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Receptor activator of nuclear factor-κB ligand (RANKL) as a novel prognostic marker in prostate carcinoma

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Summary. Combined immunodetection of parathyroid hormone-related protein (PTHrP) and receptor activator of NF-κB ligand (RANKL) has shown to successfully distinguish poorly- and well-differentiated prostate carcinoma (PCa). In the present study, we aimed to assess whether immunohistochemical evaluation of these factors, and also osteoprotegerin (OPG) and Ki67, in radical prostatectomy specimens can predict biochemical recurrence. Fifty nine PCa cases undergoing radical prostatectomy between 1995 and 1998, without history of neoadjuvant hormonal therapy, were studied. Preoperative serum prostate-specific antigen (PSA), Gleason-sum score, pathologic stage, perineural invasion, seminal vesicle involvement, and positive surgical margins were assessed in these patients. Biochemical recurrence, defined by PSA > 0.4 ng/mL at 90 days or later after prostatectomy, occurred in 32/59 patients. In these patients, positivity for OPG and RANKL in the tumoral epithelium was higher than in those patients with no biochemical recurrence. Using univariate analysis, Gleason-sum score, surgical margins, and seminal vesicle involvement, as well as OPG and RANKL immunostaining (using a score value corresponding to moderate staining as cut-off) were significant predictors of biochemical recurrence ($p<0.05$). Using the multivariate Cox model, among the evaluated factors only RANKL expression (hazard ratio 11.6; $p <0.001$) was an independent prognostic indicator. Our findings suggest that immunohistochemical evaluation of RANKL in the primary tumor is a potential risk factor in PCa patients.

Key words: Osteoprotegerin, Prognostic factors, Prostate carcinoma, PTHrP, RANKL

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Introduction

Prostate cancer (PCa) is the second leading cause of cancer deaths in men in Western countries. The high propensity of this tumor to metastasize to bone is a major cause of morbidity in PCa patients (Landis et al., 1999; Bray et al., 2002). An increasing number of biochemical factors, including chromogranin A -a neuroendocrine cell differentiation marker-, several components of the renin-angiotensin system, and cyclooxygenase-2, are currently being investigated to predict biochemical failure and/or hormone-refractoriness in these patients (Epstein et al., 2005; Kokubo et al., 2005; Cohen et al., 2006).

The receptor activator of nuclear factor-κB ligand (RANKL) -a member of the tumor necrosis factor family secreted by osteoblasts- binds to RANK in osteoclasts, and thus activates osteoclastogenesis and bone resorption; an effect modulated by RANKL decoy receptor osteoprotegerin (OPG) (Hofbauer et al., 2001). The RANKL/RANK/OPG system is also expressed in neoplastic epithelial tissues, such as breast and prostate cancer (Fata et al., 2000; Brown et al., 2001; Holen et al., 2002; Neville-Webbe et al., 2004; Chen et al., 2006; Jones et al., 2006). OPG can prevent the pro-apoptotic action of TRAIL and acts as a survival factor in these neoplastic cells (Holen et al., 2002; Neville-Webbe et al., 2004). In addition, RANKL might facilitate tumor cell seeding in bone by promoting bone resorption, and also cell migration (Hofbauer et al., 2001; Jones et al., 2006). In this regard, PCa-associated bone metastases have shown to exhibit a higher positivity for both RANKL and OPG than nonosseous metastases or the primary tumor (Brown et al., 2001; Chen et al., 2006). Moreover, RANKL has been investigated as a potential therapeutic target in PCa-induced bone disease (Zhang et al., 2003). Supporting further that RANKL is important for PCa development, we recently showed that combined immunodetection of RANKL and parathyroid hormone-

related protein (PTHrP) –which is overexpressed in PCa, apparently associated with tumor progression (Iddon et al., 2000; Asadi and Kukreja, 2005; Deftos et al., 2005; Pérez-Martínez et al., 2007)– successfully distinguishes poorly- and well-differentiated PCa based on Gleason-sum score (Pérez-Martínez et al., 2007).

In the present study, we performed an immunohistochemical evaluation of PTHrP, OPG and RANKL, as well as Ki67 (a well characterized proliferation marker) (Rubio et al., 2005), to determine their relative prognostic value as relating to prostate-specific antigen (PSA) recurrence in 59 patients with clinically localized PCa. Our results show that RANKL positivity in the primary neoplastic tissue is an independent prognostic factor for predicting biochemical failure in these patients.

