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TOPIC PAPER

Clinical relevance of gene expression in localized and metastatic prostate cancer exemplified by *FABP5*

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Abstract

Purpose Fatty acid-binding protein 5 (FABP5), a transport protein for lipophilic molecules, has been proposed as protein marker in prostate cancer (PCa). The role of *FABP5* gene expression is merely unknown.

Methods In two cohorts of PCa patients who underwent radical prostatectomy ($n=40$ and $n=57$) and one cohort of patients treated with palliative transurethral resection of the prostate (pTUR-P; $n=50$) *FABP5* mRNA expression was analyzed with qRT-PCR. Expression was correlated with clinical parameters. BPH tissue samples served as control. To independently validate findings on *FABP5* expression, three microarray and sequencing datasets were reanalyzed (MSKCC 2010 $n=216$; TCGA 2015 $n=333$; mCRPC, Nature Medicine 2016 $n=114$). FABP5 expression was correlated with *ERG*-fusion status, TCGA subtypes, cancer driver mutations and the expression of druggable downstream pathway components.

Results *FABP5* was overexpressed in PCa compared to BPH in the cohorts analyzed by qRT-PCR (radical prostatectomy $p=0.003$, $p=0.010$; pTUR-P $p=0.002$). *FABP5* expression was independent of T stage, Gleason Score, nodal status and PSA level. *FABP5* overexpression was associated with the absence of *TMPRSS2:ERG* fusion ($p<0.001$ in TCGA and MSKCC). Correlation with TCGA subtypes revealed *FABP5* overexpression to be associated with *SPOP* and *FOXA1* mutations. *FABP5* was positively correlated with potential drug targets located downstream of *FABP5* in the PPAR-signaling pathway.

Conclusion *FABP5* overexpression is frequent in PCa, but seems to be restricted to *TMPRESS2:ERG* fusion-negative tumors and is associated with *SPOP* and *FOXA1* mutations. *FABP5* overexpression appears to be indicative for increased activity in PPAR signaling, which is potentially druggable.

Keywords *FABP5* · *ERG* · *ERG* fusion · Prostate cancer · Expression · Molecular subtypes

Abbreviations

PCa	Prostate cancer
mCRPC	Metastatic castration-resistant prostate cancer
IHC	Immunohistochemistry
IQR	Interquartile range
BPH	Benign prostatic hyperplasia
pTUR-P	Palliative transurethral resection of the prostate

Introduction

Prostate cancer (PCa) is the most frequent tumor entity in men in developed countries [1]. From the perspective of the clinician, a differentiation between clinically relevant and insignificant tumors is the most urging questing in localized PCa. In metastatic and castration-resistant tumors, clinical focus moves on to the choice of the most suitable treatment approach in a growing armamentarium of therapeutic options. For both questions, molecular markers beyond prostate-specific antigen (PSA) serum level offer great opportunities.

Recent large studies have aimed to elucidate the genomic, transcriptomic and epigenetic landscape of PCa [2, 3]. Unlike other tumors, such as colorectal carcinoma or breast cancer, which typically present driver gene mutations in a broad number of cases, PCa is merely characterized by larger genomic alterations, being ETS-gene fusions (around 50%,

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with most of these cases having a *TMPRSS2:ERG* fusion), loss of *PTEN* (20–40%) and changes in the epigenetic profile [4, 5]. In addition to genetic markers, a large number of potential protein markers have also been described [6]. Gene expression analyses can be used to identify different tumor expression patterns and divide them into different molecular subtypes [3]. Such classifications can be decisive for risk stratification and therapy decision-making. For example, in urinary bladder carcinoma, tumors with a basal phenotype are more likely to respond to neo-adjuvant therapy, resulting in better clinical [7]. Such strategies are currently discussed in PCa for neo-adjuvant therapy and androgen deprivation [8, 9]. Furthermore, molecular profiling can result in the identification of novel biomarkers.

Of these, fatty acid-binding protein 5 (FABP5), which is typically associated with epithelial tissues, has been shown to be overexpressed in PCa compared to benign prostatic hyperplasia (BPH) using immunohistochemistry (IHC) [10–12]. In addition, protein-level overexpression of FABP5 in transurethral resection of the prostate (TUR-P) is associated with tumor stage and patient survival. Yet, the value of *FABP5* mRNA gene expression has only been studied in a small patient cohort [13].

In the present study, we compared *FABP5* mRNA expression determined by qRT-PCR analysis in tissue samples from patients who underwent radical prostatectomy or palliative TUR-P (pTUR-P) with benign controls. Furthermore, *FABP5* expression was correlated with *ERG* expression. To validate our findings, we reanalyzed the mRNA expression datasets and correlated *FABP5* expression with TCGA molecular subtypes and druggable downstream pathway components.

