Expression of endothelial factors in prostate cancer: A possible role of caveolin-1 for tumour progression

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Abstract. Solid tumours need to induce their own vascular supply, and microvessel density (MVD) has emerged as a prognostic factor in several tumours. We hypothesized that mRNA levels of some endothelial factors in prostate cancer tissue would correlate with histologically measured MVD, or other pathological parameters. Expression levels of the endothelial factors CD31, CD34, CD105, CD144, CD146, CAV1 and VEGFR2 were assessed by RT-qPCR in matched freshly frozen normal and tumour tissues from 69 patients that underwent radical prostatectomy. The results were compared to pathological parameters and the MVD in the corresponding paraffin-embedded material, as determined by immunohistochemistry against CD31 and CD34. Comparing mRNA expression in matched normal and tumour samples, only CAV1 showed relevant differences, being down-regulated in tumour tissues (fold change = -1.89, P<0.0001). CAV1 down-regulation correlated with pT category (P=0.006) and the Gleason score (P=0.041). In a univariate analysis, lower CAV1 mRNA expression was associated with biochemical recurrence (P=0.019). By immunohistochemistry, CAV1 was mainly localized in endothelial and stromal cells and showed a weaker staining pattern in the tumour compared to normal tissue. Furthermore, MVD significantly correlated with tumour grade and pT category. There was no significant association between endothelial mRNA expression and histologically determined MVD in tumour tissues, but only a trend for CD31 mRNA (P=0.074) and an inverse trend for CAV1 mRNA (P=0.056). In conclusion, there is only a weak correlation between the mRNA expression of endothelial factors and MVD in prostatic tumour tissue. However, loss of CAV1 mRNA expression may play a role in prostate cancer progression.

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Introduction

Numerous studies have shown that the amount of newly formed microvessels play an important role in growth and metastasis of many tumour types. Intratumoural microvessel density (MVD) was demonstrated to be a marker for the quantification of this neo-angiogenic process (1-4). In prostate carcinoma, the role of MVD is still under investigation and especially in small biopsy samples its prognostic impact remains unclear (1,3,5-14). Although the predictive value of MVD was demonstrated by several studies, others failed to show a statistically significant contribution of this parameter, and MVD was not strongly recommended for routine application in prostate cancer by the World Health Organization (WHO) and the College of American Pathologists (15-17). Usually, the demonstration of MVD is carried out by immunohistochemistry with antibodies against CD31, CD34 or von Willebrand factor, which stain endothelial cells and allow for counting even very tiny vessels in a given area under the microscope. Unfortunately, this is a very time-consuming procedure and also depends on the person that analyses the tissue sections. Other methods to assess neo-angiogenesis in solid tumour tissue are therefore desirable.

In this study, we investigated angiogenesis in prostate cancers at the mRNA level using quantitative real-time polymerase chain reaction (RT-qPCR). To this aim, we chose seven factors [CD31, CD34, CD105, CD144, CD146, caveolin-1 (CAV1) and VEGFR2], that were partly used in previous immunohistochemical MVD studies.

CD31, also known as PECAM-1 (platelet-endothelial cell adhesion molecule) is a cluster of differentiation factor that mainly occurs on the membranes of endothelial cells, but also on macrophages or platelets (18). Another factor of the vascular endothelium is CD34, a cluster of differentiation molecules located on hematopoietic stem cells, and suitable for detecting especially small, newly formed vessels (19). Compared to normal prostatic tissue, an increased number of capillaries expressing CD34 has been demonstrated in prostatic adenocarcinoma (5,20,21).

CD105 (Endoglin) is a transmembrane molecule abundantly expressed in endothelial cells, which is involved in cell proliferation since it is a receptor for the transforming growth factors TGFβ1 and -3 (22).

Table I. Oligonucleotides and probes used for qRT-PCR expression analysis.

