PAC_1-R *Null* Isoform Expression in Human Prostate Cancer Tissue

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BACKGROUND. PACAP is a member of the VIP/GHRH family of neuropeptides and has important effects on prostate cell proliferation. Here we analyze the expression and localization of PACAP and its specific receptor variants (PAC_1-R) in tissues collected from patients undergoing prostate biopsy and surgery for benign prostatic hyperplasia (BPH) and prostate cancer (PCa).
METHODS. Reverse transcriptase (RT)-polymerase chain reaction (PCR), DNA sequencing, and immunohistochemistry.
RESULTS. PACAP and PAC_1-R were localized by immunohistochemistry in the prostate tissue. While in healthy and BPH tissues PAC_1-R positive staining is present in all the epithelial cells lining the lumen of the acini and in some stromal cells (mostly in the apical portion of the cells), in PCa tissues, anti-PAC_1-R antibody stained the apical portion of the cells. We provide evidence that PAC_1-R *null* and SV_1/SV_2 variants are all present in normal and hyperplastic tissues, while in PCa tissue PAC_1-R *null* is the most relevant receptor variant expressed.
CONCLUSIONS. Our data demonstrates that the PAC_1-R *null* variant is the most relevant isoform expressed in human PCa tissue being suggestively related with the events determining the outcome of prostate cancer. *Prostate 66: 514–521, 2006.* © 2005 Wiley-Liss, Inc.

KEY WORDS: **PACAP; PAC_1-R; SV_1; SV_2; NE differentiation; prostate cancer**

INTRODUCTION

Vasoactive agents, peptide growth factors, and cytokines, produced by the complex network of epithelial and endothelial prostate cell morphotypes, are important paracrine mediators of prostate functions affecting both the physiology and the mechanisms of prostate carcinogenesis and progression [1]. Experimental evidence indicates that, among these factors, PACAP may play critical roles in cancer cells proliferation, neuroendocrine differentiation, and tumor progression [2]. There are two biological active forms of PACAP, PACAP-38, and the C-terminally truncated PACAP-27, which derive from a 176-amino-acid precursor protein by post-translational cleavage [3]. They act through three classes of membrane G-protein coupled receptors: the specific PAC_1 receptor (PAC_1-R) and the common PACAP/VIP receptors ($VPAC_1$ and $VPAC_2$) [4]. An alternative splicing of two exons of the *PAC_1-R*

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gene allows for 4 major splice variants named: the PAC_1-R *null* form, the PAC_1 SV_1, the PAC_1 SV_2, and the PAC_1 SV_3 [5]. These splice variants display different abilities to activate adenylate cyclase (AC) and phospholipase C in several cell systems studied [5,6]. Despite the expression of almost all classes of functional PACAP/VIP receptors in human prostate cancer (PCa) and healthy tissue, the potential influence of PACAP on PCa progression remains largely unknown. Recently, it has been demonstrated in the androgen dependent LNCaP prostate cancer cell line, that the sustained versus transient intracellular cAMP increase induced by the binding of PACAP to its cognate PAC_1 receptor is a crucial event determining the outcome of tumor progression [2]. However, there are little information on PAC_1-R variants expression in normal, hyperplastic, and neoplastic human prostate and about the possibility of their modifications in the evolution of PCa. In the present work, we performed PACAP and PAC_1-R expression studies and immunohistochemical experiments on tissues obtained by patients affected with different degrees of BPH and prostate cancer with the aim to add information on localization and functional expression of PACAP and PAC_1-R in healthy, hyperplastic, and neoplastic prostate.

MATERIALS AND METHODS

Reagents and Antibodies

Maloney-murine leukemia virus (M-MLV) reverse transcriptase was purchased from Life Technologies (San Giuliano Milanese, Italy). Taq polymerase was purchased from Promega Corp. (Milano, Italy). Affinity-purified polyclonal rabbit anti-human PACAP serum was from Peninsula Laboratories, Inc. (San Carlos, CA). Affinity-purified polyclonal rabbit anti-human PAC_1-receptor primary antibody was provided by Prof. A. Arimura (Tulane University Hebert Center New Orleans, Louisiana). The commercial kit for immunohistochemistry was from Zymed Laboratories, Inc., San Francisco, CA. Serum prostate specific antigen (PSA) was measured by ECLIA using a commercial kit (Roche Diagnostics Grenzacherstrasse 124CH-4070 Basel, Switzerland) and processed through an Elecsys 2010 (Boehringer Ingelheim, Germany). The sensitivity of the method was 0.002 ng/ml.