Materials and methods

Cohorts and patient samples

A cohort of 57 patients who underwent radical prostatectomy (mean age ± SD 62.7 ± 7.1 years) and 50 patients (mean age ± SD 75.9 ± 6.8 years) who underwent a pTUR-P in the Department of Urology of the Medical Faculty Mannheim of the University Heidelberg were analyzed (MA cohort). Histologically proven tumor-free prostate tissue specimen from 14 patients who underwent cystoprostatectomy or TUR-P served as controls (mean age ± SD 66.6 ± 11.9). In addition, a commercially available cDNA array (Origene, Rockville, MD, USA) consisting of 40 PCa (mean age ± SD 62.8 ± 8.2) and 8 benign control samples (mean age ± SD 64.0 ± 10.9) were used to determine the expression of *FABP5* using qRT-PCR. Patient characteristics are shown in Supplementary Table 1. All experiments conducted in this retrospective analysis were in accordance with the institutional ethics review board (ethics approvals 2013-845R-MA, 2014-592 N-MA).

For further validations, three large microarray and sequencing datasets from cBioPortal (www.cbioportal.org) were reanalyzed (MSKCC, Cancer Cell 2010 $n = 131$ localized PCa, $n = 19$ metastatic; TCGA, Cell 2015 $n = 333$ and mCRPC, Nature Medicine 2016 $n = 114$) [2, 3, 14].

RNA extraction, cDNA synthesis and qRT-PCR from patient samples

Tumor-bearing or tumor-free formalin-fixed paraffin-embedded prostate tissue specimen of patients treated in our department were sectioned, stained with hematoxylin and eosin, and reviewed by board-certified uro-pathologists (CAW, MG). Areas with at least 70% of tumor or tumor-free areas from control patients were marked and macrodissected from subsequent unstained 10-µm cuts. RNA was extracted using the XTRAKT FFPE kit (Stratifyer, Cologne, Germany), as recommended by the manufacturer. Finally, the RNA was eluted in 100 µl of elution buffer. RNA samples were stored at − 80 °C.

To receive a greater yield of target-specific transcripts and to reduce contamination with other amplified cDNA sequences, a multiplexed specific cDNA synthesis with equimolar pooling of transcript-specific reverse PCR primers (housekeeping gene *Calm* and target genes *FABP5*, *ERG* and *AR*, Supplementary Table 2) was used. As reverse transcriptase, Superscript III (Life technologies, Carlsbad, CA, USA) was used at 55 °C for 120 min, followed by an enzyme inactivation at 70 °C for 15 min. cDNA was immediately used for qRT-PCR or stored at − 20 °C. qRT-PCR analyses of the cDNA array were performed using the same primers. 40 cycles of amplification with 3 s of 95 °C and 30 s of 60 °C were conducted on a Step One Plus qRT-PCR cycler (Applied Biosystems, Waltham, MA, USA). mRNA expression in all samples was calculated using the $40-(\Delta Ct)$ method.

In silico validation and statistics

FABP5 expression was correlated with *ERG*-fusion status, TCGA molecular subtypes, known cancer driver mutations and the expression of druggable downstream pathway components. ANOVA, Mann–Whitney test, χ^2 test and Spearman correlation were used as appropriate for statistical analysis in Prism 5 (GraphPad Software, La Jolla, CA, USA).

Results

FABP5 mRNA expression is elevated in patients with localized and advanced PCa

A significantly higher expression of *FABP5* was observed in localized ($n = 56$, gene expression was not measurable in one patient, $p = 0.010$) and advanced ($n = 50$, $p = 0.002$) PCa tissue samples from the MA cohort compared to benign ($n = 14$, median expression with IQR: 39.46 (38.70–40.68) and 39.64 (38.55–40.31) vs. 38.68 (38.04–39.00); ANOVA $p < 0.001$, Fig. 1a). In these localized tumors, *FABP5* expression was independent of Gleason score, T stage, the presence of lymph node metastases and PSA level (Supplementary Figure 1A–D). Furthermore, the *FABP5* expression was analyzed in 40 patients of the Origene cohort with localized PCa by qRT-PCR in a cDNA array. *FABP5* expression was detectable in 34 patients and showed an overexpression compared to benign controls (median expression with IQR: 38.40 (37.60–40.16) vs. 36.76 (36.06–37.63), $p = 0.003$, Fig. 1b), again independent of T stage and Gleason score. To validate the expression of *FABP5* in a larger cohort, the mRNA expression microarray dataset by Taylor et al. (MSKCC) was analyzed in silico [2]. In this dataset, 131 patients with primary PCa showed an *FABP5* overexpression compared to benign controls (dashed line) independent from T stage (Fig. 1c) and Gleason score (Fig. 1d) as well as the presence of lymph node metastases and serum PSA level (Supplementary Figure 1E–F). *FABP5* expression was not significantly associated with clinical outcome in patients with localized PCa from the MA cohort (BCR-free log-rank $p = 0.151$, Supplementary Figure 1G) as well as in the MSKCC dataset (BCR-free log-rank $p = 0.483$; Supplementary Figure 1H).

