Journal of
Cancer Science & Therapy

Vlachostergios et al., J Cancer Sci Ther 2011, S1
DOI: 10.4172/1948-5956.S1-005

Research Article Open Access

CD10 and Endothelin-1 are Inversely Expressed in Early Prostate Cancer and Predict PSA Failure after Radical Prostatectomy

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Abstract

Background: Loss of the membrane endopeptidase CD10 plays an important role in the development of neuropeptide-mediated androgen-independent prostate cancer cell growth. The aim of this study was to investigate the potential prognostic value of the CD10/neuropeptide axis with regard to prostate-specific antigen (PSA) failure after radical prostatectomy in early prostate cancer patients.

Methods: Tumor samples from 70 early prostate cancer patients who underwent radical prostatectomy were immunohistochemically evaluated for expression of CD10 and endothelin-1 (ET-1). The examined parameters were prospectively correlated with time to PSA failure and combined with Gleason grade and pathological TNM stage.

Results: Membranous and apical cytoplasmic expression of CD10 was directly correlated with time to PSA failure ($P < 0.001$). Cytoplasmic ET-1 was inversely correlated with time to PSA relapse ($P = 0.002$). CD10 and ET-1 were inversely interrelated ($P < 0.001$). CD10 expression ($P = 0.012$) and stage ($P = 0.013$) were independent predictors of biochemical recurrence.

Conclusion: CD10 and ET-1 follow inverse patterns of expression in tumors of early prostate cancer patients, in accordance with their biological roles and molecular interrelations. Evaluation of CD10 expression in early prostate cancer might contribute to a better prediction of PSA relapse-free survival after radical prostatectomy.

Keywords: PSA failure; CD10; Endothelin-1; Radical prostatectomy; Prostate cancer

Introduction

The investigation of endothelins, a family of potent vasoconstrictor peptides with mitogenic properties relevant to carcinogenesis and cancer progression, has consistently provided results implicating endothelin-1 (ET-1) in the pathophysiology of prostate cancer. ET-1 has been shown to be abundantly produced and induce proliferation of prostate cancer cells *in vitro*, while i*n vivo*, high ET-1 levels were detected in the majority of plasma and tissue specimens from male patients with hormone-refractory prostate cancer [1-3]. ET-1 exerts its effects through the ET-A receptor subtype, which shows increased expression in prostate cancer. Concurrently, expression of the ET-B receptor, which mediates ET-1 clearance, is low in prostate cancer, thus contributing to reduced clearance and sustaining/amplification of ET-1 signaling and autocrine/paracrine growth effects [4-6]. Stimulation of ET-1 secretion has been related to a cytokine-mediated paracrine/autocrine mechanism, involving interleukin 1β (IL-1β), tumor necrosis factor α (TNF-α), interferon-gamma (IFNγ) and transforming growth factor β (TGF-β), notably via NFκB activation [7,8]. In contrast, it has been postulated that androgens exert a negative role in the regulation of ET-1 production by prostate cancer cells [9].

ET-1 can act alone as a mitogen but its mitogenic effects are greatest in synergy with a variety of growth factors, including basic fibroblast growth factor (b-FGF), insulin-like growth factors (IGFs) and platelet derived growth factor (PDGF) [10]. Further, ET-1 in conjunction with vascular endothelial growth factor (VEGF) appears to play a major role in tumor angiogenesis [10]. ET-1 signaling also mediates anti-apoptotic effects via activation of the ERK1/2 and PI3K/Akt pathways as well as through downregulation of the proapoptotic Bcl-2 family members, Bad, Bax and Bak [11,12]. ET-1 also appears to be a mitogen for osteoblasts and is involved in new bone formation and the induction of pain in metastatic prostate cancer [5,6,13]. The concomitant increase in ET_A receptor expression in aggressive prostate carcinoma combined with preclinical activity of ET_A receptor blockade [14] has encouraged the consideration and clinical exploitation of ET_A receptor as a promising target for therapeutic intervention. Completed phase 2 and 3 trials of atrasentan (the most well studied ET_A receptor antagonist) have demonstrated a consistent modest effect of targeting osseous prostate cancer metastases, although more questions are expected to be answered by ongoing phase 3 studies [15].