Gene	Reference sequence accession number (PubMed)	Forward (5'-3')	Reverse (5'-3')	Universal probe (UPL)
CD31	NM_000442	gcaacacagtccagatagtcgt	gacctcaaactgggcatcat	26
CD34	NM_001025109	gtgaaattgactcagggcatc	cccctgtccttcttaaactcc	1
CD105[a]	NM_001114753	ccactgcacttggcctaca	atggcagctctgtggtgtt	64
	NM_000118			
CD144	NM_001795	aagcctctgattggcacagt	ctggcccttgtcactggt	58
CD146	NM_006500	cctgctggctgctgtcctc	cacttcagaagggctgtgct	22
CAV1	NM_001753	ttccttcctcagttcccttaaa	gggaacggtgtagagatgtcc	26
VEGFR2	NM_002253	gtggaggagaagtccctcagt	tccaaggtcaggaagtccttatac	35
TUBA1B	NM_006082	ccttcgcctcctaatcccta	agcaggcattgccaatct	64

[a]For CD105, both transcript variants were detected by using the indicated oligonucleotide pair.

The adhesion molecule CD144 (vascular endothelial cadherin, VE-cadherin), a member of the cadherin family, and the melanoma adhesion molecule CD146, are both expressed in endothelial cells (23). For CD146, an association with tumour progression and metastasis in malignant melanoma has been shown (24,25). CAV1 is the main structural component of lipid raft invaginations in cell membranes of various cell types including endothelial cells, smooth muscle cells, fibroblasts and adipocytes. Moreover, CAV1 plays a role in cellular processes like endocytosis and signal transduction and was shown to mediate angiogenesis (26,27). The expression of VEGFR2 (vascular endothelial growth factor receptor 2, KDR) was also investigated. This receptor can be found on endothelial cells and is a key regulator of the VEGFA-induced angiogenetic pathway (28).

We wanted to know whether there is a differential expression of these factors between prostatic tumour tissue and matched controls, which would possibly indicate a role of these factors in tumour neo-angiogenesis. Furthermore, we were interested if there is any correlation of these factors with the MVD determined on corresponding sections from paraffin embedded tissue. Finally, we performed correlations between our findings and the basic pathological and clinical parameters to get a preliminary impression of any potential prognostic value of the markers examined.

Materials and methods

Sample collection. Tissue samples from 69 patients that underwent radical prostatectomy between 2001-2008 were included in this study. All tissues were retrieved after informed consent was obtained by the patients, and in accordance with the ethical guidelines of the Charité Berlin. The median age of the patients was 62 years (range, 46-74). The pathological examination of the prostatectomy specimen disclosed organ-confined cancer (pT2) in 46 patients and extraprostatic extension (pT3) in 23 patients. Twenty-five patients had a Gleason score of ≤6, 30 patients had a Gleason score of 7, and the remaining 14 patients had a Gleason score of ≥8. The small number of patients disallowed us any further subclassification of stage or grade for the purpose of this study. To harvest absolutely fresh tissue, the pathologist received the specimen immediately in the operating room and performed one single cut in the putative tumour region, then removed one slice of tissue while sparing the capsule, and closed the cut by a suture. Freshly frozen prostatic tissue sections were analysed for tumour foci and normal tissue areas and were then manually microdissected and stored at -80˚C until further analysis.

RNA isolation and cDNA synthesis. Total RNA from freshly frozen tumour and normal tissue was isolated using the miRNeasy Mini kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. RNA integrity was verified using the Agilent 2100 Bioanalyzer (Agilent Technologies, USA). For cDNA synthesis, 1 μg of total RNA was reverse-transcribed with the Transcriptor First Strand cDNA Synthesis Kit (Roche Diagnostics, Mannheim, Germany) with both, random and oligo(dT) primers in a 2:1 ratio. cDNAs were diluted 1:4 and 1 μl was used per amplification reaction.

Quantitative real-time polymerase chain reaction (RT-qPCR). The mRNA expression analysis was performed in a 96-well format on the LightCycler 480 instrument (Roche) using the LightCycler 480 Probes Master (Roche) with a total volume of 10 μl. Primers (Tib Molbiol, Berlin, Germany) and specific Universal Probe Library (UPL) probes (Roche) are listed in Table I. The PCR conditions were as follows: pre-incubation for 10 min at 95˚C, amplification for 10 sec at 95˚C, 20 sec at 60˚C, and 1 sec at 72˚C for acquisition. The amplification program was repeated 45 times.

