Correlation of Androgen Receptor Status, Neuroendocrine Differentiation and Angiogenesis with Time-to-Biochemical Failure after Radical Prostatectomy in Clinically Localized Prostate Cancer

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Abstract. *The aim of this study was to elucidate the prognostic value of the immunohistochemical detection of the androgen receptor (AR) status, the chromogranin A assessment of neuroendocrine differentiation (NED) and the CD34 assessment of microvessel density (MVD) with time-to-biochemical failure among surgically treated patients with clinically localized prostate cancer. Patients and Methods: Surgical specimens from 130 patients with clinically localized prostate cancer, treated with radical prostatectomy, were analyzed by immunohistochemistry on paraffin tissue sections. Full-length follow-up records were available for 94 patients. Results: Biochemical failure was observed in 37% of these patients. A statistically significant inverse relationship was observed between AR status and: (i) seminal vesicle invasion and (ii) surgical margin infiltration. Positive association was also detected between NED and: (i) Gleason's score, (ii) extracapsular extension, (iii) seminal vesicle invasion, (iv) surgical margin infiltration and (v) tumour volume. In addition, MVD was related to: (i) Gleason score, (ii) extracapsular extension, (iii) seminal vesicle invasion, (iv) pelvic lymph node metastasis and (v) tumour volume. Kaplan-Meier survival curves confirmed that Gleason score, extracapsular extension, seminal vesicle invasion, pelvic lymph node metastasis, tumour volume, NED, MVD and coexistence of increased NED and MVD may be potential biochemical failure predictors. However, in the multivariate analysis, MVD was the only independent prognostic factor for biochemical failure. Conclusion: A high MVD index can estimate the risk for biochemical failure in clinically localized prostate cancer after radical prostatectomy.*

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Prostate cancer (PC) is the most common cancer in men, excluding non-melanoma skin cancer. The significance of using prostate-specific antigen (PSA) blood testing as a tool to estimate the risk for PC has boosted early detection of PC. This has resulted in a different cohort of newly diagnosed patients with PC than that in the pro-PSA era. Thus, the employment of radical prostatectomy (RP) as a primary treatment for clinically localized PC has increased, significantly. Histological grade, stage and preoperative serum PSA values are the most useful variables for predicting PC behaviour, although not easily applied to the individual patient (1). Recently, preoperative molecular staging, using reverse transcriptase-polymerase chain reaction (RT-PCR) to detect transcripts of PSA and prostate-specific membrane antigen (PSMA) in peripheral blood and bone marrow biopsy, have shown encouraging correlations between the positive detection of these transcripts with time-to-biochemical failure (BF) after RP in patients with clinically localized PC. Nevertheless, this method is still far from being widely applied and routinely employed (2, 3). Many studies have suggested several immuno-histochemical (IHC) markers to enhance the prognostic value of the known variables (1).

In this context, androgen receptor (AR), a nuclear transcription factor that mediates the actions of androgens,

is mainly expressed in nuclei of secretory and stromal cells in the benign prostate, and variably in PC and prostatic intraepithelial neoplasia (PIN) (4). Furthermore, neuroendocrine (NE) cells, which are located in the basal cell layer of the prostatic epithelium, produce specific peptides, predominantly chromogranin A (Chr A). The NE differentiation (NED) and its IHC detection with Chr A have recently attracted considerable attention because NE cells play an important role as potential paracrine modulators of prostate growth and differentiation. Prostatic adenocarcinomas with focal NED are common and NED is apparently associated with PC progression (5).

In addition, the clinical significance of intratumoral microvessel density (MVD) in human PC was first reported in 1993 (6, 7). Since then, several conflicting studies have investigated the association of MVD with PC biology. The discrepancies noticed in results are attributed to the different antibodies used to determine MVD. The preferable antibody is CD34, a myeloid progenitor cell antigen, also present in all types of endothelial cells, including that of the capillaries (6-9).

In the present study, we examined the IHC expression of AR, Chr A and CD34 in RP specimens of 130 patients treated for clinically localized PC. We analysed the association between the IHC detection of AR, NED and MVD with the majority of pathological and clinical parameters, including time-to-biochemical failure (BF).

