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RESEARCH

Prolactin and Prolactin Receptor Expression in Cervical Intraepithelial Neoplasia and Cancer

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Abstract Prolactin receptor (PRLR) overexpression could play a role in tumorigenesis. The aim of this study was to determine prolactin (PRL) and PRLR expression in biopsies from patients with precursor lesions and uterine cervical cancer. PRLR expression was analyzed in 63 paraffin-embedded biopsies of uterine cervical tissue. In total, eleven low-grade squamous intraepithelial lesions (LSIL), 23 high-grade squamous intraepithelial lesions (HSIL), 21 uterine cervical cancers (UCC) and 8 normal epithelium (NE) were examined using immunoperoxidase staining and Western blot analysis. Additionally, PRL expression was identified in human cervical cancer serum and tissues. The PRLR expression was found to be significantly increased in cervical cancer in comparison with normal tissue and precursor lesions ($P<0.0003$). The presence of the long isoform of the PRLR was observed only in cervical cancer tissues. Serum PRL levels were normal in all samples and local prolactin expression was similar in precursor lesions and cervical cancer by Western blot analysis. Our data suggest a possible role for PRLR in the progression of cervical cancer.

Keywords Cervical cancer · Prolactin · Prolactin receptor · Expression

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Abbreviations

PRL	prolactin
PRLR	prolactin receptor
HPV	human papillomavirus

Introduction

Cervical cancer is the second most common female malignancy worldwide. Although epidemiological and molecular studies clearly indicate that persistent infection with certain types of HPV are necessary for cervical cancer development, it has also been described that other alterations in certain cofactors at the cellular level can contribute to the development and progression of cervical cancer [1].

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Prolactin (PRL) was originally identified as a lactogenic neuroendocrine hormone of pituitary origin. It is best known for its effect on mammary gland development. Also, locally-produced prolactin has been documented in several tissues, notably in humans, with both autocrine and paracrine effects [2].

The diverse activities of PRL are mediated by its receptor (PRLR) and this molecule evokes the activation of several signaling pathways including Jak2-STAT [3], PI3K [4], and MAPK [5, 6]. There are multiple isoforms of the PRLR in humans; the long form (LF), intermediate (IF), and two short forms (SF1a and SF1b) are produced by alternative splicing [7].

During the last 10 years several experimental and clinical studies have shown altered expression of PRL/PRLR in human tumors such as mammary, prostate, endometrial and laryngeal tumors [8–11].

There are few studies focused on the analysis of the PRL or PRLR expression in uterine cervical lesions [12, 13]. The objective of this study was to determine the PRLR expression and its location in UCC, HSIL, LSIL and normal cervical tissues. Our results show an overexpression of PRLR in UCC specimens (mainly located in cytoplasm and nuclei) and suggest that this signaling pathway is differentially regulated in normal/benign lesions in comparison to malignant tissues.

Methods

Cell Culture

The human breast cancer cell line MFC7 was used as a control. After cell cultures reached 80 % confluence, cells were harvested to be used in the assays.

Patients and Tissue Samples

Tissue samples were obtained from the Department of Pathology at the Institute National de Cancerología-SSA, in Mexico City; and the O.P.D. Hospital Civil of Guadalajara "Dr. Juan I. Menchaca", Guadalajara, Mexico. Tissues were fixed in 4 % formalin and embedded in paraffin. Sixty-three specimens were evaluated and characterized by experienced pathologists. Normal cervical samples (n=8) were obtained from patients undergoing hysterectomy, without malignancy. Following the guidelines of the Bethesda System, we included 11 biopsies from tissues characterized as low-grade squamous intraepithelial lesions (LSIL) and 23 high-grade squamous intraepithelial lesions (HSIL). We also included 21 uterine cervical cancer (UCC) samples classified as epidermoid [11] or adenocarcinomas [2], which were analyzed by immunohistochemistry and Western blot.