Tissue Procurement

Human prostate tissues has been collected from patients aged 51–71 yrs (n = 36) undergoing ultrasound driven diagnostic prostate biopsy or radical prostatectomy for BPH or previously untreated PCa. Normal prostate specimens (n = 3) were obtained from patients undergoing surgical treatment for pelvic and urogenital primary diseases. After prostatectomy, a wedge-shaped specimen of the fresh prostate was cut. A sample of the tissue has been submitted for a pathological examination to confirm the prostate origin, the diagnosis, and the absence of other diseases. Hematoxylin-eosin staining was used for the histopathological evaluation, diagnosis, and tumor grading. Carcinomas have been classified according to TNM score referred to the pathological T-state [7]. The Gleason histopathological grade has been evaluated on the final resected specimens. Tissues collected intrasurgically or from biopsies have been immediately frozen on dry ice and subsequently kept at −80°C until analyzed. The Ethical Committee of the University of Tor Vergata approved the collection and use of specimens and all the subjects gave their written informed consent before participating in this study.

RNA Preparation and Amplification of PACAP and PAC_1–R Isoforms cDNA

Total RNA was extracted from either normal, hyperplastic, or PCa that had been frozen in liquid nitrogen immediately after surgery by using the single-step acid guanidinium thiocyanate-phenol-chloroform method [8]. The purity and integrity of the RNA were checked spectroscopically and by gel electrophoresis before carrying out the analytical procedures. First-strand cDNA synthesis was performed as follows: 1 μg total of RNA was reverse transcribed by 200 U of M-MLV reverse transcriptase using 2.5 μM random hexamers in the presence of 250 μM deoxynucleotides triphosphate in a final volume of 20 μl. DNA contamination or PCR carryover controls were performed omitting M-MLV during RT. The reaction mixture was heat-denatured for 5 min at 75°C, then incubated for 1 hr at 42°C. Five microliters of the cDNA obtained was used to amplify $hPAC_1$-R or hPACAP. $hPAC_1$-R was PCR-and nested-amplified as follow: the first round of PCR was carried out using primers designed for amplification of PAC_1-R cDNA sequence spanning the region 872–1,626 [upstream Cat1: 5′-TGTATGCG-GAGCAGGACAGC-3′; downstream Cat2: 5′-AGGC-CAGACATGCGGATTTGGG-3′, amplified product 754 bp]. The PCR product was nested-amplified using a set of primers flanking the receptor splicing site [upstream Don1: 5′-TTAACTTTGTGCTTTTTATTG-3′; downstream Don2: 5′-GAGTCTTTCCCTTTTGCT-GAC-3′]; cDNAs were amplified using Taq polymerase (2 U per tube) with 15 pmoles of both upstream and downstream primers, 1.5 mM magnesium chloride in a final volume of 50 μl. Then, 35 cycles (94°C for 30 sec, 60°C for 30 sec, 72°C for 30 sec, with 5 min final extension for the first round of PCR, and 94°C for 30 sec, 48°C for 30 sec, 72°C for 30 sec, with 5 min finally

extension for the nested round of PCR) were applied. PACAP PCR was performed using primer designed for the amplification of the fragment 502–1,078 spanning exon 5 of the human *PACAP* gene [upstream hP1: 5′-AAACAAAGGACGCCGAATAG-3′; downstream hP2: 5′-AGACTCACTGGGAAAGAATGC-3′; amplified product 576 bp] [3]. cDNAs were amplified using Taq polymerase (2 U per tube) with 15 pmoles of both upstream and downstream primers, 1.5 mM magnesium chloride in a final volume of 50 μL. Then, 30 cycles (94°C for 30 sec, 60°C for 30 sec, 72°C for 30 sec with 10-min final extension) were applied. Finally, a 15 μl aliquot of all the amplified products was analyzed on 2% (wt/vol) agarose gel and stained with ethidium bromide. The PCR conditions were optimized for *actin* gene used as control (upstream: 5′-CGTGGACATCCG-CAAAGAC-3′; downstream: 5′-CTCGGCCACATTG-TGAACT-3′; amplified product 484 bp). For actin, 20 cycles (94°C for 1 min, 55°C for 1 min and 72°C for 1 min, with 10 min final extension) were applied. Quantitation of the signals was performed by densitometric analysis using densitometry computer software (Kodak Digital Science 1D Image Analysis software, Eastman Kodak Co., Rochester, NY).

