic hyperplasia and PCa with different Gleason patterns were selected from radical prostatectomy. The intensity, location and percentage of stained cells for PRL and PRLR were evaluated by Immunohistochemistry. Co-localization was observed by confocal microscopy.

RESULTS: PRL was expressed diffusely and with a mild intensity in the cytoplasm of normal and tumor prostate luminal cells. Its expression only augmented in the Gleason 3 pattern (p 0.0001). The immunostaining intensity and the percentage of positive cells for PRLR did not vary between normal and tumor tissues. However, the location of the PRLR was modified by the tumorigenic process. In non-tumor tissues, PRLR expression was mostly in plasma membrane in the apical zone of epithelial cells. In tumor tissues, it was expressed in intracellular vesicles. The co-localization of PRL and PRLR was demonstrated in normal and tumor tissues suggesting that PRL could be acting in an autocrine and paracrine manner.

CONCLUSION: PRL and its receptor were present in the cytoplasm of the epithelial cells of the normal and tumor prostate gland. In tumor tissues, the change in the location and appearance of cryptic PRLRs that store PRL may keep active the different signaling pathways related to cell proliferation and survival.

Keywords: *Prolactin. Prostate cancer. Prolactin receptor.*

CORRESPONDENCE

Constanza López Fontana
Adrian Ruiz Leal s/n
Parque General San Martin
C.P. 5500 Mendoza (Argentina)

clopezfontana@mendoza-conicet.gob.ar

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Resumen.- *INTRODUCCIÓN: La prolactina (PRL) se une a su receptor (PRLR) y estimula la proliferación celular, la diferenciación y la supervivencia de la líneas celulares de cáncer de próstata vía STAT5a, MAPK y AKT.*

OBJETIVO: Evaluar la expresión de la PRL y PRLR en tejido normal y tejido de cáncer de próstata con varios patrones de Gleason.

MÉTODOS: Se seleccionaron muestras de tejido benigno, hiperplasia y cáncer de próstata con diferentes patrones de Gleason de prostatectomías radicales. La intensidad, localización y porcentaje de células teñidas por PRL y PRLR fueron evaluadas por immunohistoquimica. La co-localización se observó con microscopio confocal.

RESULTADOS: PRL se presentó de forma difusa y con intensidad media en el citoplasma de células luminales normales y de tumor prostático. La expresión solamente aumentó en patrón Gleason 3 (p<0,0001). La intensidad de la tinción immunohistoquímica y el porcentaje de células positivas para PRLR no varió entre células normales y tejidos tumorales. Pero, la localización del PRLR fue modificada por el proceso generador del tumor. En tejidos no-tumorales, la expresión de PRLR fue sobre todo en la membrana plasmática en la zona apical de las células epiteliales. En tejidos tumorales, se presentó en las vesículas intracelulares. La co-localizacion de la PRL y PRLR se demostró en tejido normal y tumoral sugeriendo que la PRL funciona con un efecto autocrino y paracrino.

CONCLUSIÓN: La PRL y su receptor estuvieron presentes en el citoplasma de células epiteliales de tejido normal y glándula prostática tumoral. En tejidos tumorales, el cambio de localización y la apariencia cripticas del PRLR que guarda la PRL debe mantener activos los diferentes caminos de señalización relacionados con la proliferación celular y la supervivencia.

Palabras clave: *Prolactina. Cáncer de próstata. Receptor de prolactina.*

INTRODUCTION

Prostate development and carcinogenesis are usually characterized by their hormonal control. Prolactin (PRL) is one of the hormones that has been implicated in prostate cancer (PCa) (1). In vitro studies have shown that PRL produces a proliferative effect on PCa cell lines -DU 145- (2). In addition, PRL inhibits apoptosis induced by the androgen deprivation treatment (3) and promotes the proliferation of normal and malignant prostatic epithelial cells (2,4). Meanwhile, animal studies have shown that PRL is capable of producing lobe-specific differential effects in the prostate. The induction of moderate hyperprolactinemia produces a marked hypertrophy in the ventral lobe and hyperplasia in the dorsal (5). Additionally, local PRL regulates the homeostasis of the stem/progenitor cells that participate in the initiation and/or recurrence of PCa in mice (6). Also, PRL antagonist inhibits the initiation and growth of xenograft tumors (7). Moreover, PRL added exogenously to explant cultures of normal and malignant prostatic tissues stimulates proliferation and inhibits apoptosis of prostate epithelial cells (3). However, epidemiological studies have shown that there is no association between circulating levels of PRL and the risk of Benign Prostatic Hyperplasia (BPH) (8) or PCa in humans (9). In a prospective and case-control study, patients with and without PCa presented the same serum levels of PRL (9) suggesting that if PRL plays a role in prostate carcinogenesis, it may act through an autocrine and/or paracrine via.

