Heterogeneity and chronology of *PTEN* deletion and *ERG* fusion in prostate cancer

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***TMPRSS2:ERG* fusions, in combination with deletion of the phosphatase and tensin homolog (*PTEN*) tumor suppressor, have been suggested to cooperatively drive tumor progression in prostate cancer. We utilized a novel heterogeneity tissue microarray containing samples from 10 different tumor blocks of 189 prostatectomy specimens to study heterogeneity of genomic *PTEN* alterations in individual foci. *PTEN* alterations were found in 48/123 (39%) analyzable individual tumor foci, including 40 foci with deletions, 7 with deletion and rearrangement, and 1 focus with rearrangement only. *PTEN* was homogeneously aberrant in only 4 (8%) and heterogeneously in 44 (92%) of the foci. We found a specific sequence of molecular events from *PTEN* breakage followed by deletion of DNA sequences flanking the breakpoint, resulting in homozygous deletion. The observation that 16 of 19 foci with homogeneous *ERG* positivity had focal *PTEN* alterations but none of 10 foci with *PTEN* alterations had focal *ERG* positivity ($P < 0.0001$) suggests that *PTEN* alterations typically develop subsequent to *ERG* fusions. We demonstrate a high level of intratumoral heterogeneity of *PTEN* alterations with deletions and rearrangements that challenges potential *PTEN* routine diagnosis testing in biopsies. The observation that *PTEN* alterations develop subsequent to *ERG* fusion strongly suggests that *ERG* expression may directly drive development of *PTEN* aberrations.**

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Prostate cancer is the most frequent malignancy in males and responsible for >250 000 deaths per year worldwide.¹ These tumors typically show marked histological and molecular heterogeneity, which can be problematic if a potential biomarker or therapeutic target is found, as only minute core needle biopsies are available for the initial diagnosis. However, comparing the extension of multiple heterogeneous molecular alterations within one cancer can also serve to answer biological questions such as the sequel in which these changes occurred. A prerequisite for such an extensive

heterogeneity analysis is that the entire cancer bulk is assessable for molecular analysis. To facilitate such whole tumor analyses, we have previously developed a prostate cancer heterogeneity tissue microarray (tissue microarray) platform.² This tissue microarray platform contains samples from 10 distant tumor areas each of 189 large prostate cancers, thus enabling a high-throughput mapping of molecular features across the entire tumors. Our initial analysis of this tissue microarray revealed that the prostate cancer-specific *TMPRSS2:ERG* fusions is heterogeneous in >70% of *ERG*-positive prostate cancers.²

Inactivation of the phosphatase and tensin homolog (*PTEN*) gene by genomic deletion or rearrangement, including intragenic breakage and translocation, is another key molecular event in prostate cancer. *PTEN* deletion or rearrangement has been reported in 20–30% of prostate carcinomas, and is linked to particularly aggressive cancers.^{3–5} Mouse

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models of prostate cancer suggest that *PTEN* inactivation and *ERG* fusion may cooperate in triggering development and progression of prostate cancer.^{6,7} Studies comparing *PTEN* and *ERG* fusion status have demonstrated that these alterations frequently coexist.^{3,4,8–12} *PTEN* deletions are about three times more frequent in fusion-positive prostate cancers than in fusion-negative cancers.¹⁰ The mechanisms explaining the frequent coexistence of *PTEN* deletions and *TMPRSS2:ERG* fusions in prostate cancer are unknown. In principle, it is possible that *TMPRSS2:ERG* fusions facilitate development of *PTEN* deletions, that *PTEN* deletions facilitate development of *TMPRSS2:ERG* fusions or that a specific molecular background facilitates development of both alterations simultaneously.

In order to distinguish between these possibilities, we extended our heterogeneity tissue microarray analysis for *PTEN* alterations, including deletions and breakage, and compared the *PTEN* and *ERG* status in the same tumor areas. The data from this project show high heterogeneity for *PTEN* aberrations in prostate cancer and suggest that presence of *ERG* activation facilitates development of *PTEN* aberrations, while *PTEN* altered cancers do not show an increased risk for developing additional *TMPRSS2:ERG* fusions.

