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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 TMAs 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 34 tumors, respectively. *PTEN* deletions were present in 20.2% (458/2266) of prostate cancers, including 8.1% heterozygous and 12.1% homozygous deletions, and were linked to advanced tumor stage ($P < 0.0001$), high Gleason grade ($P < 0.0001$), presence of lymph node metastasis ($P = 0.0002$), hormone-refractory disease ($P < 0.0001$), presence of *ERG* gene fusion ($P < 0.0001$), and nuclear p53 accumulation ($P < 0.0001$). *PTEN* deletions were also associated with early prostate-specific antigen recurrence in univariate ($P < 0.0001$) and multivariate ($P = 0.0158$) analyses. The prognostic impact of *PTEN* deletion was seen in both *ERG* fusion-positive and *ERG* fusion-negative tumors. *PTEN* mutations were found in 4 (12.9%) of 31 cancers with heterozygous *PTEN* deletions but in only 1 (2%) of 59 cancers without *PTEN* deletion ($P = 0.027$). Aberrant *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. *(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[2] and encodes a dual-specificity phosphatase[3] 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 invasiveness[6] and metastasis development.[7,8]

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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 studies[16,17] 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 over 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.

Table 1. Pathological and Clinical Data of Arrayed Prostate Cancers

Variable	Study cohort receiving TMA (n = 4699)	Biochemical relapse among categories (n = 904)
Follow-up (months)		
Mean	56.6	
Median	46.7	
Age (years)		
<50	126	22
50–60	1399	227
61–70	2596	520
>70	337	84
Pretreatment PSA (ng/mL)		
<4	666	77
4–10	2559	376
10–20	894	269
>20	289	159
pT category (AJCC 2002)		
pT2	3010	272
pT3a	926	286
pT3b	489	307
pT4	42	39
Gleason grade		
≤3 + 3	1761	125
3 + 4	2055	450
4 + 3	512	261
≥4 + 4	135	68
pN category		
pN0	2317	580
pN+	151	111
Surgical margin		
Negative	3634	573
Positive	810	324

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 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 (RP11-380G5 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– two times 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 analysis[33] 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 2. Antibodies Tested for PTEN Expression Analysis

Company	Catalogue no.	Host	Clonality
Dako	M3627	Mouse	Monoclonal
Abcam	ab32199	Rabbit	Monoclonal
Abnova	MAB7764	Mouse	Monoclonal
Cell Signaling Technology	9559	Rabbit	Monoclonal
Abcam	ab31392	Rabbit	Polyclonal
Cell Signaling Technology	9188	Rabbit	Monoclonal
Abnova	PAB12724	Rabbit	Polyclonal

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 deparaffinized and incubated in pH 7.8 Tris-EDTA-citrate buffer at 121°C in an autoclave for antigen retrieval. The primary antibody was diluted 1:300 for IHC. Bound primary antibody was visualized using the DAKO EnVision Kit (Dako, Glostrup, Denmark). Nuclear staining intensity was estimated in a four-step scale: 0, negative; 1+, weak; 2+, moderate; and 3+, strong.

ERG Analysis

Immunohistochemistry (IHC) was used to detect ERG expression as a surrogate marker for *ERG* fusion. Freshly cut TMA sections were stained with a commercial anti-ERG 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 ERG staining in tumor cells were considered positive for *ERG* fusion. We have previously shown that ERG expression detected with this antibody shows 98.5% concordance with *ERG* 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 3. PTEN Exon-Specific PCR Primers