The actions of PRL are mediated by a unique transmembrane receptor (PRLR) (10). Once PRL is assembled to its receptor, a complex cascade of Jak2 tyrosine kinase-dependent phosphorylation is triggered, receptor activation occurs and, subsequently, a large variety of intracellular proteins are activated and translocated to the nucleus to perform PRL effects. There are three intracellular signaling pathways that are preferably used by this hormone and are related to differentiation, proliferation and survival processes in different tissues (11). One of these pathways is STAT5 (Signal Transducer and Activator of Transcription 5), a latent intracytoplasmic protein important for the PCa cell survival (12). It also triggers the synthesis of proteins with proliferative and antiapoptotic functions such as cyclin D1 and Bcl-XL (13,14). Another signaling pathway that is activated by PRL is the pathway of MAP Kinases (Mitogen Activated Protein Kinases) which inhibits apoptosis and induces cell proliferation participating in the metastatic process (15). Finally, the activation of the AKT (serine protein/threonine kinases) pathway by PRL has an anti-apoptotic effect promoting cell survival (16).

Since the epidemiologic and in vitro data have not provided conclusive evidence of the involvement of PRL in prostate carcinogenesis, the aim of the present study was to evaluate the expression of PRL and its receptor in normal and tumor prostate tissue with different Gleason patterns.

MATERIAL AND METHODS

In silico study

Data was programmatically extracted from the publicly available data set of prostate adenocarcinoma from The Cancer Genome Atlas Project, downloaded on May 01, 2017 using the recount2 platform (https://jhubiostatistics.shinyapps.io/recount/). Clinical data corresponding to sample patients were obtained from cBioPortal (http://www.cbioportal.org/study?id=prad_tcga_pub#clinical). Non-standardized RNASeq gene expression levels from 558

samples were downloaded. From this data 505 were tumor samples, 52 were healthy adjacent tissue and 1 sample was from a nodal metastasis. A subset of 333 tumor samples which had clinical data was used in this study. RNA expression levels were evaluated for 2 genes (PRL and PRLR), crude counts were scaled by the total coverage of the sample (area under the curve, 'AUC', of the coverage) and differential gene expression analysis (DGE) was performed using the generalized linear model method of the EdgeR R package comparing tumor vs. non-tumor samples. Log2 Fold change values were obtained associated with exact p-values and False Discovery Rate values (FDR).

Patients

This study involved 132 patients from the Province of Mendoza, Argentina. The patients recruited during the period of 2012 and 2014 were healthy volunteers (n=28), with BPH (n=23) and with PCa diagnosis with different Gleason patterns: Gleason 3 (n=23), Gleason 4 (n=32) and Gleason 5 (n=26). All patients were free of previous PCa treatments, presented operable primary tumors and were assessed to be metastasis free at the time of diagnosis. All of them signed an informed consent previously approved by the Ethic Committee of the Universidad Nacional de Cuyo in accordance with the precepts established by the Helsinki Declaration.

Tumors histology

After surgery (radical prostatectomy, adenomectomy or transurethral resection of the prostate), a piece of normal, BHP or tumor prostate tissues was processed for histopathologic studies by fixing in buffered phormol, dehydrated in ethanol and embedded in paraffin wax. Sections of 3–5 µm thickness were cut with a Hyrax M 25 microtome and stained with hematoxylin–eosin (H&E) to classify tumors according to the Gleason pattern (17).