For normalisation, α-tubulin (TUBA1B) was used as reference gene since it did not show significant expression changes between tumour and normal tissue cohorts. For quantification, a standard curve for each gene was generated. All samples were measured in triplicates and several controls were included (no-template control, interplate control, standards). Expression changes between matched tissue samples were calculated as fold changes.

Tissue microarray (TMA) analysis. For TMA construction, archived paraffin-embedded tissue blocks from 64 of the 69 cases (5 blocks were missing in this retrospective study) were

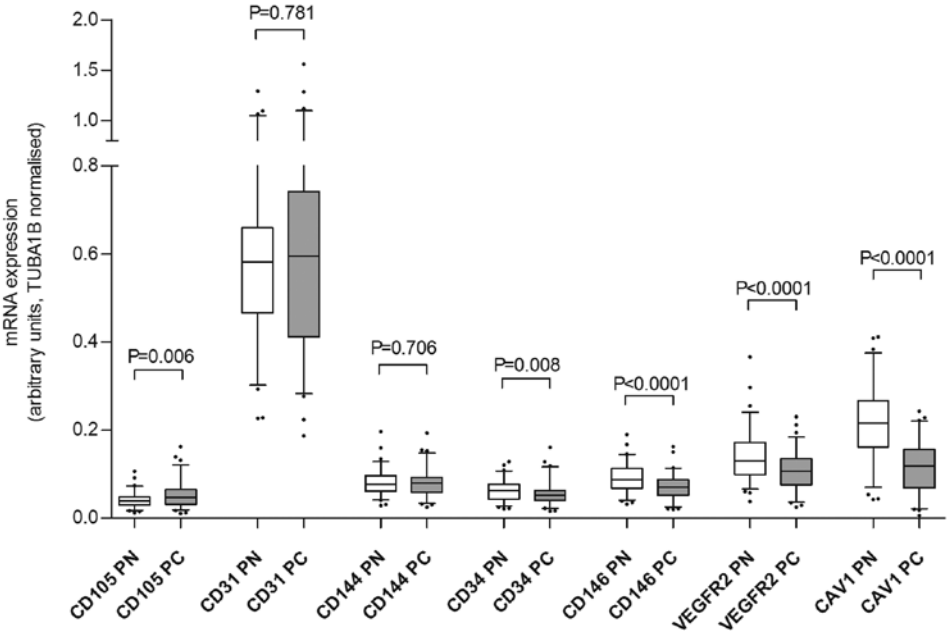

Figure 1. mRNA Expression of endothelial factors in matched prostatic normal (PN, n=69) and tumour tissues (PC, n=69). Boxes demonstrate lower and upper quartiles with medians (horizontal lines). The 5 and 95 percentiles are depicted by whiskers. P-values indicate significant differences.

used that corresponded to the area where the fresh tissue was obtained. One representative tumour tissue core (1.5 mm in diameter) of each patient was transferred in a new paraffin block as previously described (29).

Immunohistochemistry. The MVD was determined on freshly cut paraffin sections from the TMA. Endothelial cells were marked with established monoclonal antibodies against CD31 (clone QBEnd1, 1:100, Dako, Hamburg, Germany) and CD34 (clone JC/70A, 1:50; Dako) (30,31). Furthermore, non-TMA tissue sections containing tumour and normal prostatic tissue were immunostained for CAV1 protein (polyclonal, N-20, 1:500; Santa Cruz Biotechnology, Heidelberg, Germany).

Immunohistochemistry was performed using the labelled streptavidin-biotin (LSAB) method (Dako). In brief, after deparaffinizing in xylene and ethanol, the tissue sections were boiled in citrate buffer in an autoclave for 5 min. The slides were incubated with the primary antibody for 1 h followed by 20 min of incubation with the biotinylated link. The alkaline phosphatase-conjugated secondary antibody was then added for 20 min. The staining procedure was carried out using Sigma Fast Red TR/Naphthol AF/TX Tablets (Sigma-Aldrich Chemie GmbH, Munich, Germany) and was controlled under the microscope.

