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ORIGINAL ARTICLE

Myoglobin expression in prostate cancer is correlated to androgen receptor expression and markers of tumor hypoxia

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Abstract Recent studies identified unexpected expression and transcriptional complexity of the hemoprotein myoglobin (MB) in human breast cancer but its role in prostate cancer is still unclear. Expression of MB was immunohistochemically analyzed in three independent cohorts of radical prostatectomy specimens (n=409, n=625, and n=237). MB expression data were correlated with clinicopathological parameters and molecular parameters of androgen and hypoxia signaling. Expression levels of novel tumor-associated *MB* transcript variants and the *VEGF* gene as a hypoxia marker were analyzed using qRT-PCR. Fifty-three percent of the prostate cancer cases were MB positive and significantly correlated with androgen receptor (AR) expression (p<0.001). The positive correlation with CAIX (p<0.001) and FASN (p=0.008) as well as the paralleled increased expression of the tumor-associated *MB* transcript variants and *VEGF* suggest that hypoxia participates in MB expression regulation. Analogous to breast cancer, MB expression in prostate cancer is associated with steroid hormone signaling and markers of hypoxia. Further studies must elucidate the novel functional roles of MB in human carcinomas, which probably extend beyond its classic intramuscular function in oxygen storage.

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Introduction

Myoglobin (MB) is a cytoplasmatic monomeric hemoprotein, which exists in human cardiac myocytes and mitochondria-rich skeletal muscles at concentrations of ~200–300 μM. At these levels, the protein serves as a storage site for oxygen (O_2), able to buffer intracellular O_2 concentrations at different metabolic states [1, 2]. Myoglobin knockout mice are viable, but their survival depends on the activation of various compensating mechanisms, i.e., increasing capillary densities and hematocrit [3, 4]. This supports the potential, yet controversially discussed, role of myoglobin to facilitate the transport of oxygen to the mitochondria for oxidative phosphorylation

within muscle cells. Several additional functions of myoglobin have been reported. These include maintaining nitric oxide (NO) homeostasis in muscle through scavenging [5] or producing NO molecules [6], scavenging of excessive reactive oxygen species (ROS) [7], e.g. H_2O_2, and binding of fatty acids [8].

In recent years, the existence of non-muscle myoglobin has been increasingly acknowledged, demonstrating myoglobin even in various tumor entities [9]. The latter has initiated a new research field, since the role of myoglobin in tumor development and progression remains poorly understood so far. The existence of MB-positive cancer cell lines also opens up new research options permitting to analyze the functions of MB in convenient cell models. In a previous study, immunohistochemical analysis in a large human breast cancer cohort revealed endogenous expression of MB in more than 70 % of invasive breast carcinomas. Interestingly, MB expression correlated significantly with a positive estrogen receptor alpha status and a better prognosis [10]. Furthermore, MB expression showed a significant correlation with endogenous markers for hypoxia (EPAS1 (HIF2A) and GLUT1) in vivo. In breast cancer cell lines, synthesis of *MB* messenger RNA (mRNA) utilized an alternative transcription start site and *MB* expression was induced by hypoxia, in part through HIF-1-/HIF-2-mediated transactivation. In line with these findings, the alternative transcriptional start site of the human *MB* gene is flanked by a HIF-1-/-2-binding functional hypoxia response element (HRE) [10, 11]. Moreover, our recent survey expanded the human *MB* gene by seven untranslated exons, located 5′-upstream of the coding DNA sequence. From these new exons, we could deduce 16 novel alternatively spliced *MB* transcripts in addition to the three already known. Most of the new *MB* transcript variants occur predominantly in tumor tissues or cell lines and are initiated from a novel tumor-associated promoter. In human breast cancer tissue, the *MB* mRNA variants 9, 10, and 11 dominated the transcriptional profile and clearly exceeded the amount of *MB* transcript variant 2 which represents the standard muscle-associated *MB* transcript. In line with the observed expression correlation between MB and hypoxia markers in breast cancer, the tumor-associated *MB* transcript variants were significantly increased in a breast cancer cell line in response to oxygen deprivation [11, 12]. This suggests a potential role of hypoxia in regulating human MB in a non-muscular context.