Materials and methods

Patient Samples and Tissue Microarray Construction

Usage of the TMA for this study has been approved by the local ethics committee (AZ: WF-049/09; § 12 HmbKHG). The prostate cancer heterogeneity tissue microarray utilized in this study has been described in detail elsewhere.¹³ A total of 189 formalin-fixed prostatectomy specimens with tumor in at least 10 different tissue blocks were selected for tissue microarray construction. For each cancer, the number of independent tumor foci was determined according to Wise *et al.*¹⁴ 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 1 tumor focus, 48 prostates had 2 tumor foci, 28 prostates had 3 tumor foci and 37 prostates had 4 or more tumor foci. The latter group also included nine prostates that contained multiple small and very small tumor foci rather than one or several clearly distinguishable tumor masses. One 0.6 mm tumor tissue core was removed from each of the 10 tumor blocks per patient. The 10 tissue cores were distributed across 10 different tissue microarray blocks, so that the complete heterogeneity tissue microarray consists of 10 tissue microarray blocks, each containing one tissue sample of all 189 patients. The localization of each arrayed tumor sample inside the prostate was

recorded in order to distinguish between different tumor foci. Presence of cancer was histologically confirmed in each tissue spot. Normal prostate glands were immunohistochemically identified using the antibody 34BE12 (clone MA903, 1:12.5, pH7.8 DAKO, Glostrup, Denmark) for basal cell detection.

Fluorescence *In Situ* Hybridization (FISH)

FISH was used to detect genomic *PTEN* deletions and translocations. For deletion analysis, a dual-color FISH probe was constructed from two Spectrum Orange-labeled BAC clones (RP11-380G05, RP11-813O03; Source Bioscience, Nottingham, UK) and a commercial Spectrum Green-labeled centromere 10 (CEP10) reference probe (Abbott Molecular, Wiesbaden, Germany). For *PTEN* translocation analysis, a dual-color FISH break-apart probe consisting of two Spectrum Orange-labeled BACs (5'*PTEN*: RP11-659F22, RP11-79A15) and two Spectrum Green-labeled BACs (3'*PTEN*: RP11-765C10, RP11-813O03) flanking the *PTEN* gene were used. The localization of all probes and representative FISH images are shown in Figures 1a and b, respectively. Freshly cut 4 µm tissue microarray sections were deparaffinized and proteolytically pretreated using a commercial kit (paraffin pretreatment reagent kit; Abbott Molecular), followed by dehydration in 70, 80 and 96% ethanol, air-drying and denaturation for 10 min at 72 °C in 70% formamide-2x SSC solution. Hybridization was done overnight at 37 °C in a humidified chamber; slides were then washed and counterstained with 0.2 µmol/l 4'-6-diamidino-2-phenylindole in an antifade solution.

Scoring of FISH

The stained slides were visually inspected under an epifluorescence microscope. At least 30 different tumor cell nuclei were inspected for the FISH signal numbers per tissue spot. *PTEN* deletion was defined as follows: homozygous *PTEN* deletion was assumed if *PTEN* deletion probe signals were completely lacking in ≥60% of tumor nuclei, while *PTEN* FISH signals were present in adjacent normal cells. Heterozygous deletion of *PTEN* was defined as presence of less *PTEN* signals than centromere 10 probe signals of ≥60% tumor nuclei. For structural *PTEN* rearrangement analysis using the break-apart probe, tumors were defined as 'normal' when two pairs of overlapping orange and green signals were seen per cell nucleus. A *PTEN* rearrangement was assumed if at least one split signal consisting of a separate orange and green signal was observed per cell nucleus in ≥60% of the tumor cell nuclei (indicating balanced translocations) or if individual orange and green signals from the overlapping orange/green signal were lost (indicating deletions with breakpoint inside the *PTEN* gene or

Figure 1 (a) Localization of the FISH probes used for detection *PTEN* deletion and intragenic breaks. For detection of *PTEN* deletion, a *PTEN* deletion probe covering the entire *PTEN* gene and 3' adjacent DNA, and a spectrum green-labeled centromere 10 control probe (data not shown), were co-hybridized. For detection of *PTEN* breakage, two differentially labeled probes corresponding to the 5' and 3' edges of *PTEN* were co-hybridized. (b) Examples of *PTEN* aberrations as detected by FISH. (I–III) Examples of the deletion probe: (I) normal copy number with two red *PTEN* signals and two green centromere 10 signals, (II) heterozygous deletion of *PTEN* showing loss of one red signal, but two green centromere signals, (III) homozygous deletion of *PTEN* lacking *PTEN* signals but showing two green centromere 10 signals. (IV–VI) Examples of the break-apart probe: (IV) intact *PTEN* as indicated by presence of two adjacent red (3' *PTEN*) and green (5' *PTEN*) signals, (V) translocation of *PTEN* showing one normal, adjacent red/green signal and a split of the second one into clearly separated green and red signals, (VI) heterozygous deletion of one allele and translocation of the remaining allele.

imbalanced translocations). Presence of only one overlapping orange/green signal in >60% of tumor cells was considered a heterozygous deletion.