MVD determination. In each TMA spot, microvessels in an area of 0.196 mm^2 (magnification, x40) were counted using a light microscope (Leica DM2000, Leica, Wetzlar, Germany) according to the method described by Weidner *et al* (1). The MVD was extrapolated to 1 mm^2 and was then correlated with RNA expression levels and pathological parameters.

Statistical analysis. Statistical analysis was carried out using the GraphPad Prism 5.0 software (GraphPad, San Diego, CA, USA) and SPSS18 (IBM SPSS Statistics, Chicago, Il, USA). To determine significant differences in gene expression comparing normal and tumour tissues, the non-parametric Wilcoxon test was performed. The Mann-Whitney U test or Kruskal-Wallis test was used to analyze the relationship between the expression levels and pathological parameters (Gleason score, pT stage). Associations between MVD counts and pathological parameters were calculated using the Mann-Whitney U test. Expression ratios were correlated with each other using the Spearman correlation matrix. The χ^2 test was performed to analyze a putative association of MVD and CD31 or CD34 mRNA expression. Univariate Kaplan-Meier survival analysis (log-rank test) was calculated with SPSS18. P-values <0.05 were considered statistically significant.

Results

All RNA samples from tumour and normal prostatic tissues were examined by Agilent technology and showed quality values of RNA integrity numbers (RIN) >6.

RNA expression of endothelial factors in normal and tumour tissues in the prostate. Comparison of the mRNA expression levels of all analysed endothelial factors in the prostatic tissue samples, reveled the highest amount for CD31 (Fig. 1). We calculated the fold changes representing expression differences of each endothelial factor to compare the RNA levels in normal and matched tumour tissues (Table II). In spite of statistically significant differences, only fold changes >1.5 or <-1.5 were considered to be relevant in the present investigation according to the study of Chen *et al* (32).

The frequently used MVD marker CD31 as well as CD144 showed no relevant expression differences in normal vs. tumour tissues (fold changes 1.06 and 1.02, respectively). Moderate expression changes with statistical significance were observed for CD34, CD105, CD146 and VEGFR2 (fold changes -1.17,

Table II. mRNA expression changes of endothelial factors represented by fold changes and statistical analysis.

			mRNA expression and pathological parameters	
	Fold changes (normal vs. tumour)	P-value[a] (normal vs. tumour)	P-value[b,d] (pT2 vs. pT3)	P-value[c,d] Gleason score <7 vs. 7 vs. >7
CD105	1.15	0.006	0.188	0.460
CD31	1.06	0.781	0.394	0.452
CD144	1.02	0.706	0.670	0.774
CD34	-1.17	0.008	0.712	0.834
CD146	-1.25	<0.0001	0.138	0.296
VEGFR2	-1.27	<0.0001	0.321	0.627
CAV1	**-1.89**	**<0.0001**	**0.006**	**0.032**

[a]Wilcoxon test; [b]Mann-Whitney U test; [c]Kruskal-Wallis test; [d]only statistically significant results were further analysed (see Fig. 2). Bold indicates statistically significant differences using 1.5 fold-change as a cut-off for relevance.

Table III. Spearman correlation matrix of fold changes of endothelial cell marker expression.

factor	CD105	CD144	CD31	CD34	CD146	VEGFR2	CAV1
CD105	-	0.556[c]	0.547[c]	0.446[c]	0.126	0.332[a]	-0.184
CD144	0.556[c]	-	0.724[c]	0.810[c]	0.534[c]	0.676[c]	0.305[a]
CD31	0.547[c]	0.724[c]	-	0.642[c]	0.219	0.395[b]	0.124
CD34	0.446[c]	0.810[c]	0.642[c]	-	0.456[c]	0.671[c]	0.370[b]
CD146	0.126	0.534[c]	0.219	0.456[c]	-	0.579[c]	0.746[c]
VEGFR2	0.332[b]	0.676[c]	0.395[b]	0.671[c]	0.579[c]	-	0.401[b]
CAV1	-0.184	0.305[a]	0.124	0.370[b]	0.746[c]	0.401[b]	-

R_s values as well as P-values are shown. [a]P<0.05; [b]P<0.01; [c]P<0.001.

