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Marked heterogeneity of ERG expression in large primary prostate cancers

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Approximately 50% of prostate cancers are characterized by *TMPRSS2* (*transmembrane protease serine 2*)-*ERG* (*avian v-ets erythroblastosis virus E26 oncogene homolog*) gene fusions resulting in an androgen-regulated overexpression of the transcription factor ERG. Some studies have suggested prognostic or predictive relevance of ERG status in prostate cancer. Such concepts could be impaired by extensive ERG heterogeneity in analyzed tumors. The aim of this study was to analyze the extent of heterogeneity for *TMPRSS2-ERG* fusion in prostate cancer. To enable large-scale studies on the extent of heterogeneity of biomarkers in prostate cancer, a heterogeneity tissue microarray containing samples from 10 different tumor blocks of 190 large prostate cancers selected from a consecutive series of 480 radical prostatectomies was developed. ERG expression was analyzed by immunohistochemistry. Positive ERG immunostaining was found in arrayed cancer-containing samples from 103 of the 178 analyzable patients (58%). ERG immunostaining was homogeneously positive in 29 prostate cancers (16%), whereas heterogeneous ERG positivity was seen in 74 cancers (42%). ERG heterogeneity was within one tumor focus (intrafocal heterogeneity) in 69 cases (93% of heterogeneous cases) and between different tumor foci (interfocal heterogeneity) in 5 cases (7%). Marked intrafocal heterogeneity challenges the concept of *TMPRSS2-ERG* fusion always representing an early step in prostate cancer development. Marked heterogeneity also compromises the concept of analyzing ERG status for treatment decisions in diagnostic needle core biopsies.
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Approximately 50% of prostate cancers have gene fusions linking the androgen-regulated gene *TMPRSS2* (*transmembrane protease serine 2*) with transcription factors of the *ETS* (*erythroblastosis virus E26 transforming sequence*) family.[1] Fusion of these genes either occurs through translocation or more often through deletion of a 3-Mbp intervening sequence between these two genes on chromosome 21.[2–4] The androgen-responsive *TMPRSS2* gene encodes a transmembrane serine protease of unknown function. The *TMPRSS2-ERG* (*avian v-ets erythroblastosis virus E26 oncogene homolog*) fusion represents more than 90% of the currently known *TMPRSS2*-associated fusion events (reviewed in Kumar-Sinha *et al*[5]). As a result of this rearrangement, the expression of ERG becomes androgen regulated and thus overexpressed in prostatic epithelium.

Several studies have investigated clinical and molecular characteristics of fusion *versus* non-fusion prostate cancer, suggesting a potential role of the *TMPRSS2-ERG* fusion as diagnostic target.[6,7] Moreover, it has been speculated that the presence or absence of the *TMPRSS2-ERG* fusion protein could have therapeutic implications. Some studies have suggested that fusion-positive cancers might react better to anti-androgen therapy than fusion-negative tumors.[8,9]

Some studies have revealed a considerable frequency of heterogeneity for the *TMPRSS2-ERG* fusion, especially occurring between independent tumor foci in multifocal prostate cancer.[10–12] A marked heterogeneity for *TMPRSS2-ERG* fusion

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in prostate cancer might substantially affect the utility of this molecular feature as a therapeutic and diagnostic tool and would compromise the concept of defining specific therapeutic regimens for fusion-positive cancers.

To enable large-scale studies on the extent of biomarker heterogeneity in prostate cancer, we developed a heterogeneity tissue microarray containing samples from 10 different tumor blocks of 190 large prostate cancers. This approach enables a high-throughput mapping of molecular features across entire tumors. The results of this study show that ERG heterogeneity occurs in a significant fraction of prostate cancers and that this heterogeneity can often occur within individual cancer foci.

Materials and methods

Patient Samples and Tissue Microarray Construction

In all, 480 consecutive prostate cancers treated by radical prostatectomy at our center between January and March 2010 were macroscopically dissected in a standardized way. All prostatectomy specimens were completely paraffin embedded and processed totally according to a modified stanford protocol[13] as previously described.[14] In brief, the prostates were fixed in 4% buffered formalin, serially blocked at 3 mm intervals in transverse planes perpendicular to the rectal surface, and embedded in paraffin. Macroscopic images were taken from each tumor (Figure 1). The average number of tumor-containing blocks was 12.0 (standard deviation: 7.5; range: 1–42). One hundred and ninety prostate cancer patients had a tumor involvement of at least 10 different tissue blocks and were thus defined as large prostate cancers. These cancers had an average volume of 3.4 cm^3(maximum 60 cm^3). For each cancer, the number of independent tumor foci was determined according to Wise *et al*.[15] In brief, tumor areas were defined as part of a single focus if they were within 3 mm of each other in any section or within 4 mm on adjacent sections. This method identified 1–6 independent tumor foci in our prostate cancers. Seventy-six prostates had one tumor focus, forty-eight prostates had two tumor foci, twenty-eight prostates had three tumor foci and thirty-eight prostates had four or more tumor foci. The latter group also included 10 prostates that contained multiple small and very small tumor foci rather than one or several clearly distinguishable tumor masses. From each of our 190 tumors, 10 different tumor-containing tissue blocks were selected for tissue microarray manufacturing. If more than 10 blocks were available, blocks were selected to obtain an optimal representation of the entire tumor mass (ie, blocks were selected that enabled maximal distances between selected tumor areas). One core from each selected block was then taken and placed in 10 different tissue microarray blocks. This resulted in 10 different tissue microarray blocks, each containing one tissue sample from each of our 190 selected patients. For subsequent mapping of molecular findings, the exact position from where each arrayed tumor sample had been retrieved was recorded in a database also containing all macroscopical images of our tumors. Each tissue samples was assigned to a defined tumor focus. The clinical and pathological features of our tumor collection are provided in Table 1.