Exon	Forward primer	Reverse primer
1	5′-TTCCATCCTGCAGAAGAAGC-3′	5′-CCCACGTTCTAAGAGAGTGACA-3′
2	5′-CTCCAGCTATAGTGGGGAAAA-3′	5′-CTTTTTCTGTGGCTTAGAAATC-3′
3	5′-CCCATAGAAGGGGTATTTGTTG-3′	5′-CTCTACCTCACTCTAACAAGCAGA-3′
4	5′-CACATTATAAAGATTCAGGCAATGTT-3′	5′-AAGATACAGTCTATCGGGTTTAAGTT-3′
5	5′-CTTATTCTGAGGTTATCTTTTTACCAC-3′	5′-TCCAGGAAGAGGAAAGGAAAA-3′
6	5′-GGCTACGACCCAGTTACCAT-3′	5′-GGAAGGATGAGAATTTCAAGCA-3′
7	5′-CAGTTAAAGGCATTTCCTGTG-3′	5′-TGGATATTTCTCCCAATGAAAG-3′
8 fragment 1	5′-TGTTTAACATAGGTGACAGATTTTC-3′	5′-AAGTCAACAACCCCCACAAA-3′
8 fragment 2	5′-AGGTGACAGATTTTCTTTTTTA-3′	5′-AAGTCAACAACCCCCACAAA-3′
9	5′-GATGAGTCATATTTGTGGGTTTTC-3′	5′-GGTCCATTTTCAGTTTATTCAAGT-3′

Table 4. PTEN Sequencing Primers

Exon	Sequencing primer
1	5′-TTCCATCCTGCAGAAGAAGC-3′
2	5′-CTCCAGCTATAGTGGGGAAAA-3′
3	5′-CCCATAGAAGGGGTATTTGTTG-3′
4	5′-CACATTATAAAGATTCAGGCAATGTT-3′
5	5′-TCCAGGAAGAGGAAAGGAAAA-3′
6	5′-GGCTACGACCCAGTTACCAT-3′
7	5′-CAGTTAAAGGCATTTCCTGTG-3′
8	5′-AAGTCAACAACCCCCACAAA-3′
9	5′-GGTCCATTTTCAGTTTATTCAAGT-3′

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 copy number analysis. Fragmentation, labeling, and hybridization of the DNA to the SNP arrays were performed exactly as described in the Affymetrix V6.0 SNP array manual. We used our own genomic browser (FISH Oracle)[37] to map all 10q23 deletions to the human genome reference sequence (Archive 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://ajp.amjpathol.org*). Detailed analyses were performed with two amplicons using the following primers: 5′-GATTTTTTTGGGGGTATYGGAG-3′ (forward) and 5′-CTCATCCRACTCCCTTACAAC-3′ (reverse) for PTEN4.5 and 5′-GTTGTTTATAGGYGTTGAGAGG-3′ (forward) and 5′-CCTCCCCTCRATCTTCC-3′ (reverse) for PTEN10p. GSTP1 methylation was analyzed as a control using the following primers: forward, 5′-GTGTGGTTTTTATTTYGGGTTTTTTTTT-3′; and reverse, 5′-TAACCCTAATACCAACAACATAC-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 χ^2 (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 5. Primers Used for MASSArray Analysis

Variable	Forward primer	Reverse primer
PTEN		
1	5′-TGGTATTAGTTTGGGGATTTTTT-3′	5′-AAACTAATTACACAAACACCCA-3′
2	5′-TGGGTGTTTGTGTAATTAGTTTTTTA-3′	5′-AAACTACTTTCCAAAAAAAATCAC-3′
3	5′-GGAAAGTAGTTTYGATTGTGGTT-3′	5′-ATTCTCAAAAACCACCTAACCCC-3′
4	5′-GGGGTTAGGTGGTTTTTGAGAAT-3′	5′-CAAAAACCCAAAAAACACCTATCTA-3′
4.5	5′-GATTTTTTTGGGGGTATYGGAG-3′	5′-CTCATCCRACTCCCTTACAAC-3′
5	5′-TAGATAGGTGTTTTTTGGGTTTTTG-3′	5′-TTCCCCCAAATCTATATCCTCATAAT-3′
6	5′-ATTATGAGGATATAGATTTGGGGGAA-3′	5′-CCCTACAAAAAAAATACCCTCC-3′
7	5′-GGAGGGTATTTTTTTTTGTAGGG-3′	5′-ACCTCTACCCAAAAACCCAA-3′
8	5′-TTGGGTTTTTGGGTAGAGGT-3′	5′-CATACCCAATATAACTACCTAAAACTTACT-3′
9p	5′-GGAAGATYGAGGGGAGG-3′	5′-CAAACCCCAAACAACTACAC-3′
10p	5′-GTTGTTTATAGGYGTTGAGAGG-3′	5′-CCTCCCCTCRATCTTCC-3′
11p	5′-GAGAAGTYGAGGAAGAGGT-3′	5′-CCTCTCAACRCCTATAAACAAC-3′
12p	5′-TTTTTTGAAAGGGAAGGTGGAA-3′	5′-TCCCAACCCTAAAAATAATAACAAA-3′
13p	5′-TTTGTTATTATTTTTAGGGTTGGGA-3′	5′-AAATAAAAAAAAAAACRAATAATCCTCC-3′
14p	5′-GGAGGATTATTYGTTTTTTTTTTATTT-3′	5′-CTACTAATAACRAAACTTCTTCTAC-3′
GSTP1		
GSTP1_6ss	5′-GTGTGGTTTTTATTTYGGGTTTTTTTTT-3′	5′-TAACCCTAATCTACCAACAACATAC-3′