Materials and methods

Patient characteristics

PCa specimens were obtained from consecutive 59 patients [median age of 64 years (range 50-73 years)] undergoing radical retropubic prostatectomy and pelvic lymphadenectomy between 1995 and 1998 at our Institution. Patients were selected based on the following exclusion criteria: neoadjuvant hormonal therapy administration, node positivity, and clinical evidence of systemic metastases prior to radical prostatectomy; and serum prostate-specific antigen (PSA) > 0.4 ng/mL one month following radical prostatectomy. Our population had a median follow-up of 83 months (range 3 to 108). Biochemical recurrence was defined as a PSA value > 0.4 ng/mL after radical prostatectomy, according to recently suggested criteria (Amling et al., 2001). Of these 59 patients, 26 (44%) and 31 (53%) patients had biochemical recurrence at 5 and 7 years, respectively. The minimum follow up on patients free of biochemical recurrence was 81 months. Clinicopathological features, including pathological stage as determined according to TNM classification (Sobin and Wittekind, 2002), in these patients are shown in Table 1. The study was performed in accordance with the ethical standards of the Helsinki Declaration.

Immunohistochemistry

Serial PCa specimens were obtained from the extracted whole prostatic piece during prostatectomy. Paraffin-embedded tissue sections (3µm) were completely examined by a pathologist to confirm the presence of localized malignant tissue. PCa samples representing the major Gleason-sum score (Gleason, 1992), as assessed by the pathologist, were selected for immunostaining. First, samples were deparaffinized and rehydrated. Antigen retrieval was done by pressure cooker treatment in 10 mM citrate buffer, pH 6 (PTHrP, OPG, and RANKL) or microwave treatment (Ki67). For RANKL staining, PCa samples were pretreated with 100 mM glycine, pH 3, for 20 min (initial experiments showed that this maneuver improved the performance of the immunohistochemical technique). The tissue samples were then processed in an automatic device (TechMate500; Dako, Glostrup, Denmark), according to standard procedures (Pérez-Martínez et al., 2007). Briefly, endogenous peroxidase and nonspecific binding were blocked. Then, sections were incubated for 30 min at room temperature with the primary antibodies. PTHrP immunostaining was performed using rabbit polyclonal C-terminal antiserum C6, at 1:600 dilution. OPG and RANKL staining were carried out with rabbit polyclonal antibodies H-249 (OPG) and FL-317 (RANKL) (Santa Cruz Biotechnology, Santa Cruz, CA), at 1:100 and 1:40 dilution, respectively. These antibodies recognize an epitope mapping to 153-401 or 46-317 sequence in full length human OPG or RANKL molecule, respectively. Ki67 (clone MIB1) (Dako) was immunostained with a specific mouse monoclonal antibody, at 1:100 dilution. The tissue sections were subsequently incubated with a polymer-peroxidase complex (Envision+ System; Dako) and 3,3'-diaminobenzidine, and counterstained with hematoxylin. PCa specimens showing marked positivity with each primary antibody, or incubated without the primary antibody, were used as positive or negative controls, respectively.

Postivity was evaluated in five x200 microscopic fields, in both neoplastic and adjacent non-tumoral areas.

Table 1. Clinicopathologic parameters in PCa patients.

Parameter	No. of Patients (%)
Median age (range), years	64 (50-73)
Preoperative serum PSA level	
< 10 ng/mL	24 (41)
≥ 10 ng/mL	35 (59)
Gleason-sum score	
Gleason 4	3 (5)
Gleason 5-6	24 (40)
Gleason 7	22 (37)
Gleason 8-10	10 (20)
Pathologic stage	
pT2N0M0[a]	37 (63)
pT3N0M0[b]	22 (37)
Perineural invasion	
No	24 (40)
Yes	35 (60)
Seminal vesicle involvement	
No	51(86)
Yes	8 (14)
Positive surgical margins	
No	29 (40)
Yes	30 (60)
Disease status	
No progression	27 (43)
Progression	32 (57)
Median prostate weight (range), g	48 (15-90)

PSA, prostate specific antigen. [a]: Organ confined. [b]: Presence of extraprostatic involvement.