Immunohistochemical Staining

Serial sections from the formalin-fixed paraffin-embedded blocks were used for the detection of PRLR using immunohistochemical methods. Sections were deparaffinized by successive immersions in 100 % xylene, 100, 96, and 70 % ethanol for 10, 10, 5 and 5 min, respectively. Endogenous peroxidase activity was inactivated with peroxidase blocking reagent (S2001, DAKO) for 10 min. Antigens were incubated with 10 mM citrate buffer (pH 6) and autoclaved at 121 ° C for 15 min. After blockade with 50 μl of 1 % BSA (Sigma, USA) in TBST buffer (50 mM Tris–HCl, 300 mM NaCl, 0.1 % Tween 20) for 5 min at room temperature, sections were incubated overnight with 40 μl of anti-PRLR primary antibody (Santa Cruz, CA, USA clone H-300) prediluted 1:100 in TBST at 4ºC in a humidified chamber. Then sections were washed with TBST, and incubated with one drop of secondary antibody conjugated with HRP (K4061 DAKO) for 60 min at room temperature. After washing, the sections were incubated with one drop of chromogenic DAB substrate (K3468, DAKO) for 15 min at room temperature. Sections were counterstained with Mayer's hematoxylin and mounted using a hydrosoluble medium (Vectamount AQ). All sections were developed in parallel with a negative control reaction omitting the primary antibody; false positive signals were not seen.

PRL Quantification

In order to measure PRL serum levels, individuals with similar histories were selected; 30 patients with premalignant lesions, 22 UCC and eight healthy controls were analyzed. Exclusion criteria included pregnancy, endocrine, renal or any other disease and medication known to affect serum PRL concentrations. PRL was measured with ELISA according to the manufacturer's instructions (EIA-1291; DRG, International). Concentrations were calculated from a standard curve (0–200 ng/mL) and none of the samples reached values above the highest standard. Hyperprolactinaemia (HPRL) was defined as serum concentration >20 ng/mL.

Western Blot Analysis

Cellular proteins were extracted from cell lines with 300 μl of RIPA buffer (50 mM Tris, 150 mM NaCl, 1 % NP40, 0.5 % sodium deoxycholate, and 0.1 % sodium dodecyl sulfate [SDS]), with the addition of protease inhibitors (pestatin, leupeptin, aprotinin, quimostatin, antipain, and PMSF) and phosphatase inhibitors (Na3 VO4, and NAF), and clarified by centrifugation at 4 °C for 20 min. Protein concentration was determined by Lowry method (BCA Protein Assay Reagent, Pierce). Forty micrograms of total protein were mixed with

loading buffer, electrophoresed on 7.5 % SDS- polyacrylamide gels and transferred to polyvinylidene difluoride membrane (Bio-Rad). Nonspecific binding was blocked with 5 % non-fat milk and 1 % bovine serum albumin solution. Next, the membranes were incubated with 2 μg/ml polyclonal PRLR antibody (1:1000) (Santa Cruz, CA, USA clone H-300) or prolactin antibody (1:500) at 4 °C overnight. HRP-conjugated anti-rabbit secondary antibody was used to reveal the immune detection and blots were developed with a chemiluminescence system (Pierce). As an internal control to confirm that similar amounts of protein were loaded for each lane, actin levels were determined using a monoclonal anti-actin IgG (Chemicon International) 1:10000 and reveled with anti-mouse IgG peroxidase (Santa Cruz, CA, USA).

Microscopy

Specimens were analyzed with an optical microscope (Carl Zeiss, Göttingen, Germany), with the 10, 20, 40 and 100× objectives. Cells were counted when immunolabeling was clearly evident and the average of the total of photographed fields was classified as follows: negative (non immunolabeling), low, moderate and strong. Even if only in one field, when immunolabeling was clearly observed with the 10× objective it was classified as intense.

Statistical Analysis

For statistical analyses we grouped the samples according to the intensity of PRLR expression as absent and low expression (categorized to the PRLR low expression group) vs moderate and/or strong expression (categorized to the PRLR high expression group). Data was analyzed using Graph pad PRISM software (Graph pad version 5.01), the analyses of PRLR expression between the different groups were performed using the Chi-squared test. Differences were considered statistically significant for values p.

Results

To evaluate whether PRLR expression is associated with the disease progression in patients with different lesions of the uterine cervix, we used immunoperoxidase staining to determine PRLR expression in normal tissues, premalignant lesions and cervical cancer.