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*).

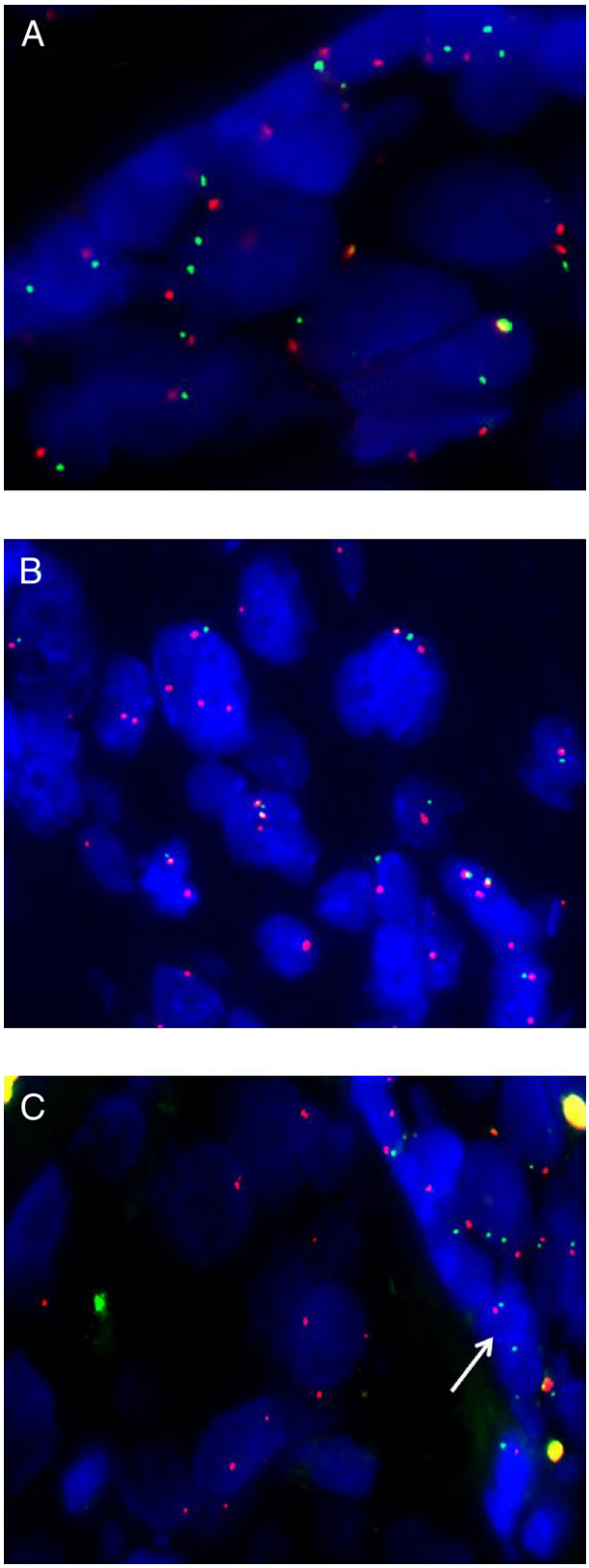

Figure 1. Examples for *PTEN* deletions in prostate cancer. **A:** Normal copy number of *PTEN* with two green *PTEN* and 2 red centromer 10 signals. **B:** *PTEN* heterozygous deletion with one green *PTEN* signal and two red centromer 10 signals. **C:** *PTEN* homozygous deletion completely lacking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

