Microvessel density and expression of vascular endothelial growth factor in clinically localized prostate cancer

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Identifying biological differences between benign lesions and malignant prostatic cancer (PC) may facilitate precise indication for more aggressive post-operative treatment. Therefore, we examined immunohistochemically histological specimens from 140 PC patients treated with radical surgery. The mean age of the patients was 62.9 ±6.2 (range 49.0-77.0) years. There were 13 (9.3%) at pTNM stage 1, 78 (55.7%) at stage 2, 40 (28.6%) at stage 3 and 9 (6.4%) at stage 4. In the analysed group there were 75 (53.6%) well-differentiated, 53 (37.8 %) moderately differentiated and 12 (8.6%) poorly differentiated tumours.
The mean pre-operative prostate-specific antigen (PSA) level was 9.9 ±0.5 ng/ml. Concentration of serum PSA was significantly increased with pTNM stage ($p = 0.011$), Gleason score ($p = 0.011$) and tumour grade ($p = 0.003$). In 34 (24.3%) tumours vascular endothelial growth factor (VEGF) expression was not shown. In the analysed group of tumours the mean percentage of positive VEGF cells was 14.8 ±1.4% and was not correlated with tumour grade ($p = 0.648$) or Gleason score ($p = 0.697$). However, significantly higher values for the protein were observed in pTNM 3 ($p = 0.035$) and pTNM 4 ($P = 0.037$) than in pTNM stage 1. In the whole series of tumours the mean microvessel density (MVD) was 97.5 ±2.4 /mm^2. A non-significant decrease in the number of microvessels was observed in the highest pathological tumour volume ($P = 0.631$), Gleason score ($p = 0.368$) and tumour grade ($p = 0.233$). Prostate-specific antigen level was not associated statistically with either MVD ($p = 0.466$) or VEGF expression ($p = 0.188$). There was also no correlation between the immunohistochemical expression of VEGF and MVD ($p = 0.925$).

Key words: vascular density, MVD, VEGF, prostatic cancer.

Introduction

Late prostate cancer (PC) recognition is the main cause of treatment failure. Earlier recognition based on biological markers could be helpful in identifying biological differences in benign and malignant lesions, which could further facilitate precise indication for more aggressive post-operative treatment (chemo/radiotherapy). Tumour growth and metastasis are dependent upon tumour angiogenesis, which relies on growth of new vessels toward and within a tumour [1]. Such vascularization may be stimulated by factors released from the tumour cells, tumour-associated inflammatory cells, and/or from the extracellular matrix. In prostate tumour, vascular endothelial growth factor (VEGF) is one of the most potent facilitators in angiogenesis and takes a direct role in oncogenesis as an important hypoxia-inducible pro-angiogenic protein [2, 3].

The proteins by auto- and paracrine mechanisms also induce proliferation, growth and dissemination of tumour cells [4]. In the case of prostatic cancer it was shown that VEGF is connected with cell malignancy and has been linked with an adverse survival outcome [5, 6]. It was also indicated that microvessel density (MVD) has been associated with tumour aggressiveness, PSA recurrence, metastatic capability after radical prostatectomy and patient survival [7-9]. The results on VEGF expression and microvessel density in PC are inconsistent; nevertheless, control of the angiogenic switch and regulation by these soluble factors is of great importance for the rational design of cancer treatment strategies. Therefore, the aim of the study was to establish whether VEGF and MVD in localized PC are associated with pathological tumour stage and grade, which could be helpful in patients' selection for more aggressive treatment (e.g. adjuvant anti-angiogenic therapy).

Material and methods

Patients

The study involved evaluation of 140 consecutive radical prostatectomy specimens obtained from patients who underwent radical prostatectomy for localized PC between 2007 and 2011. Clinical stage and tumour grade were analysed earlier [10] as this is the same group of patients. The protocol was approved by the Ethical Committee of the Centre of Oncology, and each patient submitted written consent.