Tissues have been utilized according to the Hamburger Krankenhausgesetz (§12 HmbKHG) and approved by our local ethical committee.

Validation for the Presence of Cancer on Tissue Microarray Spots

Technical issues represent a significant problem in studies analyzing heterogeneity because every false positive or false negative result will lead to a false classification as 'heterogeneous'. Every effort was thus taken in this study to avoid false interpretations including immunohistochemical confirmation of the presence of cancer for each sample. For this purpose, the antibody 34BE12 (clone MA903, Dako; 1:12.5; pH 7.8) was used for basal cell detection and p504s (clone 13H4, Dako,1:200; pH 9.0) was utilized for AMACR detection. The EnVision™ Kit (DAKO, Glostrup) was used to visualize the immunostainings. Examples of cancers verified by 34BE12 immunostaining on our tissue microarray are shown in Figure 2. For each tissue spot, presence or absence of normal prostate epithelium, high-grade PIN (prostatic epithelial neoplasia) and the proportion of cancer tissue was recorded and quantified by estimate.

ERG Immunohistochemistry

Freshly cut tissue microarray sections were analyzed in one experiment for each antibody. The antibody ERG (clone EPR3864, dilution 1:450, Epitomics) was used for ERG protein detection. Slides were deparaffinized and exposed to heat-induced antigen retrieval for 5 min in an autoclave at 121 °C at pH 7.8. Bound primary antibody was visualized using the EnVision™ Kit. Only nuclear ERG staining was considered. For each tumor sample, the staining intensity was judged from 0 to 4. Tumors with at least a weak ERG immunostaining were considered ERG positive. The specificity of our ERG immunohistochemistry protocol was validated in a previous study[16] on a prostate cancer tissue microarray known fluorescence *in situ* hybridization (FISH) data on *TMPRSS2-ERG* fusion of 453 samples. This validation resulted in a 95.8% overall concordance between FISH and immunohistochemistry on the same tumor spots in the tissue microarray. In all, 230 of the 247 immunohistochemically ERG-positive cancers showed *TMPRSS2-ERG* fusion by FISH (93.1%), whereas FISH fusions were only seen in

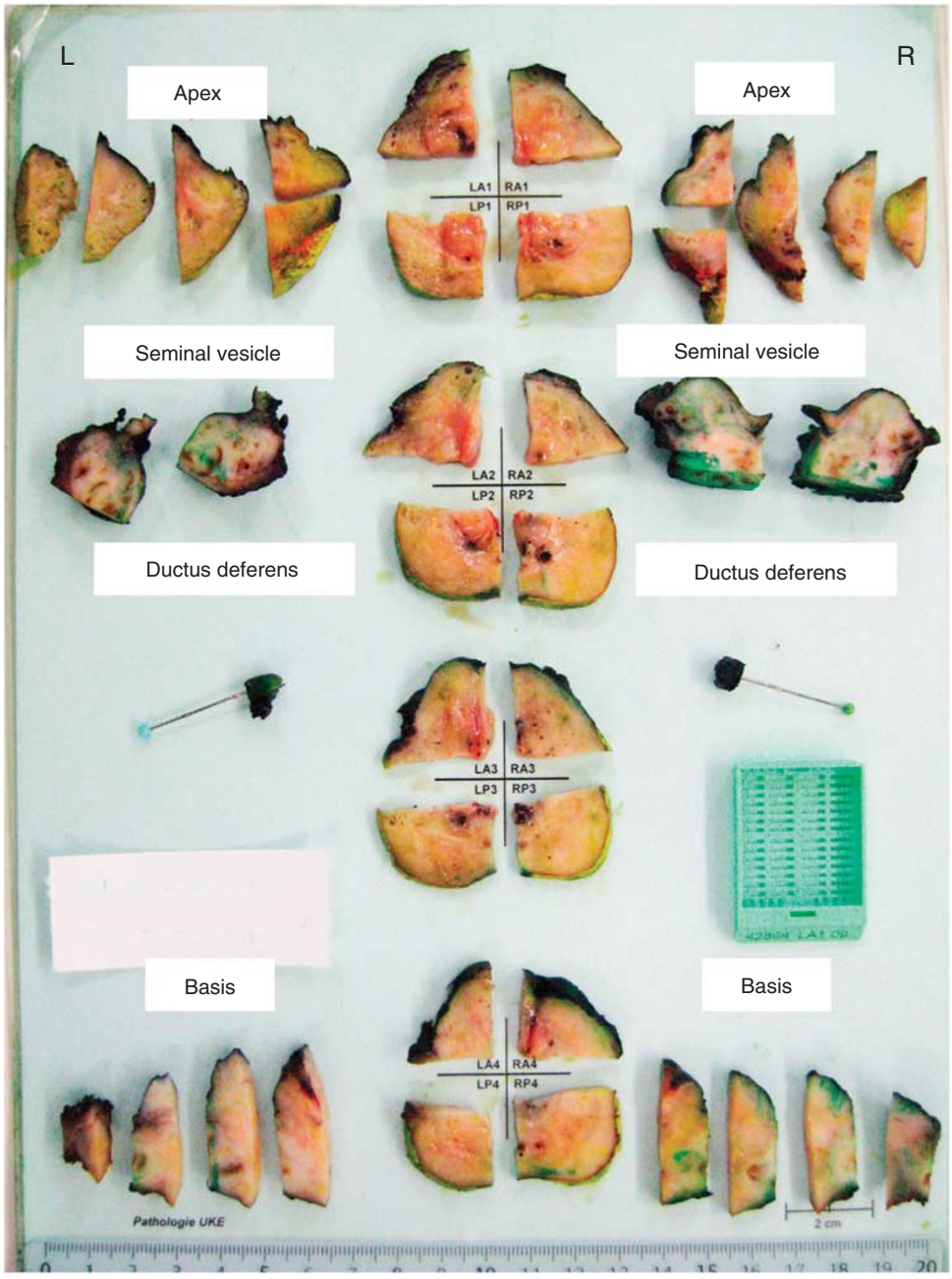

Figure 1 Macroscopic image of a cut prostate gland.

