Biomarkers, Genomics, Proteomics, and Gene Regulation

Genomic Deletion of PTEN Is Associated with Tumor Progression and Early PSA Recurrence in ERG Fusion-Positive and Fusion-Negative Prostate Cancer

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The phosphatase and tensin homolog deleted on chromosome 10 (PTEN) gene is often altered in prostate cancer. To determine the prevalence and clinical significance of the different mechanisms of PTEN inactivation, we analyzed PTEN deletions in TMA containing 4699 hormone-naïve and 57 hormone-refractory prostate cancers using fluorescence in situ hybridization analysis. PTEN mutations and methylation were analyzed in subsets of 149 and 207 tumors, respectively.

The results of this study demonstrate that biallelic PTEN inactivation, by either homozygous deletion or deletion of one allele and mutation of the other, occurs in most PTEN-defective cancers and characterizes a particularly aggressive subset of metastatic and hormone-refractory prostate cancers. (Am J Pathol 2012, 181:401–412; http://dx.doi.org/10.1016/j.ajpath.2012.04.026)

Prostate cancer is a leading cause of cancer-related mortality in men. More than 600,000 men are annually diagnosed as having prostate cancer worldwide.1 Although most prostate cancers are detected at early stages as a result of prostate-specific antigen (PSA) screening, many patients harbor advanced and metastatic cancer at diagnosis. A better understanding of the molecular biological features of prostate cancer may help to improve prostate cancer diagnosis and therapy.

Phosphatase and tensin homolog deleted on chromosome 10 (PTEN) was identified as a tumor suppressor gene on chromosome 10q23 and encodes a dual-specificity phosphatase that functions as a direct antagonist of phosphatidylinositol 3-kinase, a key kinase involved in AKT activation.4 Inactivation of PTEN causes constitutively activated levels of AKT, thus promoting cell growth, proliferation, survival, and migration through multiple downstream effectors.5 PTEN alterations may play a critical role for prostate cancer biological features. Cell line experiments led to the assumption that PTEN inactivation is capable of promoting tumor invasiveness6 and metastasis development.7,8

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PTEN promoter methylation was not detected in 34 tumors. The results of this study demonstrate that biallelic PTEN inactivation, by either homozygous deletion or deletion of one allele and mutation of the other, occurs in most PTEN-defective cancers and characterizes a particularly aggressive subset of metastatic and hormone-refractory prostate cancers.
Mouse models have further suggested that PTEN inactivation through genomic deletion or mutation leads to development of hyperplasia, prostatic intraepithelial neoplasia (PIN), and invasive carcinoma,9–15 and it has been discussed whether a reduced PTEN gene dosage (haploinsufficiency) might be sufficient to cause prostate cancer.12,14 Moreover, two recent studies16,17 suggested a cooperative effect between PTEN inactivation and ERG fusion in prostate cancer initiation and progression.

Although the importance of PTEN inactivation for prostate cancer biological features is undisputed, data on the prevalence and prognostic relevance of PTEN alterations in clinical prostate cancer specimens are inconsistent.18–24 Only a few studies have analyzed the frequency of PTEN deletions using fluorescence in situ hybridization (FISH) analysis, which is regarded as the gold standard for determination of gene copy numbers in tissue samples, or performed sequence analysis to estimate the prevalence of PTEN mutations. In these studies, PTEN deletions were reported from 17% to 68%25–29 and PTEN mutations were found in up to 21% of prostate cancers.24,30–32 To comprehensively study the prevalence and potential clinical significance of PTEN deletions and their relationship to PTEN mutations and methylation, we took advantage of a pre-existing TMA containing >4000 prostate cancers with clinical follow-up data.

Materials and Methods

Patients

Two TMAs were used in this study. The first was a prostate cancer prognosis TMA containing prostatectomy specimens from 4699 consecutive patients undergoing radical prostatectomy between 1992 and 2008 at the Department of Urology, University Medical Center Hamburg-Eppendorf, Hamburg, Germany (Table 1). This TMA is based on a previously described prostate cancer TMA consisting of 3261 samples,33 with an additional 1438 tumors and updated clinical follow-up data. Clinical follow-up data were available for 4203 of the 4699 arrayed tumors. The median follow-up was 46.7 months (range, 1 to 219 months). None of the patients received neoadjuvant endocrine therapy. Salvage therapy was initiated in cases of biochemical relapse. In all patients, PSA values were measured quarterly in the first year, followed by biannual measurements in the second year and annual measurements after the third year following surgery. Recurrence was defined as a postoperative PSA of 0.2 ng/mL, increasing thereafter. The first PSA value of 0.2 ng/mL or greater was used to define the time of recurrence. Patients without evidence of tumor recurrence were censored at the last follow-up. The second TMA was constructed from 57 hormone-refractory prostate specimens collected from palliative transurethral resections at the Department of Urology, University Medical Center Hamburg-Eppendorf, and at the Department of Surgery, University of Montreal, Montreal, QC, Canada. Hormone-refractory prostate cancer was defined as follows: serum castration level of testosterone; three consecutive increases in the PSA level, resulting in two 50% increases from the nadir; anti-androgen withdrawal for at least 4 weeks; PSA progression despite secondary hormonal manipulation; or progression of osseous or soft tissue lesion. No follow-up data were available from these patients.