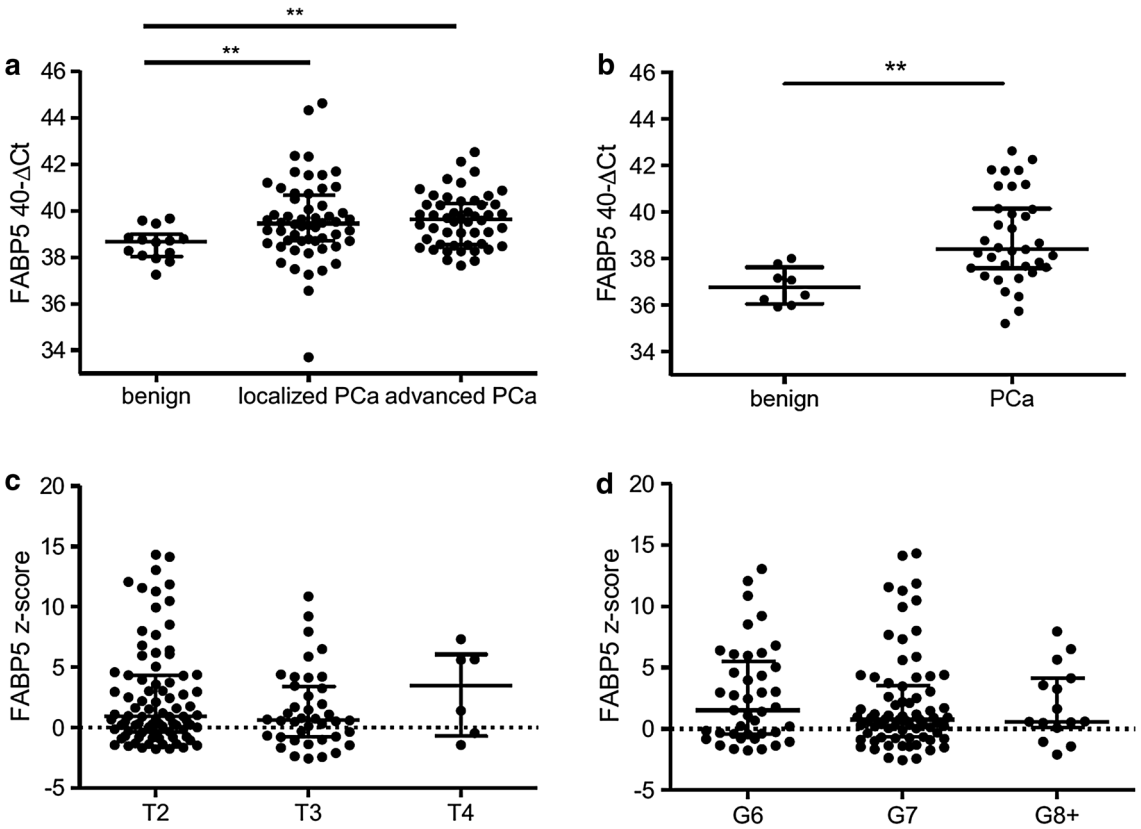

Fig. 1 *FABP5* is overexpressed in patients with localized and advanced PCa (ANOVA $p < 0.001$) (**a**) and in tissue samples with localized PCa ($n = 34$) independent of tumor stage ($p = 0.003$) (**b**). The MSKCC cohort with 131 primary PCa was reanalyzed and showed an overexpression of *FABP5* compared to benign controls, regardless of T stage (**c**) and Gleason score (**d**), (dashed line: baseline z-score = 0 as reference of average expression in benign controls). The Mann–Whitney test was used for statistical analysis (*$p < 0.05$; **$p < 0.01$)

FABP5 is overexpressed in *ERG* fusion-negative tumors which carry *FABP5* amplifications more frequently