The enzyme responsible for cleavage and inactivation of ET-1 and other bioactive neuropeptides is endopeptidase 24.11, also termed neutral endopeptidase (NEP) or CD10. CD10 is a cell surface peptidase that is normally expressed by various tissues, including prostate [16,17]. Loss of CD10 expression [18], most frequently via promoter hypermethylation [19], as well as decreased androgen-mediated

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Received October 24, 2011; **Accepted** November 09, 2011; **Published** November 11, 2011

Citation: Vlachostergios PJ, Karasavvidou F, Kakkas G, Moutzouris G, Patrikidou A, et al. (2011) CD10 and Endothelin-1 are Inversely Expressed in Early Prostate Cancer and Predict PSA Failure after Radical Prostatectomy. J Cancer Sci Ther S1. doi:10.4172/1948-5956.S1-005

transcriptional stimulation secondary to androgen deprivation therapy [20,21] is a frequent incidence in prostate cancer and has been consistently correlated with tumor progression to androgen-independence by allowing neuropeptide growth-promoting effects [18]. The regulatory role of CD10 in prostate cancer involves inhibition of cell migration via abrogation of molecular events downstream of neuropeptide signaling, involving c-Src and FAK (focal adhesion) kinases [22]. Additionally, CD10 inhibits cross-communication between ET-1-stimulated G-protein coupled receptor (GPCR) and IGF-1R signaling pathways, thus blocking Akt-mediated anti-apoptotic effects [23]. Decreased CD10 in advanced prostate cancer contributes to attenuation of phorbol-ester-induced cell death via promotion of c-Src-mediated PKC-δ degradation [24]. Reversal of CD10 loss in induced androgen receptor-expressing [25] or transfectant prostate cancer cell lines [26-28] or with the use of recombinant CD10 [29,30] suppresses proliferative, invasive and angiogenic effects, and sensitizes cells to anticancer drug-induced apoptosis [29].

The accumulation of preclinical evidence for the critical involvement of the CD10/endothelin axis in the neuroendocrine differentiation and progression of prostate cancer [31-33] necessitated the conduction of studies in the clinical setting, with the aim of elucidating its expression and potential correlations with clinical parameters that determine prognosis, particularly in the early stages of the disease. Indeed, a plethora of evidence from immunohistochemical studies of radical prostatectomy specimens has accumulated; however, the existence of conflicting results as well as the lack of an integrated comparative investigation of CD10 and ET-1 still hampers proper interpretation of these data towards refining the already existing prognostic models of the disease. In this direction, we have investigated the clinical relevance of both CD10 and endothelin-1 (ET-1) in a cohort of hormone-naïve patients subjected to radical prostatectomy for early stage prostate cancer, using the time to biochemical failure as an indicator of response, and also taking into account other known prognostic factors, including pathological stage and Gleason score.

Materials and Methods

Subjects

The study enrolled 70 patients between 47 and 75 years old (mean: 64.5 years, median: 66 years) with histologically newly diagnosed, early stage prostate cancer, admitted to the Department of Urology, University Hospital of Larissa. All patients of the study underwent an open retropubic radical prostatectomy. Patients were hormone- and treatment-naïve at the time of surgery. The study was approved by the Ethics and Scientific Committees of our Institution and written informed consent was provided by all patients before study entry. Pathological parameters including pathological TNM stage and Gleason score of the primary tumor as well as time to PSA failure and survival data were recorded. Hematoxylin and eosin stained tissue sections were examined by a single, blinded histopathologist, based on the availability of both adequate follow-up and representative pathology specimens. Cases were divided into 2 Gleason groups: low (≤3+4; n=50) and high (≥4+3; n=20). Cases were also grouped according to stage into either organ confined disease (pT≤2; n=42) or advanced tumors extending beyond the prostatic capsule (pT≥3; n=28).