2 of the 206 cancers (1.0%) with a negative ERG immunohistochemistry result.

Large Section Validation

To validate heterogeneity of ERG expression, 13 cases with documented intrafocal heterogeneity on our tissue microarray were also subjected to large section analysis. In these cases, relevant cancer-containing tissue blocks (based on which tissue microarray spots suggested heterogeneity) were cut and ERG immunostained as described.

Fluorescence *In Situ* Hybridization

One block from our tissue microarray set containing 126 tumor spots with known ERG status by immunohistochemistry was analyzed by FISH to validate the immunhistochemistry protocol. FISH was performed as described previously.[16] In brief, a two-color *ERG* break-apart FISH probe consisting of two BAC clones one each at 5′ *ERG* (spectrum green-labeled RP11-95I21 and RP11-360N24) and the other at 3′ *ERG* (spectrum orange-labeled RP11-720N21 and RP11-315E22) was made. Following pretreatment, the tissue microarray slide was hybridized

Table 1 Clinicopathological features of the entire study cohort of 190 patients

Characteristic	*No. on tissue microarray (total* n = *190)*
Age (years)	
<50	3
50–60	43
60–70	120
>70	23
pT category (AJCC 2022)	
pT2	127
pT3a	32
pT3b	29
pT4	1
Gleason score	
≤3+3	4
3+4	144
4+3	33
≥4+4	8
pN category	
pN0	130
pN1	10
pNx	50
Surgical margin	
R0	160
R1	26
Rx	3

Abbreviation: AJCC, American Joint Committee on Cancer.

overnight and counterstained with 0.2 μM DAPI in antifade solution. Tumors were defined as 'normal' when two pairs of overlapping red and green signals were seen per cell nucleus. An *ERG* translocation was assumed if at least one split signal consisting of separate red and green signals was observed per cell nucleus. An interstitial deletion of 5′ *ERG* sequences was assumed if at least one green signal per cell nucleus was lost. Tumors were defined as FISH positive if *ERG* translocation and/or interstitial deletion were present in at least 60% of the tumor cell nuclei in the corresponding tissue spot.

Statistics

Statistical calculations were performed using JMP statistical software (SAS Institute, Cary, NC, USA). Contingency tables were calculated with the χ^2 test to investigate the relationship between the degree of ERG heterogeneity and other tumor features. The significance level was defined as $P<0.05$.

Results

Tissue Microarray Validation for the Presence of Cancer and PIN

The combined HE, p504s, 34BE12 analysis of our heterogeneity tissue microarray containing 10 samples each from 190 patients revealed cancer in 1384 spots (73% of 1900 samples arrayed), pure PIN in 54 spots (3%) and PIN adjacent to cancer in 116 spots (6%). There were on average 7.2 spots containing cancer (min = 1; max = 10), and 0.9 spots containing PIN (min = 0; max = 5) per patient. There were 183 patients (96%) with at least four cancer-containing spots in our tissue microarray.

ERG expression by Immunohistochemistry *versus* *TMPRSS2-ERG* Fusion by FISH

A subset of 54 cancers was successfully analyzed by FISH on consecutive sections to those used for ERG immunohistochemistry. There was an overall concordance of 94% between FISH and immunohistochemistry. Twenty-five of the twenty-seven immunohistochemically ERG-positive cancers showed *ERG* gene rearrangements by FISH (93%), whereas *ERG* gene rearrangements were only seen in one of the twenty-seven cancers (4%) with a negative ERG immunohistochemistry result.

Heterogeneity of ERG Expression in Prostate Cancer

Twelve cases were non-informative, either due to lack of tissue samples or absence of unequivocal cancer tissue. Positive ERG immunostaining was found in cancer-containing samples from 103 of 178 patients (58%). ERG immunostaining was homogeneously positive in 29 prostate cancers (16%), whereas heterogeneous ERG positivity was seen in 74 cancers (42%). ERG heterogeneity was within one tumor focus (intrafocal heterogeneity) in 69 cases (93% of heterogeneous cases) and between different tumor foci (interfocal heterogeneity) in 5 cases (7%). The group of tumors with documented intrafocal heterogeneity also included five tumors showing also interfocal heterogeneity. These cancers were classified as cancers with intrafocal heterogeneity. In four cases, ERG intrafocal ERG heterogeneity was even found within individual tissue microarray spots measuring 0.6 mm in diameter (Figure 3). The frequency of heterogeneous cases neither increased with the number of interpretable tumor spots nor with the number of independent tumor foci observed in a prostate. Heterogeneity was found in 50% of 24 cases with 2–5 interpretable cancer spots, in 41% of 63 cases with 6–7 interpretable cancer spots and in 41% of 91 cases with 8–10 interpretable cancer spots. ERG heterogeneity was seen in 33 (33%) cancers with only one tumor focus, in 11 (42%) cancers with two foci and in 25 (46%) cancers with three or more foci. The number of cases with intrafocal and interfocal heterogeneity in prostates with different numbers of tumor foci is given in Figure 4. Intrafocal heterogeneity of ERG immunostaining was confirmed in 10 cases where a large section validation was performed (Figure 5). In 3 of these 13 cases, heterogeneity also

