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Expression of ERK1 and ERK2 in prostate cancer

Norelia Torrealba,[1] Benito Fraile,[1]
Gabriel Olmedilla,[2]
Pilar Martínez-Onsurbe,[2]
Manuel Guil-Cid,[3] Ricardo Paniagua,[1]
Mar Royuela[1]

[1]Department of Biomedicine and
Biotechnology, University of Alcalá,
Madrid; [2]Department of Pathology,
Príncipe de Asturias Hospital, Alcalá de
Henares, Madrid; [3]Department of
Urology, Príncipe de Asturias Hospital,
Alcalá de Henares, Madrid, Spain

Abstract

Prostate cancer may emerge as result of dysregulated balance between cell proliferation and death rates, increased angiogenesis and chronic. These processes are regulated by numerous signaling proteins, including the mitogen-activated protein kinases (MAPKs). JNK, p38 and extracellular signal-regulated kinase (ERK) are the three major sub-families of MAPKs. The pro-oncogenic effects of ERK isoforms (ERK1 and ERK2) lie in their aberrant activation through phosphorylation by any mutation along the pathway of receptor tyrosine kinase (RTK)-Ras-Raf-MEK-ERK1/2. Once activated, ERKs phosphorylate cytoskeletal proteins, kinases, and transcription factors. Active ERK proteins induce strong proliferative and anti-apoptotic effects. Our group has tested variations in expression, activation and localization of ERKs in human prostate. Differential ERK1/2 expression and phosphorylation status may be linked to the progression of prostate cancer. The major striking observation is that ERKs are expressed in tumors with higher proportion than normal prostate. We believe that this is an important notion because the status (expression, localization, phosphorylation and the ERK1/ERK2 ratio) of ERK in the prostate may be developed into an important prognostic marker that predicts patient responce to the anti-cancer treatment.

Introduction

Prostate cancer is the most common cancer among men worldwide, and is one of the most common causes of cancer-related deaths. It is the second most common cancer diagnosed after lung cancer.[1]

The prostate is an accessory male reproductive structure that surrounds the urethra into the pelvic cavity. The function of the prostate is to provide a series of compounds to ejaculate. Human prostate tissue is divided into two components: glands and stroma. Both are separated by a basement membrane.[2] The glands are made of two cell types: basal cells and secretory cells. Another cell type can be also observed by the means of special chromogramin A or B staining.[2]

Prostate cancer (PC) is characterized by slow-growing malignancy associated with a multifocal origin. The majority of these tumors are diagnosed as adenocarcinomas, with its origin in the prostate glands. Prostate tumors grow multifocally in the prostate and rarely produce macroscopic tumor nodules.[4] Tumor origin seems to be the secretory cells[3] although some studies suggest it could also be initiated from basal cells.[4] However, the pathological diagnosis is based on the absence of basal cells.[3,4]

The normal adult prostate is in a homeostatic state, as the result of the balance between the rates of proliferation and cell death. The abnormal growth occurs when the proliferation rate exceeds the rate of cell death, or the death rate falls below the rate of cell proliferation.[5] When the pro-apoptotic pathways are inhibited, the cells can proliferate almost continuously. Such cells lose ability to cease cell cycle, to repair their DNA, or to commit suicide if the DNA damage is not repaired.

Studies on histological-molecular aspects that predict, identify and extend our knowledge about the factors associated with the progression of PC are in high demand. Currently, prostate-specific antigen (PSA), a protein produced by cells of the prostate gland and measured in patient blood sample, serves as high sensitivity organ-specific marker. However, PSA is low specificity marker and thus results in a failure to identify the disease in a significant proportion of patients.[6] Measurements of other proteins may improve diagnostic specificity for early detection and more accurate grading of PC using the Gleason scale. The Gleason grading system assigns PC a score from 1 to 10 based upon the microscopic appearance of tumor cells. Cancers with a higher Gleason score are less differentiated, more aggressive (tending to form local and distant metastases) and have a worse prognosis for the patient survival rate. Tumors with Gleason scores 8-10 tend to be advanced neoplasms that are unlikely to be cured.