PTEN FISH Analysis

For PTEN deletion analysis, a dual-color FISH probe set was used. The set consisted of two SpectrumGreen-labeled bacterial artificial chromosome clones (RP11-380G9 and RP11-813O3; Source Bioscience, Nottingham, UK) and a SpectrumOrange-labeled commercial centromere 10 probe (06J36-090; Abbott, Wiesbaden, Germany) as a reference. Freshly cut TMA sections (4 μm thick) were deparaffinized and proteolytically pretreated using a commercial kit (paraffin pretreatment reagent kit; Abbott Molecular, Wiesbaden, Germany), followed by dehydration in 70%, 80%, and 96% ethanol, air drying, and denaturation for 10 minutes at 72°C in 70% formamide—twice timed standard saline citrate solution. Hybridization was performed overnight at 37°C in a humidified chamber; slides were then washed and counterstained with 0.2 μmol/L of DAPI in an antifade solution. Each tissue spot was evaluated, and the predominant signal was recorded for each FISH probe. A total of 659 tissue spots were excluded from FISH analysis because basal cell marker 34βE12 analysis33 indicated lack of tumor cells. Thresholds for PTEN FISH analysis were established from 0.6-mm tissue spots from seven tumors with a known

### Table 1. Pathological and Clinical Data of Arrayed Prostate Cancers

<table>
<thead>
<tr>
<th>Variable</th>
<th>Study cohort receiving TMA (n = 4699)</th>
<th>Biochemical relapse among categories (n = 904)</th>
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<tr>
<td>Mean</td>
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<tr>
<td>Median</td>
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<td>Age (years)</td>
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<tr>
<td>&lt;50</td>
<td>126</td>
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<tr>
<td>50–60</td>
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<td>&gt;70</td>
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<td>84</td>
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<td>Pretreatment PSA (ng/mL)</td>
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<td>4–10</td>
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<td>159</td>
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<td>pT category (AJCC 2002)</td>
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<tr>
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<td>pT3b</td>
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<td>573</td>
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<tr>
<td>Positive</td>
<td>810</td>
<td>324</td>
</tr>
</tbody>
</table>

Data are given as percentage or number of patients. Numbers do not always add up to 4699 in the different categories because of cases with missing data.

AJCC, American Joint Committee on Cancer.
PTEN deletion (four with a heterozygous and three with a homozygous deletion), based on single-nucleotide polymorphism (SNP) array copy number analysis. In five of these tumors, PTEN signal losses by FISH were found in all analyzed tissue blocks. The two remaining cancers had tissue blocks with and without PTEN deletion, indicating the presence of intratumoral heterogeneity. In all seven cases, tumor blocks with PTEN deletion had FISH signal losses in most (at least 60%) tumor cells. According to these findings, homozygous deletion of PTEN was defined as complete absence of PTEN FISH probe signals in ≥60% of tumor nuclei of the tissue spot, with the presence of one or two PTEN FISH signals in adjacent normal cells. Tissue spots with a lack of PTEN signals in all (tumor and normal cells) or lack of any normal cells as an internal control for successful hybridization of the PTEN probe were excluded from analysis. Heterozygous deletion of PTEN was defined as the presence of fewer PTEN signals than centromere 10 probe signals of ≥60% tumor nuclei.

PTEN IHC

We tested seven different PTEN antibodies (Table 2) for their suitability in formalin-fixed, paraffin-embedded tissues. The antibody used for this study (ab31392, rabbit polyclonal; Abcam, Cambridge, UK) was selected because it showed reproducible nuclear staining. Freshly cut TMA sections were stained with a commercial anti-PTEN antibody (clone EPR3864; dilution, 1:450; Epitomics, Burlingame, CA). Slides were deparaffinized and exposed to heat-induced antigen retrieval for 5 minutes in an autoclave at 121°C in pH 7.8 Tris-EDTA-citrate buffer. Bound primary antibody was visualized using the DAKO EnVision Kit. Tissue spots showing nuclear PTEN staining in tumor cells were considered positive for PTEN fusion. We have previously shown that PTEN expression detected with this antibody shows 98.5% concordance with PTEN rearrangement detected by FISH analysis.34

p53 and Ki-67 IHC Analysis

Nuclear accumulation of p53 was analyzed by IHC, as previously described.35 The IHC data of Ki-67 were available from a previous study.36

