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Frequent Inactivation of *PTEN/MMAC1* in Primary Prostate Cancer

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Abstract

Sporadic prostate carcinoma is the most common male cancer in the Western world, yet many of the major genetic events involved in the progression of this often fatal cancer remain to be elucidated. Numerous cytogenetic and allelotype studies have reported frequent loss of heterozygosity on chromosomal arm 10q in sporadic prostate cancer. Deletion mapping studies have unambiguously identified a region of chromosome 10q23 to be the minimal area of loss. A new tumor suppressor gene, *PTEN/MMAC1*, was isolated recently at this region of chromosome 10q23 and found to be inactivated by mutation in three prostate cancer cell lines. We screened 80 prostate tumors by microsatellite analysis and found chromosome 10q23 to be deleted in 23 cases. We then proceeded with sequence analysis of the entire *PTEN/MMAC1* coding region and tested for homozygous deletion with new intragenic markers in these 23 cases with 10q23 loss of heterozygosity. The identification of the second mutational event in 10 (43%) tumors establishes *PTEN/MMAC1* as a main inactivation target of 10q loss in sporadic prostate cancer.

Introduction

Sporadic prostate carcinoma is the most common male cancer in the Western world and the second leading cause of male cancer deaths in the United States (1). Adult sporadic cancers are known to arise through the accumulation of multiple genetic events (2). Several of these genetic events have been identified in prostate cancer, including *ras* oncogenic activation (3) and inactivation of the tumor suppressor genes *Rb*, *p53*, and *CDKN2a* (4–6). A hereditary prostate cancer gene has been localized to chromosome 1q24–25 (7), but LOH[2] of this region is rarely implicated in sporadic prostate cancer (8). The most frequent genetic events in prostate cancer have been consistently identified as LOH of 8p, 10q, 13q, and 16q in cytogenetic, allelotype, and comparative genomic hybridization studies of sporadic prostate tumors (9–14). Deletion mapping studies have unambiguously identified a region of chromosome 10q23 to be the minimal area of loss (15–17). The target of inactivation on this chromosomal arm remains to be identified.

Recently, a tumor suppressor gene on chromosome 10q23, *PTEN/MMAC1*, was cloned, and somatic mutations were identified in glioma, breast, and prostate tumor cell lines (18, 19). *PTEN/MMAC1* has also been identified as the gene predisposing to Cowden disease (20), an autosomal dominant cancer predisposition syndrome associated with an increased risk of breast, skin, and thyroid tumors and occasional cases of other cancers but not prostate cancer (21, 22). A high frequency (30–60%) of 10q LOH has been reported in prostate cancer (11, 12, 15–17), and LOH of 10q probably represents the most

frequent genetic event after 8p LOH. A candidate gene, *MXI1*, has been identified at distal 10q23; however, recent mapping studies have unambiguously identified the more proximal 10q23 region as the minimal region of loss (15–17). Moreover, clonal mutations of *MXI1* have not been described in prostate tumors with loss of 10q (15, 23).

We screened 80 prostate tumors by microsatellite analysis and found chromosome 10q23 to be deleted in 23 cases. Sequence analysis of the entire *PTEN/MMAC1* coding region and screening for homozygous deletion with new intragenic markers in these 23 cases with 10q23 LOH identified the second mutational event in 10 (43%) tumors establishing *PTEN/MMAC1* as a main inactivation target of 10q loss in sporadic prostate cancer.

Materials and Methods

Tumor and Constitutional DNA. Prostate tumor specimens were obtained from patients undergoing radical prostatectomy and frozen immediately. Normal tissue or peripheral blood collected in EDTA was obtained from each patient as a normal control. Twenty tumors were obtained from pelvic lymph node metastases. The mean Gleason score for the 60 clinically localized tumors was 7.4 (SD 1.1) with a range of 5–9. None of the patients included in the study had been treated previously with chemotherapy or hormone therapy. Neoplastic cells were microdissected from frozen sections, and leukocytes were pelleted from blood samples before extraction and purification of DNA (24).

PCR Amplification and LOH Analysis. DNA from tumor and venous blood was analyzed for LOH by amplification of microsatellite repeat-containing sequences using PCR and the conditions described previously (25). For informative cases, allelic loss was scored if the intensity of signal from one allele was significantly reduced (>30%) in the tumor DNA when compared to the normal DNA. Primer sequences for *D10S581*, *D10S537*, *D10S1744*, *D10S1687*, *D10S215*, *D10S541*, *D10S583*, *D10S185*, and *D10S221* are available from Research Genetics (Huntsville, AL) or the Genome Database (Johns Hopkins University). For analysis of heterozygosity status using the intron 8 polymorphism, normal and tumor DNAs were separately amplified with exon 8 primers (20, 26), and the PCR product was digested with *Hph*I according to the manufacturer's instructions (New England Biolabs, Inc., Beverly, MA) before separation of alleles on a 1.8% agarose gel.

