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Overexpression of vasoactive intestinal peptide receptors and cyclooxygenase-2 in human prostate cancer. Analysis of potential prognostic relevance

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Summary. Vasoactive intestinal peptide (VIP) is a potent inductor of cyclooxygenase-2 (COX-2) expression in human prostate cancer cell lines. There are conflicting data regarding the role of COX-2 in the progression of this disease. Here we examined the expression of VIP receptors ($VPAC_1$ and $VPAC_2$) and COX-2 in prostate cancer specimens. Correlations among protein levels and various clinicopathological factors and prognosis of patients were statistically analyzed. For these purposes, formaldehyde-fixed, paraffin-embedded prostate tissue specimens from 63 patients with prostate cancer and 9 control samples were used. The expression of $VPAC_1$ and $VPAC_2$ receptors and COX-2 was analyzed at mRNA levels by quantitative reverse transcriptase-PCR. The corresponding expression at protein level was studied by immunohistochemistry, scored as negative, weak, moderate, or strong, and correlated with different clinicopathological factors by means of multivariate analysis. 88% of prostate cancer tissues overexpressed $VPAC_1$-receptor at mRNA level, 72% $VPAC_2$-receptor and 77% COX-2. Simultaneous overexpression of the three genes was seen in 52% of patients. Similar overexpression patterns were observed at protein level. The correlation between $VPAC_1$ and $VPAC_2$ receptor protein levels was statistically significant. However, no significant correlations existed among protein levels of VPAC receptors and COX-2 with patient age, prostate-specific antigen (PSA) levels, tumor stage, Gleason score and survival time. The overexpression of $VPAC_1$ and $VPAC_2$ receptors and COX-2 in cancer tissue gives them a potential role as targets for diagnosis of prostate cancer but results do not support a clear value as biomarkers for the clinical prognosis of this disease.

Key words: VIP, $VPAC_1$ and $VPAC_2$ receptors, COX-2, Prognostic marker, Prostate cancer

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Introduction

Prostate cancer is the most common malignancy and the second leading cause of cancer-related death among men in industrial western countries (Jemal et al., 2010). Various signaling pathways have been involved in prostate carcinogenesis and progression, but not molecular biomarkers have yet been identified with certainty to be correlated with clinical outcome of the disease (Lopergolo and Zaffaroni, 2009).

Vasoactive intestinal peptide (VIP) is a neuropeptide present in the human prostate (Polak and Bloom, 1984; Fernández-Martínez et al., 2009). VIP exerts a wide range of biological effects which are initiated through VIP receptors ($VPAC_1$ and $VPAC_2$ coupled to adenylate cyclase stimulation, as shown in many cells and tissues, including normal and cancer prostate gland as well as prostate cancer cell lines (Juarranz et al., 2001; García-Fernández et al., 2003). A previous study on prostate cancer tissue from a small number of patients indicated a decrease in the number of VIP receptors by means of immunochemistry, but RT-PCR and western-blot experiments gave no conclusive differences in comparison with normal tissue samples (García-Fernández et al., 2003).

In prostate, VIP increases the expression of the major angiogenic factor, vascular endothelial growth

factor (VEGF) and the proinflammatory enzyme cyclooxygenase-2 (COX-2) (Collado et al., 2004; Fernández-Martínez et al., 2007). In addition, the neuropeptide induces neuroendocrine differentiation in androgen-dependent prostate cancer LNCaP cells (Juarranz et al., 2001), promotes survival and stimulates human epidermal growth factor receptor-2 (HER2) transphosphorylation in androgen-independent prostate cancer PC3 cells (Gutiérrez-Cañas et al., 2003; Sotomayor et al., 2007) and behaves as a pro-metastatic factor in both, LNCaP and PC3 cells (Fernández-Martínez et al., 2009). Angiogenesis, neuroendocrine differentiation and cell survival are steps of prostate cancer progression to androgen independence (Arya et al., 2006; Clarke et al., 2009). Thus, the consideration of the potential diagnostic and prognostic value of VIP receptors in this disease is interesting.

The role of inducible cyclooxygenase (COX-2) in prostate carcinogenesis is still controversial, since there are studies regarding the detection of enzyme expression and activity in human tissue and cell lines which report increased or even absent expression of COX-2 in prostate cancer (Castelli et al., 2010; Abedinpour et al., 2011). Moreover, some studies describe that proinflammatory atrophic lesions in prostate, which are thought to be precursors of prostate cancer, express COX-2 (Liu et al., 2000; Sotomayor et al., 2007). Thus, the association of COX-2 to prostate carcinogenesis or cancer progression has led to consider it as a rational drug target for prostate cancer prevention, although the situation remains confusing.