Table I. *Pathological characteristics of 130 radical prostatectomy specimens of patients with localized prostate cancer.*

Variable	RP specimens	(%)
Gleason score		
5	2	(1)
6	27	(21)
7a	32	(25)
7b	43	(33)
8	16	(12)
9	10	(8)
Surgical margins		
Negative	86	(66)
Positive	44	(34)
PIN		
Absent	28	(21)
Present	102	(79)
Extracapsular extension		
Absent	60	(46)
Present	70	(54)
Seminal vesicle invasion		
Absent	96	(74)
Present	34	(26)
Nodal status		
Negative	120	(92)
Positive	10	(8)

RP: radical prostatectomy; PIN: prostatic intraepithelial neoplasia.

Patients and Methods

Patients. One hundred and thirty consecutive patients with PC who underwent RP for localized PC at the Evangelismos General Hospital, Athens, Greece, were enrolled in this study. The median age of the patients was 66 (47–76) years and the median preoperative PSA was 9.23 (2.5-45.0) ng/ml. Full-length follow-up records were available for 94 patients; hence the remaining 36 were excluded from statistical analyses concerning the progression of disease. The median follow-up period was 28 (1-97) months. A cut-off of 0.2 ng/ml serum PSA, on at least two occasions, with the second elevation measured at least 6 months after the first, established the biochemical failure (BF) of the disease. Adjuvant treatment (androgen deprivation and/or radiation) was administered to patients with BF.

Tissue samples. Archival paraffin-embedded tumour sections were used for haematoxylin-eosin staining and the performance of the IHC staining. Tumour volume referred to the percentage of histological slides' surface involved by PC. Grading was established according to the Gleason score (GS) differentiation system (range 2-10). GS 7 carcinomas were stratified to 7A and 7B, according to predominate grade: GS 3+4 (7A) and 4+3 (7B). A two-scale grouping was used for the final evaluation of the histological grading, as follows: I: GS≤7A and II: GS≥7B. The area with the highest GS was selected for immunohistochemistry evaluation. High grade PIN was also evaluated. Staging was carried out according to the TNM 2002 classification (10), evaluating the following: extension of the tumour to an inked specimen margin, extracapsular extension into periprostatic tissue, seminal vesicle invasion and pelvic lymph nodes metastasis.

Immunohistochemistry. The IHC method of streptavidin-biotin-peroxidase protocol using the DAKO LSAB+Kit Peroxidase was performed, using commercially available antibodies: mouse monoclonal antibody against AR (clone AR441; DAKO, Glostrup, Denmark) at a dilution 1:10, mouse monoclonal antibody against Chr A (clone LK2H10; Monosan, Uden, The Netherlands) at a dilution 1:10, and mouse monoclonal antibody CD34 (clone QBEnd 10, DAKO) at a dilution 1:10.

Tissue marker scoring. AR nuclear staining in the benign columnar prostate epithelium was used as positive control. AR staining was evaluated in a PC area with the lowest density of positive cells. One thousand consecutive malignant cells were counted. The number of cells with any nuclear staining were recorded and then grouped. Extension of nuclear staining for AR in tumour areas was categorised according to 5 scales (<5%, 5-25%, 26-50%, 51-75% and >75%). NED, as expressed by Chr A immuno-staining, was evaluated in the area of strongest staining. One thousand malignant cells were counted and the number of cells with nuclear staining was recorded. The cases were classified as: i) negative staining or scattered positive cells up to 5% of the malignant cells, without clustering; ii) 5-10% of positive cancerous cells or small clusters of NE cells (<10 cells); and iii)

Table II. *Relationship between androgen receptor (AR) immunoexpression, clinicopathological parameters and biochemical recurrence in patients with localized prostate cancer.*