In recent years we have been monitoring variations in expression, activation and localization of MAPKs.[7] We used samples from human prostate, obtained by radical prostatectomy, transurethral resection and autopsies. We have correlated the results of immunohistochemistry with clinical data of patients, such as PSA levels (pre- and postoperative), Gleason score, presence of metastases, surgical margin

status, cancer recurrence, and patient survival at five years after prostatectomy (surgical removal of prostate).

Positive surgical margin is defined as the presence of tumor extending through the tinted area that represents the place where the urologist has cut to remove the tumor.[8] Eastham *et al.*[8] described positive surgical margins between 10% and 48% of patients treated with radical prostatectomy. A positive surgical margin has adverse impact on the probability of progression-free survival, both locally and biochemical, and the development of metastases after radical prostatectomy in multivariate analyzes.[9] The risk of biochemical progression with positive surgical margin rate has a 1.5 to 2.6 compared to a negative margin.[8] However, most studies suggest that only a third of patients with positive surgical margin have biochemical progression.[9]

The androgen deprivation therapy is the first-line treatment for metastatic prostate cancer.[10] Decreasing androgen causes tumor regression. Although metastatic patients ini-

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tially have a good response to treatment, in almost all patients there is an increase in circulating levels of androgens within 2-3 years. Some patients develop castration resistance.[10] Therefore, understanding the processes that render prostate cancer cell resistant to androgen deprivation therapy is a major challenge. Accordingly, our research is aimed at identifying prognostic markers that may predict possible relapses, development of hormone resistant prostate cancer and, thus find some prognostic factors after radical prostatectomy for preventing early recurrences.

The cell has three similar signaling pathways called mitogen-activated protein kinase (MAPKs) cascades. MAPK pathways are responsible for transmitting extracellular signals evoked by peptide growth factors, hormones, cytokines or various kinds of stress.[11] They play important roles in the regulation of cell growth, differentiation, survival, apoptosis, and inflammatory stress. The three major subfamilies of MAPKs are JNK (c-Jun NH2-terminal kinase or SAPK), p38 (CSBP) and ERK (extracellular signal-regulated kinase).[12]

JNKs are commonly associated with apoptosis induction, while ERKs are associated with mitogenesis and inversely related to apoptosis. In most biological systems ERKs are associated with proliferation, but in some cases sustained activation of ERK induces cell death or have anti-proliferative effects.[13] Multiple anti-cancer drugs are seen to induce sustained ERK signaling, as pemetrexed in lung cancer, paclitaxel in esophageal squamous cancer or cisplatin in many different cancer cells.[14] Contradictory effects on cell death have been ascribed to p38.[13] While ERK1/2 has been attributed a prominently protumoral role, the activation of JNK and p38-MAPK can exert both pro- and anti-tumor effects, which seems to depend on the cell type, stimulus, intensity and duration of the signal, and the interaction with other signaling pathways. Several studies have recently revealed the involvement of MAPKs in cancer associated with inflammation. The chronic inflammation is related to the possibility of developing prostate cancer.[11,13-16]

Among the signaling pathways most frequently dysregulated in human cancer is the RTK-RAS-RAF-MEK-extracellular signal-regulated kinase 1 and 2 (ERK1/2) pathway.[13,14] In recent years much research has focused on the development of new inhibitors signaling molecules associated with this pathway.[15,16]

ERK1 and ERK2 are involved in the processes triggered by the arrival of mitogenic growth factors and controls in turn cell proliferation and differentiation.[14] ERK1 and ERK2 are activated when the sequence TEY at their activation loop is dually phosphorylated by MEK1/2 kinases, while located in cytoplasm.[14]

