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ORIGINAL ARTICLE

A high-density tissue microarray from patients with clinically localized prostate cancer reveals ERG and TATI exclusivity in tumor cells

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BACKGROUND: Prostate cancer (PCa) is characterized by high tumor heterogeneity. In 2005, the fusion between the androgen-regulated gene *TMPRSS2* and members of the *ETS* family was discovered in prostate cancer. In particular, fusion of *TMPRSS2* with *ERG* was found in approximately 50% of prostate cancers and considered as an early event in the onset of the disease. The prognostic value of this fusion is still contradictory. Bioinformatics showed that overexpression of *SPINK1* gene in a subset of fusion-gene-negative prostate cancers was associated with a poor prognosis. In theory, overexpression of the tumor-associated trypsin inhibitor (TATI) protein encoded by *SPINK1* in fusion-gene-negative tumor cells opens the way to selected treatments for genotypically different cases. However, their expression has never been assessed at the cellular level in the same tissue samples.
METHODS: As ERG expression has been shown to be a surrogate of fusion gene occurrence in prostate cancer, we have used double immunohistochemical staining to assess expression of ERG and TATI on a large tissue microarray comprising 4177 cases of localized prostate cancer.
RESULTS: We did not detect any co-expression of ERG and TATI in the same cancer cells, which confirms previous suggestions from *in silico* studies. ERG was associated with Gleason score (GS), surgical margins and pathological stage, but had no prognostic value in this cohort. TATI was weakly associated with pathological stage but had no significant association with outcome.
CONCLUSIONS: We here provide a morphological basis for ERG and TATI exclusivity in prostate cancer cells. Future therapies should be based on a combination of different targets in order to eradicate tumor cells with gene fusions and cells expressing other tumor-associated antigens. Further studies are needed to understand why ERG and TATI are not co-expressed in the same prostatic tumor cells.

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INTRODUCTION

Prostate cancer (PCa) is the second most frequently diagnosed cancer, the sixth cause of cancer death in males worldwide and the most common cancer in developed countries.[1] At the time of diagnosis, PCa is often multifocal and highly heterogeneous, leading to difficulty in accurately determining the prognosis and the most appropriate form of therapy.[2] The disease development can range from slow-growing and localized tumors to rapidly growing and highly metastatic tumors. As a result, there is a need to find biomarkers that can identify aggressive forms of the disease. Thus far, this approach has not produced any widely used clinical tests to accurately predict the progression of the disease; however, many studies have cast light on its biological features.[3–5]

PCa, like many other malignancies, is characterized by mutations in genes that promote (oncogenes) or protect against cancer (tumor suppressors). These genetic abnormalities include point mutations and chromosomal aberrations (gain, losses, rearrangements).[6] In 2005, *TMPRSS2:ETS* family gene fusions were discovered in PCa.[7] By using cancer outlier profile analysis, members of the ETS family were found to be overexpressed in a subset of PCa types, with *ERG* being the most common fusion partner. This fusion seems to occur in approximately 50% of PCas[8] and since *TMPRSS2* is an androgen-regulated gene, this leads to androgen-regulated overexpression of the oncoprotein ERG. It seems to be an early event in the onset of PCa, but results from various studies on its prognostic value are contradicting. Rajput *et al.*[9] found that the *ERG* fusion gene was more frequent in moderately to poorly differentiated PCas than in well-differentiated tumors. Perner *et al.*[10] found a significant association between *TMPRSS2:ERG* fusions via deletion and higher tumor stage as well as the presence of metastatic disease involving pelvic lymph nodes. Additionally, Fine *et al.*[11] described an association between the *TMPRSS2-ERG* gene fusion and low Gleason score. However, others have reported no association with outcome in patients treated by prostatectomy,[12] or no association with other clinicopathological parameters.[13]

Among patients not harboring ETS rearrangements, Tomlins *et al.*,[14] using the cancer outlier profile analysis bioinformatics method, identified *SPINK1* as an outlier highly expressed in a subset of cases. Furthermore, this subset of high *SPINK1*-expressing tumors was associated with an increased risk for biochemical recurrence. Subsequent studies have also investigated the association of tumor-associated trypsin inhibitor (TATI, protein corresponding to the *SPINK1* gene) with clinicopathological