Ki67 staining was expressed as the number of stained nuclei per total nuclei (%). Positivity for PTHrP, OPG and RANKL was quantified according to published criteria (Pérez-Martínez et al., 2007). First, positive epithelial area was graded as (%): 1 (<25), 2 (25-75), and 3 (>75), using image analysis software (Optimas 6.5; Media Cybernetics, Silver Spring, Washington). Second, staining intensity was scored from 1 to 3 (most intense), based on the most frequent intensity in the five fields evaluated. The product of the two scores was considered as the final score: 0, negative; 1 to 3, weak; 4, moderate; and 6 to 9, strong. A pathologist and another independent observer evaluated all samples in a blinded fashion. Discrepancies in staining scores between both evaluations were resolved by simultaneous reexamination of those samples to yield a final score value.

Statistical analysis

Differences in both clinicopathological and immunohistochemical variables between PCa groups according to biochemical outcome were analyzed by Mann-Whitney U test. Comparison between immunostaining score values in neoplastic and adjacent non-tumoral areas was performed by simple Wilcoxon rank test. Correlations were assessed by Spearman's rho coefficient. Kruskal-Wallis test (for Gleason-sum score) or Mann-Whitney U test (for pre-operative serum PSA levels and other clinicopathological variables) were used to evaluate differences in immunostainings in the neoplastic area, according to categories as defined in Table 1. Biochemical progression-free survival regarding the different potential risk factors was evaluated by Kaplan-Meier method using the log-rank test as statistical contrast. The variables with statistical significance to predict biochemical progression-free survival in the latter univariate analysis were evaluated with multivariate Cox proportional hazard model to assess their independent importance in predicting survival. Statistical significance was considered at $p<0.05$. Statistical analysis was performed using the SPSS software program (version 13.0; Chicago, IL).

Results

Immunostaining for PTHrP and OPG mainly localized to acinar cells in neoplastic and adjacent non-tumoral areas in specimens from patients with or without biochemical recurrence (Fig. 1). The staining pattern for RANKL was similar to that for the two aforementioned factors, but patchy positivity was also evident in secretory luminal cell membranes from the more intensely stained tumors (Fig. 1). In the neoplastic epithelium, 95% (56/59) of PCa samples showed positivity for PTHrP; whereas only 51% (30/59) and 64% (38/59) samples were positive for OPG and RANKL, respectively. Score values for the latter two factors were significantly ($p<0.01$) correlated. Biochemical recurrence was not found to be associated with the presence of bone metastases in these patients, except in one, showing these metastases at 2 years after surgery (preoperative serum PSA level = 9.8 ng/ml). Primary PCa in this patient had weak Ki67 staining, and moderate positivity for PTHrP and RANKL, whereas it was negative for OPG.

Score values for OPG and RANKL in neoplastic and adjacent non-tumoral areas, but not those for Ki67 or PTHrP, were significantly higher in those patients with biochemical recurrence (Table 2). Moreover, the percentage of tumors with moderate to strong positivity for these bone-related cytokines in patients with or without biochemical failure were, respectively: 75% (24/32) and 81% (22/27) (PTHrP); 50% (16/32) and 15% (4/27) (OPG); and 62% (20/32) and 4% (1/27) (RANKL). No significant differences were found between staining score values for any of these factors in PCa groups divided according to pT stage, perineural invasion, seminal vesicle involvement, surgical margin positivity or preoperative serum PSA (Table 1).

Biochemical recurrence at 5 and 7 years for those PCa patients with score values of ≥ 4 for RANKL

Table 2. Immunohistochemical staining for Ki67, PTHrP, OPG and RANKL in PCa patients.

