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Advances in Brief

Frequent Inactivation of *PTEN* in Prostate Cancer Cell Lines and Xenografts[1]

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Abstract

Loss of chromosome 10q is a frequently observed genetic defect in prostate cancer. Recently, the *PTEN/MMAC1* tumor suppressor gene was identified and mapped to chromosome 10q23.3. We studied *PTEN* structure and expression in 4 *in vitro* cell lines and 11 *in vivo* xenografts derived from six primary and nine metastatic human prostate cancers. DNA samples were allelotyped for eight polymorphic markers within and surrounding the *PTEN* gene. Additionally, the nine *PTEN* exons were tested for deletions. In five samples (PC3, PC133, PCEW, PC295, and PC324), homozygous deletions of the *PTEN* gene or parts of the gene were detected. PC295 contained a small homozygous deletion encompassing *PTEN* exon 5. In two DNAs (PC82 and PC346), nonsense mutations were found, and in two (LNCaP and PC374), frame-shift mutations were found. Missense mutations were not detected. *PTEN* mRNA expression was clearly observed in all cell lines and xenografts without large homozygous deletions, showing that *PTEN* down-regulation is not an important mechanism of *PTEN* inactivation. The high frequency (60%) of *PTEN* mutations and deletions indicates a significant role of this tumor suppressor gene in the pathogenesis of prostate cancer.

Introduction

Prostate cancer is the most frequently diagnosed tumor in men in the United States and in western and northern Europe, and it is the second leading cause of male cancer death (1). The molecular events leading to the development and the progressive growth of prostate cancer are poorly understood. The most frequent chromosomal aberrations are the losses of chromosomes 8p, 10q, 13q, and 16q (2–4), indicating the localization of tumor suppressor genes at these chromosomal sites. Detailed allelotyping implicated deletion of chromosome 10 region 10q23–25 in prostate cancer (5–7).

Recently, the *PTEN* gene, also known as *MMAC1* or *TEP1*, which is located at 10q23.3, has been found to be frequently mutated or deleted in glioblastomas (8–10). *PTEN* encodes a dual-specific phosphatase and shows homology to the cytoskeletal proteins tensin and auxilin (8–11). Structural analysis revealed mutations of *PTEN* in many different tumor types. A high proportion of mutations was demonstrated not only in glioblastomas (8, 9, 12, 13) but also in endometrial carcinomas (14, 15). Germ-line mutations in *PTEN* have been detected in Cowden disease, an autosomal dominant cancer predisposition syndrome, associated with an increased risk of breast, skin, and thyroid cancer (16, 17).

PTEN has also been implicated in prostate cancer. Li *et al.* (8) described two homozygous deletions and one frame-shift mutation in prostate cancer cell lines. More recently, *PTEN* mutations and deletions were reported in metastatic prostate cancers (18, 19). In primary tumors, *PTEN* mutations were less frequent (18, 20).

Detailed molecular genetic analysis of prostate cancer DNA and gene expression is complicated due to the contamination by normal cells. In this study, we demonstrate a high proportion of *PTEN* mutations and deletions in 15 prostate cancer xenografts and cell lines. We characterized these mutations and studied *PTEN* mRNA expression.

Materials and Methods

Prostate Tumor Cell Lines and Xenografts. The *in vitro* growing cell lines LNCaP, PC3, DU145, and TSU were cultured under standard conditions. *In vivo* xenografts PC82, PCEW, PC133, PC135, PC295, PC310, PC324, PC329, PC339, PC346, and PC374 were propagated in male nude mice (Refs. 21 and 22 and references therein).

DNA Preparation. DNA from cell lines was isolated according to standard procedures (23). Genomic DNA from xenografts was isolated from five consecutive 5-μm cryostat tissue sections by overnight proteinase K incubation at 55°C, followed by phenol extraction and ethanol precipitation. DNA pellets were dissolved in TE [10 mM Tris-HCl (pH 7.8)-1 mM EDTA].