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variables. Leinonen *et al.*[15] showed that in a cohort of patients primarily treated with endocrine therapy, TATI-positive cases had shorter progression-free survival, with TATI falling out as an independent prognostic factor. No association with other clinicopathological variables was observed. The possibility of TATI-positive tumors being selectively targeted by antibodies for therapeutic purposes was demonstrated in an *in vitro* study showing decreased proliferation, invasion and intravasation[16] upon TATI inhibition. The relationship of TATI with other potential biomarkers has also been investigated in castration-resistant PCa. Using consecutive tissue sections and different staining techniques (fluorescence *in situ* hybridization and traditional immunohistochemistry, IHC),[17] it was reported that overexpression of *TATI* occurred in *PTEN*-deleted tumors, none of which showed androgen receptor amplification.

Based on bioinformatic analyses, it was suggested that *SPINK1* and *TMPRSS2:ERG* expression were mutually exclusive in prostatic tumors.[14] However, to our knowledge, no studies have yet compared the protein expression of TATI and ERG in the same tissue sections. It has recently been shown that ERG staining is highly sensitive and specific as a surrogate marker for *TMPRSS2:ERG* gene fusion.[18,19] Therefore, co-occurrence of TATI expression and *TMPRSS2:ERG* gene fusion can now be analyzed at the protein level, as conventional IHC can be used instead of fluorescence *in situ* hybridization to evaluate fusion gene status on tissue sections.

The aim of our study was to investigate for possibly the first time the expression of TATI and ERG in the same tissue sections by using IHC with double staining in order to determine if they are in fact expressed in different cell populations. This would further support the clinical attempts to selectively treat patients with genotypically different PCa. We have used a large tissue microarray (TMA) consisting of 4177 samples from clinically localized PCa patients who underwent radical prostatectomy.

MATERIALS AND METHODS

Patients

Tissue specimens from primary prostatic tumors were collected from 4177 patients who underwent open radical prostatectomy at the Department of Urology, University Medical Center Hamburg-Eppendorf between 1992 and 2005. Clinicopathological features included pre-operative PSA level, pathological stage (pT) as defined by the American Joint Committee on Cancer in 2002, pathological Gleason score (GS), lymph node involvement (N), surgical margins status (SMS), and, if available, also time to occurrence of metastasis (Table 1). Biochemical recurrence was defined as an increase of postoperative PSA to 0.2 ng ml^{-1} with a confirmatory value. In total, 913 patients showed BCR with an average time from operation to recurrence of 65.8 months (range 1–219 months).

None of the patients had received neoadjuvant or adjuvant therapy before the prostatectomy and additional therapy was initiated in patients with BCR.

Table 1. Patients' characteristics

	No. on TMA	*Fraction on the total (%)*
Age, years		
⩽49	119	2.9
50–60	1072	26.0
60–70	2630	63.8
>70	302	7.3
Preoperative PSA, ng ml^{-1}		
<4	642	15.7
4–10	2324	56.9
10–20	826	20.2
>20	290	7.1
pT stage (AJCC 2002)		
pT2	2745	66.4
pT3	1343	32.5
pT4	45	1.1
Gleason score		
⩽3+3	1680	40.7
3+4	1866	45.2
4+3	458	11.1
⩾4+4	124	3.0
N stage		
pN0	2094	50.8
pN+	159	3.9
pNx	1869	45.3
Surgical margin status		
R0	3299	80.2
R1	809	19.6
RX	7	0.2
PSA recurrence		
Event	913	21.9
No event	3264	78.1
Metastatic onset		
Event	137	3.3
No event	4040	96.7

Abbreviations: AJCC, American Joint Committee in Cancer; TMA, tissue microarray.
Note: Numbers do not always add up to 4177 in the different categories because of cases with missing data.

TMAs and IHC

The TMA was constructed as previously described.[20] Cones with 0.6 mm diameter were punched out from the area with largest tumor volume and/or worst GS and arranged in nine paraffin blocks. Several consecutive 4-μm-thick sections were arranged on microscope slides. One section from each block was stained with hematoxylin and eosin and another one was processed for IHC using a double-staining procedure for specific demonstration of immunoreactive ERG and TATI. The sections were then stained in a DAKO Autostainer-plus using the EnVision FLEX including Peroxidase-Blocking Reagent (DAKO, Glostrup, Denmark) with a previously characterized TATI monoclonal antibody (6E8 raised in mouse[21]) at a final concentration of 3 μg ml^{-1} and a monoclonal antibody specific for ERG (EPR3864 raised in rabbit, Novus Biologicals, Littleton, CO, USA), diluted 1:250 according to the manufacturer. Sections were deparaffinized and pre-treated in the DAKO PT-link module using a standard protocol and buffer supplied by the manufacturer (DAKO).