DNA Sequencing

The PAC_1-R *null* isoform from each sample isolate was cut from the gel and purified using the QIAquick Gel Extraction kit (Qiagen). A sample of 10 ng of purified PCR product and 3.2 pmol of the above PCR primers was directly sequenced with ABI PRISM 310 Genetic Analyzer equipment by use of the PCR cycle sequencing Big Dye Terminator protocol (Applied Biosystem). The electropherograms were assembled by the AutoAssembler Software (PE Applied Biosystems) to generate consensus nucleotide sequence. The consensus sequence was compared with the available sequences in gene bank data base with the Blast algorithm. The highest similarity was found with *null* isoform of PAC_1-R receptor.

Immunohistochemical Detection of PACAP and PAC_1-R

Immunohistochemistry for hPACAP and PAC_1-R was performed on healthy prostate, BPH, and PCa tissues, and carried out on 5-μm thick sections of the fixed tissues by the streptavidin-biotin immunoperoxidase method, using a commercial kit. Sections were deparaffinized and incubated overnight in a moist chamber at 4°C with the following antisera: 1:50 dilution of the affinity-purified polyclonal rabbit anti-human PACAP, and 1:100 dilution of the affinity-purified polyclonal rabbit anti-human PAC_1-R primary antibodies. For controls, the primary antibody was omitted in some sections. Slides were developed using amino-ethylcarbazole and 3,3′-diaminobenzidine as chromogenic substrates, which are converted by peroxidase into a red to brownish-red precipitate at the sites of antigen localization in the tissue. The preparations were counterstained with hematoxylin, dehydrated, and mounted. Assessment of PACAP and PAC1-R status was performed by two different examiners (SM and CM) through counting the percentage of cells expressing PACAP and PAC_1-R from 200 randomly sampled nuclei, which were obtained from 10 randomly selected high-powered microscopic fields.

Statistics

The nonparametric Spearman's correlation coefficient rho (also able to detect nonlinear associations) was used to assess the association between the variables in the BPH (PSA and PAC_1-R expression) and PCa (PAC_1-R *null* expression, PSA, and Gleason score) populations. Comparison between PAC_1-R *null* expression in PCa and BPH groups was evaluated by the Mann–Whitney *U*-test.

RESULTS

Clinical Remarks

The clinical details of the patients studied are reported in Table I. PSA levels assayed in the PCa subjects studied before operations were markedly variable. They ranged from 2.6 to 45.6 ng/ml with an average of 12.46 ng/ml ± 10.06 SD. The PSA levels did not correlate with the pathological stage and Gleason score.

PACAP and PAC_1-R Isoforms Expression in Normal, BPH, and Neoplastic ProstateTissues

The expression of PACAP mRNA in normal, hyperplastic, and neoplastic human prostate was examined by RT-PCR employing primers spanning the exon 5 of the human *PACAP* gene [3]. The analysis generated a product of expected size of 576 bp in all samples (Fig. 1). The identity of the PCR products was confirmed by restriction analysis.

RT-PCR was performed also in order to investigate if these tissues may synthesize PACAP-selective PAC_1-R variants. The amplification studies showed that normal and BPH tissues express both PAC_1-R containing SV_1/SV_2 cassettes and PAC_1-R (null) as predominant product (Figs. 2 and 3), while in the PCa only the PAC_1 R *null* isoform is clearly expressed (Figs. 4 and 5). The PAC_1 SV_3 isoform, a splice variant of the PAC_1 receptor containing both the SV_1 and SV_2 boxes, is occasionally albeit measurable with our method independently

