Homozygous Deletion of the *PTEN* Tumor Suppressor Gene in a Subset of Prostate Adenocarcinomas[1]

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ABSTRACT

A novel tumor suppressor gene, *PTEN*, which encodes a dual-specificity protein phosphatase, has recently been identified on chromosome 10q23. We have previously shown that both alleles of this gene are inactivated in three of four prostate cancer cell lines tested. To evaluate the role of inactivation of this gene in primary stage B prostate cancers, 60 cases were analyzed using Southern blotting with *PTEN* probes and microsatellites on 10q23. Eight of 60 cases had homozygous deletions by Southern blotting. In three of these cases, homozygous deletion was confirmed by apparent retention of heterozygosity at *PTEN* with loss of heterozygosity at telomeric and centromeric loci. In the remaining five cases, microsatellite analysis was consistent with homozygous deletion. Loss of heterozygosity at *PTEN* was found in only two cases both by microsatellite analysis and quantitative Southern blotting. No small mutations within *PTEN* exons were found in any tumors exhibiting alterations on 10q23. Thus, inactivation of the *PTEN* gene by homozygous deletion occurs in approximately 10–15% of primary stage B prostate carcinomas.

INTRODUCTION

Neoplastic transformation is characterized by the accumulation of multiple genetic alterations that lead to the malignant phenotype. Inactivations of tumor suppressor genes are the most common alterations found in carcinomas. Typically, one allele of a tumor suppressor gene is inactivated by a relatively subtle mutation that may range from alteration of a single amino acid to deletion of the entire gene. The inactivation of the second allele is frequently less precise. Commonly, large regions or the entire chromosome is deleted, which can be detected as LOH.[3] Thus, high rates of LOH are seen in malignant tumors in the region of tumor suppressor genes. In prostate carcinoma, high rates of LOH have been detected on chromosomes 5q, 8p, 9p, 13q, 16q, 18q (1), and 10q (2–5). Similar LOH has been observed on chromosome 10q in a variety of other malignant neoplasms including breast (6–8), renal (8), urothelial (9), endometrial (10), and small cell lung carcinomas (11); melanomas (12); and glioblastomas (8, 13–15).

Recently, a tumor suppressor gene designated *PTEN* (6) or *MMAC-1* (8) has been identified at 10q23. This gene encodes a dual-specificity protein phosphatase (16) and also has extensive homology to tensin, a protein that interacts with actin filaments at focal adhesions. Homozygous deletions or inactivating mutations of this gene have been identified in primary tumor samples from glioblastomas and breast cancers (6, 8, 15). Germline mutations of this gene have been found in Cowden disease (17), a familial neoplastic syndrome characterized by a variety of benign hamartomatous tumors as well as a high frequency of breast and thyroid cancer in affected kindreds.

It has been shown previously that three of four prostate cancer cell lines inactivate both alleles of *PTEN*. Both the PC-3 and NCI-H660 cell lines contain homozygous deletions of *PTEN*, whereas the LNCaP cell line contains a single allele of *PTEN* that has a frameshift mutation in codon 6. However, given that genetic alterations may arise during the passaging of cell lines, and that tumors that give rise to cell lines may not be a representative of all primary tumors of that type, it is important to examine primary tumors directly for inactivating alterations of a tumor suppressor gene. Approximately 40% of primary glioblastoma tumor samples show either inactivating mutations or homozygous deletions of the *PTEN* gene (15). Similar alterations have been found at a lower rate in primary female breast cancer (8). To evaluate the role of *PTEN* alterations in clinically localized stage B prostate carcinomas, we have evaluated a total of 60 such cases by a variety of methods including quantitative Southern blotting, LOH analysis, and sequencing. We have found that approximately 10–15% of clinically localized cancers show alterations in the *PTEN* region, with most such cases containing homozygous deletions.

MATERIALS AND METHODS

Tissue Samples and DNA Isolation. The DNAs used in this study were isolated from fresh human prostate adenocarcinomas from patients undergoing radical prostatectomy. A total of 60 matched tumor and benign DNAs were isolated from fresh radical prostatectomy specimens after frozen section analysis to

[3] The abbreviation used is: LOH, loss of heterozygosity.

insure at least 50% carcinoma in tissue used for isolation of tumor DNA as described previously (2). The percentage of tumor in the extracted tissue was recorded at the time of extraction. The normal control samples were free of carcinoma and prostatic intraepithelial neoplasia by frozen section. All cases were pathological stage T_2 or T_3, and no cases had pelvic lymph node metastasis.