PTEN Mutational Analysis

Tissue specimens were selected if at least 70% tumor cells were present. For DNA extraction, one core (0.6-mm diameter and 5-mm length) was taken from each tumor block. Paraffin was removed with xylene and 80% ethanol, followed by overnight digestion with proteinase K. DNA was isolated using a commercial kit (QIAamp DNA FFPE kit; Qiagen, Hilden, Germany). All nine PTEN exons were amplified by PCR using the AmpliTaq Gold polymerase (Applied Biosystems, Darmstadt, Germany). Primer sequences are given in Table 3. PCR cycling conditions included an initial denaturation step at 95°C for 10 minutes, followed by 35 cycles of 95°C denaturation for 20 seconds, 55°C or 53.5°C annealing for 20 seconds, 72°C extension for 40 seconds, and a final extension step at 72°C for 7 minutes. The quality of PCR products was verified by QIAxcel capillary electrophoresis (Qiagen). Sequencing was prepared by a Big Dye Terminator Kit (Applied Biosystems), and electrophoretic analysis was performed on the Genetic Analyzer 3100 (Applied Biosystems). Sequencing primers are given in Table 4.

<table>
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<tr>
<th>Exon</th>
<th>Forward primer</th>
<th>Reverse primer</th>
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</tr>
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<td>2</td>
<td>5′-CTCTGCTCTGGGAGGAAAAGC-3′</td>
<td>5′-CTTCTTCTGCTTGTTAGAATCC-3′</td>
</tr>
<tr>
<td>3</td>
<td>5′-CCCCAGAGGAGGCTTTGTG-3′</td>
<td>5′-CTCTGCTCTGGGAGGAAAAGC-3′</td>
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<tr>
<td>4</td>
<td>5′-CCATCTTTTTAAGTTCGAG-3′</td>
<td>5′-AAGATACGTTTCTAGGTTT-3′</td>
</tr>
<tr>
<td>5</td>
<td>5′-CCATCTTTTTAAGTTCGAG-3′</td>
<td>5′-TCCAGAAGAAAGAAGAGAAA-3′</td>
</tr>
<tr>
<td>6</td>
<td>5′-GGCTAGCTATCCGCTACAT-3′</td>
<td>5′-GAGAGCTGAGAAATTCAAGACA-3′</td>
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<td>7</td>
<td>5′-AGGATGAGCTGTGATT-3′</td>
<td>5′-TGATTCTCCAGGAGGAAAG-3′</td>
</tr>
<tr>
<td>8 fragment 1</td>
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<td>5′-AGGATGAGCTGTGATT-3′</td>
</tr>
<tr>
<td>8 fragment 2</td>
<td>5′-GGCTAGCTATCCGCTACAT-3′</td>
<td>5′-AGGATGAGCTGTGATT-3′</td>
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<tr>
<td>9</td>
<td>5′-GGCTAGCTATCCGCTACAT-3′</td>
<td>5′-AGGATGAGCTGTGATT-3′</td>
</tr>
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</table>
Table 5. Primers Used for MASSArray Analysis

<table>
<thead>
<tr>
<th>Exon</th>
<th>Sequencing primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5’-TTCCATCCTGGACAGAAGC-3’</td>
</tr>
<tr>
<td>2</td>
<td>5’-CTCCAGCTATTGAGGAAAAA-3’</td>
</tr>
<tr>
<td>3</td>
<td>5’-CAGTATAGCGAGTTTCTTTT-3’</td>
</tr>
<tr>
<td>4</td>
<td>5’-CTCCAGAACGGAAGAGAAAGAA-3’</td>
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<td>5</td>
<td>5’-GGTACGCAGGTTACCTTAT-3’</td>
</tr>
<tr>
<td>6</td>
<td>5’-CTGATTAGGCTTCTG-3’</td>
</tr>
<tr>
<td>7</td>
<td>5’-AAGTCAAACACCCACACCA-3’</td>
</tr>
<tr>
<td>8</td>
<td>5’-GTCGATTCATTCATTCAAAGT-3’</td>
</tr>
</tbody>
</table>

SNP Array Analysis

A total of 72 snap-frozen prostate cancer samples with at least 70% tumor cell content and five prostate cell lines (LNCaP, PC3, BPH, X22RV, and VCaP) were selected for SNP array analysis. DNA was isolated using a commercial kit (QIAamp DNA Mini Kit; Qiagen). Affymetrix SNP V6.0 arrays were used for SNP Array Analysis exactly as described in the Affymetrix V6.0 SNP array manual. DNA was isolated using a commercial kit (QIAamp DNA Mini Kit; Qiagen). Affymetrix SNP V6.0 arrays were used for SNP Array Analysis exactly as described in the Affymetrix V6.0 SNP array manual. We used our own generic browser (FISH Oracle)37 to map all 10q23 deletions to the human genome reference sequence (Archer EnsEMBL release 54, May 2009) and to define the minimally overlapping region of deletion.