PCR Amplification and Cycle Sequencing of *PTEN/MMAC1*. Fifty ng of genomic template DNA were amplified with primers for exons 1–9 of *PTEN/MMAC1* at 95°C for 30 s, 50–58°C for 1 min, and 72°C for 1 min for 30–35 cycles, with a final extension step at 72°C for 5 min. The resulting PCR product was cycle sequenced according to the manufacturer's instructions (Perkin-Elmer, Roche Molecular Systems, Inc., Branchburg, NJ) and run on a 6% acrylamide gel. The primer sequences used for amplification and sequencing of the gene were as described in Liaw *et al.* (20) and Wang *et al.* (26). Sequence changes were confirmed by reamplification and resequencing of tumor DNA and corresponding normal DNA.

Isolation of Microsatellite Markers. The human genomic BAC clones 265 and 60 containing the *PTEN/MMAC1* gene were subcloned into Bluescript and plated. Colonies were lifted onto nylon membranes and screened with the microsatellite repeat oligomer, $(GT)_{10}$. Two of the microsatellite blocks isolated were found to be polymorphic and have been designated *D10S2491* and *D10S2492*. The primer sequences used for PCR amplification are as follows: *D10S2491 F*, 5′-GTTAGATAGAGTACCTGCACTC-3′; *D10S2491 R*, 5′-TTATAAGGACTGAGTGAGGGA-3′; *D10S2492 F*, 5′-TGCAGTGAGCT-GTGAAGATG-3′; and *D10S2492 R*, 5′-TGTTTCTCTTACTACCTAT-

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GTGA-3′. Both markers have alleles in the size range of 130–150 bp and amplify well at an annealing temperature of 55°C. *D10S2491* was informative in 82% of cases and *D10S2492* in 20% of cases. Microsatellite marker *D10S2492* was used only on cases noninformative for *D10S2491*.

FISH Procedure. Frozen tissue was sectioned onto slides and dried. Slides were dehydrated in an ethanol series, denatured in formamide, dehydrated in an ice-cold ethanol series, and air dried. Digoxigenin-labeled *PTEN/MMAC1* BAC probe and biotin-labeled chromosome 10 centromere α satellite probe (Oncor, Gaithersburg MD) were hybridized to the slides overnight at 37°C. Slides were washed and detected with rhodamine-antidioxigenin and FITC-avidin and counterstained with 4′,6-diamidino-2-phenylindole. Nuclei were analyzed, and images were captured by an Oncor Image analyzing system (27). Over 200 nuclei were counted without prior knowledge of the mapping data. The copy number of each probe was determined as the predominant number of signal(s) per nuclei (chromosome mode) for neoplastic and normal cells. Two signals were observed for the chromosome 10 centromere probe, 10 α satellite, in 69% of normal lymphocytes and 55% of neoplastic cells. No signal for the *PTEN/MMAC1* BAC probe was observed in 86% of neoplastic cells.

Methylation Analysis. One hundred ng of primary tumor DNA was denatured by NaOH and modified by sodium bisulfite. DNA samples were then purified using Wizard DNA purification resin (Promega Corp., Madison, WI), again treated with NaOH, precipitated with ethanol, and resuspended in water. PCR was performed separately with methylation-specific primers and non-methylation-specific primers for each tumor sample (28). Controls without DNA and positive controls for unmethylated and methylated reactions were performed for each set of PCR. PCR reactions were analyzed on nondenaturing 6% polyacrylamide gels, stained with ethidium bromide, and visualized under UV illumination.

Results and Discussion

To determine the frequency of LOH of chromosome 10q and to identify tumors for eventual sequence analysis, we screened 80 prostate tumors with a panel of microsatellite markers (Fig. 1a) spanning the region of interest on chromosome 10q. We found LOH in 26 (32%) tumors at two or more markers on 10q. We observed 13 cases of LOH at all informative markers spanning 10q, indicative of loss of the whole chromosome arm (monosomy). Five tumors retained proximal 10q but showed LOH for the rest of the q arm including *PTEN/MMAC1*. Five tumors showed localized LOH indicative of small deletions around *PTEN/MMAC1* at 10q23. In total, 23 of 26 tumors had LOH through *PTEN/MMAC1* (2 cases of proximal 10q LOH and 1 case of distal LOH excluded *PTEN/MMAC1*).