Tumors with complete lack of overlapping orange/green signals were regarded as homozygous deletions provided that FISH signals were present in

adjacent normal cells. A final score was assigned to each tissue spot that was analyzable for both the *PTEN* deletion and the break-apart probe according to the following criteria: normal (no deletion and no rearrangement), heterozygous inactivation (heterozygous deletion or rearrangement of one allele), homozygous inactivation (homozygous deletion or heterozygous deletion combined with rearrangement of the remaining allele or rearrangement of both alleles). In case of polyploidy, presence of normal *PTEN* FISH signals and split signals was rated as heterozygous inactivation. A total of 103 tissue spots that had interpretable results with only one of the two probes were scored based on the respective FISH finding only.

ERG Immunohistochemistry

ERG immunohistochemistry data were available from a previous study.¹⁵

Large Section Analysis

To validate the *PTEN* tissue microarray findings, corresponding tumor blocks were selected from 15 tumor foci showing either a *PTEN* alteration in only one tissue core ($n = 10$) or in all but one tissue core ($n = 5$).

Statistics

Statistical calculations were performed using JMP 9.0 statistical software (SAS Institute, Cary, NC, USA). Contingency tables were calculated with the χ^2 test to investigate the relationship between the degree of *PTEN* and *ERG* heterogeneity.

Results

Technical Issues

A total of 1890 tissue spots were included in this study. A total of 887 and 907 tissue spots were interpretable with the *PTEN* deletion probe and with the *PTEN* break-apart probe, respectively. As all tumors with deletions according to the deletion probe also showed FISH signal losses with the break-apart probe, the deletion status was assigned solely based on the result of the break-apart probe in 125 tissue spots lacking results with the deletion probe. In additional 103 tissue spots that had a result with the deletion probe but were not interpretable with the break-apart probe, the *PTEN* status was assigned based on the deletion probe only, and no breakage was assumed in these cases. This assumption was based on the low overall rate of *PTEN* breaks (1%) in tumors without simultaneous deletion.¹⁶ Accordingly, the *PTEN* FISH status was available from 1008 (53%) tissue spots. Reasons for

analysis failure included missing tissue spots on the tissue microarray sections ($n = 111$), lack of unequivocal tumor cells in the tissue spots ($n = 501$) or insufficient hybridization for both FISH probe sets ($n = 270$). The 1008 interpretable tissue spots belonged to 173 different tumor foci obtained from 136 prostatectomy specimens. As different tumor foci within the same prostate represent individual cancers, and because some tumor foci had only few interpretable tissue spots, we refer to different tumor foci in our analysis (rather than to different patients) and excluded all foci with < 3 interpretable tissue cores. In summary, 821 tissue spots originating from 123 tumor foci with at least three interpretable tissue spots obtained from 118 patients were included into all subsequent analyses.

Heterogeneity of *PTEN* Alterations

PTEN alterations (including deletion and rearrangement) were found in 48 (39%) of the 123 individual tumor foci belonging to 47 patients. We observed a high degree of intratumoral heterogeneity: 44 (92%) of the foci were heterogeneously, but only 4 (8%) were homogeneously *PTEN* altered. In contrast, the inactivation status (heterozygous or homozygous) was usually identical in all *PTEN* altered tissues of the same focus, with homozygous inactivation being more frequent (25/48, 52%) than heterozygous inactivation (17/48, 35%). Only six (13%) foci showed a mixture of tissue spots with homo- and heterozygous inactivation. All results are summarized in Figure 2.

Sequel of *PTEN* Rearrangement and Deletion

Of the 48 *PTEN* altered foci, 40 foci had deletions only, 7 foci had structural *PTEN* rearrangements in addition to deletions and 1 focus had a *PTEN* rearrangement as the sole detectable *PTEN* alteration. In the 47 foci with deletion and/or structural rearrangements, deletions and rearrangements of *PTEN* were strongly linked. Rearrangements were found in 7/47 tumor foci with *PTEN* deletion, but only in 1/76 foci with normal *PTEN* copy numbers ($P = 0.003$). The presence of tissue spots with both deletions and rearrangements of *PTEN* within the same cancer focus enabled us to study the sequel of *PTEN* breakage and deletion. Of these seven foci, six had ≥ 2 interpretable tissue spots, some of which showed structural *PTEN* rearrangements, while others had partial and/or complete deletions of *PTEN*. This pattern of alterations is consistent with a specific sequel of molecular events starting with *PTEN* breakage and followed by deletion of DNA sequences flanking the breakpoint (Figure 3), including multiple 5' adjacent genes (*MINPP1*, *PAPSS2*, *ATAD1* and *KLLN*) covered by our break-apart FISH probe (Figure 1).