The PRLR immunoreactivity was semiquantitatively scored as low (<10 % of tumor cells positive), moderate (10–50 %), or strong (>50 %). The PRLR immunostaining was stronger in uterine cervical cancer samples than in intraepithelial cervical lesions and normal cervix. The expression patterns of PRLR in cervical tissues were as follows: in LSIL, low in nine samples (81.8 %), and moderate in two samples (18.2 %) (Fig. 1 C & D); in HSIL, low in 15 samples (64.2 %), moderate in 3 (13 %) and strong in one sample (4.3 %); and in UCC, low in 7 samples (33.3 %), moderate in 7 samples (33.3 %) and strong in 7 (33.3 %) (Fig. 1 G & H). The staining pattern was confined to the tumor cells, and was mostly membrane and cytoplasmic, however in several samples the localization was evident in the nuclei. Cells adjacent to the tumor showed very low PRLR expression. In normal cervical epithelium PRLR was absent in 5 (62.5 %) samples and low in the other 3 (37.5 %) (Fig. 1 A & B). Therefore, we compared absent and low expression (categorized as the PRLR low expression group) vs moderate and/or strong expression (categorized as the PRLR high expression group) and found PRLR over-expression in majority of cervical cancer samples was statistically significant when compared with normal tissues and precursor lesions ($P<0.0003$) (Table 1). We therefore examined the expression of PRLR in cervical biopsies by the Western blot method using a single antibody that recognizes long, intermediate and short isoforms. The different isoforms of PRLR can be observed in these tissues: all lesions expressed one band of 42 kDa, however one band of 100 kDa was only observed in cancer samples (Fig. 2). The MFC7 breast cancer cell line, which is known to express high amounts and different isoforms of PRLR was used as a control.

Circulating PRL concentrations were determined in sera of 30 patients with premalignant lesions, 22 UCC and 8 controls. Serum concentrations of PRL in all samples analyzed were within normal values (Fig. 3 A).

Subsequently, in order to evaluate the PRL expression at the local level of the tumors, we analyzed this expression by Western blot analysis and observed a single 60-kDa immunoreactive PRL band. However, we did not observe differences in the PRL expression between the different lesions of the uterine cervix and normal tissue (Fig. 3 B).

Discussion

There is evidence suggesting that PRL and PRLR may have a role in human breast cancers as well as prostate, ovarian and colon cancers [8–10, 14]. We previously demonstrated that increased expression of PRLR is associated with malignant laryngeal tumors [11]. There are few studies focused on the analysis of PRL or the PRLR expression in uterine cervical lesions. With respect to prolactin, two studies have shown elevated blood prolactin level in patients with cervical cancer [15]. However, in our study serum concentrations of PRL between normal, premalignant and cancer samples were similar and within normal values. Furthermore, we observed that PRL is locally produced by human cervical cells of premalignant and cancerous tissues. Our findings are correlated with