Breast cancer and prostate cancer share several biological features: both are adenocarcinomas, both are driven by steroid hormones, and both develop predominantly in the later phase of life. This prompted us to analyze the expression of myoglobin in prostate cancer and to investigate the associations of MB expression to clinicopathological parameters including follow-up data and molecular correlates of androgen signaling and tumor hypoxia. Furthermore, we investigated the transcriptional complexity of *MB* and its correlation to hypoxia in prostate cancer in comparison to breast cancer.

Material and methods

Ethics statement

The studies were approved by the Institutional Review Boards (IRB) of the University Hospital of Zurich, the Charité University Hospital, and the University Hospital of Bonn. The IRB waived the need for written informed consent from the participants.

Patients

For immunohistochemistry, two clinically well-characterised radical prostatectomy cohorts in a tissue microarray (TMA) format were used in this study. TMA #1 enclosed tissue samples from 529 patients: 409 (77.2 %) primary carcinoma specimens following radical prostatectomy in the Department of Urology of the University Hospital of Zurich (USZ), 29 (5.5 %) castration-resistant prostate cancer (CRPC) specimens, 46 (8,7 %) prostate cancer metastases, and 45 (8.5 %) benign prostate tissue specimens. Median follow-up time of the patients following radical prostatectomy was 60 months (range 0 to 136 months) and median age 66 years (range 46 to 95 years).

TMA #2 enclosed 640 patients who were diagnosed at the Institute of Pathology of Charité University Hospital, Berlin, between 1999 and 2005. Median follow-up time of the patients following radical prostatectomy was 51 months (range 0 to 129 months) and median age 62 years (range 43 to 74 years). Biochemical recurrence was defined by rising PSA levels exceeding 0.1 ng/mL from a nadir after surgery.

TMA #3 enclosed 235 patients who were diagnosed at the Institute of Pathology of the University Hospital of Bonn, between 2000 and 2008. Median follow-up time of the patients following radical prostatectomy was 73 months (range 0 to 140 months) and median age 65 years (range 45 to 83 years). Biochemical recurrence was defined by rising PSA levels exceeding 0.2 ng/mL from a nadir after surgery.

For statistical analysis, only patients with clinical follow-up data were considered. Clinical and histopathological data of the merged cohorts are summarized in (Table 1). Sixty-eight patients received hormonal therapy and no patient received chemotherapy prior to surgery.

Tissue microarray construction

Construction of tissue micro arrays was described in previous studies [13–15]. Briefly, on TMA #1, each case was

Table 1 Associations of MB expression with clinicopathological parameters. MB expression was dichotomized into MB− (0–1) versus MB+ (2–3)

Merged Cohort (*n*=1,135)

Characteristic	Patients n	MB−	MB+	*p* value
Age (year)				0.7
≤60	337	155 (46.0 %)	182 (54.0 %)	
>60	798	377 (47.2 %)	421 (52.8 %)	
Preop PSA (ng/ml)				0.4
0–10	677	303 (44.8 %)	374 (55.2 %)	
>10	331	158 (47.7 %)	173 (52.3 %)	
pT				0.9
pT2	692	316 (45.7 %)	376 (54.3 %)	
pT3	356	164 (46.1 %)	192 (53.9 %)	
Gleason				0.8
6	360	160 (44.4 %)	200 (55.6 %)	
7	485	237 (48.9 %)	248 (51.1 %)	
8–10	232	103 (44.4 %)	129 (55.6 %)	
Margin Status				0.6
R0	712	331 (46.5 %)	381 (53.5 %)	
R1	328	147 (44.8 %)	181 (55.2 %)	

represented by a single core (0.6 mm) of the dominant tumor nodule, whereas on TMA #2 and TMA #3 a minimum of two cores (1.8 and 0.6 mm, respectively) from the dominant tumor were sampled.