TABLE I. Details of Prostate Samples Used in the Study

Patient id	Age	Pre-operative PSA (ng/ml)	Tissue type	PCa stage	Gleason score
ML	51	0.4	normal	—	—
FO	55	1.2	normal	—	—
LD	57	1.6	normal	—	—
AR	64	0.8	BPH	—	—
FG	62	1.5	BPH	—	—
MR	68	2.2	BPH	—	—
RP	62	1.1	BPH	—	—
AP	59	0.8	BPH	—	—
CR	56	2.1	BPH	—	—
PL	63	3.1	BPH	—	—
MF	61	2.3	BPH	—	—
AC	58	0.9	BPH	—	—
RA	60	1.4	BPH	—	—
MDM	69	7.2	Bilateral PCa	T3b NOMx	9(4+5)
CP	61	11.7	Bilateral PCa	T3b NOMx	6(3+3)
LG	71	5.18	Bilateral PCa	T2b NOMx	7(4+3)
EA	63	9.1	Monolateral dx PCa	T3b NOMx	6(3+3)
FS	60	11	Monolateral dx PCa	T3b NOMx	7(4+3)
PR	62	45.18	Bilateral PCa	T3b NOMx	9(4+5)
LM	67	2.6	Bilateral PCa	T2b NOMx	7(3+4)
AF	68	3.44	Bilateral PCa	T2c NOMx	6(3+3)
LM	68	9.28	Bilateral PCa	T2c NOMx	7(3+4)
MD	57	6	Monolateral dx PCa	T2b NOMx	6(3+3)
RP	67	13.06	Bilateral PCa	T2c NOMx	6(3+3)
SP	62	9.7	Bilateral PCa	T2c NOMx	6(3+3)
AZ	55	20.83	Bilateral PCa	T3b NOMx	7(4+3)
FR	51	2.14	Bilateral PCa	T3a NOMx	5(2+3)
GM	61	6.21	Bilateral PCa	T3a NOMx	7(4+3)
AL	67	18.5	Bilateral PCa	T3b NOMx	7(3+4)
CP	66	6.86	Bilateral PCa	T3b NOMx	8(4+4)
RV	56	12	Bilateral PCa	T2c NOMx	6(3+3)
EL	67	13	Bilateral PCa	T3a NOMx	7(4+3)
GB	66	6.67	Bilateral PCa	T3b NOMx	7(4+3)
MD	67	8.33	Bilateral PCa	T3b NOMx	6(3+3)
RS	65	45.6	Bilateral PCa	T2b NOMx	7(3+4)
SP	66	13	Bilateral PCa	T2c NOMx	7(3+4)

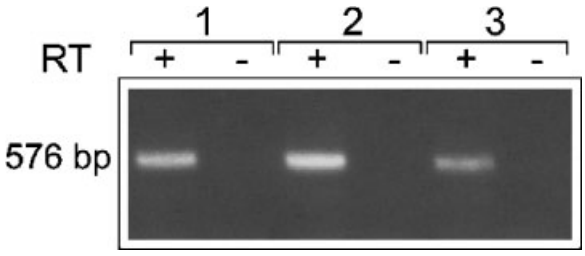

Fig. 1. PACAP expression in normal (1), hyperplastic (2), and neoplastic (3) prostate tissues. cDNAs were synthesized by RT-PCR using total RNA from tissues with hP1 and hP2 oligonucleotides as forward and reverse primers, respectively. The product obtained was of the expected size of 576 bp. Reverse transcriptase (RT) was omitted in the control experiments (-RT).

from the pathological state of the prostate tissue examined.

We investigated whether or not the densitometric levels of PAC_1-R *null* expression measured in the PCa specimens might be associated with Gleason score and/or PSA values, and whether PAC_1-R *null* densitometric levels measured in BPH samples might be associated with PAC_1-R SV_1/SV_2 expression and/or PSA values. We did observe significant association between PAC_1-R *null* versus PSA in PCa ($rho = 0.017$, $n = 23$). The mean levels of PAC_1-R in PCa and BPH were not statistically different (PCa: 0.409 ± 0.13 vs. BPH: 0.459 ± 0.09; $P > 0.07$).

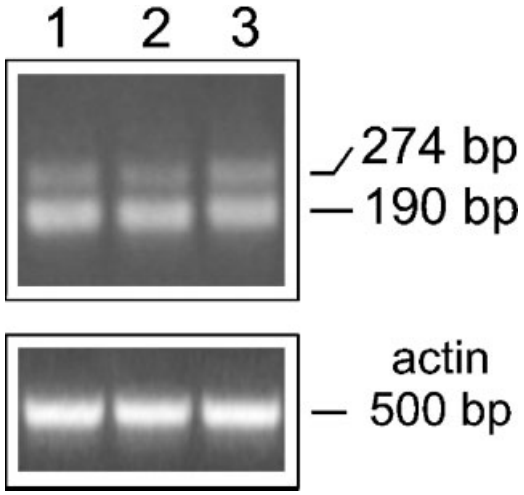

Fig. 2. Analysis of PACAP-R type I splice variants in three normal prostate tissues. cDNAs were synthesized by RT-PCR using total RNA from normal tissues with cat1 and cat2 oligonucleotides as forward and reverse primers, respectively, followed by a second round of PCR products with don1 and don2 nested primers. PCR products were electrophoresed and stained with ethidium bromide. The product of expected size (274 bp) is related to the SV1/SV2 variant. The product of expected size (190 bp) is related to the null variant. The β-actin transcript was analyzed as a control.