Previous results from our laboratory on the effect of VIP on COX-2 expression in human prostate non-neoplastic (RWPE-1), as well as cancer LNCaP and PC3 cells, showed that VIP induced higher levels of COX-2 protein expression in prostate cancer cells as compared with non-neoplastic cells (Fernández-Martínez et al., 2007). The relationship of VIP and COX-2 in a signaling network in human prostate cancer suggest that the neuropeptide may induce promotion and progression of prostate carcinoma through the activation of proinflammatory and proangiogenic signals, such as those deriving from the increased expression of COX-2 enzyme. In order to clarify a diagnostic or prognostic value, here we compared the expression levels of $VPAC_1$ and $VPAC_2$ receptors and COX-2 in human non-metastatic prostate cancers, as well as in control tissue samples; then, we searched for correlations with various clinicopathological factors.

Materials and methods

Patients

Sixty three patients with prostate carcinoma, apparently limited to the prostate gland (aged from 47 to 74 years) and subjected to radical prostatectomy with curative purposes were included in this study (Table 1). The control group consisted of 9 patients (aged from 54 to 80 years) undergoing radical cystectomy-prostatectomy due to urothelial infiltrating carcinoma. None of the patients was treated with hormones or other therapies before surgery. Clinicopathological data of the patients (Table 1) included routine determination of preoperative serum prostate-specific antigen (PSA) levels. All prostate tumors were graded according to the system of Gleason (Helpap and Egevad, 2009). The tumor pathological stage (pT) was also evaluated. Patients were regularly followed up and survival data were ascertained through patient records. Written informed consent was obtained from all patients. The study was approved by the Research Ethics Committee. Samples of the prostate proper zone and suspected tumor zones were delimited by two independent pathologists and taken from resected tissues and immediately fixed in 10 % (v/v) formaldehyde in PBS (pH 7.4) for 24 h, dehydrated, and embedded in paraffin (FFPE).

Isolation of RNA and single-step real-time quantitative RT-PCR

FFPE tissue samples were cut into 5 mm-thick sections on a microtome and subjected to RNA isolation with the Absolutely RNA FFPE Kit (Stratagene, La Jolla, CA). Deparaffinization was first performed with xylene, followed by extraction in ethanol and homogenization by overnight incubation in Proteinase K. DNase I was then used to digest residual DNA and, finally, solubilized nucleic acids were bound to a glass fiber filter in the presence of guanidine salts. Filter-bound nucleic acids were washed and RNA was eluted. RNA concentration was determined with a Nanodrop ND-100 spectrophotometer (Nanodrop Technologies, Wilmington, DE). Real-time quantitative RT-PCR analysis was performed using SYBR Green PCR master mix, in a one-step RT-PCR protocol according to the manufacturer's instructions (Applied Biosystems, Foster City, CA). Four nanograms of total RNA samples were used for each PCR amplification with a primer set which amplifies cDNAs for human $VPAC_1$ receptor (sense 5'-CTG GGT CAG TCT GGT GGG-3', antisense 5'-TCC GAG ACC TAG CAT TCG CT-3'), $VPAC_2$ receptor (sense 5'-TCA GTG CTG GTC AAG GAC GAC-3', antisense 5'- AAG ACC AGG CTC AGC TTG CA-3'), COX-2 (sense 5'- TGA CGG GGT CAC CCA CAC TGT GCC CGT CTA-3', antisense 5'- CTA GAA GCA CGG TTG ACG ATG GAG GG-3'), and ß-actin (sense 5'-AGA AGG ATT CCT ATG TGG GCG-3', antisense 5'- CAT GTC CCA GTT GGT GAC-3'). Thermal cycling parameters were 30 min at 48°C for RT and 10 min at 95°C for activation of AmpliTaq Gold DNA Polymerase, followed by 50 cycles of 95°C for 15 s and 60°C for 1 min. Negative controls with water instead of cDNA were run in parallel to exclude contamination. The relative quantification was normalized to the ß-actin gene expression level. PCR reactions were performed using ABIPrism 7000 SDS (Applied Biosystems). The mean Ct (threshold cycle; cycle at which the increase in

signal associated with an exponential growth of PCR product is first detected) value of tumor samples was compared to that of control samples using the Ct value of ß-actin as an internal reference. ΔCt was the difference in Ct values derived from genes and ß-actin gene, and ΔΔCt represented the difference between paired samples. The n-fold differential ratio was expressed as $2^{-\Delta\Delta Ct}$ (Chang et al., 2002). It should be noted that ß-actin was similarly expressed in tumor and healthy zones of tissue sections (data not shown).