Allelotyping. DNAs from prostate cancer cell lines and xenografts were allelotyped by PCR amplification of eight polymorphic markers within or flanking the *PTEN* locus at chromosome 10q23.3: *D10S1687*, *D10S579*, *D10S215*, *PTENCA*, *AFMa086WG9*, *D10S541*, *D10S1753*, and *D10S583*. *PTENCA* primers were from Ref. 12; other markers are described in the Genome Data Base or in the Genethon database. PCR amplifications, using Taq polymerase (Promega, Madison, WI) were for 30 cycles of 1 min at 94°C, 1.5 min at 50°C or 55°C, and 1.5 min at 72°C in a 15-μl reaction volume, containing 1.5 mM $MgCl_2$ and 1 μCi of [α-^{32}P]dATP (Amersham, Buckinghamshire, United Kingdom). The radiolabeled PCR products were separated over a 6% polyacrylamide sequencing gel.

Screening for Homozygous Deletions of *PTEN*. *PTEN* exons and flanking sequences were PCR amplified according to standard protocols: 30 cycles of 45 s at 94°C, 45 s at 50°C (exons 1–6), or 55°C (exons 7–9) and 45 s at 72°C in the presence of 1.5 mM (exons 1–6) or 2.0 mM $MgCl_2$ (exons 7–9). Exons 5 and 8 were amplified as two overlapping fragments. Primers were from Refs. 9 (1R,[4] 5F1, 5R2, 7F, 7R, 8F1, and 8R2) and 24 (1F, 2F, 2R, 3F, 3R, 4F, 4R, 5R1, 5F2, 6F, 6R, 9F, and 9R). Primers 8R1 (5-CTTGTCATTATCTGCACGCT-3) and 8F2 (5-GAAAATGGAAGTCTATGTG-3) are novel. Control PCRs were with primers 791B9L-A (5-GAAGGTGGCAGTCTGATCTC-3) and 791B9L-B (5-GCAACTGGTTGAAACATACTC-3), which amplify a 410-bp fragment at chromosome 8p12–p21. Amplified products were separated over a 2% agarose gel.

RT-PCR Analysis of *PTEN* Expression. RNA was isolated by standard guanidium isothiocyanate (cell lines) or LiCl (xenografts) protocols (23). cDNA synthesis, followed by PCR amplifications with *PTEN*-specific primers, was performed on 500 ng of RNA in the Access RT-PCR system (Promega) according to the instructions of the manufacturer. Primers used for cDNA synthesis were as follows: 9R-cDNA, 5-GGATGTGAACCAGTATATCACAA-3 (Fig. 3*A*); and 7R-cDNA, 5-CCGTCGTGTGGGTCCTGAATTAA-3 (Fig. 3*B*). cDNA synthesis was for 45 min at 48°C; amplification was for 35 cycles of 30 s at 94°C, 30 s at 60°C, and 1 min at 68°C. Primer combinations for PCR amplification of cDNAs were as follows: 9R-cDNA and 8F-cDNA (5-AGCCAACCGATACTTTTCTCC-3) and 7R-cDNA and 1F-cDNA (5-CCACCAGCAGCTTCTGCCATCTCT-3). Control RT-PCRs were carried out under the same conditions, using RNA polymerase II-specific primers: PolF-cDNA (5-GCTGAGAGAGCCAAGGATAT-3) and PolR-cDNA

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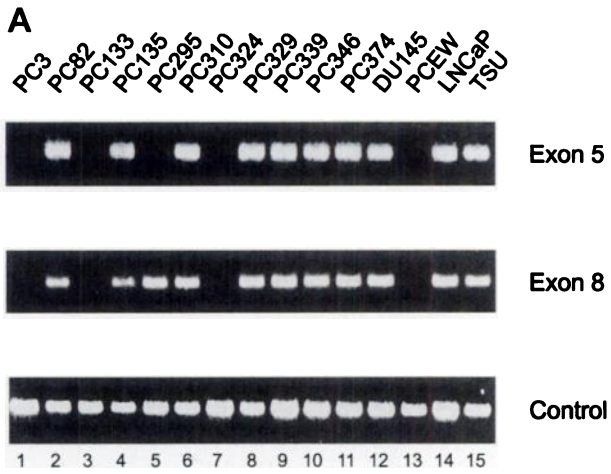
[4] The abbreviations used are: R, reverse; F, forward; RT-PCR, reverse transcriptase-PCR; SSCP, single-strand conformational polymorphism.