Localization of PACAP and PAC_1 Receptor in Normal, BPH, and Neoplastic Prostate Tissues

The localization of PACAP and PAC_1-R proteins was investigated in sections obtained from normal, BPH, and neoplastic prostate glands. Immunostaining was evaluated by an indirect immunoperoxidase technique with anti-PACAP and anti-PAC_1-R antiserum. No staining was observed in control slides (data not shown).

A specific positive immunostaining for PACAP was observed in the cytoplasm of the epithelial cells and in some cells scattered in the stroma (Fig. 6a, c and e), without significant difference in the distribution

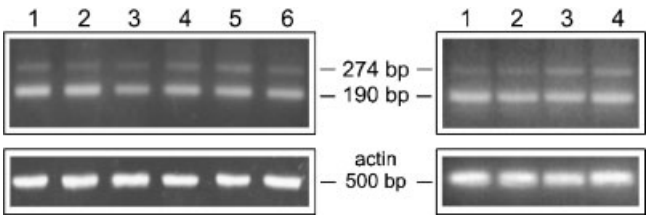

Fig. 3. Analysis of PACAP-R type I splice variants in 10 hyperplastic prostate tissues. cDNAs were synthesized by RT-PCR using total RNA from hyperplastic tissues with cat1 and cat2 oligonucleotides as forward and reverse primers, respectively, followed by a second round of PCR products with don1 and don2 nested primers. PCR products were electrophoresed and stained with ethidium bromide. The β-actin transcript was analyzed as a control.

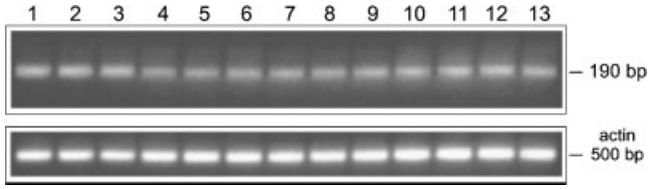

Fig. 4. Analysis of PACAP-R type I splice variants in 13 neoplastic prostate tissues. cDNAs were synthesized by RT-PCR using total RNA from neoplastic tissues with cat1 and cat2 oligonucleotides as forward and reverse primers, respectively, followed by a second round of PCR products with don1 and don2 nested primers. PCR products were electrophoresed and stained with ethidium bromide. The product of expected size (190 bp) is related to PAC1-R null isoform. The β-actin transcript was analyzed as a control.

pattern between normal, hyperplastic and tumoral tissues.

PAC_1-R was found in all the epithelial cells lining the lumen of the acini and in some stromal cells in healthy and BPH tissues with the positive staining mostly localized in the apical portion of the cells (Fig. 6b, d). In the specimens from PCa the anti-PAC_1-R antibody stained the apical portion of a large percentage of cells ($75 \pm 10\%$ SD positive cells), suggesting a different localization of the protein within the tissue without variations in staining intensity (Fig. 6f).

DISCUSSION

During androgen-independent progression, prostate cancer cells proliferation depends on various cellular pathways some involving the androgen receptor and other bypassing it. In the complex mechanisms of the androgen-refractiveness, it is clear that prostate-cancer cells develop a neuroendocrine-like behavior so that the action of many neuropeptides, secreted by epithelial and neuroendocrine cells, become a relevant factor controlling tumor growth [9]. PACAP is a neuropeptide expressed in prostate tissue [10] and in cancer cell lines [2]. We have demonstrated that it may