		No. of patients (%)					
Variable	Patients	AR (<5%)	AR (5-25%)	AR (25-50%)	AR (50-75%)	AR (>75%)	*P**
Two-scale histological grading***							0.31
≤7a	61	41 (67)	1 (2)	7 (11)	7 (11)	5 (8)	
7b-10	69	41 (59)	7 (10)	7 (10)	6 (9)	8 (12)	
Total	130	82 (63)	8 (6)	14 (11)	13 (10)	13 (10)	
Extracapsular extension							0.31
–	60	39 (65)	2 (3)	4 (7)	8 (13)	7 (12)	
+	70	43 (61)	6 (9)	10 (14)	5 (7)	6 (9)	
Total	130	82 (63)	8 (6)	14 (11)	13 (10)	13 (10)	
Seminal vesicle invasion							**0.016**
–	96	64 (67)	2 (2)	9 (9)	10 (10)	11 (11)	
+	34	18 (53)	6 (18)	5 (15)	3 (9)	2 (6)	
Total	130	82 (63)	8 (6)	14 (11)	13 (10)	13 (10)	
Lymph node invasion							0.19
–	120	76 (63)	7 (6)	11 (9)	13 (11)	13 (11)	
+	10	6 (60)	1 (10)	3 (30)	0 (0)	0 (0)	
Total	130	82 (63)	8 (6)	14 (11)	13 (10)	13 (10)	
Surgical margins							**0.021**
–	86	58 (67)	2 (2)	6 (7)	10 (12)	10 (12)	
+	44	24 (55)	6 (13)	8 (18)	3 (7)	3 (7)	
Total	130	82 (63)	8 (6)	14 (11)	13 (10)	13 (10)	
PIN							0.58
–	28	19 (68)	1 (4)	1 (4)	4 (14)	3 (10)	
+	102	63 (62)	7 (7)	13 (13)	9 (9)	10 (9)	
Total	130	82 (63)	8 (6)	14 (11)	13 (10)	13 (10)	
Biochemical recurrence							0.22
–	59	41 (70)	3 (5)	6 (10)	5 (8)	4 (7)	
+	35	21 (60)	1 (3)	4 (11)	5 (14)	4 (11)	
Total	94	62 (66)	4 (4)	10 (11)	10 (11)	8 (8)	

*Chi-square test. **A (GS≤6), B (GS=7A), C (GS=7B), D (GS=8-10); ***I (GS≤7A), II (GS=7B-10).

more than 10% positive cells or large clusters (>10 cells) of Chr A positive cells. MVD was also scored in a 'hot-spot' area, under x200 magnification (0.754 mm^2). Each endothelial cell with positive staining and group of cells with CD34 immunopositivity were regarded as one blood vessel. The results were recorded as the highest number found in a field x200. A three-scale grouping was performed (0-90 microvessels, 91-180 microvessels and >180 microvessels).

Statistical analysis. Associations between the IHC expression of the markers and the clinicopathological variables were assessed using the Chi-square and the Kruskal-Wallis test. Survival analyses were performed by constructing Kaplan-Meier progression-free survival (PFS) curves, where differences between curves were evaluated by the log-rank test, as well as by estimating the relative risks for relapse and death using the Cox proportional hazards regression model. Only patients with known status of all variables were included in the multivariate regression models. In the multivariate analysis, the clinical and pathological variables that may affect progression-free survival were adjusted. Statistical analysis was performed using SAS software (SAS Institute, Cary, NC, USA).

Results

Clinicopathological characteristics. The majority of the 130 enrolled patients showed locally advanced disease. GS ranged between 6 and 9, with a higher prevalence of GS 7 (75 cases, 58%). The pathological characteristics of the RP specimens are shown in Table I. Specimen weight ranged between 8 g and 250 g (median 45 g) and tumour volume between 1% and 100% (median 30%). BF was observed in 35/94 (37%) patients with follow-up data.

AR, NED and MVD expression. AR, NED and MVD expression is shown in Tables II-IV and in Figure 1 A, B and C, respectively.

Association of AR, NED and MVD IHC detection with clinical parameters. We documented statistically significant low AR IHC expression with seminal vesicle involvement (p=0.016) and positive surgical margins (p=0.021) at RP.