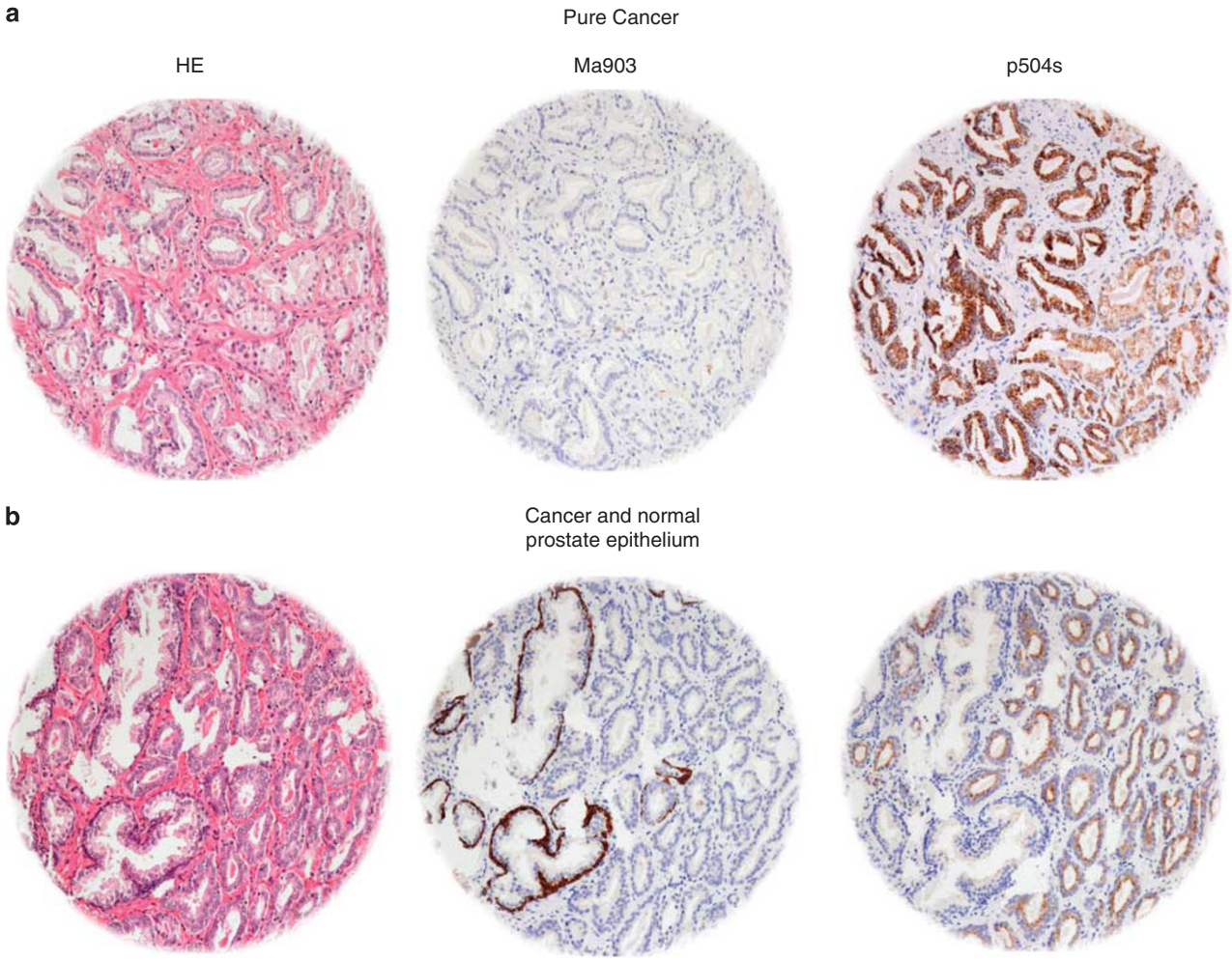

Figure 2 Validation of cancer by H&E staining, basal cell immunostaining (Ma903) and AMACR immnostaining (p504s). Representative images of tissue microarray spots (∅0.6 mm) showing (**a**) purely prostate cancer showing AMACR positivity and absence of basal cells. (**b**) Prostate cancer and adjacent normal prostate epithelium with basal cell staining and lack of detectable AMACR expression.

involved PIN (Figure 6). There was no significant association between ERG status and pT stage, tumor volume, Gleason grade and surgical margin status in our 190 patients (data not shown).

Heterogeneity of ERG Expression in High-Grade PIN

A total of 70 cases were evaluated for ERG expression in high-grade PIN: 40 cases with analyzable cancer and high-grade PIN in different tissue microarray spots, as well as 30 cases with analyzable cancer and high-grade PIN within one tissue microarray spot. In 31 cases with homogenously ERG-negative invasive cancer, high-grade PIN was ERG negative in a different tissue microarray spot of the same case. On the contrary, high-grade PIN was positive in only one of the nine cases with homogenous ERG positivity in invasive cancer.

In those cases in which invasive cancer and high-grade PIN were analyzable within 1 tissue microarray spot, 11 spots showed ERG-positive cancer (Figure 7a) and ERG-positive PIN, 12 spots showed ERG-negative cancer and ERG-negative PIN (Figure 7b), and four spots showed ERG-positive cancer and ERG-negative PIN (Figure 7c). Three other cases revealed heterogenous ERG status in high-grade PIN within one tissue microarray spot (Figure 7d). The results are summarized in Table 2.