Immunohistochemical assessment of tumour markers

Following rehydration, blocking the endogenous peroxidase, in 5 µm sections heat-based antigen retrieval was carried out (20-60 min or 1 h at 98°C with 10 mM citric acid buffer, pH 6.0). After 20 minutes, the sections were washed, flooded with 10% normal goat serum for 20 min and incubated with the appropriate antibody overnight at 4°C in a humidified chamber. For CD34 it was a mouse anti-human monoclonal antibody (DAKO, 1 : 200), and for VEGF a mouse anti-VEGF monoclonal antibody (DAKO, 1 : 25) in Tris-buffered saline (TBS, pH 7.4). After washing, slides were incubated at 37°C for 1 h with the DAKO En Vision visualisation system containing goat anti-mouse (CD34) or VECTOR ImmPRESS Reagent Kit (VEGF). The sections were stained with diaminobenzidine (DAB), counterstained with haematoxylin, dehydrated and mounted. Negative control slides omitting the primary antibody were included in each run of stains. The intensity of staining was evaluated by light microscopy at 400× magnification. Cytoplasmic VEGF expression was presented as a percentage of positively immunostained tumour cells (brown) in several (5-7) malignant areas of the tissue section. High-grade prostatic intraepithelial neoplasia foci and non-hyperplastic benign acini were not evaluated; however, they were stained heterogeneously.

For vascular density assessment 7-10 high power (400×) tumour fields, where one field is equivalent to 0.292 mm^2, were counted for each patient. We counted highlighted endothelial cells or cell clusters clearly separated from adjacent microvessels, tumour cells and other connective tissue elements regarded as a distinct countable microvessel. The mean vessel count (MVD) per 1 mm^2 of tumour volume was used in the analysis.

Statistical analysis

Statistical analysis was performed with STATISTICA vs. 9 (StatSoft Inc.). Intergroup differences in the original data were tested with ANOVA test or Student's t test. P values of less than 0.05 were considered to indicate statistical significance.

The correlation between the immunohistochemical expression of VEGF and MVD or PSA was determined using Spearman's test.

Results

We examined human histological specimens from 140 PC patients treated with radical surgery. The mean age of the patients was 62.9 ±6.2 (range 49.0-77.0) years. There were 13 (9.3%) at pTNM stage 1, 78 (55.7%) at stage 2, 40 (28.6%) at stage 3 and 9 (6.4%) at stage 4. In the analysed group there were 75 (53.6%) well-differentiated, 53 (37.8%) moderately differentiated and 12 (8.6%) poorly differentiated tumours.

Pre-operative PSA revealed a mean PSA of 9.9 ±0.5 ng/ml. Concentration of serum PSA was significantly increased with pTNM stage ($p = 0.011$), Gleason score ($p = 0.011$) and tumour grade ($p = 0.003$) (Table I). In 100 (75.7%) tumours positive VEGF expression was shown. Staining was entirely cytoplasmic (Fig. 1) and restricted mainly to secretory cells. Uniform stain intensity was seen within the cells of selected tumour acini; however, heterogeneous staining patterns were observed between different tumour foci. Very occasional positive staining was also visible in endothelial and muscle cells, macrophages and neutrophils as these cells are able to synthesize and secrete VEGF. In the analysed group of tumours the mean percentage of positive VEGF cells was 14.8 ±1.4%. The presence of positively stained cells was not correlated with tumour grade ($p = 0.649$) and Gleason scores ($p = 0.697$). However, growth of VEGF expression was observed with increase of pTNM stages. Significantly higher values for the protein were observed in pTNM 3 ($p = 0.035$) and 4 ($p = 0.037$) than in pTNM stage 1 (Fig. 2).