position →	D10S1687 (0.81) 113cM	D10S579 (0.59) 114cM	D10S215 (0.80) 114cM	PTEN CA 114cM	AFMa086WG9 114cM	D10S541 (0.78) 114cM	D10S1753 (0.74) 119cM	D10S583 (0.84) 122cM
PC 3	1	1	1	1				1
PC 82	2	1	1	1	1	1	2	2
PC 133	1	1	1				1	1
PC 135	2	1	1	1	1	1	1	2
PC 295	1	1	1	1	1	1	1	1
PC 310	2	1	2	2	1	2	2	2
PC 324	2	1	1	1			2	1
PC 329	2	1	2	1	1	2	1	1
PC 339	2	2	2	1	1	1	1	1
PC 346	2	1	1	1	1	1	2	2
PC 374	1	1	1	1	1	1	1	1
DU 145	2	1	1	1	1	1	1	1
PCEW	2					1	1	1
LNCaP	1	1	1	1	1	1	1	1
TSU	1	1	1	1	1	1	1	1

Fig. 1. Allelotyping of prostate cancer cell lines and xenografts at chromosome 10q23.3. Markers are described in more detail in "Materials and Methods." *Numbers in parentheses*, heterozygosity indices. Heterozygosity indices were not available for *PTENCA* and *AFMa086WG9*. Genetic map positions of markers are from contig 10.7 of the Whitehead Institute map. The number of bands detected is indicated in each box (0, 1, or 2). *Shaded boxes*, homozygous deletions.



B

	E1	E2	E3	E4	E5	E6	E7	E8	E9
PC 3	+	+							
PC 82	+	+	+	+	+	+	+	+	+
PC 133									
PC 135	+	+	+	+	+	+	+	+	+
PC 295	+	+	+	+		+	+	+	+
PC 310	+	+	+	+	+	+	+	+	+
PC 324	+								
PC 329	+	+	+	+	+	+	+	+	+
PC 339	+	+	+	+	+	+	+	+	+
PC 346	+	+	+	+	+	+	+	+	+
PC 374	+	+	+	+	+	+	+	+	+
DU 145	+	+	+	+	+	+	+	+	+
PCEW									
LNCaP	+	+	+	+	+	+	+	+	+
TSU	+	+	+	+	+	+	+	+	+

Fig. 2. Deletion analysis of *PTEN* in prostate cancer cell lines and xenografts. *A*, agarose gel electrophoresis of PCR-amplified exons 5 and 8. The control is yeast artificial chromosome end fragment 791B9L, located at chromosome 8p12–p21. *B*, overview of the results obtained for all *PTEN* exons. −, deleted exons (*shaded boxes*).

(5-CACCACCTCTTCCTCCTCTT-3). RT-PCR products were separated over a 2% agarose gel.

PCR-SSCP Analysis. Fragments for PCR-SSCP analysis were obtained for all exons of the *PTEN* gene, using primer sets and PCR conditions described above. PCRs were in a 15-μl volume in the presence of 1 μCi [α-^{32}P]dATP. Appropriate aliquots of the radiolabeled PCR products were separated over a 6% nondenaturing polyacrylamide gel containing 5 or 10% glycerol at 7 W overnight at room temperature.

Structural Analysis. RT-PCR fragments and amplified exons were purified over QIAquick spin columns (Qiagen, Hilden, Germany), cloned into pGEM-T Easy (Promega) and sequenced according to the dideoxy chain termination method.