1.15, -1.25 and -1.27, respectively; Table II). However, using 1.5 as cut-off for relevance, we found a significant down-regulation only for CAV1 mRNA in 87% of tumour specimens when compared to matched normal tissue samples (fold change = -1.89, P<0.0001, Table II).

Performing Spearman correlation matrix analysis, only CD146 and CAV1 did not correlate with CD105 and CD31. All remaining endothelial associated factors correlated with each other (Table III).

Associations with pathological and clinical parameters. The mRNA expression of most endothelial factors did not correlate with pathological parameters (Table II). However, CAV1 mRNA expression showed significant inverse associations with pT stage and Gleason score (Table II, Fig. 2). In a univariate Kaplan-Meier analysis and by dichotomizing the expression levels by the median, a lower rate of PSA-free survival for patients with CAV1 mRNA expression below the median could be demonstrated (log-rank: P=0.019; Fig. 3).

Immunohistochemical analysis. For TMA analysis, paraffin-embedded tissue blocks of 64 patients were available. Due to loss of tissue spots during the staining procedure, 48 tumour spots could be analysed by immunohistochemistry against

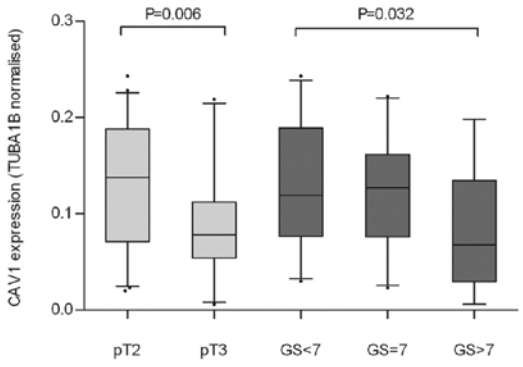

Figure 2. Associations of CAV1 mRNA expression with pT category and Gleason score. Boxes show lower and upper quartiles with medians (horizontal lines). Whiskers depict the 5 and 95 percentiles. P-values indicate significant differences.

CD31 and CD34. Microvessel counts correlated significantly between both markers (r_s=0.603, P<0.0001). MVD determined by CD31-positive vessels associated significantly with the Gleason score (P<0.001), whereas the density of CD34-marked microvessels showed significant associations with both, pT stage (P=0.017) and the Gleason score (P=0.004).

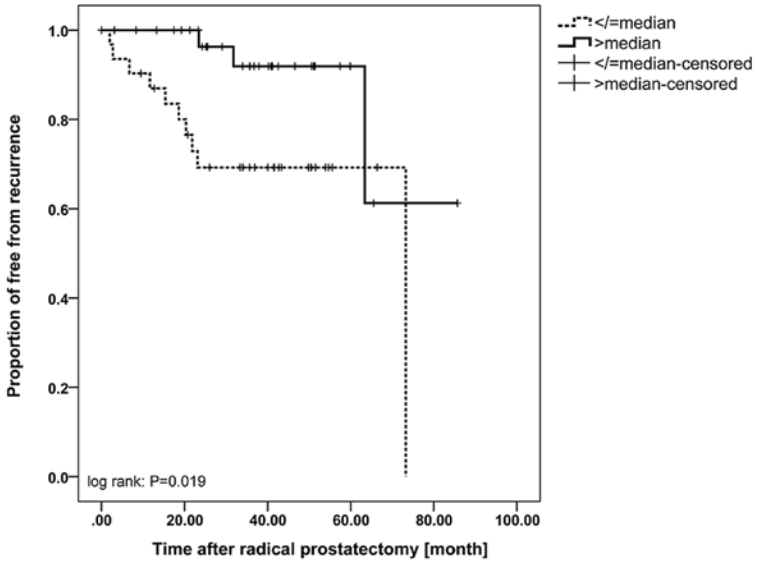

Figure 3. Univariate Kaplan Meier analysis of CAV1 mRNA expression dichotomized by the median. Time of recurrence was given, when the postoperative PSA level [that was undetectable (detection limit <0.04 ng/ml) after surgery] was >0.1 ng/ml.