Beside clinical and histomorphological classifications, PCa can be subdivided into molecular subtypes. According to the TCGA classification system [3], three of these subtypes are characterized by *ETS*-gene fusions, namely *ERG*-, *ETV1* and *ETV4*-fusions. *ERG* fusions, being the largest of these groups, typically go along with an *ERG* overexpression. Thus, the median of the *ERG* expression was determined by qRT-PCR and patients with localized and advanced PCa from the MA cohort ($n = 106$ with detectable expression) were grouped into *ERG* low and *ERG* high depending on

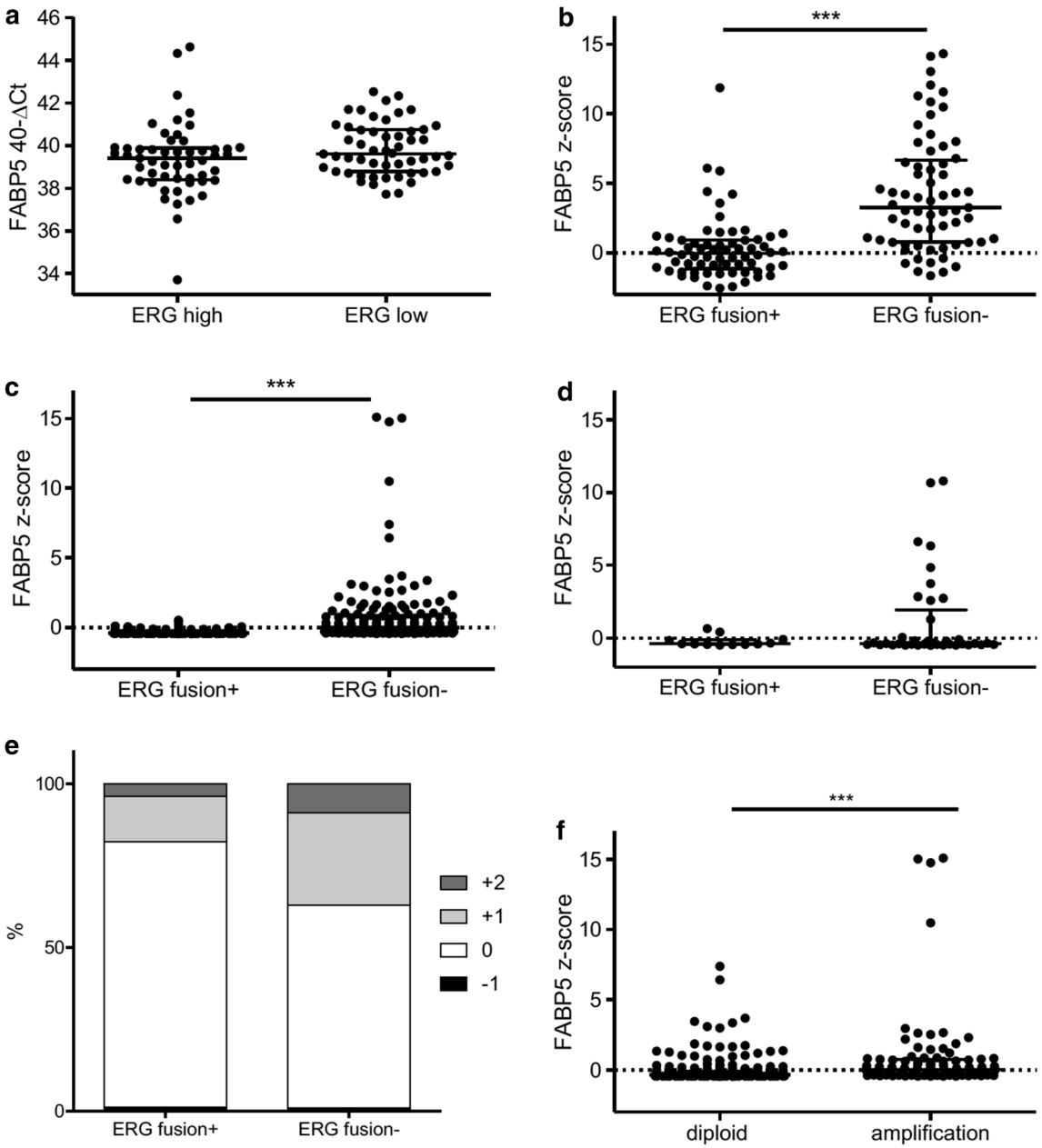

Fig. 2 In patients with localized and advanced PCa, *FABP5* showed a trend towards a higher expression in *ERG* low PCa compared to *ERG* high PCa ($p = 0.063$) (**a**). Significant overexpression of *FABP5* was observed in *ERG* fusion-negative primary tumors compared to *ERG* fusion-positive tumors in the MSKCC cohort ($p < 0.001$) (**b**). Reanalysis of 333 cases with localized PCa from the TCGA dataset showed differential expression of *FABP5* depending on *ERG*-fusion status ($p < 0.001$) (**c**). *FABP5* expression was elevated in some *ERG* fusion-negative tumor samples in mCRPC (**d**). Furthermore, *ERG* fusion-negative tumors from the TCGA dataset had a higher frequency of *FABP5* amplifications (Chi^2 $p = 0.002$) (**e**). The higher *FABP5* expression is associated with the occurrence of gene amplifications ($p < 0.001$) (**f**). The Mann–Whitney test was used for statistical analysis (***$p < 0.001$)

their *ERG* expression. PCa with low *ERG* expression showed a trend towards a higher expression of *FABP5* compared to PCa with high *ERG* expression (median expression with IQR: 39.60 (38.78–40.74) vs. 39.41 (38.39–39.88), $p = 0.063$, Fig. 2a). In a subgroup analysis, no difference in the *FABP5* expression was seen among localized tumors (median expression with IQR: 39.41 (38.46–40.85) vs. 39.46 (38.74–40.56), Supplementary Figure 2A), but among patients who underwent pTUR-P, a significantly higher *FABP5* expression was observed in PCa with low *ERG* expression (median expression with IQR: 40.25 (38.92–40.76) vs. 39.41 (38.33–39.83), $p = 0.013$, Supplementary Figure 2B). Across all tumor samples, expression of *FABP5* and *ERG* showed no correlation (Rho = − 0.139, Mann–Whitney test $p = 0.159$, Supplementary Figure 2C). To further investigate these results, the MSKCC cohort was reanalyzed for *FABP5* expression in *ERG* fusion-positive and -negative tumors. In primary PCa ($n = 131$), *FABP5* showed a significant overexpression in *ERG* fusion-negative tumors compared to *ERG* fusion-positive tumors (median expression *z*-score with IQR: 3.25 (0.77–6.66) vs. − 0.05 (− 1.12–0.93), Mann–Whitney test $p < 0.001$, Fig. 2b). In a small group of metastatic tumors from the same cohort ($n = 19$), *FABP5* gene expression was slightly higher in *ERG* fusion-negative tumors as well, but without reaching significance (median expression *z*-score with IQR: 4.97 (3.00–6.74) vs. 1.67 (− 1.07–5.30), $p = 0.149$). In primary tumors, Spearman correlation revealed a strong negative correlation between *FABP5* and *ERG* expression (Rho = − 0.5898, $p < 0.001$). To validate this observation, two additional datasets from cBioportal were reanalyzed. In the TGCA dataset, which encompassed 333 cases with primary PCa, *FABP5* was significantly overexpressed in *ERG* fusion-negative tumors (median expression *z*-score with IQR: 0.06 (− 0.30–0.82) vs. − 0.39 (− 0.43–0.32), $p < 0.001$, Fig. 2c). Figure 2d describes the *FABP5* expression pattern dependent on the *ERG*-fusion status in a cohort of mCRPC patients. The expression of *FABP5* was low in most of the tumors, yet an elevated *FABP5* gene expression (*z*-score > 2) was seen in 9 *ERG* fusion-negative tumors and not in *ERG* fusion-positive tumors (median expression *z*-score ± IQR: − 0.4 ± 0.33 vs. − 0.37 ± 2.39, $p = 0.736$).

Next, all cancer datasets ($n = 227$), listed in cBioPortal were screened for genetic and genomic alterations of *FABP5*. Among these, amplifications were far most frequent. Amplification rates of more than 10% of altered cases were almost exclusively observed in PCa and breast cancer datasets. Only datasets of the ASC project (14.29%), malignant peripheral nerve sheath tumors (13.33%) and liver cancer (10.63) showed a similar amplification rate (Supplementary Figure 3A). Mutations and deletions were rarely observed. In PCa datasets the *FABP5* amplification frequency varied between 1.52% and 40.35%. The highest frequencies were observed in highly advanced CRPC (Supplementary Figure 3B). In the TCGA dataset, *ERG* fusion-negative tumors had a higher frequency of *FABP5* gene amplifications (χ^2 $p = 0.002$, Fig. 2e). More than one-third (37.02%) of these *ERG* fusion-negative tumors were affected. In contrast, only 17.77% of the *ERG* fusion-positive tumors carried gene amplifications. In addition, compared to diploid tumors, the *FABP5* expression was significantly higher when a *FABP5* amplification was present (median expression *z*-score with IQR: amplification 0.02 (− 0.33–0.75) vs. diploid − 0.35 (− 0.41–0.11), $p < 0.001$, Fig. 2f).

