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Award Number: DAMD17-98-1-8636

TITLE: Differentially Expressed Genes in Human Prostatic Carcinoma

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REPORT DATE: February 2001

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

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20020124 286

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	February 2001	Annual (1 Aug 99 - 31 Jan 01)

4. TITLE AND SUBTITLE
Differentially Expressed Genes in Human Prostatic Carcinoma

5. FUNDING NUMBERS
DAMD17-98-1-8636

6. AUTHOR(S)
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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT ***(Maximum 200 Words)***

Unlike other major common cancers, no major tumor genes have been reported in prostate cancer, although this disease is the most frequently diagnosed cancer and the second leading cause of cancer death in American men. Identifying tumor related genes is still one of the most significant areas in prostate cancer research. We have proposed to identify the genes that are involved in prostate cancer. One type of such gene are tumor suppressor genes. In the second year of this project, we continued our effort in characterizing tumor suppressor genes in prostate cancer. Using the methods of tissue microdissection and deletion mapping, we further defined a region of deletion, that indicates the existence of novel tumor suppressor gene, at the q21 band of chromosome 13. We found that some human tumors have homozygous deletion at 13q21. In addition, the LNCaP cell line and the PC-82 xenograft of prostate cancer have hemizygous deletion at 13q21, and the region of deletion is defined to 2.8 megabase. We are currently identifying the most likely candidate tumor suppressor gene from 13q21. We also used polymerase chain reaction (PCR)-single strand conformational polymorphism (SSCP) and direct sequencing methods to examine mutations of PTEN in high grade primary tumors and metastases of prostate cancer. We found that PTEN mutation is significantly more frequent in high grade prostate cancers, indicating that PTEN is more likely involved in the progression of prostate cancer.

14. SUBJECT TERMS	15. NUMBER OF PAGES
Prostate Cancer, PTEN, deletion mapping, tumor suppressor gene, gene mutation	15
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

TABLE OF CONTENTS

Page Number

INTRODUCTION:

This project was proposed to identify the genes that are differentially expressed in prostate cancer. Tumor suppressor gene is one type of these genes. Identification of tumor suppressor gene is a significant task in prostate cancer research, because such genes are important targets for improving the diagnosis and treatment of prostate cancer. In the second year of this project, we first clarified the role of the PTEN tumor suppressor gene in aggressive prostate cancer. We also defined a region of genomic deletion, that indicates the location of novel tumor suppressor genes, at 13q21 in prostate cancer using deletion mapping methods. Again, the 13q21 tumor suppressor gene appeared to be involved in aggressive prostate cancer.

BODY:

In this budget year, we continued our effort in identifying genes that are involved in the development and progression of prostate cancer, as originally planned. We targeted two genes. One is a novel tumor suppressor gene at the q21 band of human chromosome 13, and the other is the PTEN tumor suppressor gene, the most frequently altered gene in human prostate cancer.

For the novel tumor suppressor gene at 13q21, we continued our effort in fine mapping its location. We examined a large number of prostate cancer specimens and cell lines/xenograft for genetic deletions at 13q21, using the methods of tissue microdissection and duplex PCR. Hemizygous deletion and/or homozygous deletion at 13q21 were detected in 13 of 147 (9%) prostate cancer samples. Deletion of the same region was also detected in the LNCaP cell line and the PC-82 xenograft of prostate cancer. The overlapping region of deletion in LNCaP and PC-82 spans 3.1 cM or 2.9 cR, that are equivalent to one to three megabases. The endothelin receptor B gene, a possible tumor suppressor gene at 13q21, was not located in the region of deletion. Among the 13 prostate neoplasms with deletion at 13q21, five were metastases and seven were poorly differentiated primary tumors. The only primary tumor which was not poorly differentiated but had deletion occurred in one of the youngest patients (49 years) at diagnosis. These results provide additional evidence that 13q21 harbors an unidentified gene(s) whose inactivation occurs in some aggressive carcinomas of the prostate. In addition, this study provides a framework for the cloning and identification of the 13q21 gene(s). At present, we are working to identify the tumor suppressor gene from 13q21.