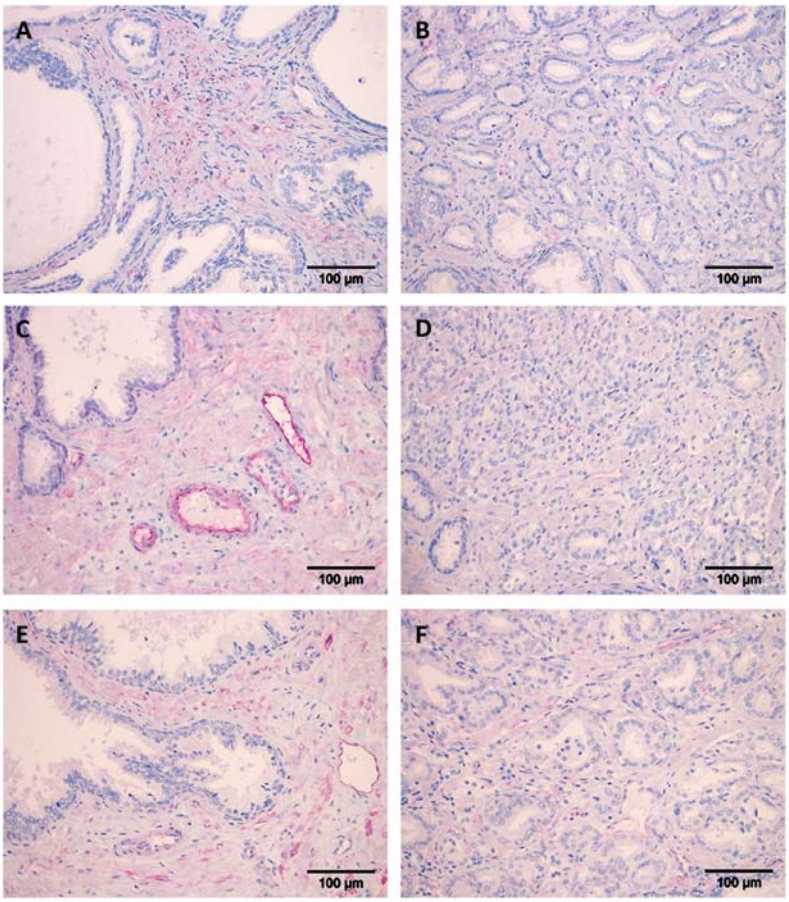

Figure 4. CAV1 protein expression in representative prostatic (A, C and E) normal and corresponding (B, D and F) tumour regions of 3 cases (magnification, x200). In a sub-group of the analysed patients, CAV1 expression was lower in stromal regions and endothelial cells of the tumour than in the corresponding normal counterparts.

In addition, we investigated CAV1 protein expression in whole tissue sections. We found expression differences in prostatic stromal cells between tumour and normal tissue in 12 out of 64 cases (Fig. 4).

Comparison of RNA expression of endothelial factors and MVD. Neither CD31 nor CD34 mRNA expression correlated significantly with histologically determined MVD. In a χ^2 test however, CD31 mRNA expression showed a trend of

association with CD31-stained MVD (P=0.074). CAV1 mRNA demonstrated a trend of an inverse correlation with MVD assessed by both endothelial cell markers [r_s(CD31)=-0.277, P=0.054; r_s(CD34)=-0.277, P=0.056]. None of the other endothelial factors investigated showed significant correlations with the MVD.

Discussion

Angiogenesis is an integral part in the progression of solid malignant tumours. Since the study of Gimbrone *et al* in 1972, it has been recognized that tumours are able to arrange their own blood supply (33). Subsequently, the field of angiogenesis came into the focus of cancer research not only for prognostic purposes, but also because specific inhibitors of angiogenesis were developed for the treatment of several solid tumour types. It is conceivable, that some sort of quantification of the angiogenic potential in malignant tumours would give valuable information regarding prognosis and response to targeted therapy.