Association of *FABP5* overexpression with molecular subtypes, genetic alterations and downstream pathway components

Next, *FABP5* expression was correlated with the PCa molecular subtypes proposed by the TCGA cohort. Overexpression of *FABP5* was mainly found in tumors with *SPOP* and *FOXA1* mutations and not in *ETS*-fusion subtypes (*ERG*, *ETV1*, *ETV4*) (Fig. 3a). In addition, tumors with a *FABP5* expression *z*-score of > + 1 showed a predominance of *SPOP* and *FOXA1* mutations. Other cancer driver mutations occurred only rarely (Fig. 3b).

Furthermore, *FABP5* was correlated with genes involved in the AR pathway and angiogenesis, which are crucial for progression and could serve as potential targets for therapeutic interventions. As in the MSKCC cohort, also in the TCGA cohort a strong negative correlation of *FABP5* and *ERG* expression was observed (rho = − 0.566, $p < 0.001$). Interestingly, there was no correlation with *AR*, but a positive correlation with the androgen signaling responsive genes *KLK2* and *KLK3* and the *ERG*-fusion partner *TMPRSS2* (Fig. 3c). As previously reported, *AR* and *ERG* showed a positive correlation. This was also the case in patients from the MA cohort (Rho = 0.219, $p = 0.025$, Fig. 3d) and the tumors of these patients showed also a positive correlation between *FABP5* and *AR* (rho = 0.347, $p < 0.001$, Fig. 3e).

In the TCGA dataset, the mRNA of two of the three *PPAR* nuclear receptor subtypes (*PPARA* and *PPARD*), whose protein products are the main ligands of FABP5, showed an inverse correlation with *FABP5* gene expression. Furthermore, *FABP5* is negatively correlated with *VEGFA*, but positively correlated with *VEGFC* (Fig. 3c).

Discussion

Recent studies have shown that FABP5 is overexpressed in PCa on the protein level [10, 11, 15]. In the present study, *FABP5* gene expression was analyzed by qRT-PCR, which is a possible alternative to IHC as it is sensitive, objective and not affected by inter-observer variability [16, 17].