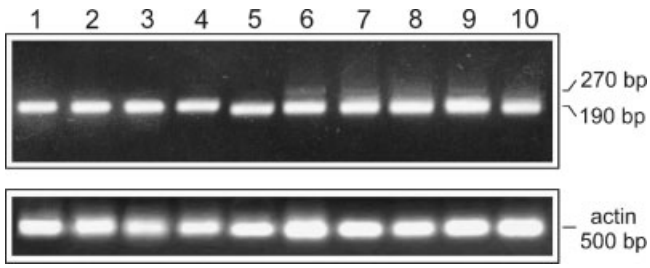

Fig. 5. Analysis of PACAP-R type I splice variants in 10 neoplastic prostate tissues. The methods used are identical to those showed in the Figure 4. In 3 out of 10 patients (see lines 6,7, and 9), in addition to the 190 bp of the PAC1-R null isoform, the products of the SV1/SV2 isoform expected size (270 bp) are also albeit measurable.

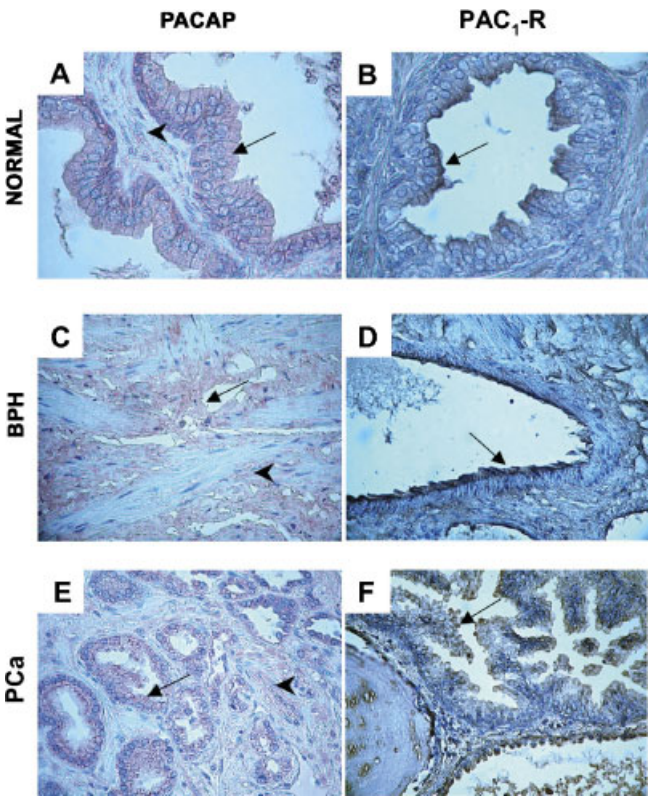

Fig. 6. Immunohistochemical analysis of PACAP and PACI-R. Representative immunohistochemical staining of PACAP (**a**, **c**, **e**) and PACI-R (**b**, **d**, **f**) in normal human prostate, BPH, and prostate cancer. PACAP staining is found both in the epithelium (arrow) and weakly in the stromal cells (arrow head), although in BPH a stronger signal in the stroma is seen. PACI-R is mostly localized in the apical region of the epithelium lining the acini (arrow), irrespective of the origin of the tissue samples either normal, BPH, or cancer. In prostate cancer the staining is restricted to a percentage of the epithelial cells. Original magnification: 40x. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

play an important role in the crucial events determining the outcome of prostatic cancer cells progression, considering its ability to increase intracellular cAMP levels and protein kinase A activity in prostate cancer cell lines [2]. In the present study, we confirm in agreement with other studies [11–13] that PACAP and PACAP type-1 receptors are expressed in normal, BPH, and PCa tissue, demonstrating their localization within these tissues. Furthermore, we show that the PAC$_1$-R *null* isoform clearly become the most important receptor present in the neoplastic prostate. This evidence is in agreement with our studies published in vitro on prostrate cancer cell lines [2] where the amplification by nested RT-PCR identifies the PAC$_1$-R isoform without SV$_1$/SV$_2$ cassettes as the predominant product. The expression of functional PACAP receptors in prostate cancer has been recently reported by other studies [10,14] that show the presence of PAC$_1$-R, VPAC$_1$, and VPAC$_2$ class of protein receptors but are inconclusive in establishing a difference in the expression of the PAC$_1$-R subtypes when comparing control and cancer tissue samples. All the variants of PACAP/VIP receptors activate the adenylyl cyclase system, whereas PAC$_1$-R activates the phospholipase C system [4,15]. This observation leads to consider the potential actions in vivo mediated by PAC$_1$-R in the neoplastic cells where it may activate the complex interplay of signal transduction pathways cooperating in the activation of the mutated androgen receptor characteristic of the androgen-independent progression of the prostate cancer. PAC$_1$-R may interfere with the key role played by the cell availability of cAMP that should reach a different outcome in the prostate neoplastic cells modulating the proliferating effect of neuropeptides and growth factors exerted through the MAPKs pathway [16]. In fact, our previous in vitro studies demonstrate that while the transient cAMP rise induces proliferation, the chronic PACAP stimulation and PACAP-Rs activation gives rise to a sustained cAMP activation which leads to proliferation arrest and neuroendocrine differentiation [2]. Neuroendocrine cells, secreting neuropeptides such as serotonin and bombesin which can increase the proliferation of neighbor cancer cells, are present in almost all prostate tissues of patients with androgen-refractory cancer [17].