Methylation Analysis

Quantitative DNA methylation analysis at single CpG units was performed on 34 prostate cancers using MassARRAY Epityper (Sequenom, San Diego, CA), as previously described.38 Briefly, bisulfite-treated genomic DNA was PCR amplified, in vitro transcribed, cleaved by RNase A, and subjected to matrix-assisted laser desorption ionization–time-of-flight mass spectrometry (Sequenom, Hamburg, Germany). An overview of MassARRAY primer sequences for PCR amplicons covering the whole PTEN CpG island is given in Table 5. The location of primers used is shown in Supplemental Figure S1 (available at http://aje.ajpmgh.org). Detailed analyses were performed with two amplicons using the following primers: 5’-GATTGTTTGGGTATYGGAGG-3’ (forward) and 5’-CTCATCCRACCTCCCTTTA -AC-3’ (reverse) for PTEN4.5 and 5’-GTTGTGTTATAGGGTGTGAGG-3’ (forward) and 5’-CCCTCCCTCCTCCTCCCTC-3’ (reverse) for PTEN10p.

GSTM1 methylation was analyzed as a control using the following primers: forward, 5’-GTTGTGTTTATTATGTTGTTGTTT-3’; and reverse, 5’-TAACTTAATTATGACACACACCCAC-3’. Y and R nucleotide codes denote wobble sites with C/T and A/G, respectively. Methylation standards (0%, 20%, 40%, 60%, 80%, and 100% methylated genomic DNA) and correction algorithms, based on custom scripts for the R statistical computing environment, were used for data normalization.

Statistics

For statistical analysis, the JMP 8.0 software (SAS Institute Inc., Cary, NC) was used. Contingency tables were calculated to study the association between PTEN deletion and clinicopathological variables, and the χ² (likelihood) test was used to find significant relationships. Kaplan-Meier curves were generated for PSA recurrence-free survival. The log-rank test was applied to test the significance of differences between stratified survival functions. Cox proportional hazards regression analysis was performed to test the statistical independence and significance between pathological, molecular, and clinical variables.

Results

Technical Issues

A total of 4040 hormone-naïve and 57 hormone-refractory cancers were included in FISH analysis in this study. Analysis failed in 45% hormone-naïve and in 14% hor-

Table 4. PTEN Sequencing Primers

<table>
<thead>
<tr>
<th>Exon</th>
<th>Sequencing primer</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>5’-GGTATATTTTTCAGTTATTTCAAGT-3’</td>
</tr>
<tr>
<td>2</td>
<td>5’-CTCCAGCTATTGAGGAAAAA-3’</td>
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<tr>
<td>3</td>
<td>5’-CAGTATAGCGAGTTTCTTTT-3’</td>
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<tr>
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<td>5’-CTCCAGAACGGAAGAGAAAGAA-3’</td>
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<tr>
<td>5</td>
<td>5’-GGTACGCAGGTTACCTTAT-3’</td>
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<td>5’-CTGATTAGGCTTCTG-3’</td>
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<tr>
<td>7</td>
<td>5’-AAGTCAAACACCCACACCA-3’</td>
</tr>
<tr>
<td>8</td>
<td>5’-GTCGATTCATTCATTCAAAGT-3’</td>
</tr>
</tbody>
</table>

Y and R nucleotide codes stand for wobble sites with C/T and A/G, respectively.
mone-refractory tumors, because of either lack of tissue spots in the TMA section or faint or lacking FISH signals. In summary, 2217 hormone-naïve and 49 hormone-refractory tumors were successfully analyzed and included in the statistical analyses.

Prevalence and Type of PTEN Deletions and Association to Prostate Cancer Phenotype

PTEN deletions were found in 20.2% (458/2266) of all prostate cancers (Figure 1, A–C). Overall, homozygous PTEN deletions (12.1%) were slightly more frequent than heterozygous PTEN deletions (8.1%). Both heterozygous and homozygous deletions were more frequent in hormone-refractory compared with hormone-naïve cancers ($P < 0.0001$ for each). The difference was particularly strong for homozygous deletions, which were found in 16 (32.7%) of 49 hormone-refractory cancers, but only in 259 (11.7%) of 2217 hormone-naïve tumors ($P < 0.0001$). The relationship between PTEN deletions and tumor phenotype and clinical parameters is summarized in Table 6. PTEN deletions (including heterozygous and homozygous deletions) were significantly linked to advanced tumor stage ($P < 0.0001$), high Gleason grade ($P < 0.0001$), presence of lymph node metastasis ($P = 0.0002$), and positive surgical margin ($P = 0.0462$). Although a significant $P$ value was obtained for the association between PTEN deletions and PSA serum level ($P = 0.0043$), we did not consider this result as indicative for a true relationship because the different PSA levels were not unequivocally paralleled by an increase or decrease of PTEN deletions.