Discussion

In this study, immunohistochemistry was used to identify prostate cancers with *TMPRSS2-ERG* fusions. In an earlier study, we have used the same experimental set-up for immunohistochemical ERG detection and found a 95.8% concordance between ERG expression and *TMPRSS2-ERG* fusions.[16] Other authors have described similar levels of concordance between immunohistochemistry and FISH for identifying fusion-positive prostate cancers.[17] ERG overexpression was found in 103 of our 178 tumors (58%). This frequency is somewhat

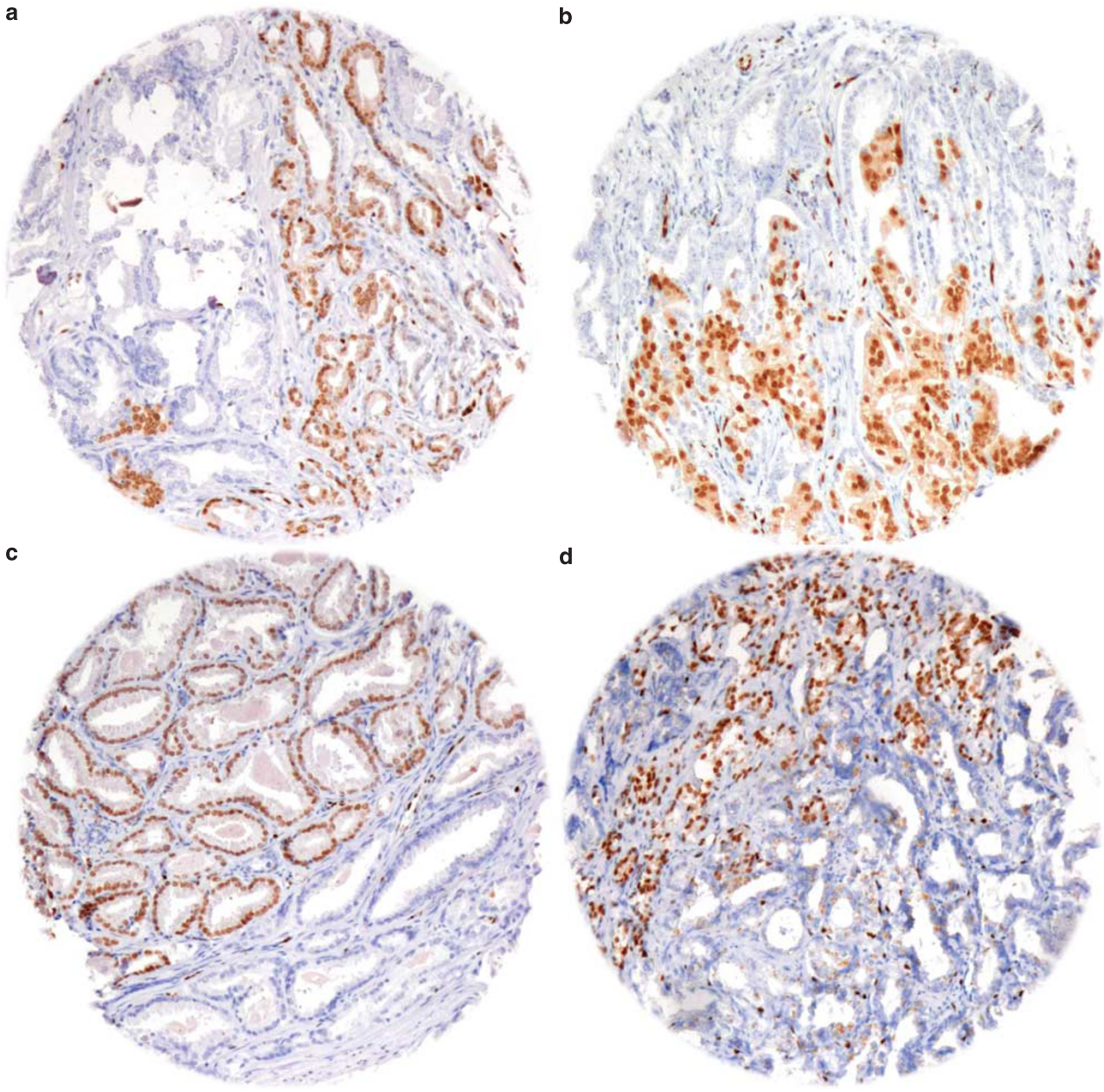

Figure 3 Intrafocal ERG heterogeneity within tissue microarray spots (∅0.6 mm). The panels (**a–d**) show cancers from four different patients showing ERG-positive and ERG-negative areas within one tissue microarray spot. Note that there is no clear difference in the morphology of positive and negative cancer areas.

higher than in our previous study analyzing one tissue microarray spot of 3261 different prostate cancers (52.4% ERG positive) and also higher than in most other previous studies. Earlier immunohistochemistry studies have described ERG positivity in 45 and 48% of cases.[17,18] FISH analyses revealed *TMPRSS2-ERG* fusions in 46–55%.[1,11,19] Others found ERG overexpression in 50–60% of prostate cancers by quantitative RT-PCR.[20,21] The somewhat high frequency of ERG positivity in our study is supposedly due to the identification of a substantial number of cancers with heterogeneous ERG overexpression, which would not have been detected in an analysis of just one tumor sample per patient.

Several studies have suggested that detection of ERG activation through *TMPRSS2-ERG* fusion may have diagnostic and/or therapeutic consequences in prostate cancer.[6,8,9,22] Some data, for example, suggest that ERG-positive cancers may respond better to anti-hormonal therapy than ERG-negative tumors.[8,9] Tumor heterogeneity is a critical issue and a major limitation for molecular diagnostics and targeted cancer therapy. Diagnostic accuracy of a