Results

Allelotyping of Prostate Cancer Cell Lines and Xenografts for Chromosome 10q23. Genomic DNAs from 11 prostate cancer xenografts and 4 *in vitro* propagated cell lines were allelotyped for eight highly polymorphic markers, spanning ~9 cM around the *PTEN* locus at chromosome 10q23 (*D10S1687*, *D10S579*, *D10S215*, *PTENCA*, *AFMa086wg9*, *D10S541*, *D10S1753*, and *D10S583*). The results are summarized in Fig. 1. For most markers, especially those mapping at 114 cM, only one amplified band was detected, suggesting hemizygosity. In DNAs from three xenografts (PC133, PC324, and PCEW) and one cell line (PC3), two or more polymorphic markers were completely deleted (see also Ref. 8 for PC3 deletion). All four

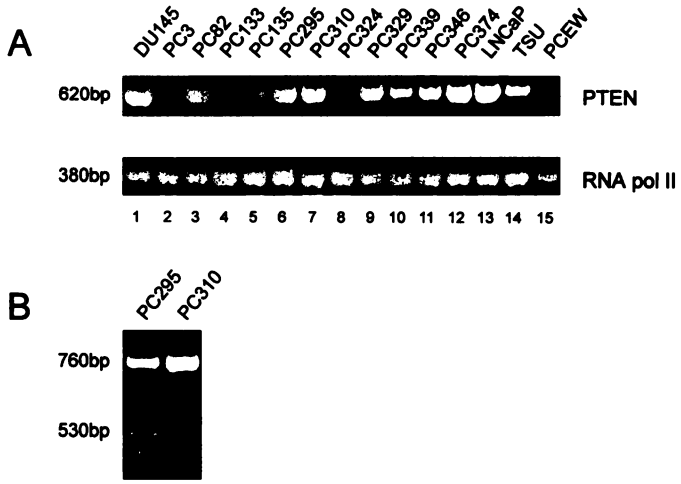


Fig. 3. PTEN mRNA expression in prostate cancer cell lines and xenografts. *A*, agarose gel analysis of a 620-bp RT-PCR-amplified fragment. Primers used are located in exons 8 and 9 and are specific for human *PTEN*. *B*, *PTEN* RT-PCR analysis of PC295 RNA. Primers used are located in exons 1 and 7. PC310 RNA was used as a control. Primers detect both human and mouse *PTEN* mRNA.

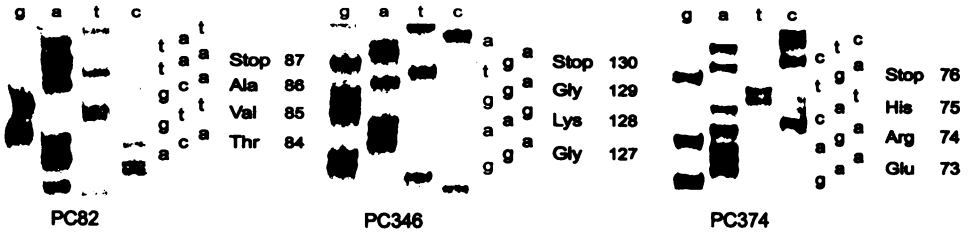


Fig. 4. Sequence analysis of *PTEN* mutations in prostate cancer xenografts. In PC82 and PC346 nonsense mutations were detected; PC374 shows a TA deletion at codon 76, resulting in a TGA stop codon.

samples were negative for *AFMa086wg9*, which is situated at 114 cM, between exons 2 and 3 of the *PTEN* gene. Therefore, these tumors are expected to be completely or partially defective of *PTEN*. The homozygous deletion in PC3 is relatively large (>5 cM), but other homozygous deletions seem to be much smaller.

Homozygous Deletion of the *PTEN* Gene. To confirm *PTEN* losses and to determine more precisely the borders of the homozygous deletions, all nine exons of the *PTEN* gene, including flanking sequences, were individually amplified. Examples of exon 5 and exon 8 amplifications are shown in Fig. 2*A*; results are summarized in Fig. 2*B*. The four tumors that lacked polymorphic marker *AFMa086wg9* showed a complete or partial deletion of *PTEN* (Fig. 2*A*, *Lanes 1*, *3*, *7*, and *13*). In PC133 and PCEW, *PTEN* was completely deleted; in PC324, *PTEN* exons 2–9 were absent, and in PC3, exons 3–9 were absent (Fig. 2*B*). Importantly, PC295 contained a very small homozygous deletion, encompassing only *PTEN* exon 5, which could not be detected by allelotyping (Fig. 2*A*, *Lane 5*).