Figure 2 Summary of all *PTEN* FISH results in 48 tumor foci with *PTEN* alterations. ‘Homogeneously’ and ‘heterogeneously affected’ indicates foci with *PTEN* alterations in all analyzable tissue spots and foci with an admixture of tissue samples with and without *PTEN* alterations. Circles represent the individual tissue cores belonging to the same tumor foci. The number of interpretable tissue cores per foci ranged between 3 and 10.

Association to *ERG*

ERG data were available from a previous study in 786 of 821 (96%) tissue spots with informative *PTEN* deletion and break-apart status. A positive *ERG* status was strongly linked to presence of *PTEN* alterations in these samples. *PTEN* aberrations were seen in 120 (36%) of 332 *ERG* positive, but only in 26 (6%) of 454 *ERG*-negative tissue spots ($P < 0.0001$; Figure 4).

Sequel of *PTEN* Alterations and *ERG* Fusion

To address the question whether alterations of *ERG* and *PTEN* occur in a specific order, we searched for homogeneously *ERG*-positive foci with focal *PTEN* inactivation and large *PTEN* inactivated tumor areas

containing focal *ERG* positivity. Figure 5 shows the *ERG* and *PTEN* findings in all 29 tumor foci that were suitable for such an analysis. Sixteen of 19 foci with homogenous *ERG* positivity had focal *PTEN* alterations but none of the 10 foci with homogeneous or heterogeneous *PTEN* alterations had focal *ERG* positivity ($P < 0.0001$).

Large Section Validation

Large section analysis of 15 tumor foci with intra-focal *PTEN* heterogeneity confirmed presence of small areas with genomic *PTEN* alterations in large *ERG*-positive tumor foci. Heterogeneous findings occurred not only between different areas within one tumor focus but also within the area represented

Figure 3 Schematic representation of the FISH findings in six cases with at least two tissue spots affected by *PTEN* rearrangements. Black dots correspond to the centromere 10 probe, yellow and green dots represent the corresponding *PTEN* break-apart FISH signals. All six cases showed translocations of all present alleles, subsequent loss of the break-apart signals mostly starting at the green 3' sequence, followed by the orange 5' sequence and finally loss of all FISH signals. For reference, the spot pattern of a normal case (no deletion, no rearrangement) is indicated in the bottom left corner.

Figure 4 Association between immunohistochemical *ERG* expression and *PTEN* alterations evaluated in a spot by spot analysis.

by a single tissue block. Examples of two cases with heterogeneous FISH findings are shown in Figure 6.

Discussion

The results of our study show maximal heterogeneity for *PTEN* alterations in prostate cancer. Almost all (92%) tumors with *PTEN* alterations also con-

Figure 5 Frequent (16/19 foci) presence of focal areas of *PTEN* alterations in *ERG*-positive tumors but lack (0/10 foci) of focal areas of *ERG* positivity in *PTEN* altered tumors indicate that *ERG* fusion precedes *PTEN* alterations but not vice versa.

tained areas without *PTEN* alterations in the same tumor focus. In about half of the tumors, *PTEN* alterations were only found in one single tumor area, suggesting that *PTEN* alterations often occur in late stages of tumor progression. Large section analysis and validation of the tumor cell content in reference tissue microarray sections excluded technical errors or misinterpretation of individual tumor spots as a possible reason for artificial heterogeneity in cases with a single *PTEN* altered tumor area. Our findings are also supported by previous studies reporting heterogeneity of *PTEN* alterations,

Figure 6 Examples of large section validation analysis of two cases with heterogeneous *PTEN* alterations. (a) Case with *PTEN* breakage detected with the *PTEN* break-apart probe. Breakage with loss of 3' *PTEN* sequences was found in the tissue punch area taken for tissue microarray construction. An adjacent area labeled by (I) contained tumor cells with an intact *PTEN* locus, while another area (II) showed homozygous breakage as indicated by the loss of green signals. (b) Case with *PTEN* deletion detected with the deletion probe. Heterozygous *PTEN* deletion was found in the tissue punch area taken for tissue microarray construction. An adjacent area labeled by (III) contained tumor cells with normal *PTEN* locus, while another area (IV) showed heterozygous deletion indicated by loss of one red signal.

including deletions and mutations, within a single prostate cancer tumor focus^{11,17} and in different metastasis of a tumor.¹⁸ *PTEN* alterations were

strongly linked to *ERG* fusion-positive tumor foci in our tumor set, which is in concordance with early studies by others^{8,9,19} and ourselves.¹⁰

In line with the marked heterogeneity, the overall frequency of *PTEN* deletions detected in our present study (48/123, 39%) analyzing 3–10 different tissue spots per tumor was significantly higher than in our previous study analyzing only a single 0.6 mm tissue microarray spot per cancer (457/2266, 20%; $P < 0.0001$).¹⁰ This discrepancy is explained by the higher likelihood for detecting deletions in heterogeneous tumors if multiple samples are analyzed. Along with others,^{3,4,20,21} we¹⁰ have previously shown that *PTEN* loss or mutation is strongly linked to aggressive and rapidly progressing prostate cancers and early tumor recurrence, making *PTEN* a potentially promising prognostic marker. However, the high degree of heterogeneity detected in our study indicates a significant problem for potential prognostic tests.