Table 6. Clinicopathological Associations of *PTEN* Deletions

Parameter	*n*	Evaluable	*PTEN* deletion status (%)		*P* value
			Heterozygous	Homozygous	
All cancers	4756	2266	8.1	12.1	
Tumor stage					
pT2	3010	1342	5.1	7.9	<0.0001
pT3a	926	491	10.6	16.3	
pT3b	489	278	16.9	21.2	
pT4	42	20	5.0	35.0	
HR	57	49	12.2	32.7	<0.0001*
Gleason grade†					
≤3 + 3	1761	715	4.5	5	<0.0001
3 + 4	2055	1039	8.6	13	
4 + 3	512	278	11.9	21.6	
≥4 + 4	135	97	14.4	20.6	
Lymph node metastasis†					
N0	2317	1111	9.6	13.4	0.0002
N+	151	99	12.1	29.3	
Preoperative PSA level (ng/mL)†					
<4	666	249	10.5	13.2	0.0043
4–10	2559	904	5.9	11.5	
10–20	894	373	9.8	10.6	
>20	289	143	12.8	10.1	
Surgical margin†					
Negative	3634	1341	7.4	11.1	0.0462
Positive	810	353	9.4	14.4	

*HR versus hormone-naïve prostate cancer.
†HR cancers excluded.
HR, hormone refractory.

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, $P = 0.0162$).

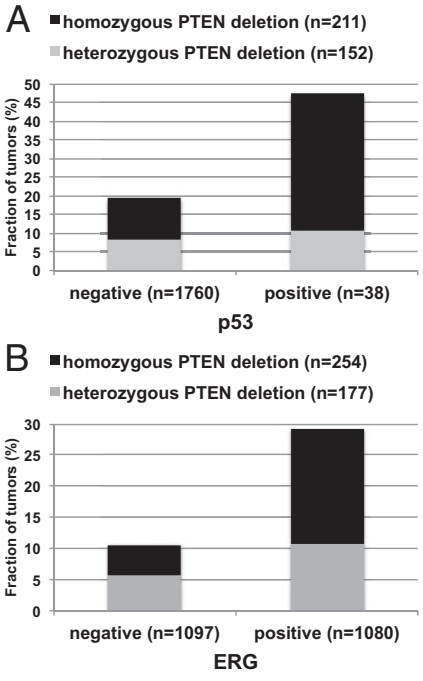

Figure 2. Association of *PTEN* deletion status to p53 expression ($P < 0.0001$) (**A**) and ERG fusion status ($P < 0.0001$) (**B**). Normal includes two *PTEN* gene copies. Heterozygous, heterozygous deletion with one *PTEN* gene copy; homozygous, homozygous deletion completely lacking *PTEN*.

PTEN *IHC*

PTEN IHC was successful in 3320 of the 4699 arrayed hormone-naïve primary prostate cancers. Immunostain-