Immunohistochemistry

TMA #1 was processed in the laboratory for in situ techniques in the Institute of Surgical Pathology, Zurich. Immunohistochemical staining of the tissue sections was performed on the Leica BondMax (Leica Microsystems GmBH, Germany) automated staining system along with Leica Reagents and the Refine DAB detection kit with Heat-Epitope-Retrieval-Buffer using the following antibodies and dilutions: myoglobin, clone Z001, Zymed Laboratories (1:300); hypoxia inducible factor 1A, clone mgc3, Abcam (Cambridge, UK) (1:400); carbonic anhydrase IX, rabbit polyclonal, Abcam (1:300); glucose transporter 1, rabbit polyclonal, Chemicon (Temecula, CA, USA) (1:1000); fatty acid synthase, clone 3 F2-1 F3, Abnova (Taipei, Taiwan) (1:2000); androgen receptor (AR), clone F39.4.4, BioGenex (San Ramon, CA) (1:500); and forkhead box A1, clone C-20, Santa Cruz Biotechnologies (Santa Cruz, CA) (1:400).

TMA #2 and TMA #3 were stained in the immunohistochemistry laboratory in the Institute of Pathology, Bonn. Immunohistochemical staining of the AR in the tissue sections was performed using the LabVision Autostainer 480S system (Thermo Scientific, Waltham, MA, USA) along with the Thermo Scientific Reagents and the N-Histofine® DAB-3S detection kit. The PT-Module was used for dewaxing and epitope retrieval (pH 6.0 at 99 °C for 20 min). Immunohistochemical staining of MB and FASN was conducted by the Ventana Benchmark automated staining system (Ventana Medical Systems, Tucson, AZ, USA) using Ventana Reagents. The following antibodies and dilutions were used: myoglobin, clone EP3081Y (Abcam, Cambridge, UK) (the clone used for cohort #1 is no longer available), (1:200); androgen receptor, clone AR441 (Dako A/S, Glostrup, Denmark) (1:400); and fatty acid synthase, clone S2 (Abgent, San Diego, Ca) (1:750).

Slides were counterstained with hematoxylin, dehydrated, and mounted.

Evaluation of the slides of TMA #1 was performed by two pathologists (GK and KI). The slides of TMA #2 were evaluated by GK and BR and TMA #3 from GK and VS. MB immunoreactivity was semi-quantitatively scored and categorized as follows: (0) negative; (1) weak; (2) moderate; and (3) strong. For statistical analysis, MB expression was dichotomized into MB− (0–1) versus MB+ (2–3).

Detection of *MB* transcript variants in prostate cancer tissue

MB splice variants were analyzed in tumor and adjacent normal tissues, obtained from surgical specimens from 16 prostate cancer patients. Patient material was collected at the University Hospital of Bonn, between 2008 and 2013. Total RNA was isolated from fresh frozen tissues by means of the RNAeasy Kit (Qiagen), following the "animal tissue" protocol. First-strand complementary DNA (cDNA) synthesis was conducted with the Superscript III RT-Kit (Invitrogen), but on 500 ng total RNA. The resulting cDNA was diluted 1:2 and quantified with the Qubit ssDNA Assay Kit (Life Technologies). For qRT-PCR reactions, the GoTaq qPCR Master Mix (Promega) was used at a total volume of 10 μL. All assays were measured in duplicates in an ABI 7500 real-time cycler (Applied Biosystems), as described in Bicker et al. 2013 [12].

Statistics

Statistical analyses were performed with SPSS, Version 21 (IBM SPSS Statistics). Fisher's exact test and chi-square test for trends were used to evaluate the statistical

significance between MB expression and clinicopathological parameters. Spearman's Rho was used for bivariate correlation analysis. Univariate survival analyses were conducted according to Univariate Cox Proportional Hazards and Kaplan–Meier analyses. P values refer to Wald test and Log-rank test, respectively. A two-sided Student's t test for independent or paired samples with an error value of 5 % (α=0.05) was performed to infer statistical significance.

Results

MB expression in prostate tissues

Matching our previous observations in breast tissues, MB showed a cytoplasmic immunoreactivity in secretory epithelium of normal and malignant glands alike, whereas basal cells and stroma were negative. In some cases, an additional nuclear immunostaining could be observed.