Progression	Ki67		PTHrP		OPG		RANKL	
	Non-tumoral area	Tumoral area	Non-tumoral area	Tumoral area	Non-tumoral area	Tumoral area	Non-tumoral area	Tumoral area
Biochemical recurrence (n=32)	Median: 2.5% (1-18%) Mean: 4.7±0.6%	Median: 5.0% (1-28%) Mean: 6.8±0.9%*	Median: 4 (0-9) Mean: 3.5±0.3	Median: 6 (0-9) Mean: 5.3±0.4*	Median: 1 (0-9) Mean: 2.1±0.4	Median: 3 (0-9) Mean: 3.5±0.4*	Median: 2 (0-6) Mean: 2.0±0.2	Median: 4 (0-9) Mean: 4.0±0.4*
No biochemical recurrence (n=27)	Median: 2.5% (0-11%) Mean: 2.5±0.2%	Median: 2.0% (0-17%) Mean: 4.3±0.6%*	Median: 4 (0-9) Mean: 4.1±0.3	Median: 4 (2-9) Mean: 5.1±0.3*	Median: 0 (0-4) Mean: 0.7±0.1†	Median: 0 (0-6) Mean: 1.1±0.2†	Median: 0 (0-4) Mean: 0.7±0.2†	Median: 0 (0-4) Mean: 1.0±0.1†

PTHrP, parathyroid hormone-related protein; OPG, osteoprotegerin; RANKL, receptor activator of NF-κB ligand. *: $p<0.05$ vs corresponding values in the non-tumoral area. †: $p<0.05$ vs corresponding values in the group of patients with biochemical recurrence. Values represent median (range) (upper line) and mean ± SEM (bottom line) in each case.

occurred in 86% (18/21) and 95% (20/21) patients, respectively; whereas for those with score values <4 for this factor, it occurred in 21% (8/38) and 29% (11/38) patients, respectively. Gleason-sum score, surgical margins, and seminal vesicle involvement, as well as OPG and RANKL staining (using a score value of 4 as