The RAS/MAPK cascade is activated by a variety of growth factors, hormones and cytokines. Upon binding to receptor tyrosine kinases (RTKs), such as epidermal growth factor receptor (EGFR) or transforming growth factor receptor I and II (TGFBRI-TGFBRII). These ligands trigger receptor dimerization and autophosphorylation on the tyrosine residues in the C-terminal region, which provides docking sited for adapter proteins SHC (Src homology 2 domain containing transforming protein 1) and GRB2 (growth factor receptor-bound protein 2). GRB2 recruits the cytosolic guanine nucleotide exchange factor SOS (Son of Sevenless) to the plasma membrane. Membrane recruitment is sufficient to activate SOS that in turn activates the membrane-bound small GTPase RAS by catalyzing the exchange of GDP with GTP.[14-17] Then, RAS phosphorylates RAF1. In this way, a MAPK cascade is initiated in which RAF1 phosphorylate sequentially MEK1/2 and ERK1/2. Later, ERK1/2 translocate to the nucleus in a process that culminates in modulation of gene transcription through the activation of several transcription factors such as ETS1, ATF-2, c-FOS, c-MYC, ELK1 or NF-kB.[18] At the same time, it can also phosphorylate cytoplasmic and nuclear kinases, such as MNK1, MNK2, MPKAP2, RSK or MSK1.[19] ERK1/2 are an important checkpoint protein, whose persistent activation may trigger the development of malignancies and play a fundamental role in cancer metastasis.[14,16,20]

At present, the inhibitors of the kinase function of RAF and MEK represent the most studied and advanced approaches for blocking ERK signaling. There are several inhibitors under evaluation but there are not many inhibitors that directly target ERK. The published data is discussed.[14,21]

Materials and Methods

All the procedures were examined and approved by the University of Alcalá and Principe de Asturias Hospital Ethics Committees (PI13/1801; 2013/003/20130214) and were in accordance with the ethical standards of the Committee for Human Experimentation, with the Helsinki Declaration of 1975 (revised in Tokyo 2004) and the Committee on Publication Ethics guidelines. This study was performed with the written consent of the patients or their relatives. All pathological, clinical or personal data were anonymized and separated from any personal identifiers. The present study included: i) transurethral resection or radical prostatectomies from 86 men (aged from 52 to 74 years) with PC (Table 1); and ii) histologically normal prostates (NP) obtained at autopsy (8-10 h after death) from 10 men (aged from 20 to 38 years) without histories or reproductive, endocrine or related diseases.

Immnohistochemical study has been performed as shown in previous publications.[22,23] The primary antibodies used were: rabbit anti-human ERK-1; mouse anti-human ERK-2 and p-ERK (Santa Cruz Biotechnology, CA, USA). The immunostaining expression is the presence or absence of staining in prostate tissue section under study. It is negative when immunohistochemical staining is absent, and positive when staining is observed in the sample. In immunostaining intensity the stain coloration ranging from light pink to deep purple and very classified crosses (+) is observed. In the tumor, the glandular component (acinar and duct) cells are counted with the help of a hematology analyzer by differences according to color staining: weak (1+), moderate (2+) intense (3+) and strong (4+).

In stroma cells are observed microscopically counting the same fields as the tumor and shown as values of percentages. Then both cases are classified depending on the result: Negative (when no staining was observed), low positive, when the result is a cross (1+) and Positive results in high when the crosses are two or more crossings (2+ or greater). These results are used for statistical calculation. The main outcome measure of the study was time to biochemical progression at 10 years, defined as the time between definitive therapy to the first of at least two consecutive elevations in the total serum PSA level above 0.2 ng/mL. Established prognostic variables

Table 1. Clinicopathological features of patients.

	Median (range)
Age (years)	66.00 (52-74)
Preoperative serum PSA (ng/mL)	11.35 (1.4-33)
	% (n)
Preoperative serum PSA (ng/mL)	
<10 ng/mL	36.0 (31)
≥10 ng/mL	64.0 (55)
Gleason score	
<7	82.6 (71)
7	9.3 (8)
>7	8.1 (7)
Clinical T stage	
I	54.7 (47)
II	45.3 (39)
Pathological T stage	
II	65.1 (56)
III	31.4 (27)
IV	3.5 (3)
Perineural invasion (yes)	26.7 (23)
Positive surgical margins	38.4 (33)
Node involvement (yes)	4.7 (4)
Biochemical progression (yes)	33.7 (29)
Survival (yes)	73.3 (63)