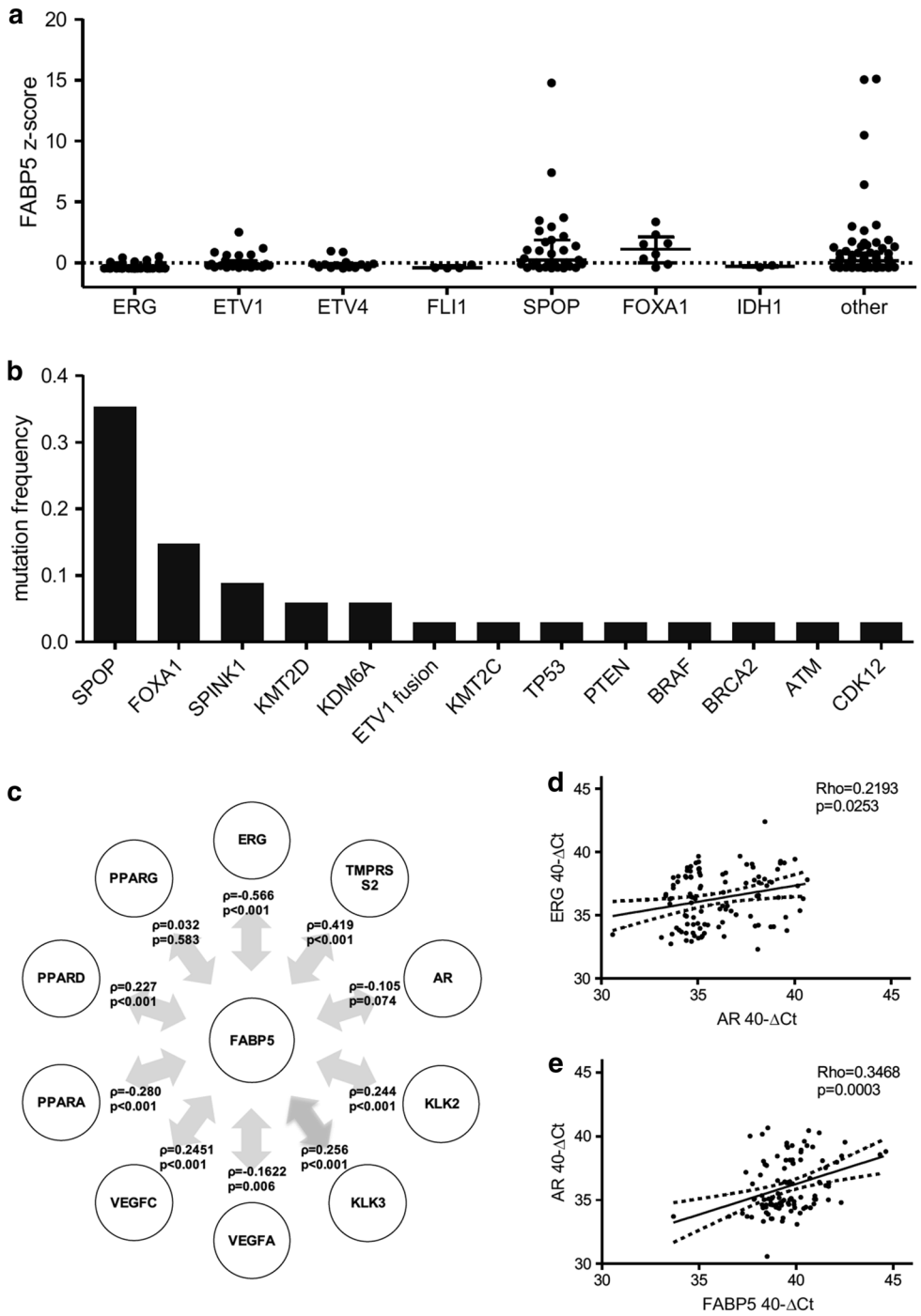

Fig. 3 Among the TCGA molecular subtypes, *FABP5* overexpression was mainly found in the *SPOP*, *FOXA1* and the subtype not associated with typical genetic alterations (**a**). Vice versa, tumors with a *FABP5* expression *z*-score of > + 1 showed a predominance of *SPOP* and *FOXA1* mutations. Other cancer driver mutations rarely occurred in these tumors (**b**). In the TCGA cohort, *FABP5* is negatively correlated with *ERG* gene expression. No correlation with *AR* was observed, but a positive correlation with androgen signaling responsive genes *KLK2* and *KLK3* and the *ERG*-fusion partner *TMPRSS2*. Furthermore, *PPARA*, *PPARD* and *VEGFC* were positively and VEGFA negatively correlated with *FABP5* expression (**c**). Spearman correlation showed that *AR* is significantly correlated with *ERG* (**d**) and *FABP5* (**e**) in the MA cohort

Overexpression of *FABP5* was observed both in prostatectomy and pTUR-P samples from our institution (MA cohort), which confirms previous studies in PCa [13].

PCa is associated with diverse genomic alterations such as fusion genes, gene amplifications and mutations. In 2005, Tomlins et al. described the fusion gene *TMPRSS2:ERG* as a predominant genomic alteration in PCa (around 50%) [4]. In contrast to relevant fusion genes in other malignant diseases such as the Philadelphia chromosome *BCR:ABL* in chronic myeloid leukemia, *TMPRSS2:ERG* does not lead to a novel fusion protein in PCa. The fusion with *TMPRSS2* results in overexpression of *ETS* transcription factors such as *ERG* and *ETV1*, which influence the regulation of cellular processes such as cell proliferation, differentiation or apoptosis and are associated with an unfavorable outcome. To date, multiple studies have reported a correlation between *ERG* overexpression and an unfavorable PCa outcome [18, 19]. The reanalysis of the 333 cases with localized PCa from the TCGA dataset showed a negative correlation of *FABP5* with *ERG*, but a positive correlation with the fusion partner *TMPRSS2*. In addition, *ERG* showed a positive correlation with the *AR* gene in the TCGA dataset and the MA cohort. The fusion gene *TMPRSS2:ERG* is mainly regulated by *AR* [4]. Interestingly, *FABP5* did not show a correlation with *AR* in the TCGA dataset, but was positively correlated with the androgen signaling responsive genes *KLK2* and *KLK3*. In contrast, *FABP5* is positively correlated with *AR* in the MA cohort. These differences may occur due to the different cohorts (localized vs. localized/TUR-P) and technical differences of gene expression analysis (RNAseq, mRNA-microarray, qRT-PCR). Besides *AR*, other hormones play also a role in PCa progression. The estrogen receptor α triggers the tumor-promoting function of the *TMPRSS2:ERG* fusion. In another study, Senga et al. could show that *FABP5* interacts with the estrogen-related receptor α (*ERRα*) [20]. This could indicate that PCa can bypass AR to promote its growth, using estrogens. In the MSKCC and the TCGA cohort, *FABP5* and *ERG* correlate negatively. The overexpression of *FABP5* in *ERG* fusion-negative tumors is associated with a higher copy number variation of *FABP5* and with *SPOP* and *FOXA1* mutations. Blattner et al. also described an inverse association of *SPOP* mutations and *ERG* rearrangement [21]. In 2012, another study postulated the *FABP5* gene itself to be potentially involved in fusion genes in PCa, with *KLK3*, which is coding for PSA, being a potential fusion partner [22]. Since this study used only a bioinformatics approach, there is no experimental evidence to support this hypothesis, yet.