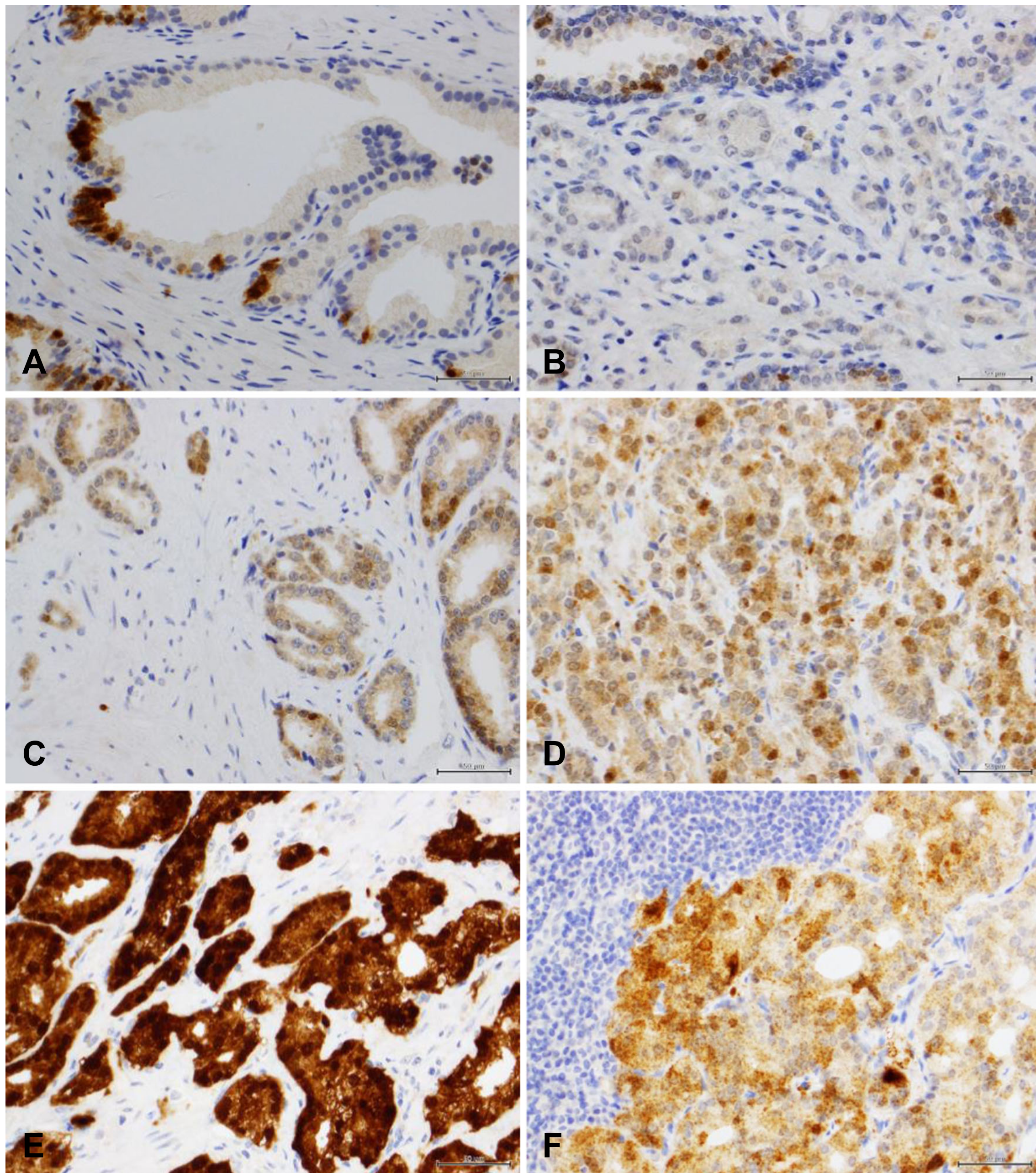

Fig. 1 Immunostaining of MB in prostate tissues. Benign glands display a heterogeneous pattern, with the majority of secretory epithelia being negative and hot spots of pronounced positivity (**a**). The expression levels in prostatic adenocarcinomas range from negative cases (**b**), weak expression (**c**), and moderate positivity (note additional nuclear staining in this example) (**d**) to intensely stained cases (**e**). An example with a moderately positive lymph node metastasis is shown in (**f**). Scale bar 50 µm

In cohort #1, 3 of 30 normal adjacent tissues (NAT) (10 %) showed no MB expression, weak expression in 46.7 % (n=14), moderate expression in 36.7 % (n=11), and strong expression in 6.7 % (n=2), resulting in a mean expression value of 1.4 and a median of 1.0. Metastases were MB-negative in 2 cases (5.1 %), 17 cases (43.6 %) were weak, whereas 20 cases (51.3 %) stained moderately to strongly, reflecting a mean value of 1.9 and a median of 2.0. A two-tailed Student's t test showed that MB expression in NAT is significantly lower compared to tumor or metastasis (p<0.001), whereas the MB expression between tumors and metastases showed no significant differences.