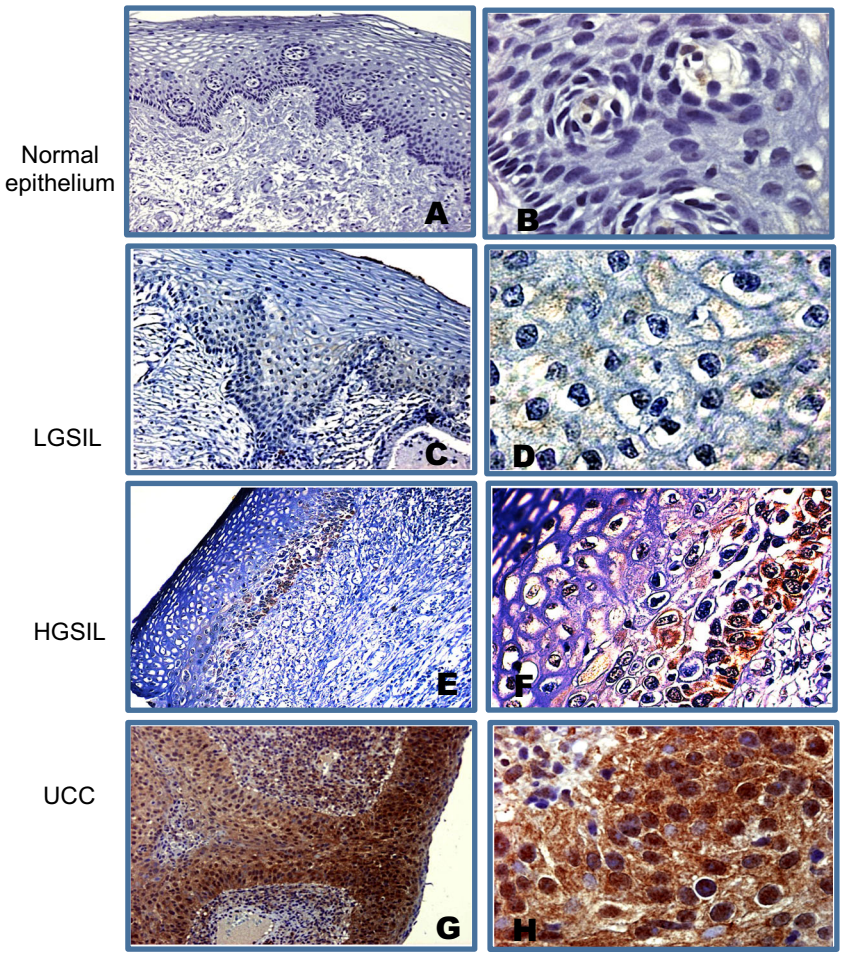

Fig. 1 An immunohistochemical expression of Prolactin receptor in normal epithelium, precursor lesion and cervical cancer. The PRLR was detected using specific antibody in paraffin-embedded tissues (4 μm) and detected as *brown* coloration in low-grade squamous intraepithelial lesion (C & D), high-grade squamous intraepithelial lesion (E & F), uterine cervical cancer (G & H) and normal epithelium (A & B). Magnification × 20 (A, C, E, G,) and × 100 (B, D, F, H)

studies that have shown ectopic PRL production by human cervical epithelium [12, 16].

The molecular diversity of PRL forms has been previously described [17] and in this work we observed expression of a large 60 kDa isoform of PRL in cervical cells. This large isoform of PRL has been previously characterized in serum, plasma, PBMC, lymphocytes and monocytes from human subjects [18–20]. The large PRL is a dimer of covalently-linked glycosylated subunits (25 kDa) [19] and has been formerly correlated with the course of several inflammatory disorders [21, 22].

Different investigations in breast and prostate cancer suggest that autocrine prolactin may play an important role in the development of cancer [23, 24]. More recently, our own data

Table 1 Expression of PRLR in normal, intraepithelial lesions and cancerous tissue of the cervix

Cervical-tissue	PRLR				%	*P*-value
	Low expression	%	High expression			
Healthy	8	100	0		0	.0003
LSIL	9	82	2		18	
HSIL	19	83	4		17	
UCC	7	33	14		67	

Abbreviation: *PRLR*, prolactin receptor; *LSIL*, low squamous intraepithelial lesions; *HSIL*, high squamous intraepithelial lesions; *UCC*, uterine cervical cancer

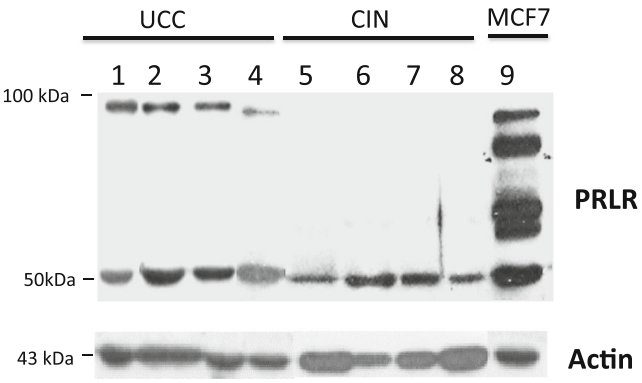

Fig. 2 Expression of PRLr isoforms in premalignant lesions and cervical cancer tissues by Western blot analysis. Lanes 1–4, cervical cancer tissues; lanes 5–8 cervical intraepithelial neoplasia (CIN); lane 9, the breast cancer cell line, MCF7. Actin was loaded as a control. A total of 40 μg of protein was loaded in each lane