Immunohistochemistry

The radical prostatectomy specimens were fixed in 10% buffered formalin solution and embedded in paraffin blocks. Serial sections (4 μm) from selected 1 or 2 paraffin blocks of each case were obtained. Tissue blocks were chosen based on the presence of both, the primary and the secondary architectural Gleason pattern of prostate adenocarcinoma, as determined on hematoxylin and eosin sections. Sections were deparaffinised in xylene and rehydrated through decreasing alcohols. Antigen unmasking for CD10 and ET-1 was achieved by boiling sections in Trilogy reagent (Cell Marque, Rocklin, Calif) for a total of 1 hour in a commercially available steamer. After quenching endogenous peroxidase with 3% hydrogen peroxide solution for 10 min, slides were incubated at room temperature for 30 minutes with mouse monoclonal anti-CD10 (clone 56C6, 1:30 dilution, DAKO, Denmark). Adjacent sections were incubated overnight at 4^{O}C with mouse monoclonal antibody against ET-1 (clone TRET-485, 1:100 dilution, SIGMA, UK). Staining was developed with substrate chromogen solution (EnVision, DAKO, Glostrup, Denmark) and diaminobenzidine for 10 minutes. Slides were counterstained with Harris hematoxylin for 1 minute, dehydrated, and mounted with DPX solution.

The normal adjacent prostate gland was used as an internal control marker for the evaluation of CD10 expression. Intensity of CD10 staining of tumor cells was evaluated and was categorized as negative, weak, moderate and strong. For statistical analysis, negative and weak staining were considered low versus moderate and strong staining which were classified as high expression. Intensity of ET-1 staining of tumor cells was categorized according to the staining of endothelial cells used as internal controls into weak, moderate, or strong categories. The weak staining category was assessed to be less than that of endothelial cells, the moderate staining category was determined to be equal to that of endothelial cells, and the intense staining category exhibited more than that of endothelial cells. Weak and moderate staining (low) versus strong (high) ET-1 expression was evaluated for statistical analysis.

Study endpoints

Our objective was to investigate possible interrelations between immunohistochemical expression of CD10 and ET-1 as well as their potential correlations with Gleason score, stage and time to PSA relapse in patients with early prostate cancer undergoing radical prostatectomy. The response variable was defined as the time from radical prostatectomy to the time of the first detectable PSA measurement.

Statistical methods

The Fisher's and χ^2 tests were used to explore associations between CD10, ET-1, Gleason score and tumor stage. The Kaplan-Meier method was used to determine the effect of each categorical variable on PSA relapse-free survival, and the log-rank test was used to compare PSA relapse-free survival differences within each variable. For PSA recurrence-free survival analysis at the univariate and multivariate level the Cox proportional hazards model was used to estimate hazard ratios (HR) with 95% confidence intervals (CI). Statistical significance was determined by using two-tailed p-values and was reported at $p<0.05$ level. Statistical analysis was performed using SPSS (SPSS for Windows, version 15.0, SPSS, Chicago, IL.).

Results

Thirty-eight patients developed PSA recurrence during follow up and 32 did not have a PSA relapse. Two patients expired. The estimated median follow up time, as calculated by the reverse Kaplan-Meier method was 30 months (range 12-86) while the median time to PSA recurrence was 56 months (range 1-74).

CD10 immunostaining was membranous and apical cytoplasmic. According to level of CD10 expression, patients were divided into a group of low (n=36) and another of high CD10 expression (n=34). In univariate analysis, a significant inverse association of CD10 with both Gleason score (P = 0.003) and pathological stage (P = 0.030) was observed (Table 1). CD10 was also was found to be directly associated with time to PSA recurrence (P < 0.001).