Representative immunostainings of MB in prostate cancer are given in Fig. 1. In the three cohorts, 1,137 primary prostate cancer cases were analysed for MB expression. Twenty-eight percent (n=318) of the samples showed no MB staining, weak expression in 23.5 % (n=267), moderate expression in 26.4 % (n=300), and strong expression in 22 % (n=250).

Correlation of MB with clinicopathological parameters and survival analysis

No significant associations of MB expression neither with clinicopathological parameters (Table 1) nor with longer recurrence free survival times in univariate Cox and Kaplan–

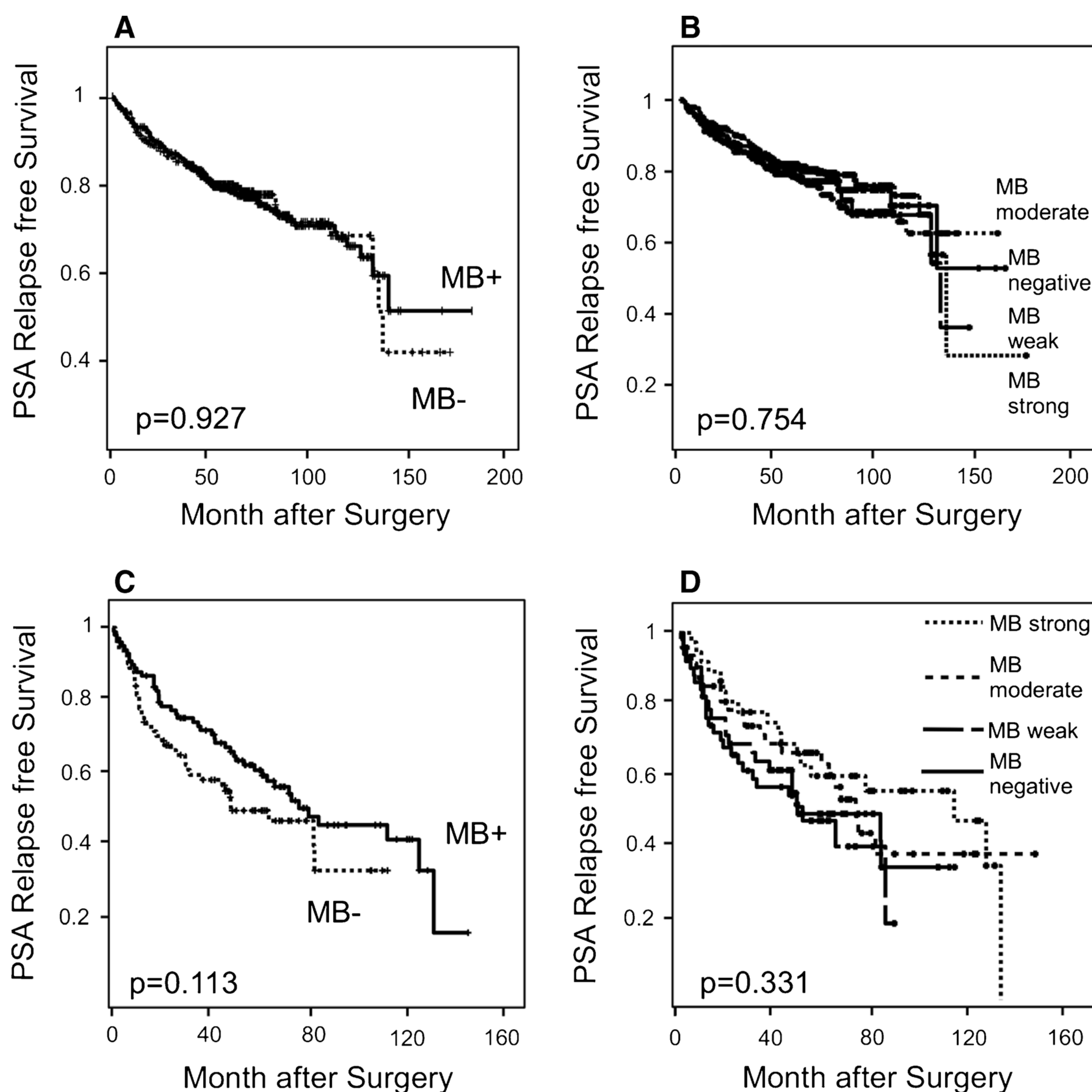

Fig. 2 Kaplan–Meier analysis (Log-rank test). **a** and **b** In 953 prostate cancer cases, no significant correlation between MB expression and longer recurrence free survival times was observed. **c** and **d** In a stratified analysis according to Gleason Scores in the subgroup of 182 Gleason 8–10 cases, a minor non-significant trend towards longer recurrence free survival times could be observed. MB expression was dichotomized into MB− (negative and weak) versus MB+ (moderate and strong)

Meier analysis (Fig. 2a, b) became apparent. Only a minimal visual trend can be appreciated in the Kaplan–Meier curve in the subgroup of Gleason 8–10 cases (Fig. 2c, d).

Correlation of MB expression with markers of hypoxia and androgen signaling

More than 200 prostate cancer cases of cohort #1 were additionally analysed for expression of hypoxia-inducible factor 1-alpha (HIF1A), the two HIF1 downstream targets glucose transporter 1 (GLUT1) and carbonic anhydrase 9 (CAIX), fatty acid synthase (FASN), the androgen receptor (AR), and forkhead box A1 (FOXA1). MB expression correlated significantly with CAIX, FASN, AR, and FOXA1, but failed significance for HIF1A or GLUT1 co-occurrence. The significant correlation between MB and the AR and FASN could be confirmed in the merged cohorts (Table 2).