Expression of *PTEN*. Because of the absence of contaminating normal cells of human origin, xenografts and cell lines are preferable sources for the study of *PTEN* mRNA expression. In all RNA preparations from cell lines and xenografts without homozygous deletion, *PTEN* mRNA was easily detectable by RT-PCR, using human *PTEN*-specific primers for amplification (Fig. 3*A*). In PC133 and PCEW, *PTEN* mRNA expression could not be visualized (Fig. 3*A*, *Lanes 4* and *15*). However, PC3 and PC324, which also lack the *PTEN* gene, showed a faint amplified band of the appropriate length (620 bp), which hybridized to a *PTEN*-specific probe (data not shown). Sequencing of the RT-PCR product from PC324 and PC3 revealed that it was not identical to *PTEN* but to a highly homologous processed *PTEN* pseudogene, located in a duplicated region of chromosome 7 to chromosome 9, near the T-cell receptor β locus (GenBank accession no. AF029308; data not shown). This band was not detectable in controls, in which reverse transcriptase was omitted from the reaction mixture (data not shown). Therefore, we concluded that, in PC324 and PC3, the pseudogene was expressed at a low level. Because *PTEN* pseudogene expression was low or undetectable in the four tumors with chromosome 10q23 homozygous deletions, we presumed that the RT-PCR product from *PTEN*-positive tumors was derived from the original *PTEN* gene. This was checked for PC295 (see below) and PC135. *PTEN* mRNA expression in PC135 is relatively low. Digestions by selected restriction enzymes showed that most, if not all, of the fragment amplified by RT-PCR was derived from wild-type *PTEN* (data not shown).

Sequencing of a 530-bp exon 1–7 RT-PCR product of PC295 *PTEN* mRNA confirmed the complete absence of exon 5 sequences in the transcript (Fig. 3*B*; data not shown). The coamplified 760-bp fragment was not derived from the human *PTEN* mRNA or the pseudogene but from *PTEN* mRNA of mouse cells present in the transplanted tumor (data not shown).

Structural Analysis of the *PTEN* Gene. From *PTEN*-positive DNAs, all nine exons were analyzed for mutations by PCR-SSCP. Aberrant SSCP bands were sequenced. In four DNA samples, *PTEN* mutations were unambiguously established (PC82, PC346, PC374, and LNCaP). The exon 1, codon 6 AAA to A frame-shift mutation in LNCaP has been described previously (8, 9). The other three mutations are depicted in Fig. 4. PC82 *PTEN* contained a CAA to TAA nonsense mutation at codon 87, PC346 contained a CGA130TGA nonsense mutation, and PC374 contained a TAT76T frame-shift mutations, directly resulting in a TGA stop codon at position 76. All mutations will lead to the synthesis of a truncated protein, lacking the phosphatase domain. In none of the samples was the corresponding wild-type sequence detected, confirming that the second *PTEN* allele was deleted.

PTEN exons from DNA samples with possible allelic loss at the *PTEN* locus (*PC135*, *PC329*, *PC339*, *DU145*, and *TSU*) but with normal SSCP patterns were all sequenced. However, in none was a mutation detected (data not shown). Both LNCaP and TSU *PTEN* were found to contain GGT(Gly) instead of GGC(Gly) at codon 44. This presumed rare polymorphism could not be detected in 34 control DNAs from healthy individuals (data not shown). The previously described ATG134TTG missense mutation in DU145 *PTEN* could not be confirmed (8).

Discussion

In this study, we describe five homozygous deletions, two frame-shift mutations, and two nonsense mutations in 15 prostate cancer cell lines and xenografts. Homozygous deletions were established by the complete absence of polymorphic markers in the *PTEN* region and by failure of *PTEN* exon amplifications. RT-PCR showed *PTEN* expression in all tumors in which the gene was not deleted.

The four tumors containing mutated *PTEN* (PC82, PC346, PC374, and LNCaP) showed one amplified band for all five polymorphic markers at 114 cM, suggesting deletion of one chromosome 10 copy at these sites. Absence of wild-type *PTEN* at the mutated site confirmed this observation. Complete or almost complete deletion of both *PTEN* copies was detected in five tumor samples. The homozygous deletion in PC3 is large (>5 cM, Fig. 1; see also Ref. 8), and that in PC295 is probably <20 kbp, as judged from preliminary data of the lengths of introns 4 and 5.[5] In PC133, PC324, and PCEW, two or more markers at 114 cM are deleted. Minimal deleted regions in these tumors are estimated to be at least 100 kbp.