Detection of ERG immunostaining in endothelial cells and macrophages served as positive control. The intensity of ERG and TATI staining in tumor areas was given a score from 0 to 3 and the percentage of immunostained cancer cells was recorded. Scoring was made by two independent observers (GL and AB) and discordant cases were re-evaluated using an open discussion procedure.

Statistical analysis

Statistical analysis was performed using SPSS (v.20, IBM, Chicago, IL, USA). Kaplan–Meier and log-rank test were used to evaluate the relationship between protein expression and BCR or metastatic disease. Crosstabs were used to show the relationship between protein expression and clinicopathological characteristics, and χ^2 test or the Fisher's exact test was used to assess the significance of differences.

RESULTS

Immunostaining for ERG and TATI

The initial number of patients included was 4177 and the design of the experiment was set to have one core from each patient. Ninety-nine cores were considered benign and 693 were damaged or missing and therefore excluded from analysis. ERG was found to be expressed in 41.7% of the cancer cases

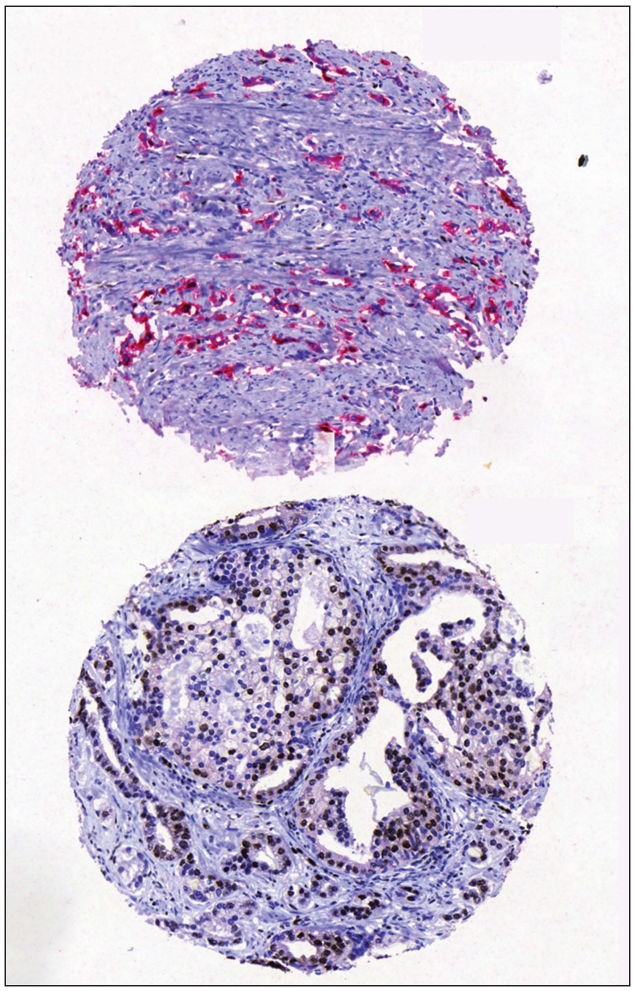

Figure 1. Immunohistochemical doublestaining for ERG and tumor-associated trypsin inhibitor (TATI). The upper panel shows a core representing a case of prostate cancer positive only for TATI (red staining). TATI is expressed in the cytoplasm of tumor cells. The lower panel shows a core with tumor cells with a cribriform growth pattern and exclusively positive for ERG, which is expressed in the nuclei of the tumor cells.