Furthermore, in publicly available expression data sets of the androgen sensitive prostate cancer cell line LNCaP, either in response to dihydrotestosterone (DHT) [16] (GEO profiles, GDS3111) or to androgen deprivation [17] (GEO profiles, GDS3358), we found that *MB* expression significantly decreased after 16 h DHT treatment (p=0.03) while androgen deprivation induces *MB* expression after 3 weeks (p=0.01).

Detection of *MB* transcript variants in prostate cancer tissue

To clarify, if the novel *MB* transcript variants we recently found in breast cancer also play a crucial role in prostate cancer [11, 12], we analyzed fresh frozen prostate cancer tissues from 16 patients. Prior IHC, staining confirmed all samples to be MB-positive. In line with the findings in breast cancer, the alternative cancer-associated *MB* transcript variants 9, 10, and 11 were expressed at significantly higher levels (copy numbers) in prostate cancer compared to normal adjacent tissue and represented by far the most common *MB* splice variants in prostate cancer tissue. The sum of these variants in tumors was on average 96 times higher expressed than the muscle-associated *MB* variant 2 (Fig. 3). Upon increased *MB* expression in tumor samples, all of these three variants (transcribed from one common promoter) were driven simultaneously.

To examine a correlation between the expression of the *MB* transcript variants and the degree of hypoxia in the tissue, the mRNA levels of the hypoxia-specific biomarker vascular endothelial growth factor A (*VEGFA)* were quantified by qRT-PCR. A significant correlation between *VEGF-A* mRNA increase and the enhanced expression of *MB* variants 9, 10, and 11 could be observed (Table 3). This correlation is stronger in the normal adjacent compared to the malignant tissue.