Immunohistochemistry (IHC)

An antigen retrieval protocol using heat was used to unmask the antigens (30 min in citrate buffer 0.01 M, pH 6.0, for PRLR and 0.5 M buffer EDTA, pH 8.0, for PRL). Tissue sections were incubated with the primary antibodies against PRL (ab11301, 1:500 dilution, Abcam plc, MA, USA) and PRLR (ab170935, 1:250 dilution, Abcam plc) overnight at 4°C in humidity chambers. A commercial kit to detect mouse and rabbit antibodies was used (Dako, Carpinteria, CA, USA). Slides were lightly counterstained with hematoxylin to reveal nuclei, examined and photo-

A

	Overall (n=333)
Age (years)	
Mean (SD)	60.6 (6.97)
Median [Min, Max]	61.0 [43.0, 76.0]
Missing	54 (16.2%)
Pre-operative PSA	
Mean (SD)	11.0 (11.2)
Median [Min, Max]	7.40 [1.60, 87.0]
Missing	146 (43.8%)
Gleason Score	
3+3	65 (19.5%)
3+4	102 (30.6%)
4+3	78 (23.4%)
>=8	88 (26.4%)
SUBTYPE	
ERG	152 (45.6%)
ETV1	28 (8.4%)
ETV4	14 (4.2%)
FLI1	4 (1.2%)
SPOP	37 (11.1%)
FOXA1	9 (2.7%)
IDH1	3 (0.9%)
other	86 (25.8%)
RACE	
Caucasian	270 (81.1%)
African descent	43 (12.9%)
Asian	8 (2.4%)
Not Available	12 (3.6%)

B

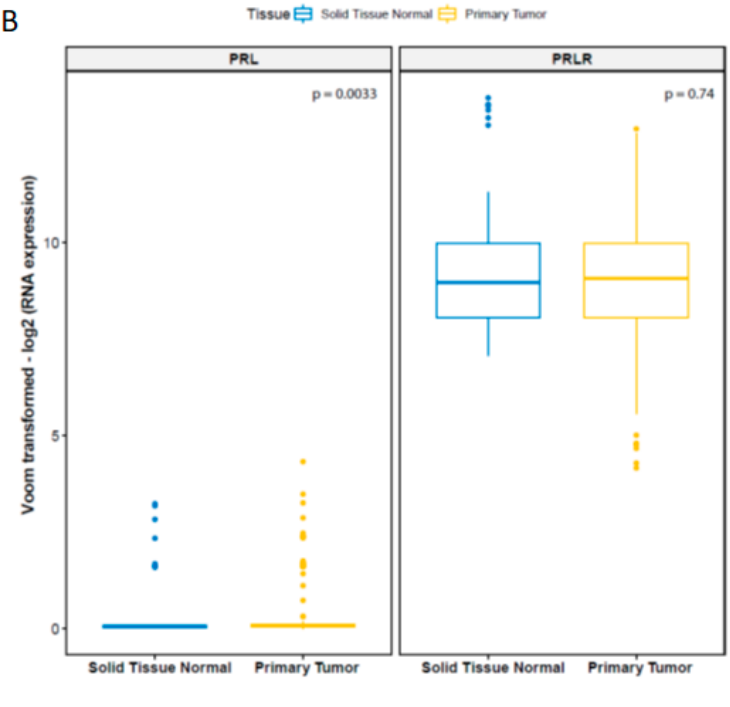

Figure 1. (A) General characteristics of the population selected for the in silico study. (B) Expression of mRNA from PRL (left) and PRLR (right) in normal and tumor tissue.

graphed. The immunostaining was evaluated in the whole sections considering the extent, intensity and localization of immunostaining independently by two experienced researchers blinded regarding the Gleason pattern and a few conflicting scores were resolved by consensus. The immunostaining of PRL and PRLR in the normal or tumor cells was semi-quantitively scored using an intensity score: 0 = no staining, 1 = weak staining, 2 = moderate staining, 3 = strong staining; and a proportion score: 0 = no staining; 1 = staining less than 10% of the cells, 2 = between 11 and 33%, 3 = between 34% and 65%, 4= between 66% and 84%, 5 = greater than 85%. The percentage of positive cells for PRL and PRLR was obtained based on an average of 700 cells counted per sample, at 400x magnification using Image J Software (http://imagej.net/) and Qupath (https://qupath.github.io/).