In the whole series of tumours the mean MVD was 97.5 ±2.4/mm^2. Single cell sprouts as well as larger

Table I. Distribution of serum PSA levels, VEGF and MVD for 140 prostate cancer patients

PARAMETER	N	PSA (NG/ML) MEAN ± SE	VEGF (%) MEAN ± SE	CD34 (/MM^2) MEAN ± SE
pTNM				
1	13	10.5 ±2.0	7.4 ±1.7	99.1 ±4.5
2	78	8.8 ±0.5	15.3 ±2.0	96.1 ±2.9
3	40	10.8 ±0.8	16.1±2.2	101.6 ±5.6
4	9	14.6 ±2.8	16.5 ±4.31	89.4 ±10.4
Gleason score				
5	11	7.7 ±1.0	20.8 ±6.6	104.9 ±10.2
6	62	8.9 ±0.6	15.0 ±2.1	96.6 ±3.6
7	54	10.4 ±0.7	13.1 ±2.1	99.6 ±3.9
8	8	15.1 ±3.7	14.9 ±3.9	94.7 ±4.1
9	5	12.6 ±2.4	17.3 ±5.1	74.9 ±11.4
Grade				
1	75	8.7 ±0.5	15.9 ±1.9	97.5 ±3.3
2	53	10.7 ±0.7	13.2 ±2.2	100.5 ±3.9
3	12	14.0 ±2.7	15.3 ±3.2	84.9 ±5.5

vessels are included in the counts (Fig. 3). A non-significant decrease in the number of microvessels was observed in the highest pathological tumour volume (P = 0.631), Gleason score (p = 0.368) and tumour grade (p = 0.233) (Table I). Preoperative serum PSA level was not associated statistically with either MVD (p = 0.466) or VEGF expression (p = 0.188). There was also no correlation between the immunohistochemical expression of VEGF and MVD (p = 0.925).

Discussion

We did not observe a significant correlation between VEGF expression, Gleason scores and tumour grade. However, we noted a statistically significant increase of VEGF expression between stages pTNM 1 and pTNM 3 or pTNM 4.

In our study, positive staining for VEGF was seen in 75.7% of cases, which is in accord with previous findings [5, 11]. However, the published data regarding VEGF association with clinico-pathological variables in prostate cancer are conflicting. We did not observe a significant association between VEGF expression and nuclear grade, and in this respect our findings contradict some earlier studies [5, 6, 11]. Although the number of negative VEGF cases decreased with tumour grade – from 19 (25.3%) in G1 to 1 (8.3%) in G3 – this had no influence on increase of percentages of VEGF positive cells. Similarly to the findings of Ferrer *et al.* [12] we observed more intense VEGF staining in well-dif-

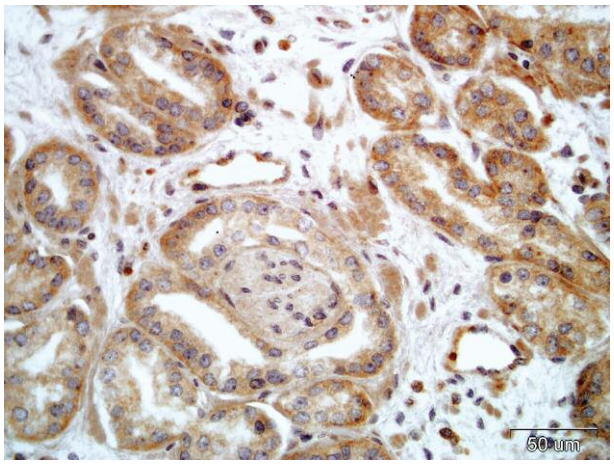

Fig. 1. Strong cytoplasmic VEGF expression in intermediate grade prostatic carcinoma