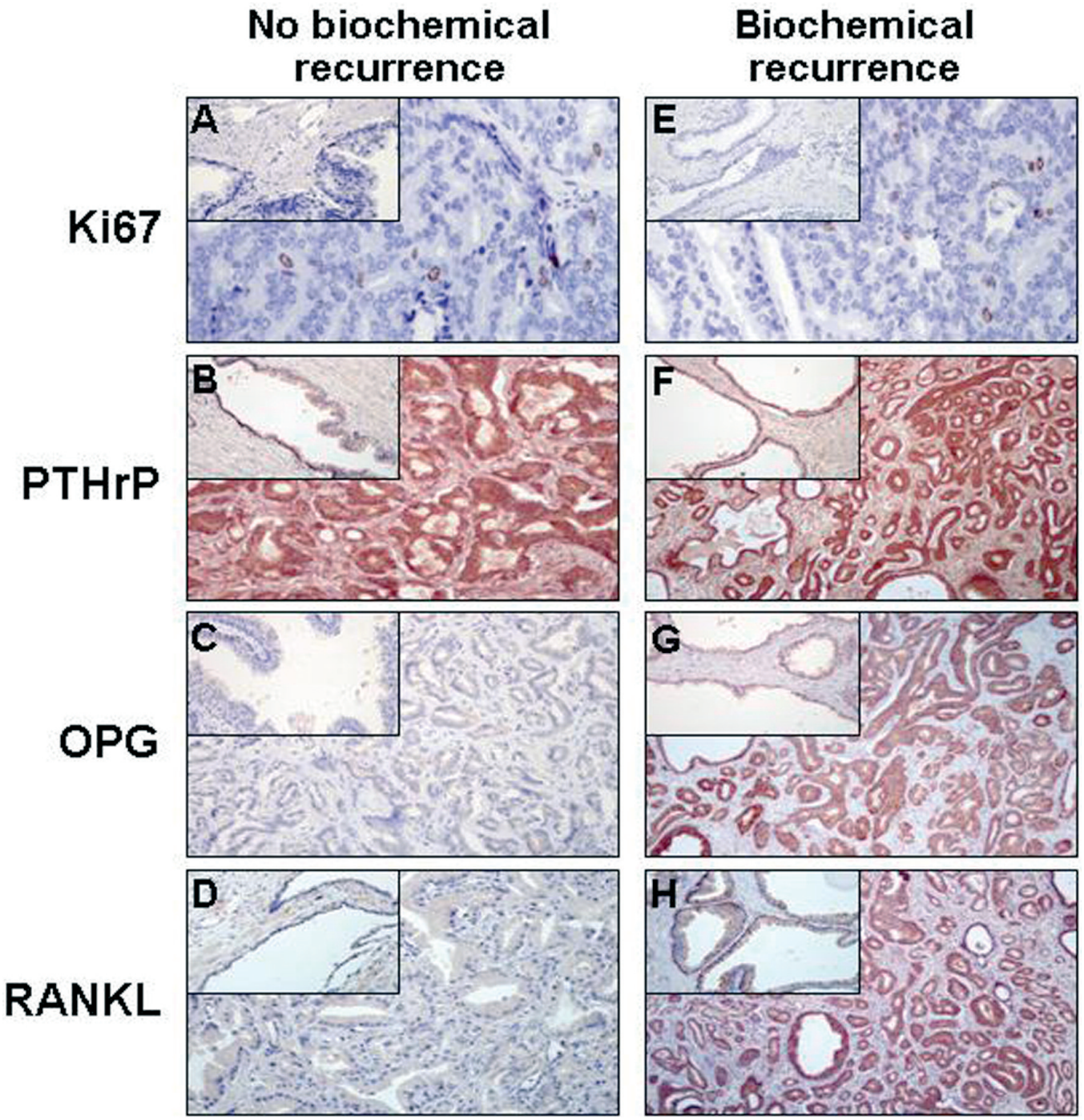

Fig. 1. Immunohistochemical evaluation of several factors in two groups of PCa patients, according to biochemical outcome. Immunostaining for Ki67 (**A, E)**, PTHrP (**B, F**), OPG (**C, G**), and RANKL (**D,H**) was performed with specific antibodies, as described in the text. Shown are representative PCa tissue samples from patients without (**A-D**) and with (**E-H**) biochemical recurrence. Adjacent non-tumoral areas are shown in the inset at the upper left corner of each panel. Negative controls (without corresponding primary antibody) exhibited no positivity (data not shown). x 200

cut-off, corresponding to moderate staining), were significantly associated with biochemical recurrence in the univariate Kaplan-Meier model (Table 3). However, using the multivariate Cox model, only RANKL retained statistical significance in estimating the hazard of biochemical recurrence, which was 11.6 for the PCa population (Table 4).

Discussion

We explored the association between immunoreactive PTHrP, OPG, and RANKL, as well as Ki67, in the primary tumor and the biochemical outcome in PCa patients. This report extends the scope of our recent study evaluating these factors in poorly- and well-differentiated PCa (Pérez-Martínez et al., 2007).

In PCa cells, PTHrP increases proliferation and inhibits apoptosis by complex mechanisms (Iwamura et al., 1994; Tovar Sepulveda and Falzon, 2002). Moreover, coexpression of PTHrP and the PTH/PTHrP type 1 receptor is a common finding in PCa metastases to bone (Iddon et al., 2000). It has been hypothesized that PTHrP, by increasing bone resorption, might promote malignant cell seeding to bone (Deftos et al., 2005). In the present study, PTHrP staining intensity was high in the neoplastic tissue in PCa patients, but it was similar in those with or without biochemical recurrence. Moreover, univariate Kaplan-Meier analysis showed that PTHrP overstaining in PCa tissue failed to predict biochemical recurrence in these PCa patients. In the population studied, only one patient -with moderate PTHrP staining- displayed skeletal involvement. Our findings, however, do not rule out that an increased PTHrP production by PCa cells in the bone microenvironment might facilitate the formation of bone metastases in patients with more advanced PCa tumors.

The present report, consistent with our previous study (Pérez-Martínez et al., 2007), further confirms that OPG and RANKL are present in the majority of primary PCa samples, mainly in those from patients with biochemical recurrence in the period of study after prostatectomy. In fact, the staining score levels for both factors were significantly higher in the latter patients than in those with no biochemical recurrence. Several studies support the concept that OPG expression by tumor cells might confer a cell survival advantage (Holen et al., 2002; Neville-Webbe et al., 2004). Of note, in this regard, OPG-overproducing gastric carcinoma has been reported to be associated with poor prognosis (Ito et al., 2003). In the present study, only one patient in our cohort showed biochemical recurrence related to bone metastases, and had undetectable OPG and moderate RANKL and PTHrP positivities in the primary tumor. In this regard, results using an experimental PCa model indicate that OPG injection into the mouse tibia prevents the tumor growth at this site (Zhang et al., 2001). On the other hand, previous studies have found OPG overexpression in metastatic PCa, mainly in bone metastases (Brown et al., 2001; Chen et al., 2006). As recently suggested (Inoue et al., 2005), OPG might be unable to interact with PCa products other than RANKL (i.e., PTHrP) which would promote skeletal metastases by RANKL-independent mechanisms. Therefore, the true role of OPG in PCa development is presently unclear.