Immunofluorescence staining for confocal microscopy

Normal, BHP or tumor prostate tissue sections were dewaxed, antigen retrieved at pH6 and incubated with primary antibodies against PRL (ab11301, 1:500 dilution, Abcam plc, MA, USA) and PRLR (ab170935, 1:100 dilution, Abcam plc) overnight at 4°C and stained with Alexa Fluor® 488 goat anti-mouse IgG and 555 goat anti-rabbit IgG (1:400, Invitrogen Thermo Fisher Scientific) for 2h at room temperature. Slides were counterstained with DAPI (4´,6-Diamidine-2´-phenylindole dihydrochloride, 10236276001, Sigma), cover-slipped and visualized using confocal microscopy.

Statistical Analysis

Values are given as means ± SEM of 23-32 patients per group. All statistical analyses were performed using GraphPad Prism 5.01 software (GraphPad Software Inc., CA, USA). Differences in the distribution of the variables were assessed using one-way analysis of variance (ANOVA I) or the Kruskal Wallis test depending on the normality of the variables as evaluated by the Kolmogorov-Smirnov test. Post hoc comparisons between means were conducted by Bonferroni´s test or Dunn´s multiple comparison test. Co-localization of PRL and PRLP was analyzed by Pearson Correlation Coefficient. Differences were considered significant if the probability was 5% or less.

RESULTS

In silico analysis of PRL and PRLR expression in normal and malignant prostate tissues

The general characteristics of the population selected for the in silico study are shown in Figure 1 A. The expression of PRL mRNA was very low in