Quantitative Southern Blot Analysis. Ten μg of each benign or tumor high molecular weight DNA were digested with *Hin*dIII restriction endonuclease, subjected to electrophoresis on 1% agarose gels, blotted onto nylon membranes, hybridized with ^{32}P random-primed probes, and washed, and autoradiography was performed as described previously (2). To evaluate the *PTEN* gene, blots were hybridized with the JL25 genomic probe (6), which resulted in a single 6-kb band on blots of *Hin*dIII-digested DNAs. As a control for variation in DNA amount in each lane, blots were subsequently hybridized with a *Tyk-2* cDNA probe, kindly supplied by Dr J. Krolewski (Columbia University). The *Tyk-2* gene is located on chromosome 19 (18), which rarely shows LOH in prostate cancer as assessed by comparative genomic hybridization (1). To determine the presence and extent of loss at the *PTEN* locus, all autoradiograms were quantitated using video densitometry as described previously (2). The absorbance of the JL25 band for each carcinoma was compared to benign DNA in the same blot after correction for slight variations in DNA amount using the absorbance of the 12-kb *Tyk-2* cDNA band. All cases with losses at *PTEN* were evaluated on two or more independent blots if sufficient DNA was available. A subset of samples was also analyzed in a similar manner using a 5' *PTEN* cDNA probe on blots of DNAs digested with *Hin*dIII or *Bgl*II restriction endonuclease.

LOH Analysis. LOH was determined by PCR of microsatellite repeats using matched benign and tumor DNAs as described previously (2). Most primers were obtained from Research Genetics (Huntsville, AL), except for PTENCA. The primers for PTENCA are as described in Cairns *et al* (15).[4] After autoradiography, the relative band intensity was determined by video densitometry as described previously (2). All cases showing allelic imbalance were confirmed at least in duplicate. Map position of all microsatellites was determined from the Whitehead Genome Database, except D10S608, which was mapped as described previously (2). LOH at the intron 8 sequence polymorphism was determined by direct sequencing as described by Wang *et al.* (15)

Mutation Analysis. Sequencing of all nine exons of *PTEN* was performed on all samples exhibiting LOH at 10q23 by microsatellite analysis or with loss at the *PTEN* locus by Southern blotting. The nine exons, including the intron-exon splicing junctions, were amplified by PCR and sequenced by cycle sequencing (Amersham Life Science) as described by Wang *et al.* (15).

[4] P. Cairns, E. Evron, K. Okami, N. Halachmi, S. Bose, S. I. Wang, R. Parsons, and D. Sidransky. Mutation analysis of *PTEN/MMAC1* in primary bladder and renal cell cancers, submitted for publication.

RESULTS

Quantitative Southern Blot Analysis of *PTEN*. To detect loss of the *PTEN* gene in primary stage B prostate cancers, a total of 60 cases were analyzed at the *PTEN* locus by quantitative Southern blotting. *Hin*dIII-digested DNAs were used for Southern blotting and hybridized with a genomic probe, JL25, located within the *PTEN* gene, and the resulting autoradiograms were analyzed by video densitometry. The absorbance of the JL25 band in each carcinoma band was compared to that of benign DNA in the same blot. To correct for slight variability in DNA amount between lanes, the blots were subsequently hybridized with a *Tyk-2* cDNA probe (18), and the resulting 12-kb band was quantitated in a similar fashion. *Tyk-2* is located on chromosome 19, and this chromosome is rarely altered in prostate cancers, as assessed by comparative genomic hybridization (1). All cases with losses at *PTEN* for which sufficient DNA was available (8 of 10 cases) were analyzed on 2–4 independent blots to confirm the results of the initial blot. Half of such cases were also analyzed in a similar manner in at least one blot using a 5'*PTEN* cDNA probe. Examples of such blots are shown in Fig. 1, and the results of the quantitative analysis are summarized in Table 1. As can be seen in Table 1, there is a good correlation between the loss seen with the JL25 probe and the 5' cDNA probe. A total of 10 cases showed losses at the *PTEN* gene. Eight of these cases (cases 6, 31, 35, 45, 61, 119, 140, and 145) had losses of a magnitude consistent with a homozygous deletion. A homozygous deletion should have a loss of *PTEN* signal approximately equal to the percentage of tumor in the sample. A hemizygous deletion would have a decrease in *PTEN* signal intensity approximately equal to one-half the percentage of tumor in the extracted tissue. Case 46 showed a loss midway between that of a homozygous and a hemizygous deletion and thus could represent either a hemizygous or homozygous deletion. In contrast, case 87 is clearly a hemizygous deletion by quantitative Southern blot analysis.