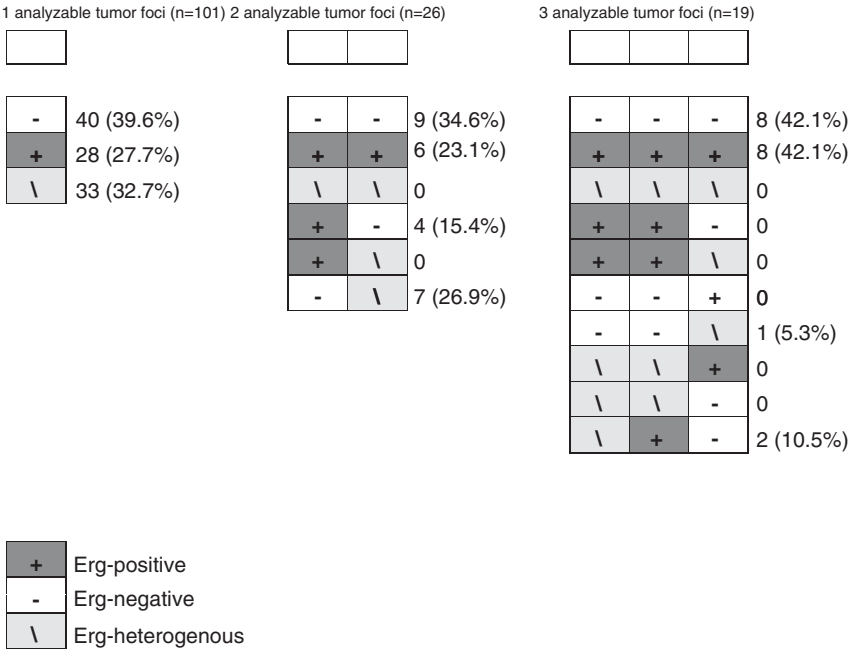

Figure 4 ERG findings in tumors with different numbers of tumor foci. The graph shows the different combinations of negative, positive and heterogeneous cancer foci in prostates with one, two or three distinguishable cancer foci. The number of analyzable tumor foci differs from the number of tumor foci found for each prostate, as some of the tumor foci were not analyzable.

molecular assay may be limited if the analyzed biomarker is only present in a fraction of a tumor. Absence of a drug target structure in a cancer subpopulation of a patient tested 'positive' for a specific drug target may cause drug resistance after outgrowth of the target-negative population under therapy. Considering its importance, the number of studies analyzing target heterogeneity in cancer is relatively small. If tumor heterogeneity is analyzed and quantified, this is often only based on the analysis of one slide/block per tumor/patient. However, one tissue section may not completely represent the biology of a large cancer. For example, one tissue section containing a tumor area of $2 \times 1\,\text{cm}$ represents only 1/75 000 of a cancer measuring $60\,\text{cm}^3$ (the maximal volume among the prostate cancers included in this study).

A new tool for studying molecular cancer heterogeneity was thus manufactured for this study. The tissue microarray analysis of one sample each from 10 different tumor-containing blocks distributed across the entire tumor enables a comprehensive three-dimensional analysis of molecular features in a large series of tumors. The heterogeneity tissue microarray concept introduced in this project differs markedly from previous attempts to increase the representativity of prostate cancer tissue microarrays by sampling multiple cores from just one tumor block.[23,24] The number of tumor-containing blocks per prostatectomy specimen was 10 in this study. It is therefore conceivable that arraying three and more cores from just one tumor block may not substantially improve the sampling representativity with respect to the entire cancer, although the concordance of tissue microarray results with corresponding large section analyses will improve.[23]

The data of our study demonstrate that ERG expression heterogeneity occurs in the majority of ERG-positive prostate cancers. Our study cohort of 190 consecutive cancers operated at our prostate center, which were large enough to have cancer in at least 10 different tumor blocks, revealed heterogeneous ERG overexpression in 42% of all tumors and in 72% of ERG-positive cancers. The data suggest a particularly high fraction of cases with intrafocal heterogeneity, a finding that was previously suggested to only occur rarely.[25,26] It is well possible that the number of tumors with intrafocal heterogeneity was overestimated to some extent in our study because of an unknown number of collision tumors meeting our criteria for 'unifocality'. The distinction of different foci is often difficult in large prostate cancers, because tumors may be irregularly shaped and contact between potentially different foci can often not be excluded on adjacent sections.

Earlier studies had mostly compared the *ERG* fusion status in between specifically selected tumor foci in multifocal cancers that were previously determined as fusion positive. For example, Miyagi *et al*[27] found interfocal heterogeneity in 5 of 11 (45%) analyzed multifocal fusion-positive tumors. Similarly, Barry *et al*[11] found interfocal heterogeneity in 41% of 32 fusion-positive tumors. Furusato *et al*[28] described interfocal ERG heterogeneity in 14 of 27 multifocal cancers (52%). Zhang *et al*[29] found 28% heterogeneous cases in 61 tumors