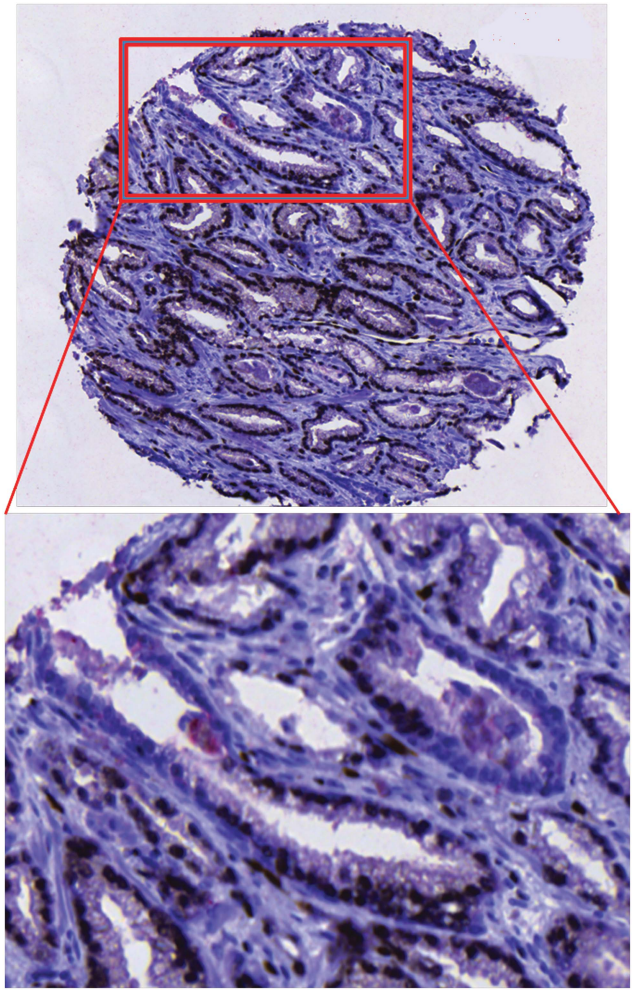

Figure 2. Immunohistochemical doublestaining for ERG and tumor-associated trypsin inhibitor (TATI). TATI is not expressed in this case. Only ERG is present in the nuclei of epithelial cells. In the red square, two glandular structures show expression only in some of the cells. This might represent a case of transition towards a malignant lesion.

(1411/3385), with intensity scores of +1 in 13.9%, +2 in 20.4% and +3 in 7.4%. Staining was found in the nuclei of cancer cells (Figure 1) and in some areas of prostatic intraepithelial neoplasia (PIN). As expected, endothelial cells and macrophages also stained positive for ERG in both benign and malignant areas. We did not observe expression of ERG in any of the benign epithelial structures. Expression of TATI in tumor cells was observed in 5.2% of the cores (175/3385) with the following distribution: +1 in 2.2%, +2 in 1.9% and +3 in 1.0%. As previously demonstrated,[22] TATI protein was exclusively localized in the cytoplasm of epithelial cells (Figure 1). A very weak immunostaining for TATI was often found in the cytoplasm of benign luminal epithelial cells.

Interestingly, we identified areas showing transition from benign to PIN and malignant epithelium with ERG expression as the markers of transition (Figure 2). TATI was not found to be overexpressed in any of these areas. Representative immunostainings for ERG and TATI is shown in Figure 1.

Associations between ERG and TATI expression and clinicopathological characteristics

Associations of ERG and TATI with preoperative PSA, pathological GS, pT stage, SMS and N-stage were investigated. ERG intensity, dichotomized as negative and positive expression, was not significantly associated with PSA or N stage (χ^2 test, $P=0.078$ and $P=0.792$, respectively), but there was a significant association with SMS ($P=0.004$), GS ($P<0.0001$) and pT stage ($P<0.0001$), although there was no linear trend. ERG was significantly more often expressed in tumors with GS 3+4 and pT3 stages than in the other subgroups (Table 2). When TATI was tested as a dichotomized variable (positive vs negative) for association with clinicopathological characteristics, TATI turned out to be weakly associated with pT stage ($P=0.0496$) without a clear linear pattern. However, TATI positivity was not associated with other characteristics (PSA $P=0.119$, Gleason $P=0.948$, lymph node $P=0.205$, surgical margins $P=0.769$, Table 3).

Association of TATI with ERG is presented in a crosstab format (Table 4). TATI was expressed exclusively in ERG-negative cases.