FABP5 is correlated with two of the *PPAR* receptors *PPARA* and *PPARD* as well as *VEGFA* and *VEGFC*, which are involved in the angiogenesis and are essential for tumor growth and progression [12, 23]. Pan et al. were able to show that *FABP5* is higher expressed in hepatocellular carcinoma and that mRNA expression is positively correlated with *VEGFA*. Downregulation of *FABP5* inhibits the IL6/STAT3/VEGFA pathway and angiogenesis [24]. Another study from Al-Jameel et al. showed that the chemical inhibitor SBFI26 of FABP5 suppresses proliferation, migration and invasiveness in vitro by affecting the signal axis of FABP5-PPARγ-VEGF [25]. FAPB5 appears to indicate increased activity in PPAR and VEGF signaling, which may act as a potential drug target in PCa. In preclinical studies, knockdown of the coding gene *FABP5* resulted in a reduced growth of prostate cancer cells and xenografts [10, 23, 26]. This could be confirmed in stable knockout cell lines [11]. A suppression of *FABP5* gene expression, along with suppression of cell growth and invasion, was also one of the main effects induced by several procyanidins, members of the tannin family, used as anti-neoplastic drugs in in vitro prostate cancer models [27]. Furthermore, FABP5 was shown to be a regulator of lipid composition and metabolism in highly aggressive prostate and breast cancer [28]. Both tumor entities are highly depending on stimulation with steroid hormones. Fitting to this FABP5 was shown to be a direct interaction partner of ERRα. This interaction leads to an increased expression of ERRα target genes with impact on the cellular energy metabolism [20]. Unfortunately, nothing is known yet about the expression of *FABP5* and subsequent signaling cascades in dependency of antihormonal therapy.

A proteomic study identified FABP5 as a differentially expressed marker in lymph node-positive PCa tumor samples, confirming its potential relevance as a predictive marker [13]. A study by Fujita et al. identified FABP5 as a potential extracellular vesicle-based protein marker for the detection of high-risk PCa in urine samples [29]. In our own analyses, we could identify FABP5 to be present on extracellular vesicles of PCa cell lines [30].

In summary, the analysis of different cohorts with localized PCa revealed that *FABP5* gene expression is not associated with clinical outcome, and therefore does not seem to be suitable as a single marker for risk stratification or outcome prediction. However, on the protein level data from the literature point to a potential role of FABP5 as a marker for PCa diagnosis and prediction of high risk localized PCa and the presence of lymph node metastases. Further studies, especially liquid biopsy based, are warranted to prospectively validate these findings.

Besides this, the characterization of new therapeutic targets, especially for the treatment of advanced PCa after failure of first- and second-generation antihormonal therapy is of clinical relevance. Due to its role in tumor cell metabolism and as it is part of the FABP5-PPAR-VEGF signaling axis, relevant for angiogenesis and tumor progression, FABP5 might serve as a novel therapeutic target, especially in ETS fusion-negative tumors, in which its coding gene *FABP5* was shown to be frequently overexpressed.

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Author contributions KN: qRT-PCR experiments, data analysis, data collection, manuscript writing. PE: data analysis, manuscript writing. FW: qRT-PCR experiments, data analysis, data collection. AA: qRT-PCR experiments, data analysis. C-AW: tissue fixation, embedding, sectioning, staining and image acquisition. MG: tissue fixation, embedding, sectioning, staining and image acquisition. SW: qRT-PCR experiments. PN: project planning, manuscript writing. MB: project planning. MSM: project planning. JH: scientific advice, manuscript writing. TSW: project development, data collection, data analysis, manuscript writing.

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Compliance with ethical standards

Conflict of interest All authors declare no conflict of interest.

Ethical approval The study includes data and tissue from human participants in a retrospective study (ethics approval 2013-845R-MA, 2014-592 N-MA).

Informed consent All patients gave informed consent for participation. All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards.

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