included in the study were preoperative serum PSA levels, pathological and clinical T stages, postoperative Gleason score, perineural invasion, lymph node involvement, surgical margin status and overall survival. To evaluate the association between clinicopathological and immunohistochemical variables Spearman's test was performed. Log-rank test and Kaplan-Meier curves were used for survival comparisons. To explore the correlation of the studied immunohistochemical parameters and the established prognostic variables with biochemical progression, univariate and multivariate Cox proportional hazard regression analyses were performed. All statistical analyses[23] were performed using the SPSS 22.0 software (SPSS Inc. Chicago, IL, USA). P values <0.05 were considered as significant.

Results and Discussion

According to immunostaining data, that our group has previously published[22] ERK1 was detected in the cytoplasm of epithelial cells in the 90% of the samples obtained from PC patients (Figure 1A), which was similar to that obtained from normal prostate (Figure 1B) glands (94%).[22] In PC samples, ERK1 optical densities were increased with Gleason scores. ERK2 was detected in cytoplasm of the epithelial cells in 100% of samples obtained from normal prostate (Figure 1D) while in PC group (Figure 1C) appeared in 68 % of patients.[22] Optical density was the highest in PC, but no differences between Gleason groups. The p-ERK was found in the cytoplasm in 40% of normal prostate (Figure 1F) and 30% of PC samples.[22] The highest optical density was observed in PC (Figure 2E) but no differences were found in Gleason score.[22] Several authors showed than ERK is important in the progression of the malignancy in prostate cancer. We saw in our patients with PC a significant expression of ERK, perhaps related with cancer progression. Imada et al.[24] described the biochemical relevance of ERK to the progression of PC, while according to our results Gioeli et al.[25] found the correlation between the increase in ERK activation and advanced PC grade and stage. The normal prostate is composed of glands and stroma. We analyzed the differences of ERK expression in the prostatic stromal and tumor cells. The presence of ERK was indicated as positive result, whereas absence of antibody reactivity with ERK1, ERK2 or their phosphorylated forms (p-ERK) was considered as negative result (Table 2 - Expression). Then, the staining intensities of ERK1, ERK2 and p-ERK were evaluated between stroma and tumor locations (Table 2 - Intensity).

We see as ERK1 is expressed in tumor with

Table 2. Distribution of ERK-1, ERK-2 and p-ERK in PC samples (86) based on immunostaining score for expression and intensity.

Expression		Immunostainig score		
		N % (n)		P % (n)
Stroma	ERK-1	4.7 (4)		95.3 (82)
	ERK-2	38.4 (33)		61.6 (53)
	p-ERK	70.9 (61)		29.1 (25)
Tumor	ERK-1	5.8 (5)		94.2 (81)
	ERK-2	31.4 (27)		68.6 (59)
	p-ERK	69.8 (60)		30.2 (25)
Intensity		**Immunostainig score**		
		N % (n)	P-L % (n)	P-H % (n)
Stroma	ERK-1	4.7 (4)	10.5 (9)	84.9 (73)
	ERK-2	38.4 (33)	16.3 (14)	45.3 (39)
	p-ERK	70.9 (61)	15.1 (13)	14.0 (12)
Tumor	ERK-1	5.8 (5)	1.2 (1)	93.0 (80)
	ERK-2	31.4 (27)	8.1 (7)	60.5 (52)
	p-ERK	69.8 (60)	9.3 (8)	20.9 (18)