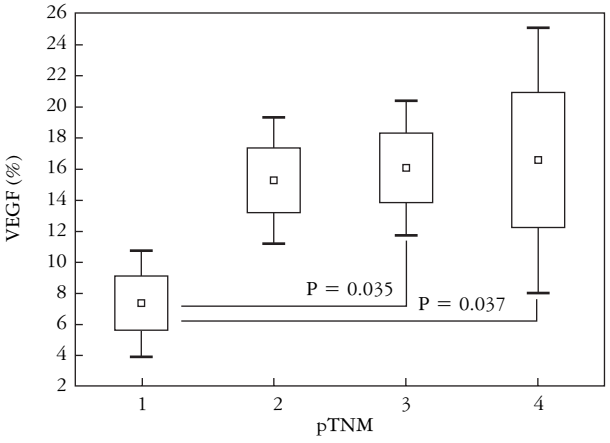

Fig. 2. Association between VEGF expression and PC pathological tumour stage. Symbols represent mean values ± SE

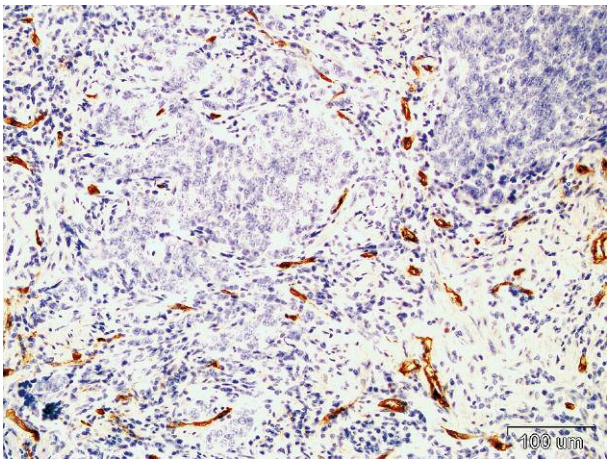

Fig. 3. Positive expression of CD34 in endothelial cells of high grade prostatic carcinoma

ferentiated than in poorly differentiated tumours. Therefore, our data are in agreement with those authors who noted no difference in the intensity or distribution of VEGF immunoreactivity between well-, moderately and poorly differentiated tumours [4, 12]. This may indicate that VEGF expression might not be related to malignancy, which could confirm no difference in VEGF immunoreactivity between malignant and benign prostatic epithelium shown by some studies [7, 11]. However, similarly as in earlier studies [11], we observed increased VEGF immunoreactivity with pathological stage. We noted elevation of VEGF expression in stages pTNM 3 and pTNM 4, in which there was seen a subsequent decrease of vascular density.

The mean value of MVD obtained in our study (97.5/mm^2) is within the range given by other authors [8, 9, 13-15]. The lowest microvessel density was observed in the highest Gleason scores, tumour grade and pT classification. There was no significant relationship between MVD and pTNM. Lack of correlation between tumour volume and MVD has also been reported by other authors [13, 14, 16, 17]. However, some authors found that MVD is associated with tumour stage [8, 13, 18-20]. Gleason score was associated with MVD in some studies [9, 17, 19-21], but in others, similarly to our results, such a correlation was not indicated [8, 16]. Several key studies concerning MVD in PC are summarized in Table II.

This indicates that data are inconclusive and the differences may depend upon several parameters, such as the kind of endothelial marker (CD31, CD34, factor VIII antibody) used, the way in which the microvessels are analysed [Chalkley point eyepiece graticule, "hot spot" automated digital image analysis, tissue microarray (TMA)] or size of tumour sample analysed (surgical or tumour biopsy) [8, 20, 24], and the small biopsy specimens (TMA) seem to be critical. Counting MVD on large sections appears to be superior to the counting of all microvessels in each TMA spot [20].