Immunohistochemistry

For immunohistochemistry studies, deparaffinized sections of prostate tissue representative of the tumor (5 μm thickness) were hydrated and incubated for 30 min in 3% H_2O_2 diluted in methanol to reduce endogenous peroxidase activity. For antigen retrieval, sections were incubated with 0.1 mol/L citrate buffer (pH 6) for 2 min in a conventional pressure cooker. After rinsing in TBS, slides were incubated with normal donkey serum (NDS) at a 1:5 dilution in TBS (TBS/NDS) for 60 min, to prevent nonspecific binding of the primary antibody. Then, primary antibodies against $VPAC_1$ and $VPAC_2$ receptors (Thermo Fisher Scientific, Rockford, IL) or COX-2 (Cayman Chemical, Ann Arbor, MI) were applied at 1:500, 1:500 or 1:50 dilution, respectively, diluted in blocking solution 1:9, at 4°C overnight. Afterwards, sections were washed twice in TBS and detection was done by the conventional labeled-streptavidin-biotin method (LSAB-kit, Dako, Barcelona, Spain). Peroxidase activity was detected using the glucose oxidase-3,3'-diaminobenzidine (DAB) nickel intensification method kit (Zymed Laboratories, San Francisco, CA). Sections were lightly counterstained with hematoxylin, dehydrated and mounted in DePex (Probus, Barcelona, Spain). Sections of normal human tissues (skin, testes and cerebellum) that express $VPAC_1$ and $VPAC_2$ receptors and COX-2 were used as positive controls. In negative control experiments, serial sections of each specimen were processed as described, but the corresponding primary antibody was omitted. Immunoreactivity of each focus of interest was semiquantitatively graded by two independent observers as negative (0), weakly positive (1), moderately positive (2), or strongly positive (3) (Fig. 1). In order to strengthen the immunological score and gain in reproducibility, the samples were reviewed and scored again by two independent pathologists.

Statistical analysis

The SPSS 17.0 software package (SPSS Inc., Chicago, IL) was used for data retrieval and analysis. To perform a differential analysis of the positive tissue specimens for $VPAC_1$ and $VPAC_2$ receptors and COX-2, Dunn's Multiple Comparison test was used. Univariate analysis comparing categorical variables ($VPAC_1$ and $VPAC_2$ receptors and COX-2 expression, and clinicopathological data: patient age, tumor pathological stage, Gleason score, and pre-operatory PSA levels) was performed using chi-square tests. We tested for the presence of a linear trend when there were more than two categories of staining using the Mantel-Haenszel chi-square test. Continuous variables were compared using the Mann-Whitney test. For all these tests we computed *P*-values using an exact method due to small sample sizes. This analysis was completed with multiple regression analyses (Durbin-Watson test) to evaluate the possible dependence between $VPAC_1$ receptor

Table 1. Association of $VPAC_1$-receptor immunoexpression with expression of $VPAC_2$ receptor, COX-2 and several clinicopathological data of patients with prostate cancer.

			VPAC1 expression				
			Negative	Weak	*P*-value	High	*P*-value
Age	≤65	38	6	5		27	
	>65	24	1	4	0.1967	20	0.1524
PSA, ng/ml	3.5-10	43	5	8		30	
	>10	16	1	1	0.7565	14	0.4474
pT status	pT2	40	5	6		29	
	pT3	23	2	3	0.8385	18	0.6191
Gleason score	<7	41	4	6		31	
	≥7	22	3	3	0.6963	16	0.4568
$VPAC_2$	Negative		3	3		7	
	Cytoplasmic	Weak	4	6		19	
		High	0	0	0.6963	21	0.0140*
COX-2	Negative		2	2		7	
	Cytoplasmic	Weak	1	3		13	
		High	4	4	0.8792	27	0.649

Fisher's exact test (*P*<0.05).

expression and the other variables analyzed. Survival time of patients was calculated from the date of diagnosis until death or last follow-up. Survival curves were estimated using the Kaplan-Meier method. The unadjusted difference in these estimates was assessed by the log rank test. All tests were performed in the two tail form and a value of $P<0.05$ was considered statistically significant.

Results

Quantitation of $VPAC_1$ and $VPAC_2$ receptor and COX-2 mRNAs

The expression levels of $VPAC_1$ and $VPAC_2$ receptor and COX-2 genes were significantly higher in carcinomatous samples (n=27) than in matched non-malignant samples. Figure 2A shows the quantitative reverse transcriptase-PCR results for the levels of expression of $VPAC_1$ and $VPAC_2$ receptors and COX-2 in prostate cancer tissue in comparison with healthy tissue from the same patient. In order to interpret the results, we defined three levels of expression: negative, low and high, for $2^{-\Delta\Delta Ct}$ values below one, between one and two, or more than two, respectively. Figure 2B represents the percentage of patients at each level of expression and indicates that the tumor area overexpresses frequently one or more of the three genes analyzed. In particular, the overexpression ($2^{-\Delta\Delta Ct} \geq 1$) of $VPAC_1$ receptor gene was seen in 24/27 patients (88%) whereas the simultaneous overexpression of the three genes studied was seen in 14/27 patients (52%).