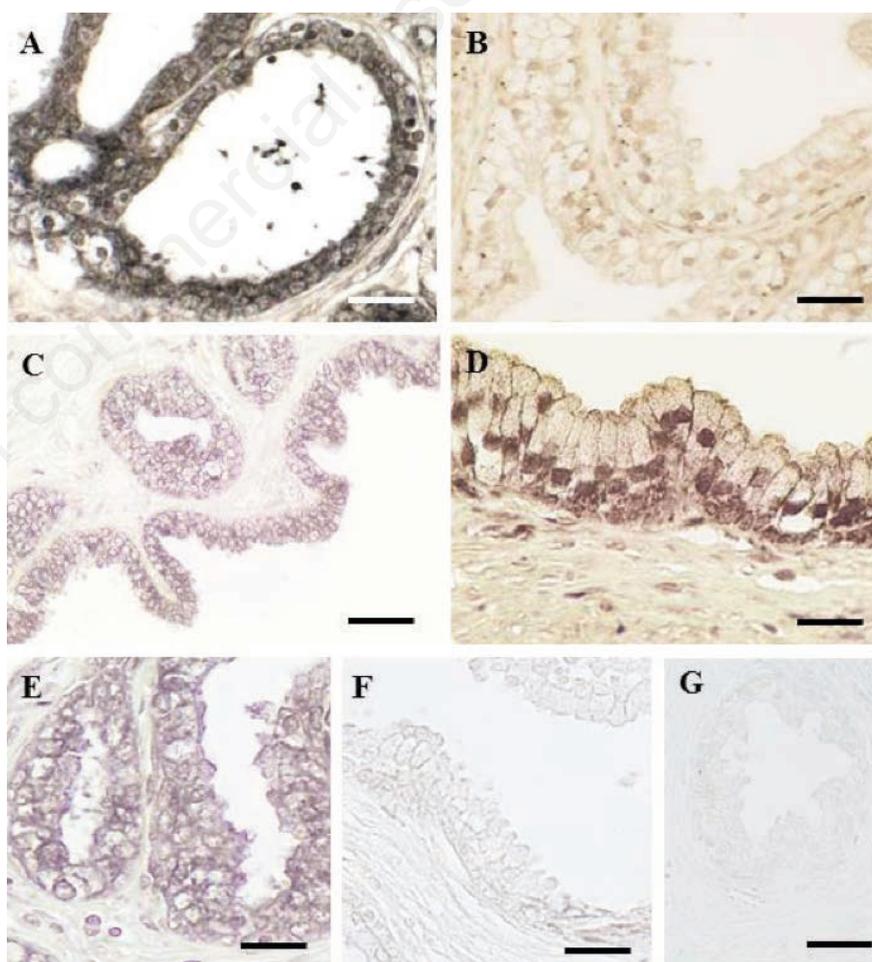N, negative; P-L, Positive-low; P-H, Positive-high. Modified by Rodriguez-Berriguete et al., 2010.[22]

Figure 1. ERK-1 (A, B) ERK2 (C, D), p-ERK (E, F) and negative control (G) in cancer (A, C, E) and normal prostate (B, D, F, G). Scale bars: 30 μm(C, G), 25 μm (A), 20 μm (B, E, F) and 15 μm (D).

higher proportion than ERK2 and p-ERK. Also, we see the intensity in tumor for ERK1 has a higher proportion to ERK2 and p-ERK. For the stroma, the proportions are similar with those described above. Several authors have described as p-ERK is significantly decreased in PC. The authors see that in PC3 and DU145 cells (hormonal independent advanced prostate cancer cells) levels of expression for the members RAS/RAF/MEK/ERK cascades are low. The deletion of PTEN gene in PC could play a role in the suppression of this pathway.[26]

In this analysis, *r* is the correlation coefficient and P marks a statistical significance. The correlation coefficient may range from –1 to 1, where –1 or 1 indicates a *perfect* relationship. The further the coefficient is from 0, regardless of whether it is positive or negative, the stronger the relationship between the two variables. Therefore, a coefficient of –0.213 is almost as strong as a coefficient of 0.241. Positive coefficients tell us that there is a direct relationship: when one variable increases, the other increases. Negative coefficients tell us that there is an inverse relationship: when one variable increases, the other one decreases.