In our study, the lack of correlation between MVD and tumour volume is not surprising. In a higher tumour volume with lower oxygen concentrations tumour cells can remain viable, and they can exist at greater distances from the vasculature because they acquire the ability to take up glucose and perform glycolysis. In the same series of tumours we found a significantly larger number of GLUT-1 positive cells in higher than in lower pTNM stages [10]. This may suggest that in pT3 and pT4 tumours the lower vascular number was compensated by

Table II. Overview of methods, MVD values and their correlation with pathology results in prostatic cancer

Author	N	Antibody	Magnification	MVD	Correlation with	
				Mean (range)	Gleason score	pT stage
Weidner *et al.* 1993 [9]	74	FVIII	200×	39.2 (10-110)	+	+
Brawer *et al.* 1994 [13]	32	FVIII	400×	81.2 (45.7-116.9)	–	+
Silberman *et al.* 1997 [17]	109	CD31	400×	47.1 (18.2-108.8)	+	–
Arakawa *et al.* 1997 [21]	101	FVIII		95.6 (22-274)		
		CD34	400×	151.6 (64-302)	+	+
Rogatsch *et al.* 1997 [18]	36	CD31	400×	35.5 (13-70)	+	+
Bettencourt *et al.* 1998 [22]	149	CD34	200×	116.7 (18-315)	+	+
Rubin *et al.* 1999 [16]	87	CD31	400×	78.0 (15-324)	–	–
Borre *et al.* 2000 [15]	221	FVIII	400×	43.0 (16-151)	+	+
De la Taille *et al.* 2000 [8]	102	CD34	400×	80.3 (21-179)	+	+
		CD31		60.1 (6-184)	+	+
Mucci *et al.* 2009 [23]	572	CD34	200×	76.0 (13-491)	+	+
Erbersdobler *et al.* 2010 [20]	2508	CD31	100×	16 (1-89)		
				(TMA)*	+	+
Present study	140	CD34	400×	97.5 (32.4-190.3)	–	–

**Tissue microarray*

glucose uptake for cell metabolism. It is known that in hypoxic conditions, the cells produce hypoxia-inducible factor (HIF), a transcription factor which stimulates the release of VEGF. Circulating VEGF then binds to VEGF receptors on endothelial cells, triggering a tyrosine kinase pathway leading to angiogenesis. In higher tumour stages we observed significant increase of VEGF expression; therefore expression of this protein seems to reflect better tumour progression in PC than MVD.

Recently it has been considered that microvessel density is not a measure of the angiogenic dependence of a tumour. It rather reflects the metabolic burden of the supported tumour cells [25]. Contrary to common belief, microvessel density does not reflect the angiogenic activity but the intercapillary distance. Oxygen and nutrients consumption sets a limit on how far away from the vasculature tumour cells can remain viable. It is approximately 150 μm and viable cells form a viable cuff around a vessel. However, cuff size tends to vary with the tumour metabolic demand [25]. Tumours with low metabolic demand have relatively large cuff sizes with many cell layers and relatively low vascular density. As the metabolic needs of cancer cells vary with the tissue of origin and change with tumour progression [25], MVD may not be an indicator of antiangiogenic efficacy. Current opinion is that a proportion of primary PCs may progress via a non-angiogenic pathway without neo-vascularisation, and be clinically more aggressive than angiogenic tumours [26].

We did not observe a significant correlation between VEGF and PSA level, although such a correlation was found by some authors [11, 27]. In our series, patients who had higher PSA levels manifest lower VEGF expression. This might confirm earlier studies showing that VEGF expression may be controlled by androgens [2, 28]. Also PSA was not related to MVD, similarly as in other authors' studies [16, 23]. Additionally, we did not observe a correlation between MVD and VEGF expression. However, contradictory results were obtained in other studies [6], where VEGF expression was correlated with MVD.

The assessment of the examined proteins' expression may be important not only for prediction of biological tumour behaviour but also for development of therapeutic strategies incorporating antiangiogenic drugs with chemotherapy. Perhaps it should be remembered that MVD is not a measure of the angiogenic dependence of a tumour, and may not be an indicator of angiogenic treatment efficacy [20, 25]. Low microvessel density within tumours might not be a sufficient criterion to exclude patients from treatment with angiogenesis inhibitors [26]. Furthermore, rapid tumour growth may not imply high MVD [24, 25].

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