Figure 3 compares the expression of $VPAC_1$ and $VPAC_2$ receptor and COX-2 genes in prostate cancer tissue (n=27) and control prostates (n=9). As shown by $2^{-\Delta\Delta}Ct$ values, the expression of each gene was higher in

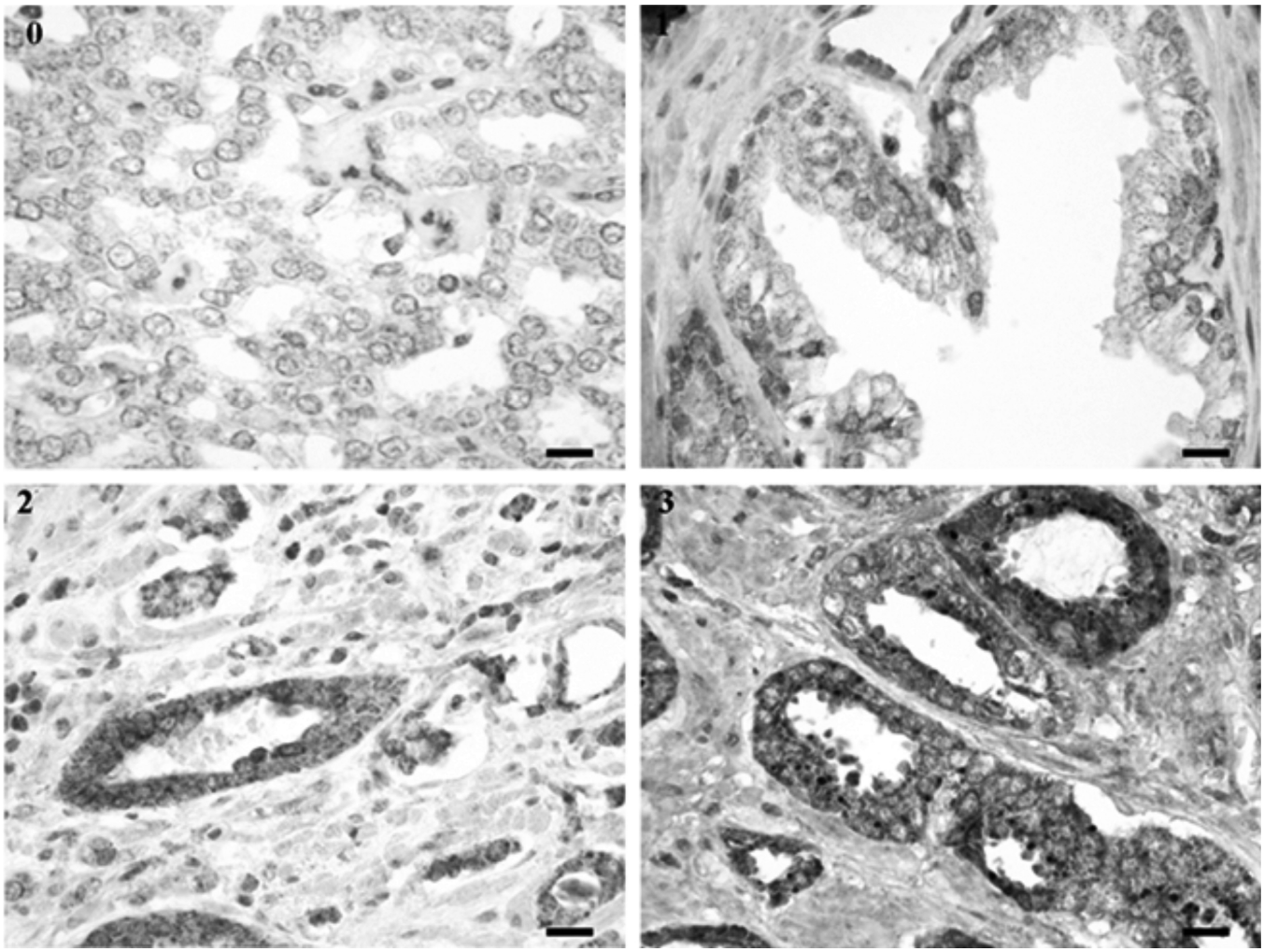

Fig. 1. Examples of semiquantitative grading of immunostained tissue sections. Two independent observers classified the samples as negative (0), weakly positive (1), moderately positive (2), or strongly positive (3). $VPAC_1$ and $VPAC_2$ receptors, and COX-2 were independently studied. Bar: 20 μm.

patients with prostate cancer than in control subjects.