ERK1 expression in stroma has indirect relation with nodal involvement (Table 3 - Stroma) and with positive margins surgical in stromal intensity (Table 3 - Stroma). ERK activation is correlated with increasing of Gleason score, and these patients have a malignancy progression.[26,27] Uzgare *et al.*[27] and Junttila *et al.*[18] demonstrated in TRAMP (transgenic adenocarcinoma of the mouse prostate), that ERK activation has effects in prostatic epithelial cells, and it produces the tumor. ERK has differentially expressed and activated during prostate cancer progression. These authors think that the inactivation of ERK has a relationship with the emergence of a poorly differentiated metastatic and androgen-independent phenotype. Koul *et al.*[28] showed in PC3 cell line that ERK signal transduction pathway has essential paper for cell migration, invasion and clonogenic activity. This clonogenic activity causes cells with the capacity to proliferate a colony of genetically identical cells.

In our patients, p-ERK is related in a direct manner with pathological T stage and indirectly with the survival of patients in the case of the expression in the stroma (Table 3 - Stroma) and epithelium (Table 3 - Tumor). Intensity in tumor patients also stores relationships with the presence of p-ERK in the same manner (Table 3 - Tumor).

After evaluating the correlation of our MAP kinase, we studied the survival curves of Kaplan-Meier. Survival curves for classic markers confirmed the forecast for biochemical recurrence, T value pathological stage (P=0.051), Gleason score (P=0.003) and lymph node metastasis (P=0.000) but not for preoperative serum PSA and perineural invasion, positive surgical margins.[23]

The regression model for univariate Cox proportional hazards were used to study the association between each variable separately study the event of interest (time of biochemical recurrence) providing hazard ratio (Hazard ratio) which estimates differences in risk of experiencing the effect of interest between different groups of patients. It also allows us to corroborate the results of the Kaplan-Meier.[23] In this review we have added charts Kaplan-Meier classical markers to demonstrate that the values of Log rank are corroborated in the study of univariate Cox provided contrary to the values obtained in the survival curves of cytokines study showing no significant values.[23]

We performed a Univariate Cox analysis of classical markers; also we performed analysis for biochemical recurrence.[23] We note that the pathological T stage, Gleason score and node involved relate to biochemical recurrence-free time (Table 4), thus fulfilling our patient group some classical predictions.

We performed survival curves of Kaplan-Meier for the cytokines of the study and we evaluated the univariate Cox. In this group of patients, we did not obtained significant results for any cytokines. We also did not find data in this regard in the literature.

Ricote *et al.*[29] demonstrated that p-ERK has an important role in the increase of proliferation in LNCaP cells; in this study p-ERK was notably increased by TNF-α.

Table 3. Correlation between immunohistochemical variables and clinicopathological features for expression and intensity.

| | Stromal expression | | | | | | Stromal intensity | | | | | |
| | ERK-1 | | ERK-2 | | p-ERK | | ERK-1 | | ERK-2 | | p-ERK | |
	r	p	r	p	r	p	r	p	r	p	r	p
Preoperative serum PSA	-0.501	0.642	-0.094	0.387	0.107	0.325	-0.113	0.301	-0.018	0.873	0.076	0.486
Pathological T stage	-0.102	0.322	0.075	0.491	**0.241**	**0.025**	-0.050	0.647	0.163	0.134	0.201	0.063
Clinical T stage	-0.021	0.850	0.142	0.191	-0.017	0.874	0.054	0.624	0.134	0.219	-0.054	0.623
Gleason score	-0.189	0.081	-0.141	0.194	-0.024	0.824	-0.076	0.485	-0.099	0.363	-0.009	0.931
Perineural invasion	0.009	0.937	0.099	0.366	0.192	0.077	0.034	0.756	0.036	0.740	0.147	0.176
Node involvement	**-0.213**	**0.049**	-0.053	0.629	0.102	0.351	-0.077	0.482	-0.015	0.895	0.144	0.186
Positive surgical margins	-0.053	0.629	0.033	0.766	-0.031	0.775	**-0.259**	**0.016**	0.045	0.681	-0.013	0.407
Biochemical progression	0.041	0.710	0.006	0.953	-0.023	0.831	0.029	0.793	0.056	0.608	-0.015	0.209
Survival	0.133	0.221	-0.009	0.931	**-0.213**	**0.049**	0.045	0.680	0.043	0.693	-0.188	0.084