Discussion

Until recently, MB has solely been considered an O_2 storage or transporter protein within cardiac or skeletal muscle cells. Meanwhile, it has been demonstrated that MB is also endogenously expressed in different tumor entities, i.e., breast, non-small cell lung, colon, and ovary cancers and in medullomyoblastomas [18–20]. However, the biological function and pathophysiological role of ectopically expressed MB remain unclear. The aim of the present study was to analyze the prostate cancer for MB expression and to correlate the resultant data to clinicopathological parameters. Our findings indicate that MB expression in prostate cancer might be regulated by androgen signaling and possibly also by hypoxia. Growth and differentiation of the prostate gland during development are dependent on androgens acting through the

Table 2 Correlations of MB expression with markers of hypoxia and androgen signaling. Upper values represent the correlation coefficients, p values according to Spearmen Rho

	Cohort #1						Merged cohorts	
	HIF1A	CAIX	GLUT1	AR	FOXA1	FASN	FASN	AR
MB	0.055; 0.348	0.246; 0.001***	0.053; 0.371	0.158; 0.006**	0.274; 0.001***	0.153; 0.007**	0.087; 0.008**	0.216; 0.001***
HIF1α		0.255; 0.001***	0.180; 0.001***	0.124; 0.014**	0.261; 0.001***	0.165; 0.001**		
CAIX			0.333; 0.001***	0.253; 0.001***	0.384; 0.001***	0.403; 0.001***		
GLUT1				0.218; 0.001***	0.176; 0.001***	0.244; 0.001***		
AR					0.543; 0.001***	0.279; 0.001***		

*p values <0.05 were considered statistically significant

**p values <0.01 were considered statistically significant

***p values <0.001 were considered statistically significant

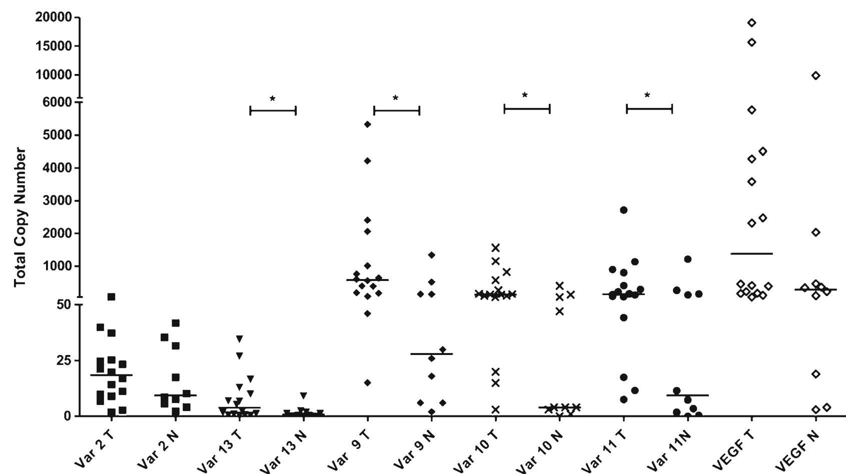

Fig. 3 Expression analysis of *MB* splice-variants (named Var 2, 13, 9, 10, and 11) and the hypoxia marker *VEGF* in prostate cancer biopsies (T) and normal adjacent tissue (N). cDNA copy numbers inferred by qRT-PCR are plotted on the *y*-axis. Shown are individual measurements and corresponding median values. *Asterisks* refer to *p* values according to the paired *t* test. (* p values <0.05 were considered statistically significant)

androgen receptor (AR). The growth of the majority of primary adenocarcinomas is androgen-dependent. Therefore, it is not surprising that many prostate cancer biomarkers are androgen-regulated genes, demonstrating the enhanced activity of AR in prostate cancer. For the treatment of advanced prostate cancer, anti-hormonal therapy is therefore a common and initially effective adjuvant strategy. However, most cancers become refractory after a few years, resuming growth despite anti-androgen application, and are considered as castration-resistance prostate cancers (CRPC) [21]. There is strong evidence that CRPC remain AR signaling pathway dependent [22, 23]. Therefore, it is of clinical and scientific interest to understand the AR downstream effectors, which might trigger the hormone independent AR functions and might offer new biomarkers or therapeutic targets. Massie et al. conducted ChIP to identify new functional AR binding sites and observed the relative enrichment of the *MB* promoter region in androgen-treated LNCaP cells [24]. Our in silico analysis in the prostate cancer cell line LNCaP showed that androgen starvation is able to induce, while androgen application suppresses *MB* expression. Possibly, MB expression in PCa is partly regulated by a hitherto unknown AR-dependent section of its promoter. We further noted that the tumor-associated *MB* transcript variants 9, 10, and 11, recently identified in breast cancer samples [11, 12], also occur at significantly upregulated abundances in prostate cancer tissues and in correlation with the increased expression of the hypoxia responsive *VEGF* gene. We speculate that the higher correlation between the hypoxia marker *VEGF* and the MB splice variants in the benign tissue compared with the tumor samples could thus be explained that in the normal tissue MB expression is only induced by hypoxia, whereas in the malignant tissue a deregulated AR signaling become more important.