Immunohistochemical analysis

The percentage of positive samples for $VPAC_1$ and $VPAC_2$ receptors and COX-2 after specific immunostaining is indicated in Figure 4. No background immunoreaction to any of the three proteins studied was observed in the corresponding negative controls (Fig. 5A). Samples of skin, testes and cerebellum showed an intense staining for these antibodies. Ganglion presented in the samples was considered as internal positive control (Fig. 5A, inset). According to the immunohistochemical score, an intensity score ≥2 was chosen to classify tumor samples in the $VPAC_1$ and $VPAC_2$ receptor and COX-2 high-expression group, and an intensity score <2 was used to classify them in the low-expression group. The number of positive samples for $VPAC_1$ receptors in prostate cancer was higher (56, 88.9%) than that for $VPAC_2$ receptors and COX-2; the immunolabeling was always located in the nuclei of the neoplastic cells (Fig. 5B). Normal prostates were also positive to $VPAC_1$ receptor but the immunohistochemical staining was lower than that seen in carcinoma (Fig. 5C). $VPAC_2$ receptor was detected in 50 prostate cancer specimens (79.4%); the labeling was observed in the nuclei of the neoplastic cells (Fig. 5D); however, in neuroendocrine cells, the immunoreaction was intense and located in the cytoplasm (Fig. 5E). The expression of COX-2 was located in most (52/63, 82.6%) prostate neoplastic cells (Fig. 5F); the immunolabeling was always confined to cytoplasm.

Statistical analysis

The Fisher's exact tests performed in order to search for relationships among the expression levels of $VPAC_1$ and $VPAC_2$ receptors and COX-2 with several clinicopathological factors including patient age, preoperatory PSA levels, Gleason score, tumor grade and

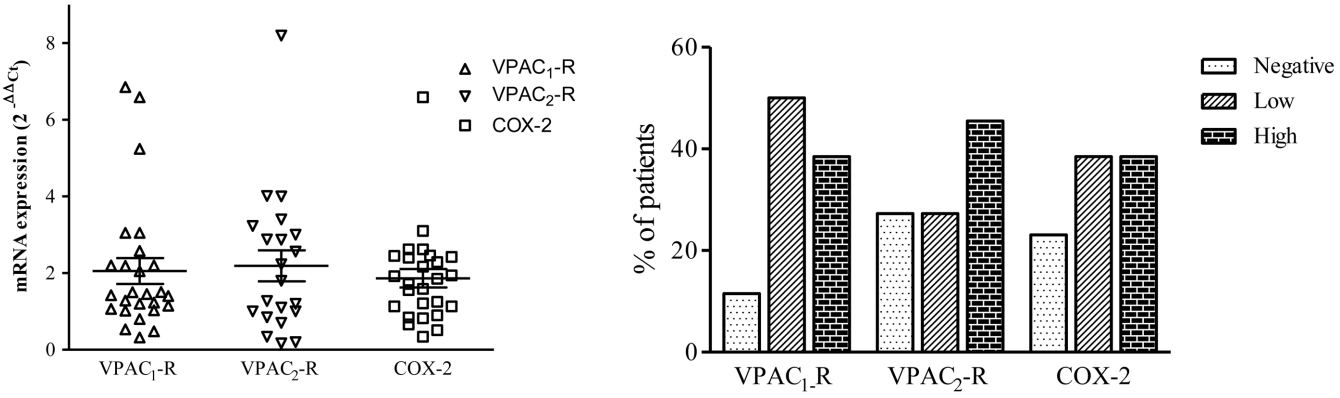

Fig. 2. A. Quantitative reverse transcriptase-PCR results for the levels of expression of $VPAC_1$ and $VPAC_2$ receptor and COX-2 genes in prostate cancer tissue in comparison with healthy tissue from the same patient. **B.** Three levels of expression: negative, low and high, for $2^{-\Delta\Delta Ct}$ values below one, between one and two, or more than two, respectively. The percentage of patients at each level of expression is shown.

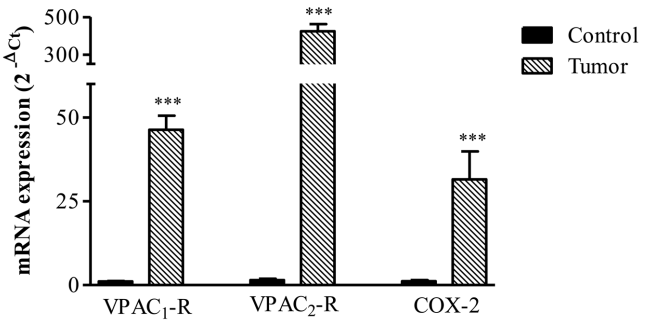

Fig. 3. Quantitative reverse transcriptase-PCR results for the levels of expression of $VPAC_1$ and $VPAC_2$ receptor and COX-2 genes in prostate cancer tissue (n=27) and control prostates (n=9).