Table III. *Relationship between neuroendocrine differentiation as determined by chromogranin A immunoexpression, clinicopathological parameters and biochemical recurrence in patients with localized prostate cancer.*

Variable	Patients	No. of patients (%)			P**
		<5% without clustering*	5-10% or clustering (<10 NE cells)	>10% or large clusters (>10 NE cells)	
Two-scale histological grading****					**0.003**
≤7a	61	49 (80)	12 (20)	0 (0)	
7b-10	69	40 (58)	20 (29)	9 (13)	
Total	130	89 (68)	32 (25)	9 (7)	
Extracapsular extension					**<0.001**
–	60	53 (88)	7 (12)	0 (0)	
+	70	36 (51)	25 (36)	9 (13)	
Total	130	89 (68)	32 (25)	9 (7)	
Seminal vesicle invasion					**<0.001**
–	96	74 (77)	21 (22)	1 (1)	
+	34	15 (44)	11 (32)	8 (24)	
Total	130	89 (68)	32 (25)	9 (7)	
Lymph node invasion					0.12
–	120	85 (71)	27 (22)	8 (7)	
+	10	4 (40)	5 (50)	1 (10)	
Total	130	89 (68)	32 (25)	9 (7)	
Surgical margins					**0.048**
–	86	65 (75)	16 (19)	5 (6)	
+	44	24 (55)	16 (36)	4 (9)	
Total	130	89 (68)	32 (25)	9 (7)	
PIN					0.92
–	28	20 (71)	6 (21)	2 (7)	
+	102	69 (68)	26 (25)	7 (7)	
Total	130	89 (68)	32 (25)	9 (7)	
Biochemical recurrence					**<0.001**
–	59	48 (81)	10 (17)	1 (2)	
+	35	19 (54)	10 (29)	6 (17)	
Total	94	67 (71)	20 (21)	7 (8)	

*Detailed analysis in Patients and Methods, **Chi-square test; **A (GS≤6), B (GS=7A), C (GS=7B), D (GS=8-10); ***I (GS≤7A), II (GS=7B-10); NE: neuroendocrine cells.

Levels of AR IHC positivity did not show any association with the other clinicopathological factors examined in this study (Tables II and V).

A strong association was found between NED and GS ($p=0.003$). All 9 cases which showed extensive Chr A IHC positivity (NED) were of GS≥7B (2 cases of 7B and 7 cases of GS≥8). Strong associations with variables related to pathological stage were found. High nuclear Chr A expression was associated with extracapsular extension ($p<0.001$), seminal vesicle involvement ($p<0.001$), positive surgical margins ($p=0.048$), and tumour volume ($p<0.001$). We did not detect any association with the other clinicopathological factors examined in this study (Tables III and V).

Increased neovascularization showed statistical association with GS ($p=0.021$). Additionally, strong associations between high MVD and variables related with the stage of PC were identified. Extracapsular invasion ($p=0.005$), seminal vesicle involvement ($p=0.012$), metastatic disease to pelvic lymph nodes ($p=0.004$) and increased tumour volume ($p=0.003$) were significant findings in cases with high MVD index. However, there was no statistical association of CD34 IHC expression with the other clinicopathological factors (Tables IV and V).

Preoperative serum PSA values were not associated with any of the three IHC markers studied.

Univariate analysis of AR, NED, MVD and the other clinicopathological variables with regard to PFS. Evaluating the follow-up data of 94 patients, BF occurred in 6/7 cases with extensive NED, in 10/20 with moderate NED and 19/67 with low NED (Table III). Regarding MVD, 9/12 patients with high MVD, 17/28 with moderate MVD and 9/54 with low MVD relapsed (Table IV).

Table IV. *Relationship between microvessel density (MVD), as determined by CD34 immunoexpression, clinicopathological parameters and biochemical recurrence in patients with localized prostate cancer.*

		No. of patients (%)			
Variable	Patients	MVD<90	MVD 90-180	MVD>180	*P**
Two-scale histological grading***					**0.021**
≤7a	61	41 (67)	18 (30)	2 (3)	
7b-10	69	33 (48)	25 (36)	11 (16)	
Total	130	74 (57)	43 (33)	13 (10)	
Extracapsular extension					**0.005**
–	60	41 (68)	18 (30)	1 (2)	
+	70	33 (47)	25 (36)	12 (17)	
Total	130	74 (57)	43 (33)	13 (10)	
Seminal vesicle invasion					**0.012**
–	96	61 (64)	29 (30)	6 (6)	
+	34	13 (38)	14 (41)	7 (21)	
Total	130	74 (57)	43 (33)	13 (10)	
Lymph node invasion					**0.004**
–	120	71 (59)	40 (33)	9 (8)	
+	10	3 (30)	3 (30)	4 (40)	
Total	130	74 (57)	43 (33)	13 (10)	
Surgical margins					0.24
–	86	52 (60)	28 (33)	6 (7)	
+	44	22 (50)	15 (34)	7 (16)	
Total	130	74 (57)	43 (33)	13 (10)	
PIN					0.47
–	28	13 (46)	11 (39)	4 (14)	
+	102	61 (60)	32 (31)	9 (9)	
Total	130	74 (57)	43 (33)	13 (10)	
Biochemical recurrence					**<0.001**
–	59	45 (76)	11 (19)	3 (5)	
+	35	9 (26)	17 (48)	9 (26)	
Total	94	54 (57)	28 (30)	12 (13)	