In the present study, we present the immunohistochemical localization of PACAP and PAC$_1$-R in normal, BPH, and neoplastic prostate tissue samples.

The immunolocalization of PACAP and PAC$_1$-R in these prostate specimens confirmed previous reports in which an epithelial and weak stromal distribution of PACAP ligand and PAC$_1$ receptor in the gland was seen. Furthermore the immunohistochemical data reported here are in line with the results of the RT-PCR analysis of the corresponding mRNAs. Interestingly, we observed that the PAC$_1$-R positive staining in the PCa specimens, localized in the epithelium lining the acini of the gland, was restricted to a subset of the epithelial cells, suggesting a different expression of the PAC$_1$-R isoforms in the tumor compared to normal and hyperplastic tissue. Finally, although the anti-PAC$_1$-R antibody used in our experiments did not distinguish between the different PAC$_1$-R splice variants, it is reasonable to speculate that the positive staining might be referred to the PAC$_1$-R *null* isoform which is the only PAC$_1$-R mRNA variant detected in the human PCa tissues analyzed. In fact, PAC$_1$-R *null* and PAC$_1$-R containing SV$_1$ and SV$_2$ cassettes genes are present in the normal and BPH tissues while in the PCa only the PAC$_1$ *null* isoform is clearly expressed.

We have not enough elements to affirm that the PAC$_1$-R *null* variant expression might be associated with malignancy of the prostate, but the in vitro studies may suggest that the presence of this PAC$_1$-R isoform

correlates with the microenvironmental cAMP availability, remarking the importance of the signaling mechanism mediating proliferation, degradation of extracellular matrix and invasion sustained by PACAP autocrine loops [18,19].

In our study, there is no correlation between PSA preoperative levels and intensity of PAC_1-R expression, except for a significant association observed between PAC_1-R *null* densitometric levels and PSA values in PCa patients. This latter observation may be considered as preliminary original data that need to be validated studying a larger number of subjects. Taking into account the recent finding of surprisingly high rates of prostate cancer in men with low (4.0 ng per milliliter or less) PSA levels [20] this observation might open new insight on the importance of neuropeptide-mediated cAMP increase which is considered a crucial event determining tumoral cell progression [2]. Furthermore, it has been recently reported that more than a single assay, the rate of rise in the PSA levels during the year before radical prostatectomy predicts the risk of death from prostate cancer [21]. In our PCa patients the pre-operative PSA levels ranged between 2.6 and 45.18 ng/ml and were significantly stable in two samples collected before the operation. Furthermore, the Gleason score was also not related to the expression of PAC_1-R in the prostate tissues examined.

In summary, we have demonstrated that the PAC_1-R *null* variant is differentially expressed in normal, hyperplastic and neoplastic prostate being the only one expressed in PCa. The loss in the expression of the other variants of the PAC_1-R, the SV_1 and SV_2 isoforms generated by the alternative splicing of the PAC_1-R gene, which present either one or two cassettes of 28 aminoacids in the third intracellular loop, may lead to the activation of more complex signal transduction pathways in the cancer tissues comparing to normal and BPH. These pathways in PCa might be related to the sustained PACAP-induced cAMP availability and then to the activation of signals that induce neuroendocrine differentiation and negative PCa progression. Therefore, PACAP may be considered an important epithelium-derived paracrine factor mediating prostate carcinogenesis and progression. Considering this point, studies performed on a large number of patients will be necessary to establish if PAC_1-R *null* and/or its signaling pathway might become a potential therapeutic target for patients with aggressive forms of PCa.

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