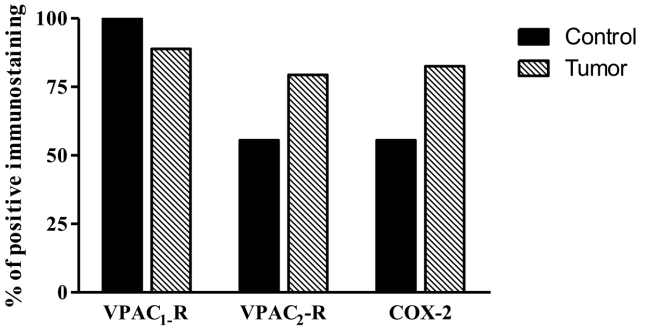

Fig. 4. Immunohistochemical analysis. Percentage of positive samples for $VPAC_1$ and $VPAC_2$ receptors and COX-2 after specific immunostaining of tissue sections.

survival time (Tables 1-3) showed only a positive association between the expression of $VPAC_1$ and $VPAC_2$ receptors. This association was confirmed with the non-parametric Spearman correlation (r=0.494, P=0.014). Patient's age was homogeneous and independent of $VPAC_1$-receptor results. $VPAC_1$ expression was not associated with pT status, Gleason score or pre-operatory PSA value. $VPAC_1$ and $VPAC_2$ receptor and COX-2 expression levels were not correlated with survival time (Fig. 6).

Discussion

It is important to find useful biological markers with the potential to define the aggressiveness of prostate cancer and give prognostic information which will allow stratifying patients into appropriate treatment regimens (Sánchez-Chapado et al., 2003; Slater et al., 2003; Mol et al., 2007; Fritzsche et al., 2008; Niu et al., 2008; Evans, 2009).

PSA is unquestionably the most commonly used circulating biomarker for prostate cancer but its revolutionary role as screening tool is now subjected to controversy (Bensalah et al., 2008). In addition to PSA, a plethora of circulating prostate cancer biomarkers have been considered as promising candidates for prognosis and analysis of disease progression and response to therapy, including insulin-like growth factor-I (IGF-I), urokinase plasminogen activation system, transforming growth factor-ß (TGF-ß), interleukin-6 (IL-6), chromogranin A and prostate cancer autoantibodies (Bensalah et al., 2008). Among other molecules, COX-2, TGF-ß, IL-10, and Ki67 have evolved as potential tissue biomarkers that can better identify the biological nature of prostate tumors and predict which will act more aggressively; however, contradictory results warrant further studies (Howell and Rose-Zerilli, 2007; Evans, 2009).

The expression of VIP receptors in tissue specimens from patients with prostate cancer has been described previously by our group (García-Fernández et al., 2003). We have also shown that VIP action through these

Table 2. Association of $VPAC_2$-receptor immunoexpression with expression of COX-2 and several clinicopathological data of patients with prostate cancer.

			$VPAC_2$ expression				
			Negative	Weak	*P*-value	High	*P*-value
Age	≤65	38	7	17		14	
	>65	25	6	12	1.000	7	0.4913
PSA, ng/ml	3.5-10	43	9	25		9	
	>10	16	2	4	1.000	10	0.1213
pT status	pT2	40	7	19		14	
	pT3	23	6	10	0.5097	7	0.4913
Gleason score	<7	41	10	18		13	
	≥7	22	3	11	0.4852	8	0.4653
COX-2		Negative	5	2		3	
		Weak	2	12		4	
		High	5	16	0.0668	14	0.0852

Fisher's exact test ($P<0.05$).

Table 3. Association of COX-2 immunoexpression with several clinicopathological data of patients with prostate cancer.

			COX-2 expression				
			Negative	Weak	*P*-value	High	*P*-value
Age	≤65	38	6	9		23	
	>65	25	5	8	1.000	12	0.7216
PSA, ng/ml	3.5-10	43	8	14		21	
	>10	16	1	3	1.000	12	0.2319
pT status	pT2	40	7	13		20	
	pT3	23	4	4	0.6715	15	1.000
Gleason score	<7	41	8	13		20	
	≥7	22	3	4	1.000	15	0.4865

Fisher's exact test ($P<0.05$).

receptors induces the expression of COX-2 (Fernández-Martínez et al., 2007). In the present study, our aims were to extend knowledge on the functional role of VIP in the etiopathogenesis of prostate cancer, as well as to contribute to the identification of early diagnostic and prognostic markers for this disease. For these purposes, we studied the correlation of the expression of $VPAC_1$ and $VPAC_2$ receptors and COX-2 in control and cancer prostates as well as the possibility of clinicopathological and prognostic significance of the expression levels of these molecules.