*Chi-square test; **A (GS≤6), B (GS=7A), C (GS=7B), D (GS=8-10); ***I (GS≤7A), II (GS=7B-10).

On univariate analysis high NED and MVD were strongly associated with BF ($p<0.001$ and $p<0.001$ respectively – Figures 2 and 3). The hazard ratio (HR), estimated from a Cox proportional hazard regression model, taken as equal to 1.00 for patients with low Chr A IHC expression, was found to be 2.21 for patients with high expression ($p=0.001$, 95% CI=1.41-3.47). The HR was found to be 2.79 for patients with high CD34 IHC expression ($p<0.001$, 95% CI=1.82-4.28). Levels of AR IHC detection did not show any correlation with clinical outcome ($p=0.22$). High NED and MVD co-expression was proved to be a strong BF predictor, as 6/7 patients with extensive NED and 9/12 with high MVD were documented with BF ($p<0.001$). GS, extracapsular extension, seminal vesicle involvement, metastatic disease in the pelvic lymph nodes and tumour volume were strong predictors for BF ($p=0.046$, $p=0.002$, $p=0.001$, $p=0.008$ and $p<0.001$ respectively) (Table VI).

Multivariate analysis of AR, NED and MVD expression adjusted with other clinicopathological variables with regard to PFS. Multivariate analysis (Cox regression) of the studied markers and clinicopathological parameters revealed that MVD, as measured by CD34 IHC expression, was the only independent prognostic factor for BF ($p=0.010$) (Table VII).

Discussion

Prostate adenocarcinoma is a heterogeneous tumour with generally low but constant growth rate. Androgens play a key role in both growth regulation of the normal gland and PC. Androgens act *via* the AR, a nuclear transcription factor. Androgen depletion therapy is the worldwide mainstay of PC treatment. Unfortunately, it usually fails after a period of time due to the development of androgen ablation refractoriness. Angiogenesis, NED and AR

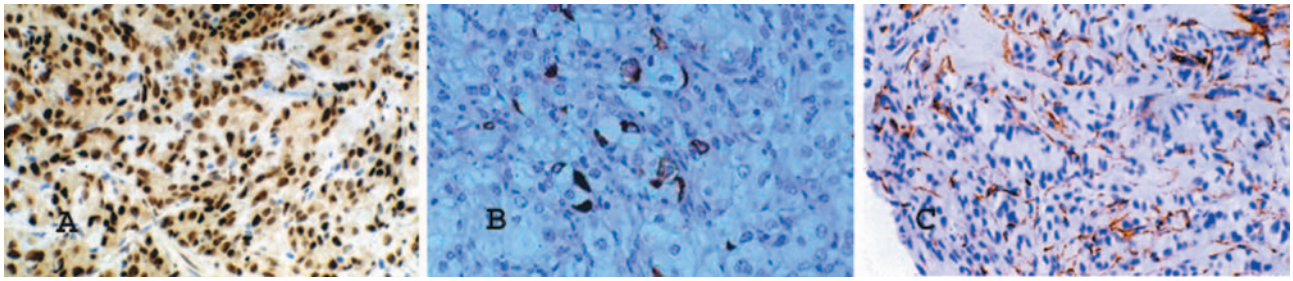

Figure 1. *Panel A. High androgen receptor (AR) immunohistochemical expression in prostate cancer. Panel B. High neuroendocrine differentiation, assessed by chromogranin A (Chr A) immunohistochemical expression in prostate cancer. Panel C. High microvessel density assessed by CD34 immunohistochemical expression in prostate cancer.*