Expression of ERG and TATI to predict the outcome after radical prostatectomy

We also investigated if expression of ERG or TATI could predict BCR or metastatic events. Kaplan–Meier curves were built on dichotomization where expression of ERG and TATI was either positive or negative. Neither ERG nor TATI predicted BCR (log rank

Table 2. ERG association with clinicopathological parameters

	Total no. of patients	*ERG negative (%)*	*ERG positive (%)*	P *value*
Preoperative PSA, ng ml^{-1}				
<4	505	298 (59.0)	207 (41.0)	0.0777
4–10	1842	1043 (56.6)	799 (43.4)	
10–20	697	429 (61.5)	268 (38.5)	
>20	259	161 (62.2)	98 (37.8)	
Surgical margin status				
Negative	2664	1586 (59.5)	1078 (40.5)	0.0037
Positive	662	353 (53.3)	309 (46.7)	
Gleason score				
$\leqslant 3+3$	1299	818 (63.0)	481 (37.0)	1.2324×10^{-8}
$3+4$	1552	820 (52.8)	732 (47.2)	
$4+3$	387	236 (61.0)	151 (39.0)	
$\geqslant 4+4$	106	76 (71.7)	30 (28.3)	
pT stage				
pT2	2177	1351 (62.1)	826 (37.9)	1.6054×10^{-8}
pT3	1132	580 (51.2)	552 (48.8)	
pT4	40	23 (57.5)	17 (42.5)	
N stage				
Negative	1826	1022 (56.0)	804 (44.0)	0.7919
Positive	141	77 (54.6)	64 (45.4)	

Table 3. TATI association with clinicopathological characteristics

	Total no. of patients	*TATI negative (%)*	*TATI positive (%)*	P *value*
Preoperative PSA, ng ml^{-1}				
<4	505	485 (96)	20 (4)	0.1189
4–10	1842	1742 (94.6)	100 (5.4)	
10–20	697	653 (93.7)	44 (6.3)	
>20	259	251 (96.9)	8 (3.1)	
Surgical margin status				
Negative	2664	2527 (94.9)	137 (5.1)	0.7693
Positive	662	626 (94.6)	36 (5.4)	
Gleason score				
$\leqslant 3+3$	1299	1229 (94.6)	70 (5.4)	0.9482
$3+4$	1552	1475 (95)	77 (5.0)	
$4+3$	387	366 (94.6)	21 (5.4)	
$\geqslant 4+4$	106	101 (95.3)	5 (4.7)	
pT stage				
pT2	2177	2049 (94.1)	128 (5.9)	0.0496
pT3	1132	1088 (96.1)	44 (3.9)	
pT4	40	38 (95.0)	2 (5.0)	
N stage				
N0	1826	1740 (95.3)	86 (4.7)	0.2054
N+	141	138 (97.9)	3 (2.1)	

Abbreviation: TATI, tumor-associated trypsin inhibitor.

Table 4. Crosstable illustrating association of ERG with TATI based on immunohistochemical analyses of slides with doublestaining

	TATI		
	Negative	*Positive*	*Total*
ERG			
Negative	1799	175	1974
Positive	1411	0	1411
Total	3210	175	3385

Abbreviation: TATI, tumor-associated trypsin inhibitor.
Note: Values represent number of cases.