| | Tumoral expression | | | | | | Tumoral intensity | | | | | |
| | ERK-1 | | ERK-2 | | p-ERK | | ERK-1 | | ERK-2 | | p-ERK | |
	r	p	r	p	r	p	r	p	r	p	r	p
Preoperative serum PSA	-0.083	0.447	-0.143	0.190	**0.231**	**0.033**	-0.020	0.856	-0.161	0.139	**0.219**	**0.043**
Pathological T stage	-0.055	0.613	0.070	0.520	**0.271**	**0.012**	-0.020	0.854	0.056	0.611	**0.239**	**0.026**
Clinical T stage	0.027	0.807	0.163	0.133	0.011	0.923	0.064	0.559	0.106	0.333	-0.009	0.932
Gleason score	-0.148	0.175	-0.151	0.165	-0.002	0.348	-0.117	0.282	-0.185	0.088	-0.103	0.347
Perineural invasion	-0.074	0.496	0.069	0.527	0.174	0.109	-0.043	0.693	0.029	0.793	0.173	0.111
Node involvement	-0.181	0.095	-0.089	0.418	0.095	0.348	-0.159	0.144	-0.064	0.556	0.116	0.288
Positive surgical margins	-0.110	0.311	0.019	0.865	0.001	0.991	-0.157	0.149	0.074	0.498	-0.029	0.793
Biochemical progression	-0.033	0.763	-0.047	0.664	0.012	0.909	0.000	1.000	-0.036	0.745	0.009	0.937
Survival	0.038	0.721	-0.044	0.687	**-0.226**	**0.036**	0.061	0.576	-0.049	0.654	**-0.224**	**0.038**

Bold values indicate significant correlations.

Table 4. Univariate Cox proportional hazard regression analysis for biochemical progression according to classical markers.

	P value	Hazard ratio (95% CI)
Preoperative serum PSA	0.146	1.879 (0.802-4.401)
Pathological T stage	**0.017**	**2.017 (1.132-3.595)**
Gleason score	**0.000**	**2.966 (1.818-4.838)**
Perineural invasion	0.077	1.971 (0.930-4.177)
Node involvement	**0.001**	**6.166 (2.068-18.318)**
Positive surgical margins	0.404	1.367 (0.657-2.844)

P<0.05 is considered as statistically significant. Bold values indicate significant correlations. CI, confidence interval.

Shimada *et al.*[26] has been described the status of activated phosphorylated ERK in human prostate cancer cells with pathological parameters; ERK cannot be activated without being phosphorylated at the same time unless it is a mutant. They suggested that the activation of ERK signaling is related with prostate cancer progression. ERK is commonly considered as anti-apoptotic, cell growth-related kinase, and as such ERK activation favors clinical tumor progression. ERK is associated with malignancies and for this is investigated in several studies. In other tumors as breast, lung, colon and stomach, the role of ERK in the progression and tumor development also has been described.[30,31]

Our research group showed the important role of the IL6/ERK transduction pathway in the activation of NF-kB in these patients.[26,27] IL6/ERK transduction pathway stimulated the translocation of NF-kB to the nucleus in PC. NF-kB in the nucleus promotes the expression of inhibitors of apoptosis proteins and BCL2, and this is relation with the malignance of the tumor.[13,22,23] Our large experience makes us think that the use of these parameters could be a useful tool in clinic-pathological practice.

In the last decade, researchers have progressed in the knowledge of prostate cancer initiation, progression and biomarkers associated with progression of disease. Several studies associate ERK with a pro-tumor role. ERK is involved in several transduction pathways such as IL6/ERK/NF-kB related with advanced malignance and the molecular roles of ERK are not known at all. The aim of future studies might be directed in the knowledge of the different mechanisms that provoke pro-tumor effect of ERK, in order to finding effective therapeutic targets that could reduce the effect of ERK in prostate cancer.

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