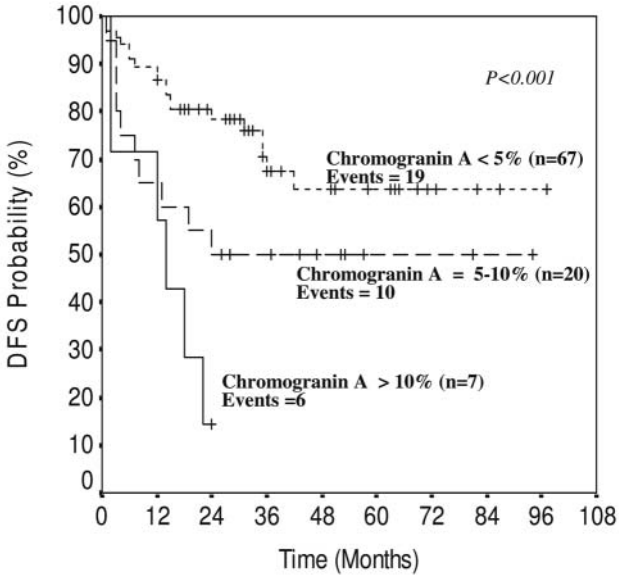

Figure 2. *Relation between chromogranin A (Chr A) immunoexpression and time-to-biochemical failure in 94 patients after radical prostatectomy specimens for localized prostate cancer.*

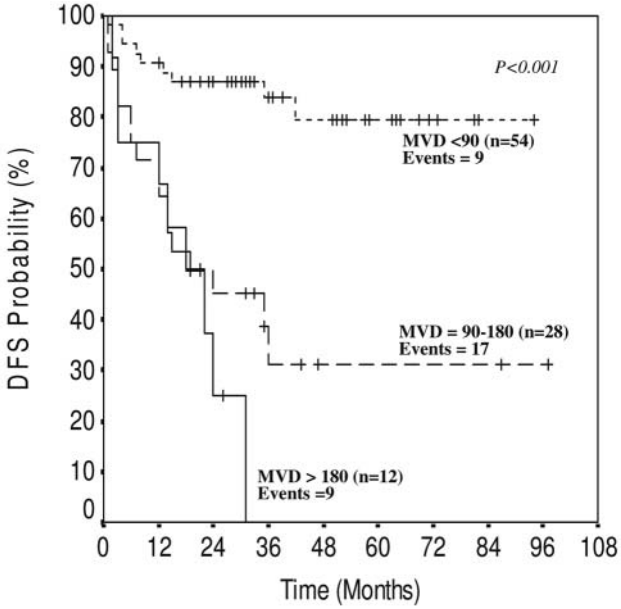

Figure 3. *Relation between microvessel density (MVD) and time-to-biochemical failure in 94 patients after radical prostatectomy specimens for localized prostate cancer.*

expression are basic facets implicated in tumour progression to metastatic disease and androgen refractoriness (5, 11, 12).

In normal prostate, AR immunostaining is strong in nuclei of secretory and stromal cells and mostly negative in basal cells. ARs are variably expressed in PIN, PC and metastatic disease (4). Recent studies suggested that heterogeneous AR IHC expression is associated with PC of high GS and advanced stage disease. Some investigators reported that reduced IHC expression of AR is correlated with high grade, poor response to androgen ablation and decreased PFS (4, 13). Other studies found that high AR expression was associated with aggressive clinicopathological features and reduced PFS in PC patients treated with RP of curative intent (14-16). However, Dunsmuir *et al.* did not observe any association between AR expression and GS, but reported a weak correlation between reduced AR levels and metastatic status (11). Henshall *et al.*, studying the expression of AR in the malignant epithelium and adjacent stroma found that concurrent overexpression of AR in the malignant cells and loss of the AR immunoreactivity in the adjacent peri-epithelial stroma was associated with higher clinical stage, high levels of pre-treatment PSA and earlier relapse after RP (17). Linja *et al.*, using molecular features, found a 6-fold higher AR gene expression level in androgen-refractory PC than in androgen-sensitive tumours or benign prostate hyperplasia (18). Fuelled by this prospect, Latil *et al.* found an increased AR expression in androgen refractory PC,

Table V. *Relationship of AR, chromogranin A (Chr A) and CD34 immunoexpression and tumour volume in patients with localized prostate cancer.*

	Tumour volume mean rank	No. of cases	*P**
AR			0.68
<5%	59.65	78	
5-25%	70.79	7	
26-50%	71.14	14	
51-75%	56.17	12	
>75%	67.29	12	
Chr A			
<5%	54.93	84	**<0.001**
5-10%	70.48	31	
>10%	103.38	8	
CD34			
1(<90)	53.66	71	**0.003**
2(90-180)	68.64	38	
3(>180)	86.18	14	

*Kruskal Wallis test.