In the present study, we analysed the mRNA expression of several angiogenic markers in tumour and normal prostatic tissues and compared the results to the histologically determined MVD and to pathological and clinical factors.

Analysing the MVD by immunohistochemistry, comparable results could be demonstrated with the endothelial cell markers CD31 and CD34, validating the suitability of both of these markers to assess MVD in prostatic cancer. The intratumoural MVD associated significantly with prostatic tumour grade and stage. This confirmed the results of a previous study (15) and made MVD in our specimens a suitable parameter for the comparison with the mRNA expression experiments.

Correlation between mRNA expression and immunohistochemically determined MVD was not strong. Only a trend for a correlation between CD31 mRNA expression and CD31-stained vessels was identified, as well as an inverse trend for CAV1 mRNA and MVD determined by CD31 and CD34 staining, respectively. This is in contrast to some other malignancies where investigators were able to show a correlation between the expression of endothelial factors and MVD. For example, the expression of the endothelial factors CD144 and VEGFR2 by RT-qPCR were proposed to be reliable markers for MVD determination in esophageal carcinoma (34). Moreover, CD144 was shown to correlate with the tumoural vasculature in breast cancer (35). An explanation for this observation might be the fact that the growth kinetics differ considerably between prostate cancer and the more aggressive cancers in the esophagus and female breast. In a slow-growing tumour like prostate cancer, it is conceivable that the dynamics of angiogenesis is also quite low. The subtle alterations of many endothelial mRNAs might not be detectable by quantitative methods like RT-qPCR, and by a definition of fold changes >1.5 or <-1.5 as relevant.

In our experimental setting, four of the seven mRNA factors we analysed showed only moderate changes in expression between tumour and normal tissue and only CAV1 showed a relevant expression difference, manifested by a decrease in its mRNA expression in nearly 90% of the cancerous tissue specimens. This structural component of lipid raft invaginations in cell membranes of various cell types is known to be involved in cellular processes like endocytosis and signal transduction (26). CAV1 is discussed to be both, a putative tumour suppressor gene and a tumour promoting factor (36,37). In 2005, Liscovitch *et al* proposed a complex hypothesis on the role of CAV1 expression for tumour development and cell survival (37,38).

Most of the recent studies on CAV1 focussed on its expression at the protein level in tissue or sera samples (39,40), whereas only few dealt with its mRNA expression. In accordance to our findings, Bachmann and colleagues also found a down-regulation on the transcript level when they analysed 35 specimens of prostate cancer (41). They suggested that DNA promoter methylation could play a role in the regulation of CAV1 gene expression and detected a putative regulating region consisting of seven CpG dinucleotides embedded in a CpG island upstream of the transcriptional start codon. Loss of CAV1 mRNA was also detectable in breast cancer compared to normal tissue samples, further supporting its role in glandular neoplasias (42,43).

We found evidence that loss of CAV1 mRNA might also have some prognostic value in prostate cancer. We demonstrated a negative association with pathological stage and Gleason score. Furthermore, it correlated with PSA-free survival in a univariate analysis. However, this question was not the primary goal of our study and further investigations are clearly needed, since the number of cases is certainly too small to make a binding conclusion.

By immunohistochemistry, we were able to identify the compartment where CAV1 expression is down-regulated in prostate cancer to the stromal cells between the normal or tumour glands. At least in 12 out of the 64 analysed specimens, stromal and endothelial CAV1 in tumour regions showed a lower expression as compared to stromal regions surrounding normal prostatic glands. Differences in the stromal distribution of CAV1 protein were also reported by Di Vizio *et al* (44). They demonstrated that loss of stromal CAV1 represents a marker of advanced prostate cancer and metastatic disease.

In conclusion, we could show that the mRNA quantification of the endothelial factors CD31 and CD144 is not suitable to distinguish between normal and tumour tissues, whereas CD34, CD105, CD146, and VEGFR2 demonstrated only moderate expression differences, respectively. Furthermore, these factors are not able to give significant information about MVD in prostate cancer. However, CAV1 might be a promising marker for further studies, since it seems to be down-regulated in a significant proportion of prostate cancers, and especially in the more aggressive ones.

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