Microsatellite Analysis at 10q23 in Stage B Prostate Carcinomas. All 60 cases were analyzed for LOH on 10q23 by PCR at 10 microsatellite repeats centered on the *PTEN* gene and its immediate vicinity. Cases with alterations of 10q23 by either quantitative Southern blotting or microsatellite analysis were also analyzed for LOH at a sequence polymorphism in intron 8 of the *PTEN* gene by direct sequencing. Results are shown in Fig. 2. Nine of 10 cases with loss of *PTEN* by Southern blotting showed LOH at 1 or more 10q23 loci. Three cases (cases 35, 45, and 145) showed LOH at markers both telomeric and centromeric of *PTEN* but showed no loss at the PTENCA repeat and/or the intron 8 polymorphism, which are located within the *PTEN* gene. Such a pattern of apparent retention of heterozygosity embedded within an area of LOH is strongly correlated with the presence of a homozygous deletion (19). Apparent retention of heterozygosity is due to residual benign DNA in a tumor containing a homozygous deletion, which gives rise to PCR products from the benign DNA only. Examples of PCR microsatellite reactions illustrating this pattern of apparent retention of heterozygosity are shown in Fig. 3. Four other cases (cases 6, 31, 61, and 119) had apparent retention of heterozygosity at the PTENCA and/or intron 8 loci and LOH at either telomeric or centromeric markers. This pattern is
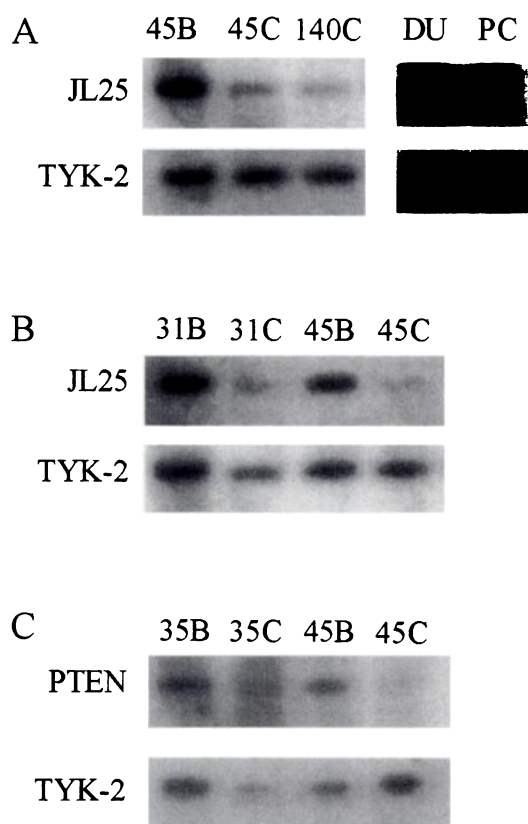
Fig. 1 Representative autoradiograms of Southern blots with homozygous deletions of the *PTEN* gene. Each panel (*A–C*) represents an independent blot hybridized with either the JL25 probe or a 5'*PTEN* cDNA probe. Each blot was subsequently hybridized with a *TYK-2* cDNA probe as a control for variability of the DNA amount in each lane. The case number is indicated: *B*, DNA from benign tissue; and *C*, DNA from the carcinoma sample. In *A*, *DU* is DNA from the DU145 cell line, whereas *PC* indicates DNA from the PC-3 prostate cancer cell line, which we have previously shown to have a homozygous deletion of the *PTEN* gene (6), included as a positive control.