Association of PTEN Deletions to Other Molecular Markers of Prostate Cancer

Data on PTEN and p53 status were available from 1798 hormone-naïve cancers. PTEN deletions were significantly more frequent in p53-positive tumors (heterozygous, 10.5%; and homozygous, 36.8%) than in p53-negative tumors (heterozygous, 8.4%; and homozygous, 11.2%; $P < 0.0001$; Figure 2A). This overall significant association resulted from homozygous deletions ($P < 0.0001$), whereas the difference was not significant for heterozygous deletions ($P = 0.2758$). Data on PTEN deletion and ERG fusion status were available from 2177 tumors. PTEN deletion was strongly associated with ERG fusion-positive tumors (29.1% versus 10.7%; $P < 0.0001$ overall) and for separate analysis of heterozygous and homozygous deletions (Figure 2B). PTEN and Ki-67 labeling index (Ki-67 LI) data were both available from 1802 tumors. Ki-67 LI was significantly higher in PTEN-deleted (average Ki-67 LI, 6.3) than in -undeleted (average Ki-67 LI, 5.5; $P = 0.0321$) cancers, if all cancers were jointly analyzed, but there was no statistically significant association found in tumors of identical stage and grade (see Supplemental Table S1 at http://ajp.amjpathol.org).

Figure 1. Examples for PTEN-deletions in prostate cancer. A: Normal copy number of PTEN with two green PTEN and 2 red centromer 10 signals. B: PTEN heterozygous deletion with one green PTEN signal and two red centromer 10 signals. C: PTEN homozygous deletion completely lacking PTEN signals but showing two red centromer 10 signals. Arrow, normal prostate cells showing normal PTEN copy number.
Clinical Significance of PTEN Deletions, ERG Fusion, and p53

PTEN FISH was analyzable in a subset of 1931 cases with follow-up data. In this subset, Gleason grade, pT stage, and preoperative serum PSA levels were significantly linked to poor prognosis (P < 0.0001, data not shown). PTEN deletions were significantly linked to early PSA recurrence in univariate analysis (P < 0.0001, Figure 3A). No difference was seen between tumors with heterozygous or homozygous deletion (P = 0.6970). In a multivariate cyclooxygenase regression proportional hazard analysis including pT stage, Gleason grade, preoperative PSA level, and PTEN deletion status, PTEN deletion was identified as an independent predictor of PSA recurrence-free survival (P = 0.0158, Table 7). ERG fusion was analyzable in 3751 tumors with follow-up data. The presence of ERG fusion was unrelated to patient prognosis (P = 0.7346, Figure 3B). A combined analysis of PTEN and ERG in 1895 tumors revealed no prognostic differences between tumors with PTEN deletion and ERG fusion, compared with tumors with PTEN deletion but lack of ERG fusion (P = 0.9459, Figure 3C). There was a significantly worse prognosis for ERG-negative compared with ERG-positive tumors in the subset of 1524 cancers with normal PTEN copy numbers (P = 0.0044). A combined analysis of PTEN deletion and presence of nuclear accumulation of p53 in a subset of 1545 cancers revealed that 35 cancers with p53 accumulation (irrespective of the PTEN deletion status) had a significantly worse prognosis than tumors with PTEN deletion but lack of p53 alteration (Figure 3D, P = 0.0162).

PTEN IHC

PTEN IHC was successful in 3320 of the 4699 arrayed hormone-naive primary prostate cancers. Immunostain-
ing was considered negative in 83 cases (2.5%), weak in 903 cases (27.2%), moderate in 2192 cases (66.0%), and strong in 142 cases (4.3%) (see Supplemental Table S2 at http://ajp.amjpathol.org). No meaningful associations were found between the PTEN staining levels and tumor phenotype or presence of PTEN deletions (see Supplemental Table S1 and Supplemental Figures S2 and S3, A and B, at http://ajp.amjpathol.org). The intensity of cytoplasmic staining was markedly reduced with higher (1:150) antibody dilutions (see Supplemental Figure S3 at http://ajp.amjpathol.org). PTEN staining was also unrelated to patient prognosis in a Kaplan-Meier survival analysis (P = 0.5251, data not shown).

**Table 7. COX Regression Multivariate Analysis for Predictive Factor Biochemical Recurrence**

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<tr>
<th>Parameter</th>
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<td>pT3b versus pT2</td>
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<td>PSA level</td>
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<td>10–20 versus &lt;4</td>
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<tr>
<td></td>
<td>&gt;20 versus &lt;4</td>
<td>1.8</td>
<td>1.18–2.66</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>PTEN</td>
<td>Deleted versus not deleted</td>
<td>1.3</td>
<td>1.05–1.60</td>
<td>0.0158</td>
</tr>
</tbody>
</table>

HR, hazard ratio.