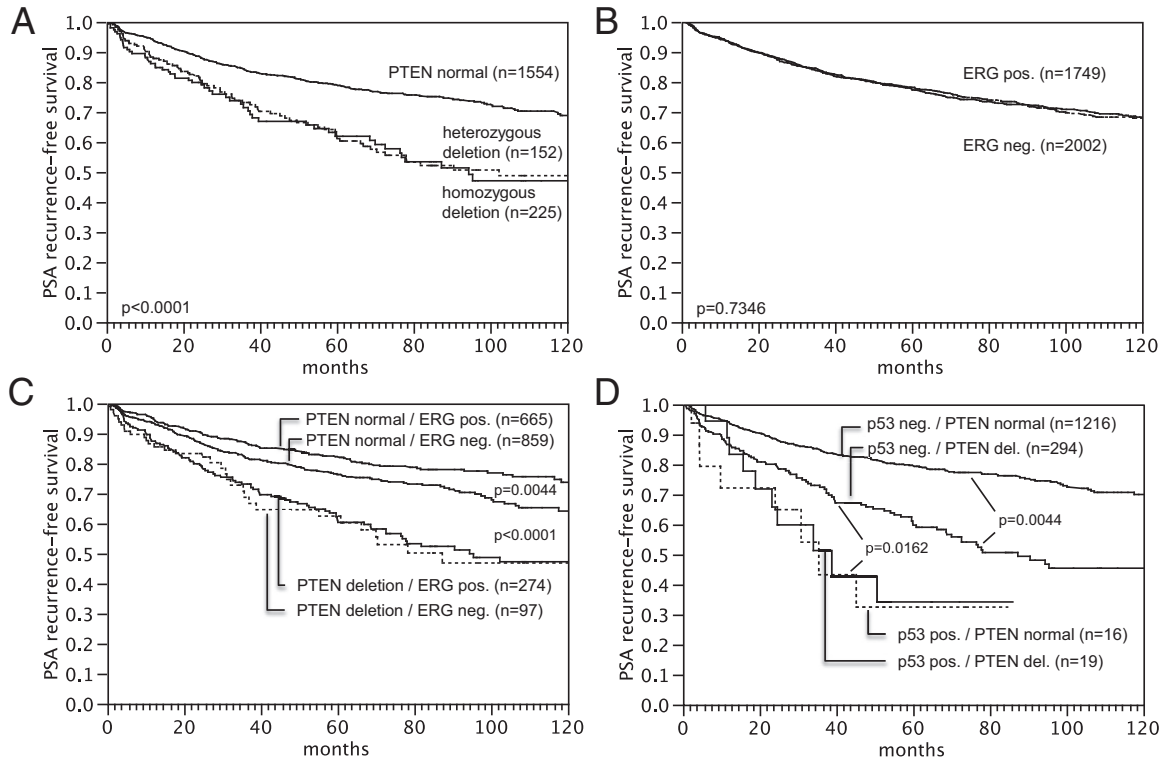

Figure 3. Association between *PTEN* deletion (**A**), ERG fusion (**B**), and the combination of *PTEN* deletion and ERG fusion (**C**) or nuclear p53 accumulation with biochemical recurrence in prostate cancer (**D**).

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

Parameter	Factor	HR	95% CI	*P* value
Gleason grade	3 + 4 versus ≤3 + 3	2.3	1.71–3.76	<0.0001
	4 + 3 versus ≤3 + 3	5.7	4.01–8.10	
	≥4 + 4 versus ≤3 + 3	6.1	3.76–9.69	
pT stage	pT3a versus pT2	1.9	1.47–2.43	<0.0001
	pT3b versus pT2	3.2	2.42–4.14	
	pT4 versus pT2	5.9	3.37–9.83	
PSA level	4–9 versus <4	0.9	0.64–1.29	<0.0001
	10–20 versus <4	1.4	1.00–2.05	
	>20 versus <4	1.8	1.18–2.66	
PTEN	Deleted versus not deleted	1.3	1.05–1.60	0.0158

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 asparaginic acid (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.

Table 8. Association between *PTEN* Mutations and *PTEN* Deletions

Variable	*n*	*PTEN* not mutated*	*PTEN* mutated*	*P* value
PTEN not deleted	59	58 (98)	1 (2)	0.027†
PTEN heterozygous deleted	31	27 (87)	4 (13)	
Deletion status unknown	7	5 (71)	2 (29)	
Total	97	90 (93)	7 (7)	

*Data are given as number (percentage).
†*PTEN* heterozygous deleted versus not deleted.