Table 1 Summary of quantitative Southern blotting

The percentage of tumor was estimated by examination of frozen sections at the time of tumor DNA extraction. Quantitative Southern blotting was performed as described. Results are expressed as the percentage loss of either JL25 or 5' *PTEN* cDNA relative to benign DNA in the same blot ± SE of the mean in those cases with multiple blots. The number of independent blots is indicated in parentheses.

| Case | % tumor | % loss ± SE (no.) | |
		JL25	cDNA
6	50	40	ND[a]
31	70	71 ± 4 (4)	83 ± 9 (2)
35	50	53	68
45	60	65 ± 8 (4)	71
46	50	38 ± 9 (3)	ND
61	60	39 ± 7 (3)	72
87	90	51 ± 2 (2)	ND
119	60	53 ± 2 (4)	79
140	90	88 ± 6 (2)	ND
145	50	40 ± 9 (3)	ND

[a] ND, not determined.

consistent with either no loss of *PTEN* or homozygous deletion of *PTEN*. However, given that these samples all showed losses on quantitative Southern blots consistent with homozygous deletion (Table 1), the reasonable interpretation is that the retention of heterozygosity at the *PTEN* loci supports the presence of homozygous deletion in these cases. One case (case 140) showed apparent retention of heterozygosity at both the PTENCA and intron 8 loci without LOH at adjacent markers, again consistent with the homozygous deletion of the *PTEN* gene detected by Southern blotting. Two cases (cases 46 and 87) showed LOH at the PTENCA and/or intron 8 loci as well as at adjacent markers. These results are consistent with the hemizygous deletions seen by quantitative Southern blotting. Three additional cases were found that had no loss of *PTEN* by either microsatellite analysis or quantitative Southern blotting (data not shown) but had LOH at one or two markers proximal or distal to *PTEN*. These cases may represent random losses due to genomic instability or could be related to inactivation of as yet unidentified tumor suppressor genes on 10q.

In summary, of the 60 cases analyzed, 8 cases (cases 6, 31, 35, 45, 61, 119, 140, and 145) had losses on quantitative Southern blotting consistent with homozygous deletion. In all of these cases, there was apparent retention of heterozygosity at one or more loci within the *PTEN* gene, supporting the idea that homozygous deletion of *PTEN* had occurred in these cases. Two cases (cases 46 and 87) had LOH at intragenic *PTEN* polymorphisms and losses on Southern blotting consistent with hemizygous deletion.

Mutation Analysis of the *PTEN* Gene. All 10 cases with alterations in the 10q23 region, either by microsatellite analysis or by quantitative Southern blotting, were analyzed for small mutations in all of the exons of the *PTEN* gene, including the intron-exon borders, using the tumor DNA. No mutations were found within exons in any case. The absence of mutations of *PTEN* in the eight cases with losses on quantitative Southern blotting consistent with homozygous deletion supports the idea that these cases are indeed homozygous deletions. Thus, homozygous deletion of *PTEN* seems to be the major mechanism of inactivation in prostate cancer.

DISCUSSION

The presence of a homozygous deletion of a gene in a significant fraction of tumors of a given type is a strong indication that the gene is a tumor suppressor for that tumor. We have found that 8 of 60 stage B prostate carcinomas have a homozygous deletion at the *PTEN* locus as determined by quantitative Southern blotting. The conclusion that these cases had homozygous deletions is supported by LOH analysis showing apparent retention of heterozygosity at the PTENCA and/or intron 8 loci, surrounded by areas of LOH in three cases, and apparent retention of heterozygosity at one or both of these intragenic loci, with or without LOH at adjacent loci, in five other cases. Thus, 10–15% of stage B carcinomas have a homozygous deletion of the *PTEN* gene. An additional two cases showed LOH at *PTEN* without clear evidence of inactivating genetic changes.

Fig. 2 LOH analysis of prostate carcinomas with allelic losses of the *PTEN* locus by Southern blotting. Microsatellites were analyzed by PCR as described in "Materials and Methods." The polymorphism at intron 8 was analyzed by sequencing. ■, LOH; □, informative, no LOH; ▢, not informative.