**Mutation Analysis of PTEN**

All nine exons of PTEN were analyzed in 97 prostate cancers containing 71 hormone-naïve and 26 hormone-refractory prostate cancers. PTEN mutations were found in 7 cases, including 5 (7.0%) hormone-naïve and 2 (7.7%) hormone-refractory tumors (P = 0.943). Mutations were found in tumors with heterozygous PTEN deletion in 12.9% (4/31, one hormone refractory) and in tumors with normal PTEN copy numbers in 2% (1/59, one hormone refractory, P = 0.027, Table 8). No FISH result was available for two additional cases with mutation. The five hormone-naïve mutated tumors had a Gleason score of 6 to 7. Based on the type of mutation identified in our analysis, at least four of the seven mutations inevitably cause PTEN inactivation. These include three tumors with small deletions and insertions in exons 1 and 8, causing frameshift mutations, and another tumor with a truncating mutation (E201end) in exon 8. The remaining three tumors showed point mutations in exons 3, 5, and 8, including amino acid changes from tyrosine to asparagine (T68G) in exon 3; from asparaginic acid to asparagine (D326N) in exon 8; and from histidine to tyrosine (H118Y) in exon 5 (see Supplemental Figure S4, A–E, at http://ajp.amjpathol.org). Most likely, the latter two mutations also led to inactivation, because exon 5 contained the functionally relevant WDP and P loops that formed the active pocket of the phosphatase domain.
Architecture of 10q23 Deletions Harboring PTEN

Deletions involving the long arm of chromosome 10 were found in 14 of the 77 analyzed prostate cancer samples. The largest deletion spanned >23 Mb. The smallest deletion marked a region of 809 kb that were commonly deleted in all 14 tumors. This region contained the PTEN gene and two adjacent genes (ATAD1 and RNLS). The minimal overlapping region of deletion also contained both bacterial artificial chromosome clones used for generation of our FISH probe (Figure 4).

Epigenetic Changes at the PTEN Promoter

We designed 15 primer pairs to cover the whole CpG island associated with PTEN for quantitative DNA methylation analyses using MassARRAY technology. Initial analyses indicated overall low methylation and did not reveal any differences between tumor and normal samples (data not shown). We selected two amplicons (PTEN4.5 and PTEN10p) covering 6 and 7 CpG units, each representing one or two individual CpG sites, to further analyze PTEN promoter methylation in 34 prostate cancer samples and 5 normal prostate tissues (Figure 5A). Median methylation was <10% in both amplicons and did not differ between tumor and normal samples (Figure 5B). In contrast, all tumor samples were highly methylated at the GSTP1 promoter CpG island analyzed as a positive control, with median methylation of 79% in tumor samples and 5% in normal controls.

Discussion

Our study shows that PTEN deletions occur more frequently (20%) than mutations (8%) in prostate cancer. PTEN deletions are strongly linked to important biological and clinical features, such as rapid tumor progression, hormone-refractory state, and early PSA recurrence. The low rate of muta-
tions fits well with previous reports showing that 2.5%, 31
5.1%, 32 and 7.5% 39 of unselected, localized prostate can-
cers harbor
PTEN
mutations. Higher mutations rates were
only found in cancer sets selected for metastatic disease
(34%) 32 or loss of heterozygosity at 10q23 (43%), 24 and if
multiple tumor sites of individual cancers were screened for
mutations (21%). 30 In our study, 
PTEN
mutations were
found in 4 (12.9%) of 31 cancers carrying heterozygous
PTEN
deletions, compared with 1 (2%) of 59 cancers with
normal 
PTEN
copy numbers, suggesting a strong selection for complete 
PTEN
inactivation in cancers with defective 
PTEN.
The absence of promoter methylation in 34 randomly
selected cancers is in line with previous studies 24, 40, 41 and
confirms that epigenetic mechanisms do not play a signifi-
cant role for 
PTEN
inactivation in prostate cancer. Our data
also demonstrate that approximately two thirds of clinical
cancer specimens with genomic 
PTEN
alterations show in-
avitration of both alleles (typically by homozygous deletion),
suggesting that 
PTEN
haploinsufficiency may be less im-
portant in prostate cancer than previously thought. 42

The main purpose of this study was to determine the
relationship between 
PTEN
alterations and tumor pheno-
type, as well as clinical outcome. The many tumors included
in our study enabled us to identify 458 tumors with 
PTEN
deletions. The strong link between 
PTEN
delusions and ad-
verse tumor features suggests that 
PTEN
alterations confer
substantial malignant potential to prostate cancer cells. This
is in line with previous studies using FISH to assess 
PTEN
deletions in cohorts containing between 59 and 322 tu-
mors. 25, 28, 29, 43, 44 These studies suggested associations
between 
PTEN
deletion and metastatic prostate cancer phenotype, 25
hormone-refractory state, 28 early biochemical relapse, 43
overall survival 44 and cancer-specific death in hormone-refractory prostate cancers. 29 In addition, several
studies 22–24 analyzing loss of heterozygosity at 10q23 (in-
cluding the 
PTEN
locus) found the highest loss of heterozy-
gosity rates in advanced and metastatic tumors.