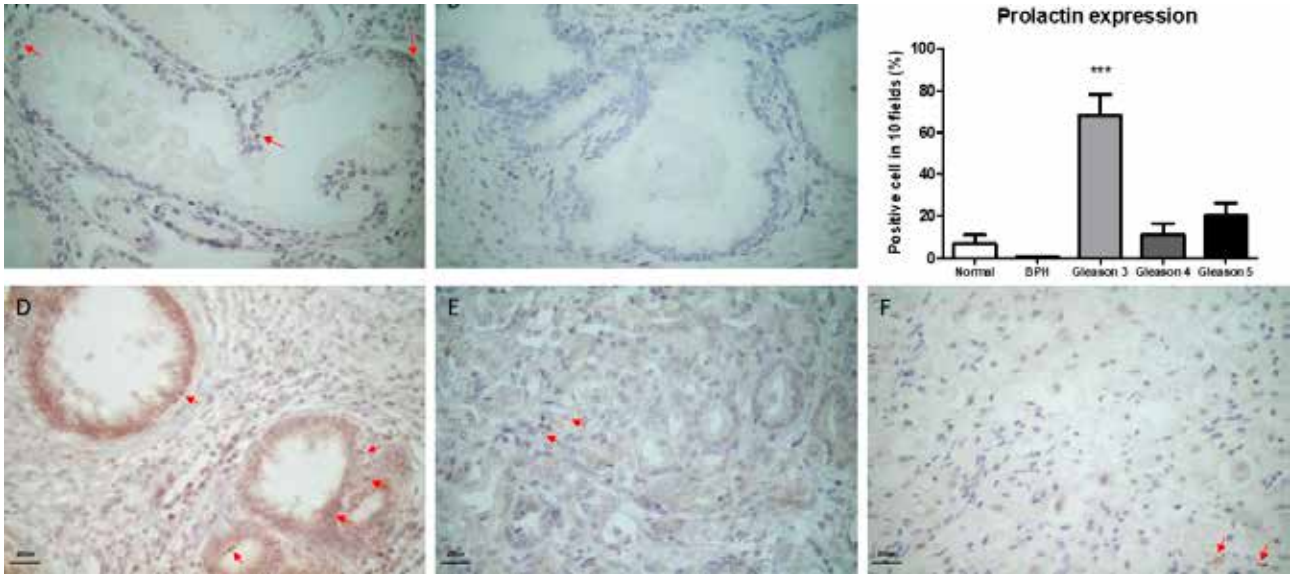

*Figure 2. Representative microphotographs of PRL immunostaining in normal tissue (A); BHP (B); Gleason pattern 3 (D); Gleason pattern 4 (E) and Gleason pattern 5 (F). Red arrows indicate representative cytoplasmic vesicles of PRL. For comparative purposes, all photographs are shown with the same magnification (40x); bar = 200px. PRL was diffusely expressed and in the form of vesicles with a mild intensity in the cytoplasm of normal and tumor prostate luminal cells. Only in some cells the presence of vesicles in cytoplasm was observed. Expression only increased in intensity and proportion in the Gleason 3 pattern. (C) The Gleason 3 showed a larger percentage of PRL positive cells compared with the other prostate tissues. Values represent mean + S.E.M. of 8-10 fields of each preparation. Comparisons were performed by ANOVA I and Bonferroni's Multiple Comparison Test as post hoc. *** p 0.0001*

normal and tumor tissue. On one hand, there was a significant difference in the expression of PRL in tumor vs non-tumor (p=0.003). Nevertheless, the majority of the patients did not express the PRL mRNA and the difference was biased by the presence of outliers. On the other hand, no significant differences were observed in the expression of PRLR mRNA between normal and tumor tissues (Figure 1 B).

PRL in normal and tumor prostate tissue

PRL immunostaining was poor in normal tissue where it appeared as vesicles in the cytoplasm (Figure 2 A). In BPH, PRL expression was almost non-existent in the luminal cells (Figure 2 B). In Gleason pattern 3, PRL expression was diffuse in the cytoplasm and some vesicles were observed in intracytoplamastic locations (Figures 2 D). In the Gleason 4 and 5 patterns, the cytoplasmic distribution of the hormone decreased significantly compared to Gleason 3 and only a few cytoplasmic vesicles were observed (Figures 2 E and F). In the stroma, PRL was expressed in a mild and diffuse form in normal and tumor tissues. However, its expression was more intense and abundant in the Gleason 3 (Figure 2 D). The Gleason 3 showed a higher percentage of PRL positive cells (Figure 2 C) than the other tissues (p 0.0001).

PRLR in normal and tumor prostate tissue

The expression of PRLR in normal tissue was diffuse and intense in cytoplasm, predominantly in the luminal cells, where it also presented vesicles in the apical plasma membrane of these cells (Figure 3 A). The PRLR staining in BPH was less intense than in normal tissue but its expression was also as vesicles in the plasma membrane (Figure 3 B). In the Gleason 3 pattern, a mild and diffuse immunostaining was observed mostly in cytoplasm although in some cells the expression was more intense and intracellular (Figure 3 D). In the Gleason patterns 4 and 5, the expression of PRLR was mainly as intracellular vesicles with moderate intensity (Figure 3 E and F). In all samples, PRLR was expressed slightly in the prostate stroma. Finally, no significant differences were observed in the percentage of PRLR positive cells (Figure 3 C).

Although the immunostaining intensity for PRLR and the percentage of cells positive for PRLR did not vary between normal, BPH and tumor prostate tissues; the location of the receptor was modified with the tumorigenic process. In non-tumor tissues, its expression was mostly in plasma membrane with the presence of vesicles in the apical area of epithelial cells. In tumor tissues, the expression of the receptor