Table VI. *Univariate analysis of AR, chromogranin A (Chr A), and CD34 and other variables with regard to PFS.*

Variable	HR	95% CI	*P*-value
AR (ordinal)	1.14	0.92-1.43	0.22
Chr A (ordinal)	2.21	1.41-3.47	**0.001**
CD34 (ordinal)	2.79	1.82-4.28	**<0.001**
Gleason score			
≤7a	1.00		
7b-10	2.07	1.01-4.24	**0.046**
Surgical margins			
Negative	1.00		
Positive	1.59	0.81-3.10	0.17
PIN			
Absent	1.00		
Present	0.72	0.35-1.51	0.38
Extracapsular extension			
Absent	1.00		
Present	3.23	1.51-6.90	**0.002**
Seminal vesicle invasion			
Absent	1.00		
Present	3.22	1.65- 6.29	**<0.001**
Nodal status			
Negative	1.00		
Positive	3.76	1.42-9.94	**0.008**
Tumour volume	1.024	1.013-1.035	**<0.001**

PFS: progression-free survival; HR: hazard ratio estimated from Cox proportional hazard regression model; CI: confidence interval of the estimated HR.

Table VII. *Multivariate analysis of AR, chromogranin A (Chr A), and CD34 adjusted with other clinicopathological variables with regard to PFS.*

Variable	HR	95% CI	*P*-value
AR (ordinal)	1.18	0.91-1.52	0.20
Chr A (ordinal)	1.12	0.65-1.94	0.17
CD34 (ordinal)	1.96	1.17-3.27	**0.010**
Gleason score			
≤7a	1.00		
7b-10	1.31	0.55-3.09	0.54
Surgical margins			
Negative	1.00		
Positive	0.85	0.33-2.21	0.73
PIN			
Absent	1.00		
Present	1.04	0.43-2.49	0.92
Extracapsular extension			
Absent	1.00		
Present	2.01	0.65-6.19	0.22
Seminal vesicle invasion			
Absent	1.00		
Present	1.51	0.52-4.34	0.45
Nodal status			
Negative	1.00		
Positive	2.44	0.77-7.73	0.12
Tumour volume	1.008	0.99-1.03	0.31

PFS: progression-free survival; HR: hazard ratio estimated from Cox proportional hazard regression model; CI: confidence interval of the estimated HR.

simultaneously with reduced expression of alpha and beta estrogen receptors (ER). Hence, it has been speculated that increased AR gene expression could mirror PC progression and serve as a valuable tool for BF (19). In the present study, we did not find any association between AR expression and GS. We identified only statistically significant associations of reduced AR IHC detection with seminal vesicle involvement and positive surgical margins. In univariate analysis, AR IHC expression did not show any prognostic value in BF prediction.

NE cells in normal and malignant prostate show no proliferative activity, are androgen-refractory and are considered to be 'postmitotic', as they lie in close vicinity to PC cells of increased proliferative activity (5, 20). Nowadays, NED in PC attracts increasing attention. NE prostatic cells produce a number of hormone-regulated growth factors that may act through endocrine, paracrine and autocrine mechanisms. The main product of prostatic NE cells is Chr A. Wu *et al.* reported that elevated serum Chr A plays a potential role in the early detection of PC progression (21). Chr A IHC expression has been widely used to reveal NE cells in the prostate (22, 23).