Because the initial reports of *PTEN/MMAC1* mutation also described frequent homozygous deletion of the gene in tumor cell lines (18, 19), we searched for these homozygous deletions in primary tumors. To reliably detect homozygous deletion in primary tumors, we preferred to assess apparent retention of heterozygosity at the gene of interest in tumors with LOH of flanking markers (6). The apparent retention of heterozygosity is due to amplification of DNA from normal cells contaminating the tumor specimen and correlates with homozygous deletion assessment by Southern and FISH analysis (6). We screened the prostate tumors with the closest mapped flanking markers to *PTEN/MMAC1* (*D10S215* and *D10S541*); however, in one of the initial reports, approximately one-half of the homozygous deletions did not extend to these flanking markers (18). We, therefore, obtained two overlapping human BAC clones that together contained the entire genomic *PTEN/MMAC1* gene (18) and screened for microsatellite blocks with a GT oligomer. We isolated several microsatellite blocks, two of which were found to be polymorphic. We then screened the prostate tumors using the new markers (*D10S2491* and *D10S2492*), which map within *PTEN/MMAC1,* and a single bp polymorphism in intron 8 (26) to detect homozygous deletion in the 23 tumors with 10q LOH.

We found six cases of homozygous deletion of *PTEN/MMAC1* in these 23 primary prostate tumors (26%), as indicated by apparent retention of heterozygosity (Fig. 1a). The homozygous deletions were small, <2 cM of a chromosome that is 181.7 cM in size (29), and
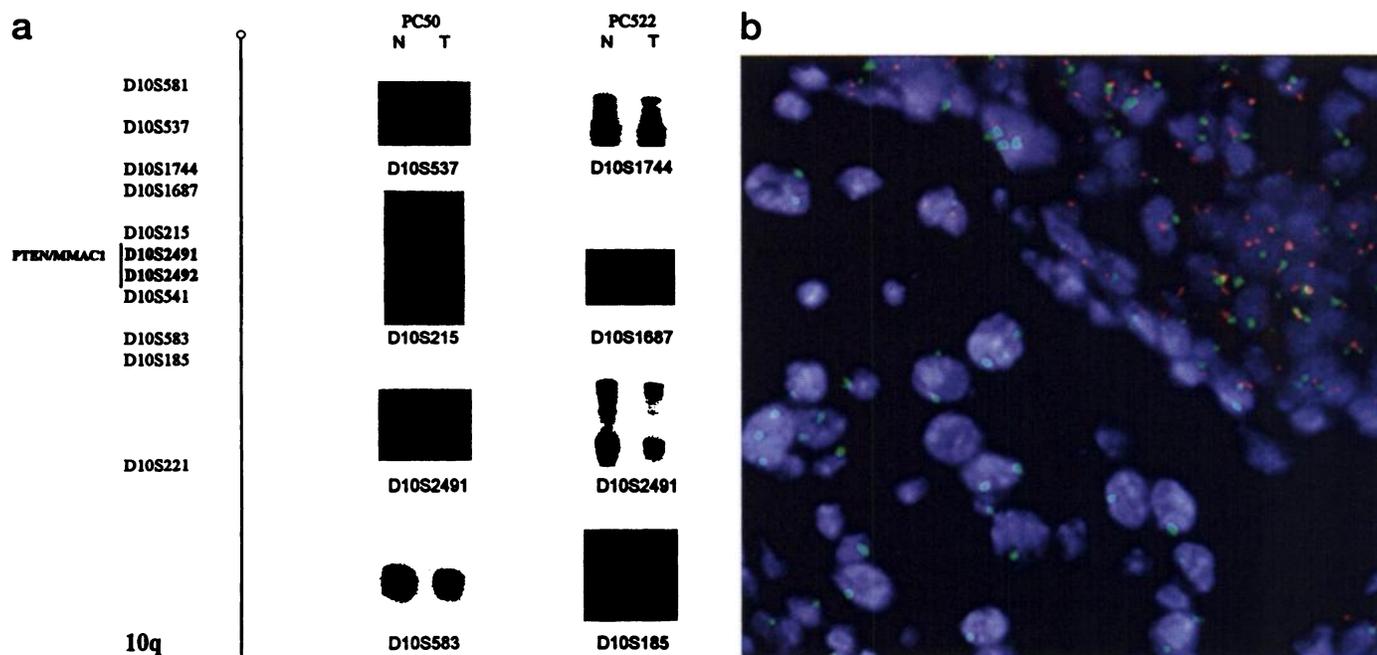