Fig. 3 Homozygous deletion at *PTEN* detected by retention of heterozygosity. Cases 35 and 45 are presented, and only representative polymorphic markers are illustrated. Dinucleotide repeat polymorphisms are indicated with the prefix D10S deleted. *Dotted lines*, missing allele.
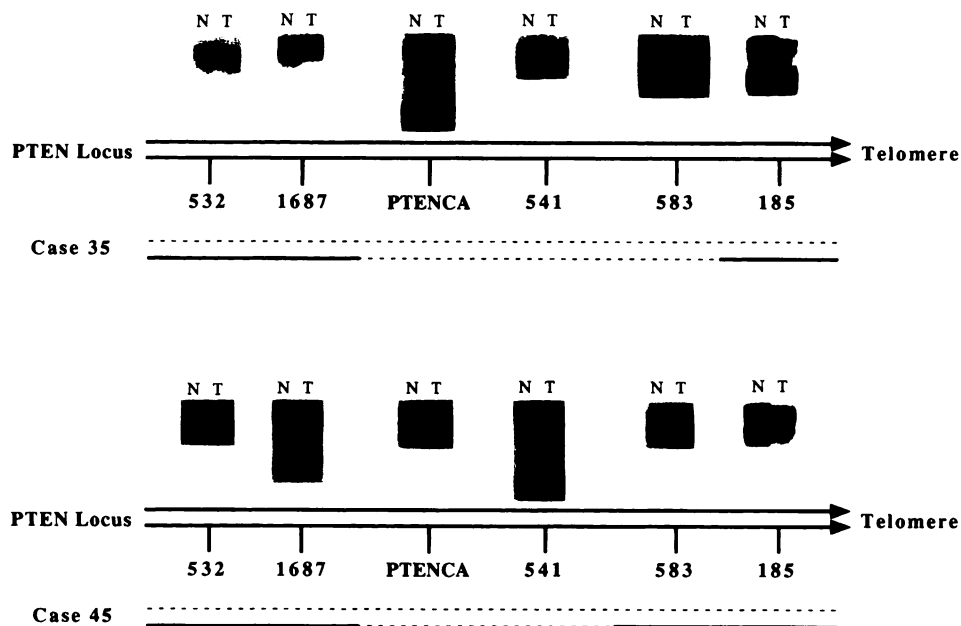
The failure to detect mutations within the exons of the *PTEN* gene in our 10 cases with losses in this region is explained for the most part by the fact that 8 of the cases had homozygous deletions of the *PTEN* gene. There are a number of possible explanations for our failure to detect inactivating genetic lesions in the two cases with LOH in the 10q23 region without homozygous deletions. In these cases, a small homozygous deletion, such as a deletion of one or more exons, would not be detected by our mutation analysis or Southern blotting unless it involved part of an exon or a substantial portion of the region corresponding to the probe used for Southern blotting. Another possibility is methylation or other forms of transcriptional inactivation of the retained allele in these cases. Finally, the LOH found in these two cases might reflect the inactivation of another tumor suppressor or nonspecific genomic instability. Many groups have presented data consistent with the presence

of one or more tumor suppressor genes telomeric to 10q23 (2, 4, 7, 10, 13). Ultimately, further analysis, particularly immunohistochemistry of primary tumors, will be needed to clarify the exact role of *PTEN* in stage B prostate carcinoma.

Although only 10–15% of stage B prostate cancers were found to have inactivation of the *PTEN* gene, it is likely that metastatic and extensively invasive prostate cancers will have a much higher rate of *PTEN* inactivation. We and others have reported a significantly higher rate of LOH at 10q23 in stage C and D prostate cancers. Stage B cancers show a 0–40% rate of LOH at 10q23, whereas stage C and D cancers have a 40–75% rate of loss in this region (2–5). A similar association of LOH on 10q with high grade (7, 9, 14, 20), invasion (9), and metastasis (12) has been observed in other malignant neoplasms including astrocytic brain tumors (14), urothelial carcinomas (9), melanomas (12), breast carcinomas (7), and malignant meningiomas

(20). Additional investigations are needed to evaluate the frequency of *PTEN* inactivation in advanced prostate cancers and to understand the biological reasons for inactivation of *PTEN* in malignant neoplasms.

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ADDENDUM

After the submission of this manuscript, Cairns *et al.* (21) published an analysis of alterations of the *PTEN* gene in 80 prostate cancers. They found inactivation of *PTEN* in 5% of localized cancers and in 35% of pelvic lymph node metastases, including a high proportion of homozygous deletions, consistent with our results.

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