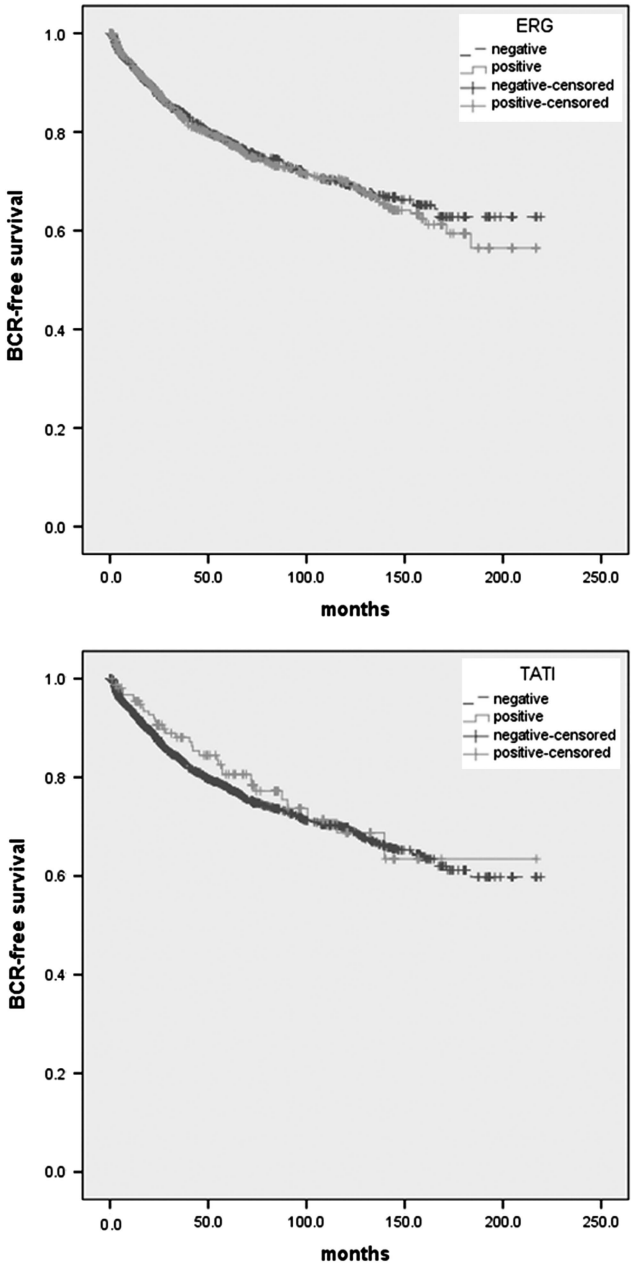

Figure 3. Kaplan–Meier curve representing biochemical recurrence (BCR) for patients stratified according to ERG (upper panel) and tumor-associated trypsin inhibitor (TATI) (lower panel) expression. Neither ERG (log rank (Mantel–Cox), $P=0.689$) nor TATI (log rank (Mantel–Cox), $P=0.447$) turns out to be a significant predictor of BCR.

(Mantel–Cox), $P=0.689$ and $P=0.447$, respectively, Figure 3) or development of metastatic disease (log rank (Mantel–Cox), $P=0.681$ and $P=0.530$, respectively Figure 4). In a univariate Cox regression model, ERG and TATI intensity as a continuous or as a dichotomized variable was not a significant predictor of BCR or of metastatic disease.

DISCUSSION

Previous studies[18,19,23] have shown that ERG expression analyzed by IHC is strongly correlated with ERG gene fusion as detected by fluorescence *in situ* hybridization analysis. Here we applied IHC of ERG on a high-density TMA ($n=4177$) in order to explore a