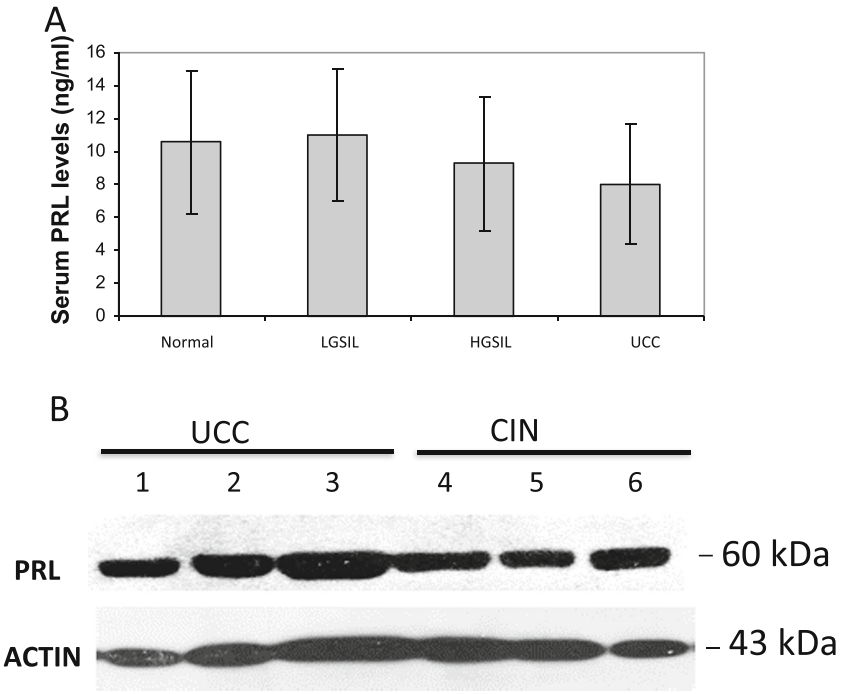

Fig. 3 PRL levels in normal women, women with LGSIL, HGSIL and UCC **a**) Serum PRL levels (ng/ml). No significant difference was detected when we compared with normal controls or between the patient groups. Results represent the mean of ± SD of three replicate experiments. (**b**) Western blot analysis of immunoreactive PRL-like molecules in biopsies from patients with pre malignant lesions and cervical cancer. Lanes 1–3 patients with cervical cancer; lanes 4–6 are patients with cervical intraepithelial neoplasia (CIN). A total of 40 μg cell lysate was loaded per lane. Actin was loaded as a control

showed an autocrine PRL synthesis in cervical cancer cells through the PRL transcript and protein. Despite, we did not find an effect over the proliferation of the cervical cancer cells using blocking anti-PRL or anti-PRLR antibodies, we found that treatment with PRL had a protective effect against cell death induced by etoposide, decreasing the number of apoptotic cells in all the cervical cancer cell lines [25].

At the present, it is known that PRL exerts its activity through at least six recognized PRL receptor isoforms and that the biological actions of PRL are dependent on the PRLR isoforms expressed. The long PRLR isoform is able to activate several signaling pathways including Jak-Stat, MAPK, and PI3K; while short forms are considered as negative regulators of the long forms and do not activate the Jak-Stat pathway [26].

In this study, we observed that high levels of PRLR in the UCC patients correlated to the degree malignancy of the cervical tissues and we found one PRLR isoform, of approximately 100 kDa, expressed only in UCC tissues suggesting that the presence of this isoform in these samples activates signaling pathways that may contribute to the malignant phenotype of the cell. These results agree with our previous data, which showed a high expression of multiple PRLR forms in cervical cancer cell lines compared with non-tumorigenic keratinocytes [25]. A previous work in breast cancer shows an increased expression of PRLR long forms when compared with normal samples suggesting that augmented long form expression in patients with cancer could contribute to breast tumor development and progression [27]. It has also been shown that the long term increased expression of the PRLR short form 1b in PC-3 human prostate cancer cells decreases cell growth and reduces invasive capacity [28]. It is known that the proteolytic degradation of PRLR is usually induced through ubiquitination of PRL, but it is possible that the prolactin receptor accumulation is due to a defect in its degradation [29]. Recently, it has been demonstrated that the stabilization of PRLR in breast cancer is due to the decreased activity of GSK3b, as a result of the constitutive activation of a Ras-dependent oncogenic pathway [30]. The accumulation of PRLR in cytoplasm and its translocation to the nucleus could explain our observations in the same manner that has been described for other non-ubiquitinated proteins in malignant processes [31].

In summary, our results show an important role for the activation of the PRLR signaling pathway in uterine cervical cancer.

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Authors' Contributions RAC and EILP: experimental work; JFMV and CECH: assistance with the writing; ADN, NVS, AGC interpretation of data; STA assistance with ELISAS; RFT, DPM processing samples; ALPS design of the study.

All authors read and approved the final manuscript.

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