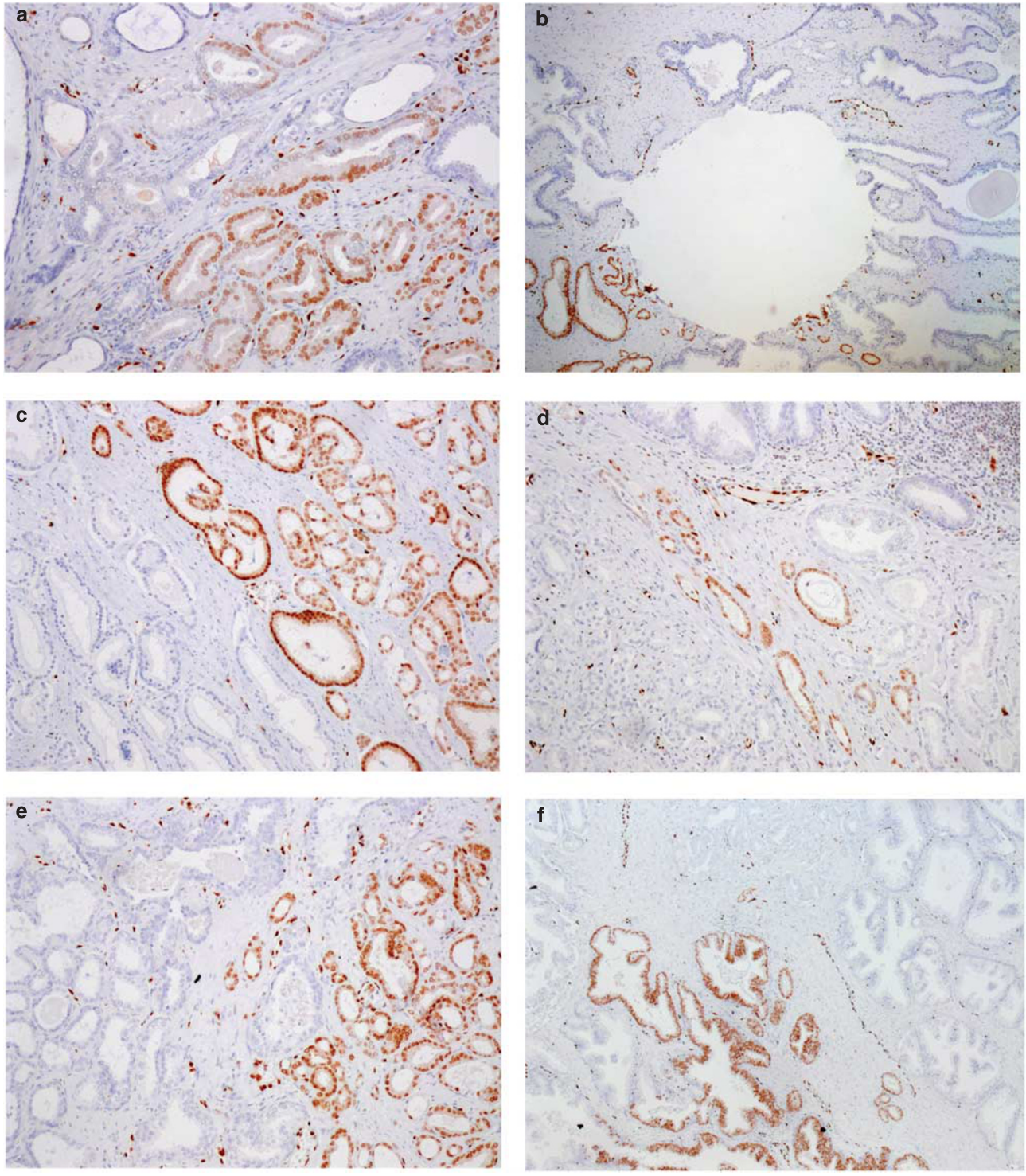

Figure 5 Intrafocal ERG heterogeneity (validation on large tissue sections). The panels (**a–f**) show examples of cancers with intrafocal ERG positivity. Panel (**b**) contains a tissue defect derived from tissue microarray manufacturing. Panel (**f**) shows ERG heterogeneity in cancer and high-grade PIN.

analyzing large tissue sections by FISH. Although none of the previous studies have systematically analyzed all large sections from individual tumor foci to systematically search for and exclude intrafocal heterogeneity, there are studies mentioning occasional cases of intrafocal heterogeneity. In a study by Svensson *et al*,[26] where interfocal heterogeneity was detected in 20 of the 44 (45%) multifocal prostate cancers, some cases showing intrafocal heterogeneity were discussed without providing exact number.

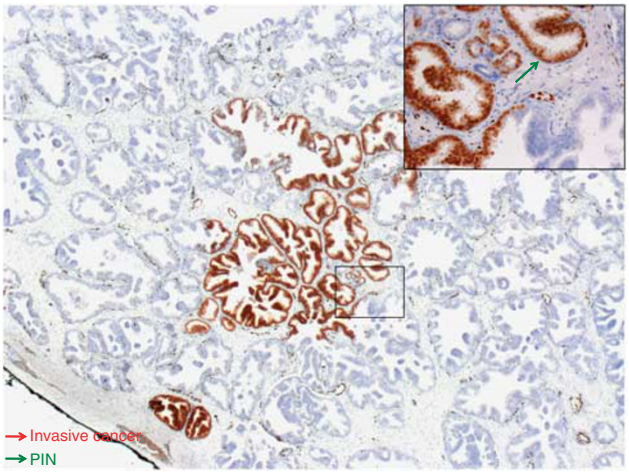

Figure 6 ERG heterogeneity in a high-grade PIN with a small heterogenous tumor focus.

Furusato *et al*[10] found interfocal heterogeneity in 35 of 81 multifocal cancers (43%) and also described three cases with intrafocal heterogeneity.

Irrespective of issues in the distinction of unifocal *vs* multifocal prostate cancers in patients with large tumor masses, our data raise two important points. First, it is evident that heterogeneity of the ERG status is rather a rule than an exception on a patient level at least in these patients having large and significant cancer masses. This limits the potential of a differential therapy based on the ERG status and challenges the possible concept to determine the ERG status on one single cancer biopsy. Second, our data show that intrafocal ERG heterogeneity is far from being an exception. This is also underscored by our finding of intrafocal heterogeneity in 4 of the 1384 cancer-containing tissue microarray spots having a diameter of 0.6 mm each (Figure 3). Experimental proof of intrafocal heterogeneity in