For IHC analysis, we selected the antibody ab31392 (Ab-
cam) from a series of seven tested anti-
PTEN
antibodies
(Table 2) because it showed reproducible nuclear staining.
Nuclear accumulation of 
PTEN
is mediated by monoubiq-
uitination and has been essential for tumor suppression. 45
Nuclear 
PTEN
contributes to maintaining chromosomal sta-
bility and promotes apoptosis, whereas cytoplasmic 
PTEN
negatively regulates AKT signaling. 46 For the remaining six
antibodies, it was not possible to develop a robust IHC
protocol or they were not suitable to detect reduced or
lacking expression in 
PTEN
-deleted cancers. This study
also included one relatively new antibody (Cell Signaling
Technology, Danvers, MA) that may detect 
PTEN
protein
loss in 
PTEN
-deleted tumors only recently. 47, 48 Our IHC
analysis detected at least weak nuclear staining in
95% of
analyzable prostate cancers. There was also no meaningful
association between IHC staining levels and tumor pheno-
type or patient prognosis. These findings argue against the
suitability of the 
PTEN
antibody ab31392 for IHC in formalin-
fixed tissues. This is even more true because tumors with
homozygous deletion by FISH, which would be expected to
completely lack 
PTEN
protein, showed a positive IHC result.
The difficulty of studying 
PTEN
expression by IHC is also
reflected by the highly discrepant findings in the published
literature, with the frequency of reduced 
PTEN
expression
ranging from 4% to 88%. 18–21, 28, 49, 50 Accordingly, associ-
ations between the level of 
PTEN
expression and clinico-
pathological parameters vary. For example, McCall et al 28
reported a link between low-level cytoplasmic 
PTEN
ex-
pression and tumor recurrence, and between loss of nu-
clear 
PTEN
staining and patient survival in 68 hormone-
naive prostate cancers. However, Koumakpayi et al 15 and
Bedolla et al 21 could not confirm such associations in sim-
ilar tumors.
similarly sized patient cohorts. A link between loss of PTEN expression and high Gleason grade or advanced tumor stage was reported from some studies,\(^{18,19}\) but not corroborated by other studies.\(^{20,21,50}\)

PTEN deletions were approximately three times more frequent in ERG-positive compared with ERG-negative cancers. This association was not because of a higher fraction of advanced tumors in the subset of ERG fusion-positive cancers, because stage and grade distribution was comparable in both subsets (see Supplemental Table S3 at http://ajp.amjpathol.org). This is in line with recent studies\(^{16,17,25,27}\) reporting a link between PTEN deletion and ERG fusion in prostate cancer. Our findings suggest a selection advantage for tumor cells harboring both PTEN deletion and ERG fusion. Several studies using transgenic mice to monitor the effects of PTEN inactivation and/or ERG expression suggested a cooperative effect of these genes for prostate cancer initiation and progression. For example, Kwabi-Addo et al\(^{12}\) found that PTEN levels corresponding to heterozygous PTEN deletion caused PIN, and Trotman et al\(^{14}\) reported that particularly low PTEN levels (ie, 25% of wild-type expression) were sufficient for development of invasive cancer. Similarly, overexpression of ERG\(^{51,52}\) or ETV1\(^{53}\) alone resulted in PIN in some studies, although King et al\(^{16}\) observed PIN only if ERG was overexpressed in mice that were either PTEN deficient or had high AKT activity. ERG overexpression in PTEN-deficient mice of Carver et al\(^{12}\) caused PIN and led to invasive cancer. Although the link between PTEN deletion and ERG fusion found in our study supports the existence of such cooperative effects, it also suggests that ERG fusion is not required for PTEN loss to determine aggressive tumor behavior, because PTEN deletion in both ERG fusion-positive and fusion-negative cancers was independently linked to poor prognosis. Two previous studies\(^{27,44}\) have analyzed the association between co-alterations of PTEN and ERG and patient prognosis. The results differed in that Yoshimoto et al\(^{27}\) found the worst prognosis for patients with both PTEN deletion and ERG fusion in a study on 125 patients, whereas Reid et al\(^{44}\) suggested, in their analysis of 308 patients, that tumors with PTEN loss but lack of ERG fusion had a particularly poor outcome. Our study demonstrates that PTEN is a major strong driver of patient prognosis, independent of ERG status. Given the strong association seen between PTEN deletions and high Ki-67 labeling index, the aggressive behavior of PTEN-deleted cancers may be driven by increased cell proliferation. Such a scenario would also be concordant with the known role of PTEN as a key regulator of the AKT growth-signaling pathway.\(^{54}\) Remarkably, ERG-negative cancers had earlier PSA recurrence than ERG-positive cancers if tumors were PTEN wild type, whereas the ERG status had no detectable impact on clinical outcome if all tumors were analyzed together. This observation may be related to both the higher fraction of PTEN-deleted tumors in ERG-positive compared with ERG-negative cancers and to the presence of other molecular alterations that may drive poor prognosis, particularly in ERG-negative cancers.