Next-generation sequencing studies performed by us¹⁶ and others²² revealed that *PTEN* can be disrupted by chromosomal breakage, resulting in partial deletions, inversions or translocations. We have previously used a break-apart FISH probe to demonstrate presence of *PTEN* breakage in 4% (including cases with simultaneous deletion of the remaining allele) of prostate cancers using a large prostate cancer prognosis tissue microarray containing a single tissue microarray core per donor tumor.¹⁶ The results of our current heterogeneity study suggest that *PTEN* breakage may be much more frequent (18%), but may be a transient event during the development of complete *PTEN* loss. This assumption is based on the observation that all tumor foci with *PTEN* rearrangements also had tissue spots with partial and/or complete deletion of the broken *PTEN* allele, which can only be explained by initial *PTEN* breakage and subsequent deletion of chromosomal material flanking the breakpoint. Importantly, such additional deletions occurred even in cases where *PTEN* was already completely inactivated, for example, by deletion of one allele and breakage of the other. This finding suggests additional selection pressure acting beyond sole inactivation of *PTEN*. It is possible, that the nearby *KLLN* gene (*KLLN*), encoding a p53-dependent inducer of apoptosis²³ drives these additional deletions. The small (537 bp) *KLLN* locus is located directly adjacent to the 5' edge of the *PTEN* locus²³ and, therefore, included in 10q23 deletions detected by 'typical' *PTEN* deletion probes including ours (Figure 1a).¹⁰ It is tempting to speculate that tumors with co-deletion of *PTEN* and *KLLN* may escape the p53-dependent fail-safe mechanism resulting in growth arrest and senescence in *PTEN*-deficient cells.²⁴ Such a co-deletion model would also be compatible with earlier reports demonstrating accumulation of tumor cells with homozygous deletion during tumor progression.²¹

Although cancer heterogeneity limits the applicability of diagnostic, prognostic and predictive test assays, it can be useful to monitor the sequel of two or more molecular events occurring during tumor

progression. Depending on the sequel of development of *PTEN* alterations and *ERG* fusion, one can expect a small area of cancer having both alterations within a larger area having only one (the earlier) of these changes. We found small *PTEN* altered areas in 16 of 19 homogeneously *ERG*-positive tumors, but we failed to see any *ERG*-positive areas within 10 *PTEN*-deficient cancer foci, suggesting that *ERG* fusion typically precedes alterations of *PTEN*. These findings would be consistent with *ERG* fusions facilitating the development of *PTEN* alterations rather than a sole cooperative effect that had been earlier suggested from mouse models with prostate-targeted *ERG* expression and *PTEN* depletion.^{6,7} In a recent study, Rickman *et al*²⁵ demonstrated that *ERG* expression is associated with broad changes in chromatin topology, giving rise to specific genomic rearrangements driven by a particularly high density of *ERG*-binding sites located close to the breakpoints. It may be speculated that also the *PTEN* locus may be prone to such *ERG*-driven rearrangements.

In summary, the heterogeneity tissue microarray approach used in this study did not only allow estimating the degree of heterogeneity of individual markers, but also provided insights into the sequel of *PTEN* and *ERG* alterations in prostate cancer. The results of our study show a marked degree of heterogeneity of *PTEN* alterations, including deletions and gene breakage, and outline a stepwise molecular progression of *PTEN* alterations from heterozygous breakage to homozygous deletion. In addition, our data strongly suggest a driving role of *ERG* fusion to promote development of *PTEN* alterations.

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Disclosure/conflict of interest

The authors declare no conflict of interest.

Author contributions

AK, FF, SH, MK, SF and LB performed and analyzed the tissue microarray and large section FISH experiments; PS, MCT, MB and SS reviewed all tissue samples, determined tumor foci and selected tumors for molecular analyses, PT, MG and TS provided cancer specimens; and AK, HS, TS, GS, RS and SM interpreted all data and wrote the manuscript. All contributors were involved in approving the final version of the manuscript before submission.

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