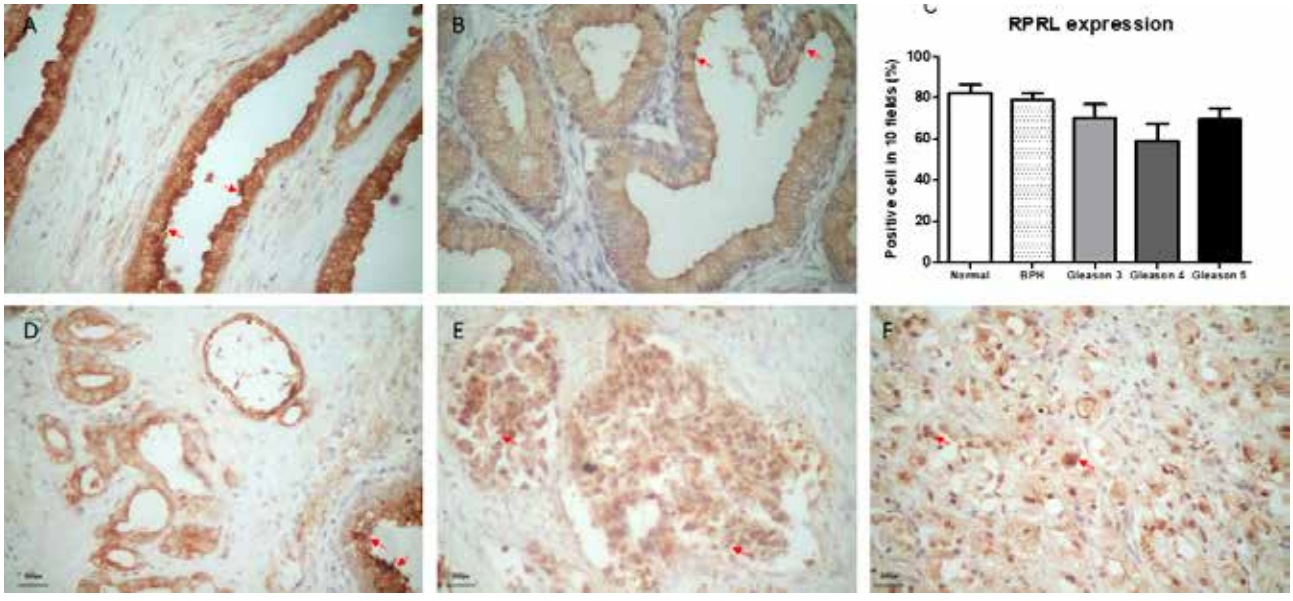

Figure 3. Representative microphotographs of PRLR immunostaining in normal tissue (A); BHP (B); Gleason pattern 3 (D); Gleason pattern 4 (E) and Gleason pattern 5 (F). Red arrows indicate illustrative vesicles of PRLR. For comparative purposes, all photographs are shown with the same magnification (40x); bar = 200px. PRLR was present as vesicles in the plasma membrane of epithelial cells in non-tumor tissues while it was internalized in tumor tissues. (C) The percentage of PRLR positive cells was similar in all prostate tissues. Values represent mean + S.E.M. of 8-10 fields of each preparation. Comparisons were performed by Kruskal Wallis and Dunn's Multiple Comparison Test as post hoc.

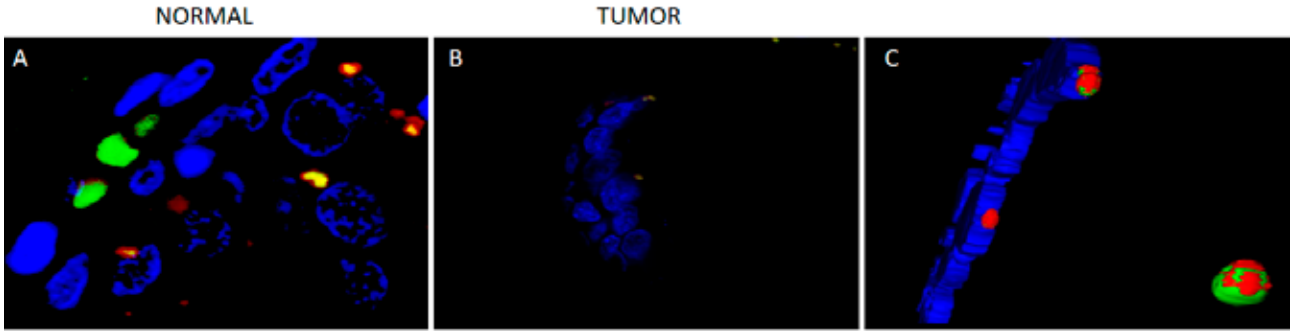

Figure 4. Representative images of PRL (red) and PRLR (green) expression in normal (A) and tumor (B) prostate tissue. Co-localization of the hormone and its receptor (yellow) can be observed in both tissues (C) 3D illustration of the binding of PRL to its receptor indicating co-localization.

was intracellular, accentuating its internalization with the aggressiveness of the Gleason pattern.