Nuclear accumulation of p53 is strongly linked to presence of inactivating p53 mutations,\(^{35}\) which is an important reason for failure of cellular repair systems and development of genetic instability.\(^{55}\) The association between PTEN deletions and nuclear p53 accumulation suggests that development of PTEN deletion may be caused by p53-mediated genetic instability in a subset of prostate cancers. The particular striking association between homozygous PTEN deletion and nuclear p53 accumulation further suggests a selection advantage for complete PTEN inactivation in a p53-deficient background. This finding fits well with a previous report\(^{36}\) describing a functional link between complete PTEN inactivation and subsequent activation of a p53-dependent failsafe program, which triggers a proliferation block and induces cellular senescence. In their prostate cancer mouse model, Chen et al\(^{56}\) found that invasive tumors developed only in mice with concurrent inactivation of both p53 and PTEN. The comparison of impacts of PTEN and p53 on patient outcome emphasizes the striking prognostic relevance of p53 alterations on prostate cancer outcome, as previously described by us\(^{35}\) and other groups.\(^{57–59}\) Our data do not suggest an additional prognostic impact of PTEN deletions in p53-altered cancers, whereas PTEN deletions remain of high prognostic importance in p53-negative cancers.

Data from our SNP array copy number analysis demonstrate that the minimal commonly deleted region at 10q23 in prostate cancer contains only one gene, PTEN. We took advantage of the fact that the minimal region of deletion always extended the PTEN gene locus and constructed a large (360-kb) FISH probe, including flanking regions of PTEN, to obtain bright FISH signals that can be scored with high reliability. By using this probe, the fraction of PTEN deletions detected in our study in hormone-naïve (18%) and in hormone-refractory (45%) cancers is in the lower range of previous studies\(^{25–29,39,43,44}\) reporting 17% to 68% PTEN deletion in localized and 41% to 77% in PTEN deletion in hormone-refractory cancers. We believe that the comparatively low frequency of deletions in our study is mainly caused by stringent criteria for defining PTEN deletions. We expected FISH signal loss in at least 60% of tumor cells to call a tumor deleted. This threshold was based on FISH findings in seven tumors with known heterozygous or homozygous deletions, according to an SNP array-based copy number analysis. In 0.6-mm tissue spots obtained from these tumors, virtually all tumor cells showed PTEN signal losses, including two cancers that had both tumor blocks with and without PTEN deletion. These findings confirm that cancer foci with and without PTEN deletions may exist within the same prostate,\(^{25,60}\) but also demonstrate that it is unlikely that such heterogeneity becomes visible within an area of 0.6-mm cancer tissue analyzed per TMA spot. Our cutoff is substantially more stringent than in most previous FISH studies,\(^{25,29,43,44}\) in which the rate of artificial FISH signal losses caused by nuclei truncation was first determined in normal prostatic epithelium and then used as a threshold for deletion in cancer samples. In such a scenario, false deletion calling can occur because the larger nuclei of cancer cells will more often lose FISH signals because of truncation than the smaller normal cell nuclei. Accordingly, the highest frequencies of heterozygous deletion (44% to 59%) were reported from studies using less stringent thresholds (eg, ≥20% to 30% of tumor cells with FISH signal loss required to define deletion).\(^{29,43}\) We found a slightly lower fraction of heterozygous deletions (8.1%) compared with homozygous deletions (12.1%). This is dif-
fherent from most previous studies25,26,29,39,43,60 that usually reported more heterozygous (12% to 62%) than homozygous (5% to 25%) deletions. However, PTEN FISH results are highly variable in the literature, and some studies reported markedly higher rates of heterozygous (30% to 62%) compared with homozygous (5% to 6%) deletions,26,43 whereas others25,29,39 found more similar frequencies of heterozygous (12% to 34%) and homozygous (9% to 25%) deletions. These differences may be related to different scoring criteria but also to the comparatively few samples and the presence or absence of hormone-refractory cancers in individual studies. The results of our analysis fit best to the findings reported by Han et al,25 who applied a comparable definition for heterozygous deletion (>50% of tumor cells with signal loss), as used in our study. The authors reported 12.6% heterozygous and 9.2% homozygous deletions in a set of 251 hormone-naive and 41 hormone-refractory cancers, which is close to the 8.1% heterozygous and 12.1% homozygous deletions found in our study.

In summary, our data demonstrate that PTEN deletions are found in approximately 20% of prostate cancers, and represent a major driver of patient prognosis independent of ERG status. The frequent finding of homozygous deletions or combinations of a heterozygous deletion and mutation, in two thirds of PTEN-defective cancers, suggests a strong selective advantage for tumor cell clones with complete PTEN inactivation. These tumors account for approximately 15% of all prostate cancers and are characterized by particularly aggressive features, including hormone-independent and metastatic growth.

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References