ET-1 immunostaining was cytoplasmic. ET-1 expression was distributed in two groups of either low (n=36) or high (n=34) immunoreactivity. High ET-1 expression was directly correlated with more advanced disease, evidenced by both Gleason score (P = 0.008) and pathological stage (P = 0.003) (Table 1). ET-1 expression correlated with a shorter time from radical prostatectomy until PSA relapse (P < 0.001).

A significant inverse correlation was found between expression of CD10 and ET-1 (P < 0.001). Immunohistochemical expression patterns of CD10 and ET-1 are depicted in Figure 1.

Expectingly, both pathological stage (P < 0.001) and Gleason score (P < 0.001) were inversely related with PSA-relapse free survival. Stage and grade were also directly interrelated (P < 0.001).

In multivariate analysis, there was a significant inverse association between CD10 expression and time to PSA recurrence after controlling for tumor stage, Gleason score and ET-1 expression (P = 0.012; 95% [CI] = 0.230 [0.073-0.723]). Pathological stage also retained its significance as direct predictor of time to PSA failure (P = 0.013; 95% [CI] = 2.784 [1.241-6.246]) (Table 2).

Discussion

The observation of ET-1 overexpression and simultaneous loss of CD10 expression during transition to the androgen-independence phase of prostate cancer and the evidence that there is a causative relation between these events [18] has provided ground for conducting immunohistochemical studies with the aim of testing their prognostic significance at the clinical level. Nonetheless, despite the presence of numerous relevant studies, there is not a uniform agreement on how to

Parameters	Low CD10		High CD10		Low ET-1		High ET-1		Patients
	n	%	*n*	%	*n*	%	*n*	%	*n*
Gleason score ≤7 (3+4)	20	40	30	60	31	62	19	38	50
Gleason score ≥7 (4+3)	16	80	4	20	5	25	15	75	20
p value	0.003				0.008				
pathologic TNM stage ≤2	17	40.5	25	59.5	28	66.7	14	33.3	42
pathologic TNM stage ≥3	19	67.9	9	32.1	8	28.6	20	71.4	28
p value	0.03				0.003				

n: number of patients.

Table 1: Correlations between CD10, ET-1 and pathological characteristics.

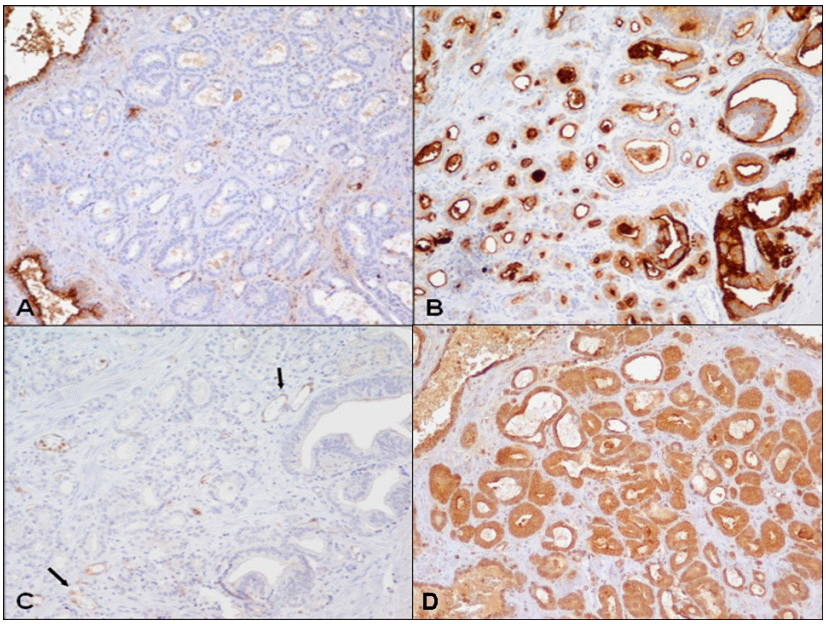

Figure 1: Prostate adenocarcinoma Gleason pattern 3. **A.** Negative immunoreactivity for CD10. Positive marker the normal prostate glands (x 200). **B.** Strong membranous and apical cytoplasmic IHC staining for CD10 (x 200). **C.** The same area as in B. Negative immunoreactivity for ET-1. Positive marker the capillary endothelium (arrows) (x 200). **D.** The same area as in A. Strong cytoplasmic IHC staining for ET-1 (x 200).