Fig. 1. *a*, approximate map location of microsatellite markers on chromosome 10. The previously mapped 10q markers *D10S581, D10S537, D10S1744, D10S1687, D10S215, D10S541, D10S583, D10S185,* and *D10S221*[29] are indicated, together with the newly cloned *PTEN/MMAC1* polymorphic markers *D10S2491* and *D10S2492. Right,* homozygous deletion of *PTEN/MMAC1* by microsatellite analysis. Prostate tumor 50 shows apparent retention of heterozygosity indicating homozygous deletion at *D10S2491,* flanked by LOH indicated by loss of the upper allele at *D10S537* and loss of the lower allele at *D10S215* and *D10S583* in the tumor (*T*) lane. Prostate tumor 522 shows apparent retention of heterozygosity, indicating homozygous deletion at *D10S2491,* flanked by LOH indicated by loss of the upper allele at *D10S1744, D10S1687,* and *D10S185* in the tumor (*T*) lane. *N,* normal DNA lane. *b,* FISH analysis of interphase nuclei from metastatic lymph node of prostate cancer 522. Homozygous deletion of *PTEN/MMAC1* is indicated by the absence of signal from the *PTEN/MMAC1* BAC probe (*red*) in neoplastic cells (*bottom left*) and presence of signal in normal lymphocyte cells (*top right corner*). Retention of chromosome 10 is indicated by the signal from the centromeric α satellite probe (*green*) showing two copies in both neoplastic cells and normal lymphocytes (see "Materials and Methods").
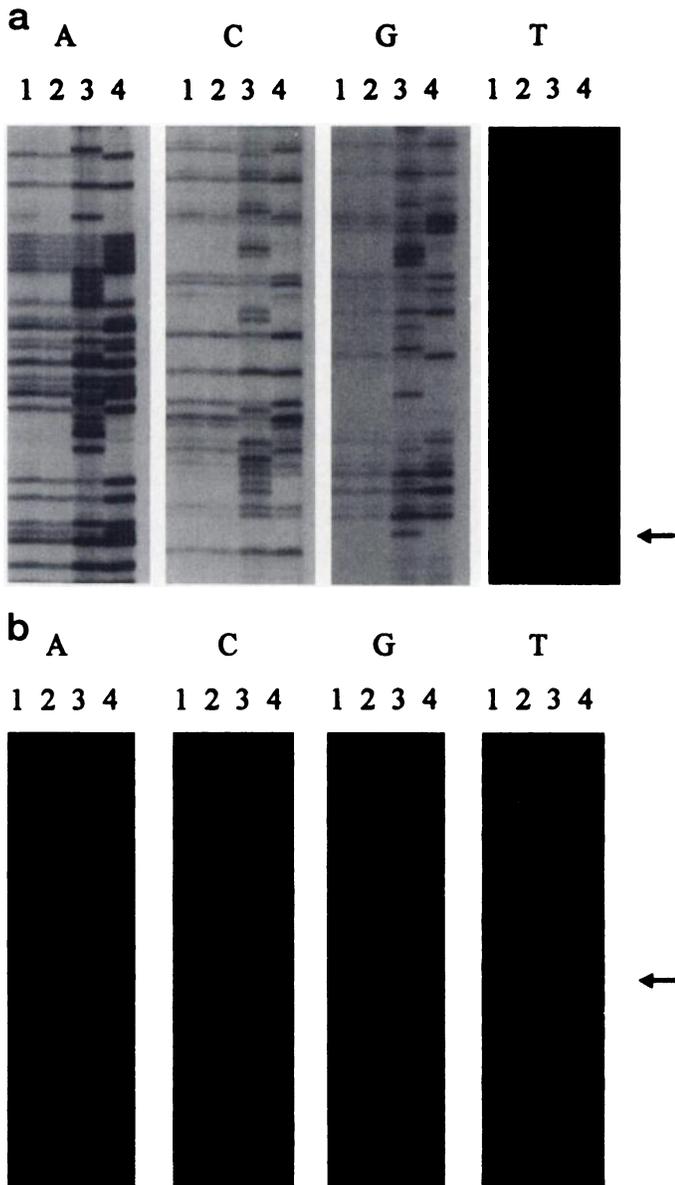
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a

b

Fig. 2. Sequence mutations of *PTEN/MMAC1* in primary prostate tumors. *a: Lanes 1, 2,* and *4,* tumor DNAs with wild-type sequence. *Lane 3* is tumor DNA from patient 273 showing a 5-bp deletion at nucleotides 761–765 in exon 7, resulting in a frameshift (*arrow*). *b: Lanes 1, 2,* and *4,* tumor DNAs with wild-type sequence. *Lane 3* is tumor DNA from patient 155 showing a nonsense change of T to A (antisense strand is shown) at nucleotide 564 in exon 6 (TAT-TAA, tyr-stop; *arrow*). Diminution of the substituted base is clearly visible. The mutations were confirmed by tumor and corresponding normal DNA reamplification and resequencing.