Present quantitative RT-PCR results on matched malignant and normal prostate tissue samples show that $VPAC_1$ and $VPAC_2$ receptors and COX-2 enzyme were overexpressed in a high number of cases (up to 88% for $VPAC_1$ receptors or 52% for the combined three

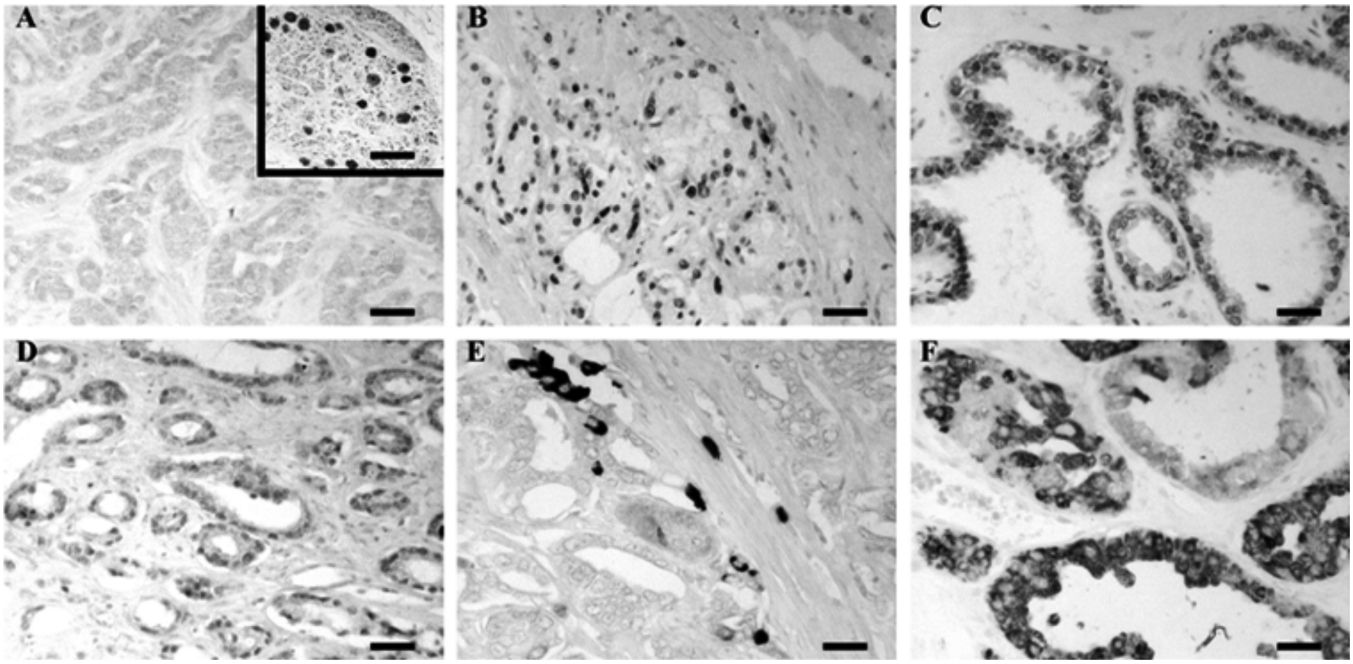

Fig. 5. Immunohistochemical analysis. **A.** Negative control section of prostatic carcinoma was obtained when it was incubated without the primary antibody. Inset: ganglion neurons were positive to $VPAC_1$ receptor. **B.** Prostate cancer tissue displaying an intense immunoexpression to $VPAC_1$ receptor in the nucleus of neoplastic cells. **C.** $VPAC_1$ receptor was also located in the nuclei of glands from control prostates. **D.** Tumoral prostate tissue displaying positive immunolabeling to $VPAC_2$ receptor in the cellular nuclei. **E.** Neuroendocrine cells showing an intense cytoplasmic reaction to $VPAC_2$ receptor; in this sample, the carcinomatous tissue showed no reaction for $VPAC_2$ receptor antibody. **F.** A strong cytoplasmic immunoreaction to COX-2 can be observed in neoplastic glands. Bar: 20 μm