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-

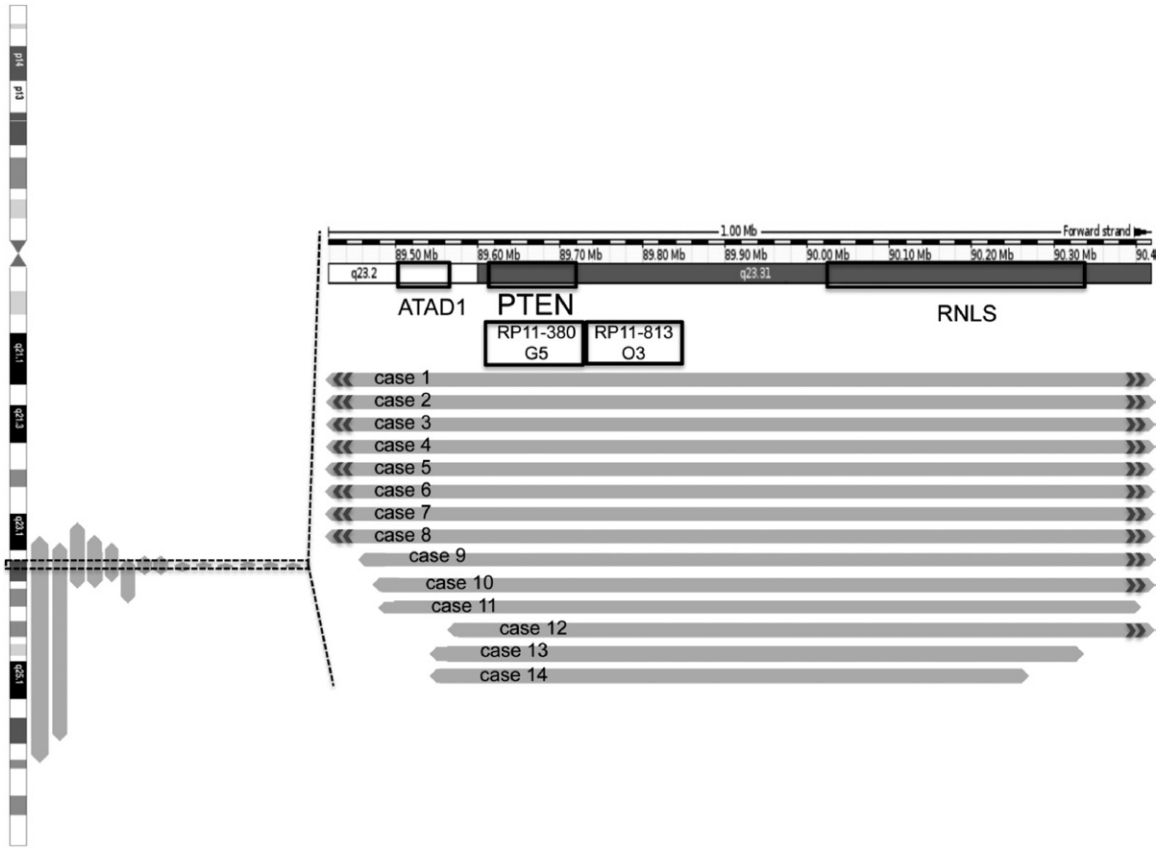

Figure 4. Genomic *PTEN* deletions detected in 14 of 77 prostate cancer samples in SNP-array analysis. **Left panel:** An overview of position and size of genomic deletion relative to the 10q region in 14 prostate cancers. **Right panel:** Detailed view of genomic deleted region relative to 10q23.2 to 10q23.31 region. The position of bacterial artificial chromosome clones (RP11-380G5 and RP11-813O3) relative to the *PTEN* locus is indicated. ≪ and ≫, the deletion exceeds the genomic area depicted in the figure.