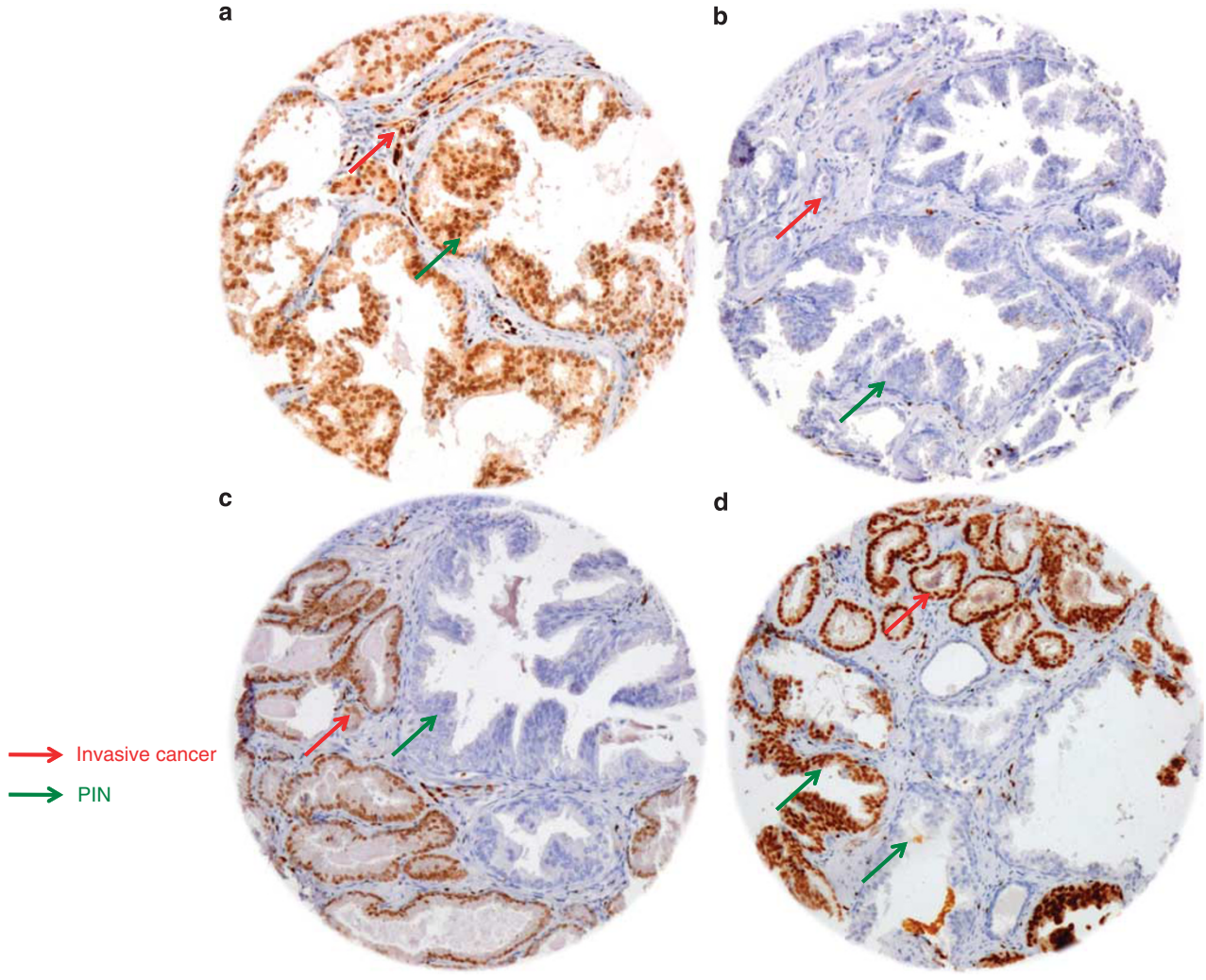

Figure 7 Differential ERG expression in PIN and invasive prostate cancer within tissue microarray spots (Ø0.6 mm). These panels show different combinations of ERG findings in PIN (green arrows) and invasive cancer (red arrow). (**a**) PIN and cancer positive. (**b**) PIN and cancer negative. (**c**) PIN negative and cancer positive and (**d**) PIN heterogenous and cancer positive.

Table 2 Heterogeneity of ERG expression in high-grade PIN

	High-grade PIN		
	N *(total)*	*ERG negative*	*ERG positive*
Prostate cancer			
ERG negative	43	43	0
ERG positive	24	12	12
N (total)	67	55	12

0.29% of such small tissue spots representing on average 0.00003% of the individual tumor masses argues for a significant fraction of cases with intrafocal heterogeneity. Moreover, the successful large section validation in 14 cases with suspected intrafocal heterogeneity demonstrated strong evidence for intrafocal heterogeneity with adjacent strongly ERG-positive and clearly ERG-negative areas without a significant difference in morphology between these areas (Figure 5).

A significant frequency of intrafocal ERG status heterogeneity challenges the concept of *TMPRSS2-ERG* fusion always representing an early step in prostate cancer development,[25] as this would mean the alteration could be seen in the entire tumor focus. It is obvious that this alteration can also occur later during prostate cancer progression. This is also emphasized by a significant number of cases identified in this project with ERG heterogeneity involving PIN and PIN next to invasive prostate cancer (Figure 6). Heterogenous finding with ERG-positive cancer adjacent to ERG-negative PIN also supports the fact that ERG-positive and ERG-negative neoplastic subclones of individual tumor foci exist. A total of 18% of our PINs detected on our heterogeneity tissue microarray differed molecularly from the invasive cancer. In contrast to this heterogenous finding, Furusato *et al*[10] found in 97% (82/85) ERG-positive tumor foci ERG-positive PIN lesions, and in all cases with ERG-positive PIN lesions an ERG-positive tumor.

In summary, the results of this study show that ERG overexpression is often heterogeneous in prostate cancer. This observation may compromise the previously suggested utility of ERG measurement as a predictive biomarker for response to antihormonal therapy. It also challenges the concept of determining the ERG status by analyzing just one tissue biopsy per patient.

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Disclosure/conflict of interest

The authors declare no conflict of interest.

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