At the protein level, increased amounts of MB correlate with the hypoxia marker CAIX. These findings suggest that expression of MB in prostate cancer cells is induced in response to longer periods of oxygen paucity and might be driven by alternative, tumor-specific hypoxia/HIF-dependent transcription machinery.

For breast cancer, we already showed that MB-positive tumors reflect the luminal molecular subtype, which is characterized by a higher degree of differentiation, hormone receptor positivity, and a better prognosis [10]. In this study, only a trend towards a better prognosis within the patients with high Gleason scores could be observed. So far, we assume that the possible beneficial effect of MB expressed in cancer is independent of its O_2 binding properties, since the amount of expressed MB protein would be too low to sustain O_2 supply. In human normoxic breast cancer cells, amounts of

Table 3 Associations of *MB* transcripts with levels of *VEGFA* mRNA. Upper values represent the correlation coefficients, *p* values according to Spearmen Rho

		Var 2	Var 13	Var 9	Var 10	Var 11	Σ Var 9, 10, and 11
VEGF	tumor	−0.156	0.365	0.606	0.512	0.500	0.579
		0.564	0.165	0.013*	0.043*	0.049*	0.019*
	benign	0.261	0.576	0.976	0.976	0.964	0.976
		0.467	0.082	0.001***	0.001***	0.001***	0.001***

* *p* values <0.05 were considered statistically significant

***p* values <0.01 were considered statistically significant

****p* values <0.001 were considered statistically significant

65 ng MB/10^6 cells (i.e., low μM concentrations) were measured. Thus, it is doubtful that endogenous MB in cancer cells, at levels several hundred-fold lower than in striated muscles, does confer meaningful O_2 storage or buffering capacities [10]. The correlation of tumors MB with the FASN, a key player in the de novo synthesis and homeostasis of fatty acids, could be a hint to the fatty acid binding properties of MB [8, 25]. MB could support active lipogenesis or deliver fatty acids to the mitochondria. In this way, MB may decrease glycolysis and therefore a metabolic state that has been proposed to facilitate the growth and invasion of cancer cells [26]. Vice versa, Flögel et al. [27] showed that the absence of Mb in hearts of Mb-deficient mice yielded a biochemical shift in cardiac substrate utilization from fatty acid to glucose oxidation which suggests that Mb is involved in FA transport or turnover in vivo [27]. In further experiments, it would be interesting to investigate if differences in the amount or spectrum of fatty acids can be observed in different MB knock down/out cancer cell lines grown under different hypoxic and normoxic conditions.

Tumoral MB levels appear to be particularly high in steroid hormone-dependent tumors as breast and prostate cancer [12, 18] indicating a possible regulation of MB expression by steroid signaling. In our earlier study on the luminal type breast cancer cell line MCF7, we found that MB transcript levels could be repressed in a dose-dependent manner by estrogen (E2) treatment [10]. This is furthermore endorsed by the correlation of MB with FOXA1, since FOXA1, often termed as "pioneer factor", is known to bind to the androgen receptor and to regulate AR-specific gene expression [28]. Interestingly, a highly significant correlation of FOXA1 with markers of hypoxia could also be observed in this study, which is an incidental novel finding that also deserves further investigations.

In summary, we found MB expression in a larger proportion of prostate cancer, where it appears to be regulated by AR signaling and hypoxia and, as in breast cancer, displays significant transcriptional complexity. This unexpected finding sheds further light on the role of non-muscle MB in human tumors and also provides additional support for the notion of MB as a hemoprotein with putative tumor-suppressive capacities.

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Conflict of interest The authors declare that they have no conflict of interest.

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