Frequent Inactivation of PTEN in Prostate Cancer Cell Lines and Xenografts

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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 DNA samples (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).

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).

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, AFM6086WG9, D10S541, D10S1753, and D10S583. PTEN CA 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 MgCl₂ and 1 μCi of (α⁻³²P)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 MgCl₂ (exons 7-9). Exons 5 and 8 were amplified as two overlapping fragments. Primers were from Refs. 9 (1R, 4F1, 5R2, 7F, 7R, 8F1, and 8R2) and 24 (1F, 2F, 3F, 3R, 4F, 4R, 5R1, 5F2, 6F, 6R, 9F, and 9R). Primers 8R1 (5-CTTCTATTAGCCATGCTT- GCACCT-3) and 8F1 (5-GAATGGAGATCTCTAATG-3) are novel. Control PCRs were performed on 500 ng of RNA in the Access RT-PCR system (Promega) 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 guanidinium 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-GGATGTGAAACGTATATCACA-3 (Fig. 3A); and 7R-cDNA, 5-CCCGCGCGGTGCGGCGGAAATTC-3 (Fig. 3B). 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-AAGCCAACGTATCCTTCTCC-3) and 7R-cDNA and 1F-cDNA (5-CCACGACGTCGGTCCTCCTC-3). Control RT-PCRs were carried out under the same conditions, using RNA polymerase II-specific primers: PolI-cDNA (5-GCTGAGAGGGCAAAAGTAT-3) and PolII-cDNA (5-AGGAGAGGCAAAAGTAT-3)

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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 AFM086WG9. 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.

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).

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.
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. 2A; results are summarized in Fig. 2B. The four tumors that lacked polymorphic marker AFMe086wg9 showed a complete or partial deletion of PTEN (Fig. 2A, 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. 2B). Importantly, PC295 contained a very small homozygous deletion, encompassing only PTEN exon 5, which could not be detected by allelotyping (Fig. 2A, 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. 3A). In PC133 and PCEW, PTEN mRNA expression could not be visualized (Fig. 3A, 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).

Sequence 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. 3B; 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. 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 MXII gene at 10q24–25 might be a candidate, as suggested previously (25). However, others exclude a major role of MXII in prostate cancer (5).

In addition to deletions and mutations, down-regulation of expres-
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 RTPCR experiments (Fig. 3A) 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 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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