There are currently few reliable markers as accurate predictors of PCa recurrence besides Gleason-sum score (Swindle et al., 2003). In those patients with Gleason-sum score of 5-7, accurate prognostic indicators might particularly improve the physicians' ability to identify PCa aggressiveness, so that more individualized treatments could be offered. In this study, including a majority of patients with Gleason-sum score in the latter range, this score and other clinicopathologic parameters failed to provide independent prognostic information, consistent with other studies with a similar cohort of patients (Kokubo et al., 2005; Cohen et al., 2006). However, our present results using multivariate Cox analysis demonstrate that RANKL immunostaining in

Table 3. Kaplan-Meier analysis to estimate biochemical recurrence in PCa patients stratified by various clinicopathologic and immunohistochemical parameters.

Parameter	Chi-Square	p-value
Gleason-sum score (4/5-6/7/8-10)	3.852	0.050*
Pathologic stage (pT2/pT3)	0.898	0.343
Preoperative serum PSA level (<10 / ≥ 10 ng/ml)	0.580	0.446
Surgical margins (negative/positive)	4.052	0.044*
Perineural invasion (no/yes)	2.509	0.113
Seminal vesicle involvement (no/yes)	7.631	0.011*
Ki67 (< 4%/ ≥ 4 %)	0.550	0.458
PTHrP (< 4/ ≥ 4)	0.001	0.994
OPG (< 4/ ≥ 4)	9.532	0.002*
RANKL (<4 / ≥ 4)	32.399	<0.001*

PSA, prostate specific antigen; PTHrP, parathyroid hormone-related protein; OPG, osteoprotegerin; RANKL, receptor activator of NF-κB ligand. *: Statistically significant.

Table 4. Multivariate analysis using COX regression model in PCa patients.

			95% CI for hazard ratio	
Parameter	p-value	Hazard ratio	Lower	Upper
Gleason-sum score	0.260	0.840	0.001	6.246
Surgical margins (negative/positive)	0.315	1.505	0.678	3.340
Seminal vesicle involvement (no/yes)	0.182	0.989	0.974	1.005
OPG	0.621	1.513	0.293	7.806
RANKL	<0.001*	11.651	2.748	49.389

OPG, osteoprotegerin; RANKL, receptor activator of NF-κB ligand; *: Statistically significant; CI: Confidence interval.

PCa tissue, in contrast to the other evaluated parameters, had a statistical significance to predict biochemical failure in PCa patients. The finding that RANKL staining was increased in both non-neoplastic and neoplastic areas in these patients with biochemical recurrence might suggest a paracrine mechanism as responsible for RANKL induction. Thus, RANKL could be considered as a potential risk factor in PCa patients. However, a larger amount of patients than the relatively small cohort of patients included in the present study is needed to confirm the usefulness of RANKL as a risk factor in PCa.

RANKL can exert a variety of actions unrelated to its effects on bone turnover: it stimulates mammary epithelial cell differentiation and lymphocyte development, and has pro-inflammatory features (Fata et al., 2000; Seshasayee et al., 2004). Interestingly, inflammation related to NF-κB activation is emerging as a putative mechanism of malignant transformation and progression (Lucia and Torkko, 2004). In addition, RANKL increases the expression of several Bcl-2 anti-apoptotic family members, and thus enhances dendritic cell survival (Wong et al., 1997). Whether RANKL might act as a pro-survival factor in PCa cells remains to be determined. RANKL has shown to promote the migration of several cancer cell types, including PCa cells, related to their preferential spread to bone (Jones et al., 2006). Interestingly, in this regard, the only patient in our PCa population who had bone metastases was negative for OPG but showed moderate staining for RANKL in the primary tumor.

In summary, PCa prognosis is now evolving towards new molecular systems to predict more accurately tumor recurrence. Present findings suggest that RANKL immunoreactivity in the primary tumor appears to independently predict PCa recurrence. Based on the present data, we think that RANKL should be assessed thoroughly in primary PCa samples to validate it as an independent prognostic factor in PCa patients.

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