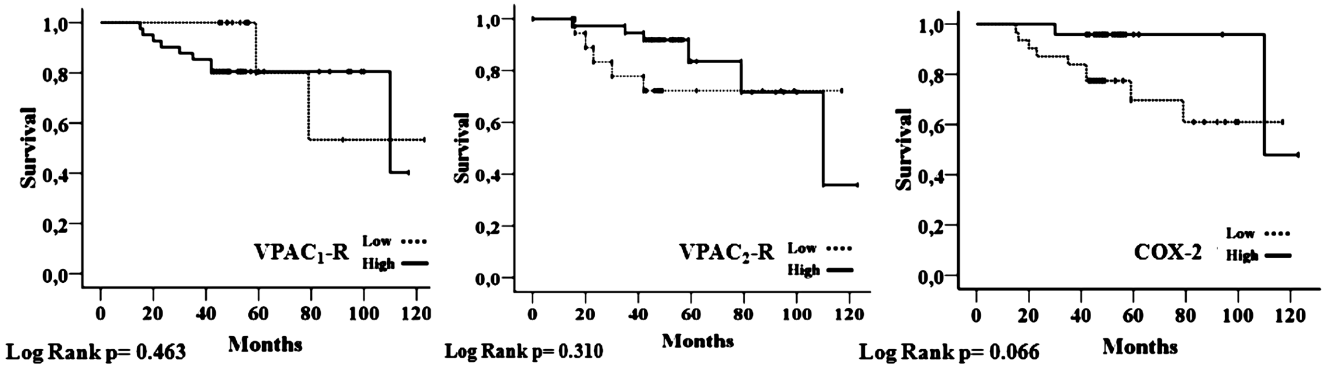

Fig. 6. Kaplan-Meier analysis of the correlation of $VPAC_1$ and $VPAC_2$ receptor and COX-2 expression levels with survival time (n = 63 patients). Marks represent censored data. No statistically significant differences were found with the Log-Rank test, as shown by the corresponding P values.

molecules). These high levels of expression of the three genes were confirmed when comparing cancer and normal tissue pieces from different patients. Moreover, immunohistochemical studies on the corresponding protein levels led to similar observations on overexpression of $VPAC_1$ and $VPAC_2$ receptors and COX-2. The detection of VPAC receptors in intracellular locations is no surprise, since there is an increasing number of reports on the cytoplasmatic and/or nuclear presence of plasma membrane receptors, including VPAC receptors in human breast cancer cells (Valdehita et al., 2010). The localization of VPAC receptors at intracellular levels in both normal and tumor prostate samples does not discount their presence in the plasma membrane. We have previously observed this dual presence of VPAC receptors in the normal human prostate epithelial cell line RWPE-1 after VIP-induced malignant transformation (Fernández-Martínez et al., 2010). Also, we have shown in breast carcinoma subcellular fractions that nuclear VPAC receptors are functional since VIP stimulated cAMP production (Valdehita et al., 2010). A previous study from our laboratory (García-Fernández et al., 2003) showed some increase of immunostaining of VIP receptors but no differences in VIP receptor expression at mRNA and protein levels as measured by means of RT-PCR and western-blot, respectively. Moreover, only a small number of samples were studied so that they must be considered as inconclusive results. We have previously shown the overexpression of the main VIP receptor ($VPAC_1$ receptor) and COX-2 proteins in a xenograft model of tumors derived from prostate cancer PC3 cells, as well as the blocking of $VPAC_1$ receptor expression by a COX-2 inhibitor (Fernández-Martínez et al., 2009). Other studies have shown COX-2 overexpression in prostate cancer (Chang et al., 2002; Fujita et al., 2002; Dandekar and Lokeshwar, 2004; Evans, 2009) but there are reports on high expression levels of this enzyme in benign lesions of the prostate and normal levels in prostate carcinoma (Helpap and Egevad, 2009). Thus, our results support the consideration of $VPAC_1$ and $VPAC_2$ receptors and COX-2 among biomarkers associated with prostate cancer growth.

In multivariate analysis, we could not find any significant association of the overexpression of $VPAC_1$ and $VPAC_2$ receptors and COX-2 with clinico-pathological factors or prognosis of patients with prostate cancer. The intensity of immunostaining for the three proteins in tumor areas was not significantly associated with patient age, preoperatory PSA circulating levels, tumor stage, Gleason score and overall survival time. Only the expression levels of $VPAC_1$ and $VPAC_2$ receptors showed a statistically significant correlation, which reinforces the role of VIP in prostate cancer (Juarranz et al., 2001; García-Fernández et al., 2003; Collado et al., 2004, 2006; Fernández-Martínez et al., 2007). Thus, our study does not support a value of VIP receptors as independent prognostic indicators in this disease. Something similar occurred with the expression level of COX-2 that did not correlate significantly with the clinicopathological features studied; however, the correlation between COX-2 levels and survival time approached the level of statistical significance (p=0.066). An association of COX-2 expression with Gleason score and tumor stage, but not with age or PSA, has been found in Chinese patients with prostate cancer (Chang et al. 2002). Furthermore, overexpression of COX-2 protein has been observed in metastatic prostate tumors (Khor et al., 2007; Sooriakumaran et al., 2009) and in prostate cancer patients who later metastasized (Evans, 2009). In contrast, other reports have dismissed any association with prostate cancer progression (Izawa and Dinney, 2001; Helpap and Egevad, 2009). Interobserver variations, low number of patients and other methodological flaws, as well as race differences can contribute to this controversy. In conclusion, the present study indicates that $VPAC_1$ and $VPAC_2$ receptors and COX-2 may be considered targets for diagnosis of prostate cancer in view of their overexpression. However, it does not support their role as molecular biomarkers for the clinical prognosis of this disease.

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