Many studies have tried to shed light on the relationship of NED with grade, stage and clinical outcome, and have reported controversial data. However, most authors have reported statistically significant positive associations of NED with the above parameters (24-28). In patients with clinically localized PC treated with RP, Weinstein *et al.* reported that

NED was a statistically significant unfavourable marker in univariate analysis, but not when GS was considered in the analysis model (25). Bostwick *et al.*, in a series of node-positive prostate adenocarcinomas, failed to establish any statistically significant association of NED and disease outcome. However, they established an association between the reduced Chr A expression with lymph node metastases, suggesting that reduction of NED may be involved in tumour progression (26). In the present study, high NED was strongly correlated with high GS and pathological parameters related to advanced stage disease. In univariate analysis, NED was a strong predictor of BF, but was not an independent prognostic marker in multivariate analysis.

Angiogenesis is directly stimulated by the tumour, through angiogenetic substances secreted or activated within the extracellular matrix. Angiogenesis promotes tumour enlargement and metastases through perfusion and paracrine effects. A variety of antibodies including von Willebrand factor or factor VIII (FVIII), CD31 and CD34 have been used for MVD evaluation. FVIII is the oldest IHC marker applied in vascular tumours and tumour angiogenesis. Later studies comparing FVIII, CD31 and CD34 microvascular staining concluded that anti-CD34 antibodies were the most sensitive endothelial markers (8, 9, 29). Bigler *et al.*, were the first to report that the MVD, as determined by FVIII staining, is increased in tumourous areas as compared to benign areas (7). Weidner *et al.* showed statistically significant higher MVD in patients with metastatic disease (6). Most studies reported positive correlation of MVD, as determined by the above antibodies, with GS and pathological stage in RP specimens of patients with localized PC (8, 9). However, conflicting results have also been reported (30). De la Taille *et al.* reported that MVD/CD34 and MVD/CD31 were strongly associated with BF on a univariate level, but only MVD/CD34 was an independent predictor in multivariate analysis (9). Bettencourt *et al.* found that MVD/CD34 was significantly related to time-to-biochemical recurrence. Controlling for stage, GS and nuclear grade, MVD remained significant in predicting BF, but was no longer significant when pre-treatment PSA was included in the model (8). Rubin et al could not find any independent prognostic value of MVD in PC outcome (30).

In our study, MVD determined by CD34 immunostaining, showed a statistically significant association with GS, extracapsular extension, seminal vesicle involvement, tumour volume and lymph node metastasis. In univariate analysis MVD was a strong predictor of disease recurrence and was proved to be the only independent prognostic factor of BF in multivariate analysis. It is well known that GS is an established marker for PC prognosis. In our study, GS was found to be a statistically significant prognostic marker for PFS in univariate analysis. Nevertheless, we did not find the same results in multivariate analysis. Our findings in multivariate analysis do not reduce the prognostic significance of GS but are due to the statistically significant relationship between MVD and GS ($p=0.021$). Hence MVD "removes" a part of the prognostic value of GS in the multivariate model. GS remains significant in multivariate analysis when we do not include the MVD in the multivariate model. In addition, expression of vascular endothelial growth factor in Chr A positive NE PC cells has been reported by Grobholz *et al.* who found that neovascularization in high grade tumours was associated with extensive NED (31). Our findings agree with these studies, revealing strong positive association between NED and angiogenesis.

The results of the present study reflect many of the previous reports and show that MVD measurement using CD34 IHC may be helpful as a prognostic marker in PC. Patients with a high MVD index could be considered as high risk for disease progression and could be candidates for adjuvant therapy (androgen deprivation and/ or radiation) after RP for local or locally advanced PC. Adjuvant androgen deprivation and postoperative radiation appear to increase local control preferably in locally advanced PC and positive surgical margins. However, whether these therapies prolong survival when administered before there are symptoms caused by disease progression remains controversial (32-36). Furthermore, as these strategies may be associated with significant side-effects, some urologists advise a "wait and watch" policy for high-risk postprostatectomy patients and administration of radiation therapy +/– androgen ablation only if and when the PSA rises (37, 38). Once positive margins and/or locally advanced disease have been diagnosed, we prefer a delayed treatment once biochemical relapse has actually occurred. This attitude spares unfavourable effects of irradiation in some patients. However, as angiogenesis is associated with micrometastatic dissemination early in the disease process, patients with high MVD, independently of the co-existence of any other high risk factors, could benefit from immediate adjuvant treatment, including a combination of local and systemic therapies. Angiogenesis inhibitors incorporated in a multimodality treatment strategy may impact the course of localized PC (39, 40).

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