Variable	HR [95%CI]	*p* value
NEP	0.230 [0.073-0.723]	0.012
ET-1	0.761 [0.259-2.237]	0.620
Gleason score	1.818 [0.816-4.048]	0.143
pathologic TNM stage	2.784 [1.241-6.246]	0.013

HR: hazard ratio; CI: confidence interval.

Table 2: Multivariate Cox regression analysis.

interpret data of immunohistochemical expression of these markers in the context of prognostic models following radical prostatectomy. The major reason for this is the lack of reproducibility of results, which may be mostly attributed to different size of studied populations as well as variations in immunohistochemical techniques and scoring used.

Clinical studies of ET-1 in prostate cancer have revealed a widespread, mostly cytoplasmic pattern of expression, with loss of immunostaining in completely regressed areas of tumors from patients subjected to "neoadjuvant" hormonal pretreatment followed by radical prostatectomy [34]. When tested in a wider range of distinct histopathologies of prostatic origin, positive cytoplasmic ET-1 staining did not differ between groups of benign prostate hyperplasia (BPH) and prostate cancer, bone metastasis (BM) and non-BM, and highly and moderately differentiated prostate cancer, but the staining intensity for ET-1 was significantly higher in the poorly compared to the highly and moderately differentiated prostate cancer [35]. The simultaneous expression of ET-1 and its receptors, ET_AR and ET_BR has been demonstrated to be a more general finding in prostatic tissues of both late and early phase malignant disease, including high-grade prostatic intra-epithelial neoplasia (HGPIN) and incidental prostate cancer in cysteoprostatectomies (CyP) [36]. The strongest degree of expression of ET-1, ET_AR and ET_BR expression was observed in high grade cancer [36], in line with previous studies that separately correlated the immunohistochemical expression of ET-1 receptors with advanced stage and grade of the disease [1,37,38].

In our study we confirmed the direct association of ET-1 expression with pathological TNM stage and Gleason score, with the majority of high ET-1-expressing tissues correlating with high stage and Gleason score. The evaluation of ET-1 extent and intensity of immunoreactivity was recently correlated with biochemical recurrence in a large scale study of 287 radical prostatectomy specimens [39], demonstrating that both the intensity and the combination of intensity and extent of ET-1 immunoreactivity (IRp) but not the staining extent alone predicted biochemical recurrence. Recurrence-free survival in patients with strong ET-1 staining was shorter than in those with weaker expression [39]. In our study, we also observed a statistically significant inverse association of ET-1 intensity with time to PSA recurrence. Therefore, low ET-1 expression predicted a significantly longer time to PSA relapse, whereas high ET-1 was a negative predictor of biochemical progression of the disease.

The cleavage of ET-1 by CD10 is a process that takes place at the extracellular membrane and is therefore critical for preventing the pro-survival signaling induced by intact ET-1. Accordingly, immunohistochemical studies of CD10 have repeatedly shown an apical plasma membrane localization pattern in normal epithelial cells and BPH tissue, which is shifted to a cytoplasmic pattern in prostate cancer specimens in a direct Gleason grade-dependent manner [40,41]. This differential sub-cellular localization of the protein might be at least partially attributed to several identified motifs in the primary sequence of prostate cancer cells' CD10 [42]. However, a more deep insight into the pattern of CD10 sub-cellular localization in hyperplastic and neoplastic conditions of the prostate has revealed a distinct subset of high Gleason score specimens with no decline in either cytoplasmic or membrane CD10 expression [43]. This unexpected finding was assumed to have resulted from accumulation of mutations causing reexpression of CD10 [43].