Co-location of the PRL and its receptor in prostate cells

Figures 4 shows the expression of PRL and PRLR in normal and tumor tissue determined by immunofluorescence and observed by confocal microscope. The tissues were scanned and, in some areas, color overlap was observed which could indicate co-localization of PRL and its receptor (Figure 4; r=0.99).

DISCUSSION

Since the locations and functions of PRL are increasingly wide opening an extensive field of research, and there is not a very complete understanding of PRL-mediated effects in prostate tumorigenesis, we evaluated the expression of PRL and its receptor in normal and tumor prostate tissue with different Gleason patterns.

First, we performed an *in silico* analysis of the gene expression of PRL and PRLR in normal and tumor prostate tissue, and we observed a poor expression of PRL in normal and tumor tissues, while its receptor was abundant in both tissues. Subsequently, we evaluate the expression of the hormone and its receptor in normal and prostate tumor tissues by IHC. Our results indicated that PRL was expressed diffusely and with a mild intensity in the cytoplasm of normal and tumor prostate luminal cells. The expression only increased in intensity and proportion in the Gleason 3 pattern. On the other hand, we observed a tendency to a greater expression of PRLR in normal tissues and with BPH than in tumors. Accordingly, Leav et al. (18) demonstrated that PRLR was strongly expressed in BPH and prostatic intraepithelial neoplasia (PIN) decreasing as the tumor was more undifferentiated, suggesting that PRL would act in early stages of carcinogenesis.

Interestingly, in our study, although the expression of PRLR did not vary significantly in quantity or intensity between normal and tumor tissues, the location of the receptor was modified with the neoplastic transformation. An internalization of PRLR was observed in the form of vesicles in tumor tissues. These results are the first to show a change of PRLR location in PCa. In mammary tumor cells, only 5-10% of the PRLR of tumor cells are normally on the cell surface in a way that recognizes and binds to the PRL, while the rest are cryptic associated with intracellular vesicles (19,20). The number and distribution (surface vs. cryptic) of PRLR are not altered with the concentration of circulating PRL (21). There is consistent evidence that surface receptors could be related to PRL absorption while cryptic receptors would be involved in PRL accumulation (19). Our results in prostate tumors showed that total receptor levels were similar in normal and tumor tissues. However, the amount of cryptic receptors increased in tumors compared to normal tissues suggesting that the appearance of cryptic receptors may arise from tumorogenesis. This would be reflected as a loss in the ability to transport PRL through the cell while retaining the ability to absorb PRL through receptor-mediated endocytosis. Persistent occupation of internal receptors by PRL could result in the maintenance of sufficient PRL-induced signals and explain why prostate tumors grow at low physiological levels of PRL.

Finally, we were able to determine that the same cells expressed the hormone and its receptor suggesting a possible paracrine effect of the hormone secreted by some tumor cells stimulating the proliferation of others that possess the receptor; and a potential autocrine effect of the hormone acting as a

growth factor on the same tumor cells that produce it. The functional relevance of the autocrine / paracrine effects of PRL in prostate carcinogenesis has been demonstrated by the ability of PRLR antagonists to reduce the viability of PCa cells in the absence of exogenous PRL stimulation (22).

For all these reasons, we propose that one of the molecular mechanisms responsible for the initiation and progression of PCa could be that PRLRs accumulating PRL are located in intracellular vesicles probably maintaining PRL-proliferative and -antiapoptotic pathways active.

CONCLUSION

PRL and its receptor were present in the cytoplasm of the epithelial cells of the normal and tumor prostate gland. In tumor tissues, the change in the location and appearance of cryptic PRLRs that store PRL may keep active the different signaling pathways related to cell proliferation and survival.

ETHICAL APPROVAL

All of the volunteers approved and signed an informed consent previously approved by the Ethic Committee of the Universidad Nacional de Cuyo in accordance with the precepts established by the Helsinki Declaration.

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DECLARATIONS

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