Five of six tumors, in which we could not find *PTEN* homozygous deletions or mutations (PC135, PC329, PC339, TSU, and DU145), showed apparent allelic losses for the markers *PTENCA* and *AFMa086WG9*, which are located within the *PTEN* gene. Three of these DNAs even showed one band for all five markers that map at 114 cM (Fig. 1). Sequencing of all exons showed that, in these tumors, a second *PTEN* deletion or mutation has not yet occurred. Alternatively, in these tumors, a gene different from *PTEN* is mutated. For tumors with a large deletion, the *MXI1* gene at 10q24–25 might be a candidate, as suggested previously (25). However, others exclude a major role of *MXI1* in prostate cancer (5).

In addition to deletions and mutations, down-regulation of expres-

[5] C. Cleutjens, unpublished observation.

sion by promoter methylation, mutation, or other processes can be a mechanism of gene inactivation. Xenografts and cell lines have the advantage that mRNA expression can easily be monitored. The RT-PCR experiments (Fig. 3*A*) clearly showed that down-regulation of *PTEN* expression is not a common mechanism of *PTEN* inactivation. An exception might be PC135, which shows a considerably lower *PTEN* mRNA level than the other samples. Additional experimental evidence must be collected to prove the physiological implication of this observation. The processed *PTEN* pseudogene, which most likely is expressed at a low level, complicates expression studies. Previously, it has mistakenly been described as a mutated *PTEN* gene in breast cancer (26).

Mutations or deletions were found in six of nine xenografts/cell lines derived from metastatic sites and in three of six cell lines derived from primary tumors or obtained by prostatectomy or transurethral resection. Original tumor DNAs were available for PC346 (primary tumor) and PC374 (metastasis). In both samples, the mutations as shown in Fig. 4 could be confirmed (data not shown), indicating that they were not introduced during propagation in nude mice.

During the course of our study, *PTEN* mutations were reported in primary and metastatic prostate cancer tissues (18–20). In the study of Cairns *et al.* (18), mutations or deletions were found in 3 of 60 primary cancers and in 7 of 20 lymph node metastases. Teng *et al.* (20) could not detect *PTEN* mutations in six primary tumors; Suzuki *et al.* (19) described *PTEN* abnormalities in 12 of 19 tumors obtained from metastatic sites during autopsy. These numbers might be underestimations because small deletions would have been missed. As we found in this study, deletions, point mutations, and frame-shift mutations were detected. In prostate cancer, *PTEN* mutations lead, almost without exception, to the synthesis of a truncated protein. The CGA130TGA nonsense mutation and the TAT76T frame-shift mutation have now been reported in different studies in both glioblastomas and prostate cancer, indicating hot spots of mutation (this study and Refs. 9, 12, 13, and 20).

Most frequently, *PTEN* aberrations were found in DNAs from metastatic disease (Refs. 18 and 19 and this study). However, *PTEN* abnormalities do also occur at the primary tumor site (18). Not unexpectedly, in the cell lines and xenografts derived from the primary tumor site, as studied here, the percentage of *PTEN* abnormalities is much higher than that found at the primary tumor site by Cairns *et al.* (Ref. 18; 3 of 6 and 3 of 60, respectively). The difference might be explained by a growth advantage of the original xenografted material in case of the absence of functional *PTEN*.

Because *PTEN* is frequently completely absent or severely truncated, immunohistochemical staining using specific antibodies could be applied to address in more detail the question of whether *PTEN* is a tumor progression marker. Specific antibodies are also additional tools for the detection of the complete spectrum of *PTEN* mutations and deletions in patient samples, including small deletions such as that found in PC295. Both complete absence and aberrant cellular distribution of the PTEN protein can be expected.

Thus far, *PTEN* is the most widely mutated tumor suppressor gene in prostate cancer. Therefore, further elucidation of its function in prostate cancer is of utmost importance.

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