Two later studies, despite being confirmatory of a progressive loss of the protein's expression in prostate tissues of benign prostate, prostatic intraepithelial neoplasia (PIN) and prostate cancer samples, they were not supportive of any association between CD10 expression and Gleason score, stage or biochemical failure after radical prostatectomy [44,45]. However, both these studies retrospectively included a significant proportion of patients from 1984 to 1990, the pre-PSA era, when evaluation recurrence was based on developing gross metastatic disease from imaging studies [46]. Further to that, one study included patients who received neoadjuvant hormone treatment [45], which is known to influence both time to PSA relapse and CD10 expression [46]. The challengers of the above-mentioned studies reported, in their own work, a statistically significant direct correlation between complete loss of CD10 expression and time to PSA relapse after controlling for Gleason grade, stage, preoperative PSA level and race [46]. This was shown, in the same patient population, to be more likely observed in histological specimens with positive expression of phosphorylated Akt, providing at least one plausible molecular explanation of the clinical data [47].

Further studies with both comparable [48] and greater [49] number of patients disclosed that positive CD10 expression is an independent predictor of biochemical failure in addition to correlating with elevated preoperative PSA levels, higher Gleason score and stage [48,49]. This was in accordance with the CD10 expression profile of lymph node metastases -where present- of the same tumours that was strongly positive [48], thus making an argument in favour of a relationship between CD10 expression and a more aggressive prostate cancer phenotype [48,50] although in one of these studies, CD10 was present in lymph nodes but absent in tumours [50]. When the expression of CD10 was examined together with that of endothelin receptors ET_A and ET_B, a combination pattern of high ET_AR and low ET_BR/CD10 correlated with shorter time to PSA progression. Moreover, the majority of incidents of early biochemical failure occurred in the low CD10 expression subgroup, whereas prolonged time to PSA relapse was rarely combined with elevated CD10 [39].

The comparability of results among all these studies may be limited due to differences between the evaluated cohorts concerning the distribution of Gleason patterns, immunohistochemical assessment criteria and sampling variations. Despite discrepancies, it is a uniform suggestion that PSA recurrence-free survival declines from cytoplasmic over membrane-cytoplasmic to exclusively cytoplasmic CD10 expression. However, no existing molecular model could explain the favorable role of CD10 negativity in terms of outcome in the large cohort of Fleischmann et al. [49].

After the study of Godara et al. [39] who investigated the expression of ET-1 receptors together with that of CD10 in prostate cancer samples of radical prostatectomy, this is the second attempt for an integrated approach of the role of the CD10/endothelin axis in the clinical course of prostate cancer patients. Our results concur with their suggested pattern of high ET_AR with low ET_BR/CD10 expression correlat-

ing with decreased time to PSA failure, and they are both in line with the underlying biology. The latter has been extensively studied *in vitro* by our [51] and other groups [12,14,21-23,30] and offers a strong preclinical model of prostate cancer evolution which is reflected in certain clinical studies, including ours.

Limitations of the present study should be acknowledged. The small number of patients, retrospective review of our prospectively collected data and the relatively short median follow-up of patients may have biased our results. Further, there was no comparison of prognostic values of CD10 and ET-1 to predicted outcomes of validated nomograms. Finally, our study was not intended to include all current prognostic markers [52].

Conclusion

The challenge of treating prostate cancer in the context of an enhanced prognostication approach beginning from early stages might at least partially be met with success with evaluation of the CD10/neuropeptide axis in tissues of patients treated with radical prostatectomy. The incorporation of this assessment into the current model of predictive factors might help to better classify patients into groups of low and high risk, thus enabling the latter to benefit from a more aggressive radical treatment and a closer follow-up.

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This article was originally published in a special issue, **Prostate Cancer** handled by Editor(s). Dr. Gary Guishan Xiao, Creighton University, USA; Dr. Sreenivasa R. Chinni, Wayne State University, USA