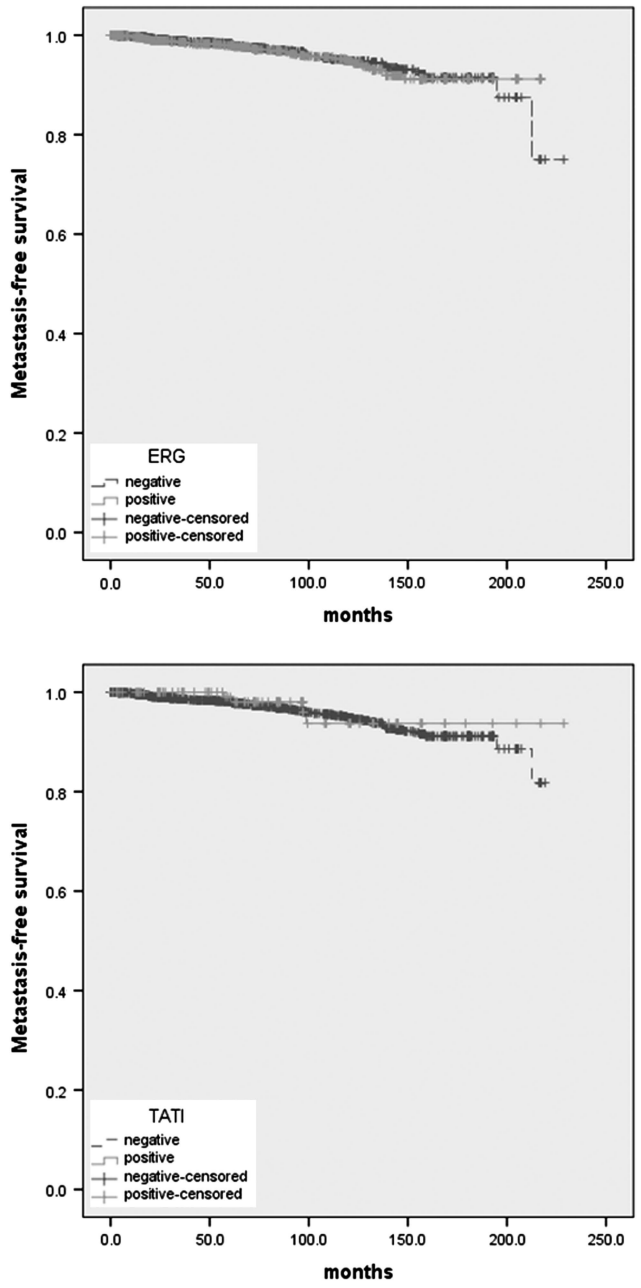

Figure 4. Kaplan–Meier curve representing metastatic onset for patients stratified according to ERG (upper panel) and tumor-associated trypsin inhibitor (TATI) (lower panel) expression. Neither ERG (log rank (Mantel–Cox), $P = 0.681$) nor TATI (log rank (Mantel–Cox), $P = 0.530$) turns out to be a significant predictor of metastatic event.

previously generated hypothesis that *TMPRSS2:ERG* fusion-positive PCas do not express *SPINK1* (TATI protein).[14] We successfully performed immunohistochemical double staining for ERG and TATI and demonstrated that these proteins are expressed in a mutually exclusive manner.

The TMA used in our study contains tumor samples obtained from PCa patients who underwent radical prostatectomy at a tertiary referral center (Hamburg) and none of the patients received hormonal treatment prior to surgery. This TMA has proved to be useful for biomarker evaluation and is described in several reports.[20,24,25] It also extends the TMA used in a previous study by Minner *et al.*[26] The observed expression of ERG (positive in 41.7%) and TATI (positive in 5.7%) is in accordance with results from previous publications, although the frequency is slightly lower. This can be related to the fact that in the TMA used, only one core was available from each patient. If we assume that ERG IHC is a good surrogate marker for *TMPRSS2:ERG* fusion in PCa, our present results favor the view that the occurrence of this gene fusion in PIN is an early event in tumor development. Our data also confirm the findings reported by Furusato *et al.*,[27] who observed the presence of ERG in PIN and found a strong concordance of ERG-positive foci in PIN with ERG-positive carcinoma. However, our results do not display its usefulness as a prognostic biomarker as previously suggested.[26]

Our data show that ERG did not predict the course of the disease in radical prostatectomy-treated patients, since it was neither related to BCR nor related to metastatic onset. ERG positivity was significantly associated with pT stage, SMS and GS but not with N stage or with the preoperative PSA value. Even if the expression of ERG was significantly different in the groups with various GSs ($\leqslant 6$, $3+4$, $4+3$, $\geqslant 8$) and pT stages (pT2, pT3, pT4), there was no clear linear trend. ERG seemed to be more often expressed in tumors with pT3 stage and a Gleason score of $3+4$ than in other stages and grades, as previously described.[26] As for the association with SMS, it must be interpreted with caution until it has been confirmed in subsequent studies. Of note, our data set is larger than earlier-described ones and seems to exclude the use of ERG staining for stratification of patients for the risk of relapse.

TATI has previously been shown to identify a subgroup with more aggressive cancer. Our data show a significant association ($P = 0.0496$) with pT stage, but the association is weak and it is difficult to draw conclusions. No association was found with BCR or metastatic event.

In silico data from studies on different PCa cohorts have suggested that *SPINK1*/TATI and *TMPRSS2:ERG* are expressed in a mutually exclusive manner.[14] In this study, we aim to clarify if this pattern of expression is observed at the protein level. Our presented data seem to show that ERG and TATI are expressed in separate tumor cell populations and that further studies are needed to elucidate the underlying tumor biology. Another confirmatory observation is that expression of ERG-positive cells may indicate a transition from benign to PIN or from PIN to malignancy as illustrated in Figure 2.

In conclusion, by using immunohistochemical double staining, we showed that ERG and TATI are exclusively expressed in separate tumor cell populations. However, in this setting, neither ERG nor TATI was a useful predictor of outcome in PCa patients undergoing radical prostatectomy. The results provide a morphological basis for future PCa therapy using a combination of different targets in order to eradicate tumor cells expressing different markers. Further studies are needed to elucidate why ERG and TATI are not co-expressed in the same prostatic tumor cells.

CONFLICT OF INTEREST

The authors declare no conflictof interest.

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