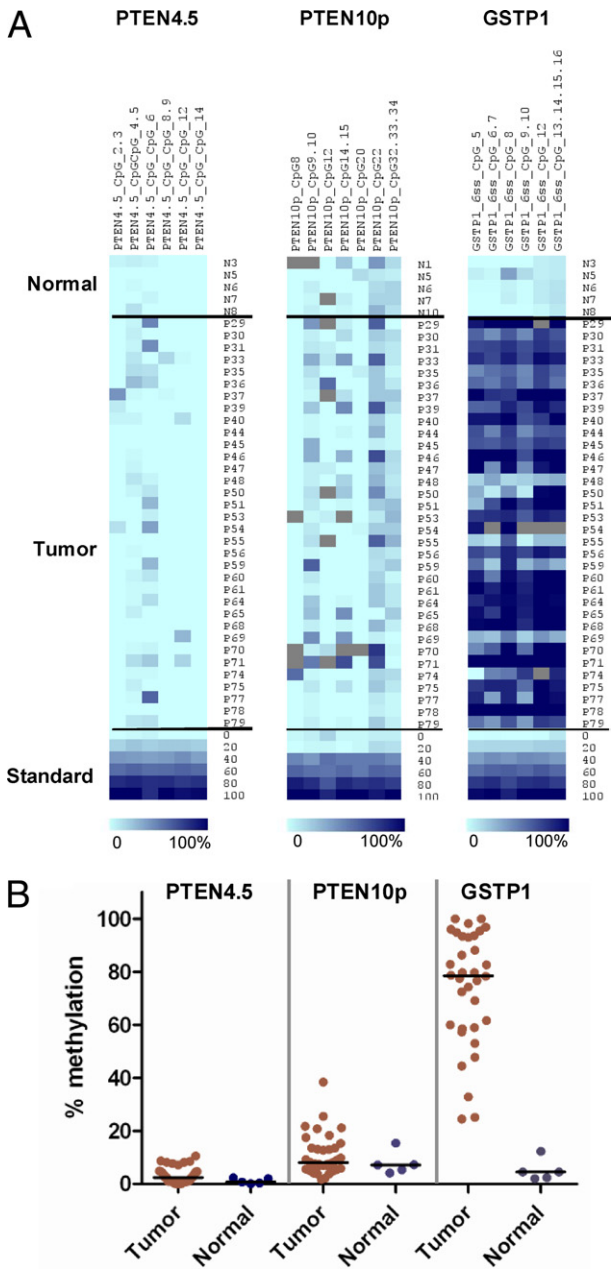

Figure 5. *PTEN* promoter methylation. **A:** Representative heat map of quantitative CpG methylation analysis using MassARRAY technology. Each row represents a sample of normal prostate tissue (N) or prostate cancer (P). Each square represents a single CpG site or a group of one to two CpG sites analyzed together. Methylation frequencies extend from light blue (0%) to dark blue (100%) and are corrected, compared with a methylation standard with 0% to 100% methylation. GSTP1 is analyzed as a methylation control. **B:** Dot blot of average methylation across amplicons. Horizontal lines, median methylation in tumor and normal samples, respectively.

tions fits well with previous reports showing that 2.5%,[31] 5.1%,[32] and 7.5%[39] of unselected, localized prostate cancers 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 significant role for *PTEN* inactivation in prostate cancer. Our data also demonstrate that approximately two thirds of clinical cancer specimens with genomic *PTEN* alterations show inactivation of both alleles (typically by homozygous deletion), suggesting that *PTEN* haploinsufficiency may be less important in prostate cancer than previously thought.[42]

The main purpose of this study was to determine the relationship between *PTEN* alterations and tumor phenotype, 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* deletions and adverse 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 tumors.[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 (including the *PTEN* locus) found the highest loss of heterozygosity rates in advanced and metastatic tumors.

For IHC analysis, we selected the antibody ab31392 (Abcam) from a series of seven tested anti-PTEN antibodies (Table 2) because it showed reproducible nuclear staining. Nuclear accumulation of PTEN is mediated by monoubiquitination and has been essential for tumor suppression.[45] Nuclear PTEN contributes to maintaining chromosomal stability 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 phenotype 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, associations between the level of PTEN expression and clinicopathological parameters vary. For example, McCall et al[28] reported a link between low-level cytoplasmic PTEN expression and tumor recurrence, and between loss of nuclear PTEN staining and patient survival in 68 hormone-naïve prostate cancers. However, Koumakpayi et al[49] and Bedolla et al[21] could not confirm such associations in sim-

ilarly 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[17] 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[56] 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-

ferent from most previous studies[25,26,29,39,43,60] 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 (39% to 62%) compared with homozygous (5% to 6%) deletions,[26,43] whereas others[25,29,39] 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-naïve 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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