nested around the highly informative marker *D10S2491*, which maps to the 5' end of the gene. Primary tissue was available for FISH analysis in one case with a homozygous deletion detected by microsatellite analysis. Absence of signal from a *PTEN/MMAC1* BAC probe (red) after FISH confirmed a homozygous deletion in tumor 522 (Fig. 1*b*) by this approach. Two copies of a control chromosome 10 centromeric probe (green) were visible in both the neoplastic cells and normal lymphocytes (Fig. 1*b*).

After exclusion of tumors with homozygous deletion, we then proceeded with complete sequence analysis of the coding region of *PTEN/MMAC1* and the intron/exon boundaries. In the remaining 17 prostate tumors with 10q LOH, we found 4 tumors with somatic mutation, 1 tumor showed a 5-bp deletion, and a second demonstrated a single-bp change resulting in a nonsense mutation (Fig. 2). Another

tumor contained a 4-bp deletion at the donor splice site of intron 3, and the fourth tumor demonstrated a 9-bp deletion in exon 8. All four mutations are predicted to result in a truncated protein (30).

Tumor suppressor genes in general, and *CDKN2a* in particular, can be inactivated by epigenetic methylation of the promoter, resulting in complete blocking of transcription (31). We also investigated promoter methylation as a possible inactivation mechanism of the retained allele of *PTEN/MMAC1* in all the tumors with 10q LOH but without homozygous deletion or point mutation. However, using methylation-specific PCR (28) with appropriate controls, we found no evidence of *PTEN/MMAC1* promoter methylation (Fig. 3).

According to Knudson's two-hit hypothesis (32), tumor suppressor gene function is lost by independent inactivation events of both parental alleles. We detected the second inactivation event at *PTEN/MMAC1* in 10 of 23 (43%) prostate tumors with LOH of 10q23. This result is still likely to be an underestimation, because we did not search for sequence mutations in the promoter or regulatory regions, did not sequence tumors without LOH (potentially harboring point mutations of both alleles), and almost certainly missed some small homozygous deletions. We have shown previously at the *CDKN2a* tumor suppressor locus on chromosome 9p21 that the frequency of homozygous deletion increases when markers near or within the gene are used because homozygous deletions are nested in size around the target gene (6). A nonpolymorphic marker, such as WG9 (18), can be used to detect homozygous deletion by the simple presence or absence of signal in tumor cell lines that are composed of neoplastic cells only. In primary tumor specimens, normal cells complicate or render impossible this method of detecting homozygous deletions. The newly cloned markers *D10S2491* and *D10S2492* will thus be invaluable in assessing the true rate of homozygous deletions in many tumor types. As shown here, apparent retention of these markers correlates with homozygous deletion by FISH analysis (Fig. 1*b*).

Previous reports suggested that mutation of *PTEN/MMAC1* is a late genetic event associated with advanced cancers (18, 19). For prostate cancer, we observed LOH of 10q in 11/60 clinically localized tumors and 12/20 pelvic node metastases. Seven of the 10 tumors with homozygous deletion or point mutation were found in neoplasms with pelvic lymph node metastases. Thus, as in other cancer types, prostate tumors of high grade and stage are more likely to harbor 10q loss and *PTEN/MMAC1* mutations. The frequent detection of the second mutational event clearly establishes *PTEN/MMAC1* as the main inactivation target of 10q deletion in sporadic prostate cancer. The majority of prostate cancer families do not show linkage to chromosome 1q24–25 (8), and it will be interesting to assess linkage to *PTEN/MMAC1* in these families. The identification of *PTEN/MMAC1* as a frequent target in sporadic prostate cancer highlights the gene for novel diagnostic and therapeutic approaches and represents a significant advance in our knowledge of the molecular biology of prostate cancer.

Fig. 3. Methylation-specific PCR of *PTEN/MMAC1* in primary prostate tumors. *Left,* PBR322 *Msp*I-digested DNA. Primary prostate tumors (1–6) showing a PCR product in the unmethylated DNA lane (*U*) and no product from the methylated DNA lane (*M*). *In vitro* methylated DNA as a positive control for methylation (*IV*) and normal lymphocyte DNA (*NL*) as a negative control for methylation and a water control for the PCR reaction are also shown.

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