For the PTEN tumor suppressor gene, we analyzed its mutation in prostate cancer from Chinese patients. *PTEN/MMAC1* is a putative tumor suppressor gene located on 10q23, one of the most frequently deleted chromosomal regions in human prostate cancer. While mutations of *PTEN* have been often detected in metastases of prostate cancer, localized tumors have shown lower rates of mutation, which have varied from zero to 20% among different studies. It is unknown whether the rate of *PTEN* mutations is different in prostate cancer from Asian compared with Western men. To further clarify the role of *PTEN* in prostate cancer and to examine the gene for mutations in Asian men, we analyzed 32 cases of primary prostate cancers from Chinese patients, each of whom was not diagnosed by screening with serum PSA, for *PTEN* mutations using the methods of tissue microdissection, single strand conformation polymorphism, and direct DNA sequencing. Seventy percent of the tumors were Gleason score 8-10, while the remainder were Gleason score 7. Six metastases of prostate cancer from American patients were also analyzed. Five of 32

(16%) primary prostate cancers from Chinese men and two of six metastases from American men showed mutations in a total of 10 codons of *PTEN*, which involved exons 1, 2, 5, 8, and 9. Two of the mutations were truncation type, while the rest were missense mutations. The mutation frequency in these cases from Asian patients was higher than that in our previous study of cases in radical prostatectomy specimens from American men, in which the 40 primary tumors were lower grade and had been detected by serum PSA test. We conclude that mutation of *PTEN* occurs more often in primary prostate cancers of Chinese men, whose tumors are high grade and reflective of an unscreened population.

The above studies have been published in two papers:

1. Dong JT, Chen CC, Stultz BG, Isaacs JT, Frierson HF, Jr. (2000) **Deletion at 13q21 is associated with aggressive prostate cancers.** *Cancer Res* 60:3880-3883.
2. Dong JT, Li CL, Sipe TW, Frierson HFJ (2001) **Mutations of PTEN/MMAC1 in primary prostate cancers from Chinese patients.** *Clin Cancer Res* in press.

Both papers are included in the appendix.

KEY RESEARCH ACCOMPLISHMENTS:

- Mutations of the PTEN gene occurs more frequently in high grade tumors and metastases of prostate cancer. PTEN thus plays a role in the progression of this disease.
- A novel tumor suppressor gene exists at the q21 band of chromosome 13.
- The EDNRB gene, a tumor suppressor gene located at 13q21, is not the target gene of 13q21 deletion.
- The tumor suppressor gene at 13q21 remains to be identified.
- Alterations at 13q21 is responsible for the aggressive behavior of prostate cancer.

REPORTABLE OUTCOMES:

The second year of this project has generated another two papers, one has been published and one is in press. They are:

1. Dong JT, Chen CC, Stultz BG, Isaacs JT, Frierson HF, Jr. (2000) **Deletion at 13q21 is associated with aggressive prostate cancers.** *Cancer Res* 60:3880-3883.

2. Dong JT, Li CL, Sipe TW, Frierson HFJ (2001) **Mutations of PTEN/MMAC1 in primary prostate cancers from Chinese patients.** *Clin Cancer Res* in press.

In addition, based on the data generated in this project, the P.I. has been awarded two R01 grants by the National Cancer Institute, NIH:

(1). Period: 4/1/00 - 3/31/05; Title: *Molecular dissection of 13q14 in prostate cancer.*
Agency: NIH/NCI; P.I.: Dong JT
Award Number: 1 R01 CA 85560; Current year direct cost: $202,500; Total direct cost: $922,500; Total award amount: $1,349,737 (this grant received a priority score of 113 and a percentile of 0.2)
The goal of this project is to clone and characterize a new tumor suppressor gene from the q14 region of chromosome 13, whose homozygous deletion has been detected in malignant prostate cancers.

(2). Period: 8/18/00 - 7/31/05; Title: *A tumor suppressor gene at 13q21 in prostate cancer.*
Agency: NIH /NCI; P.I.: Dong JT
Award Number: 1 R01 CA 87921; Current year direct cost: $172,500; Total direct cost: $804,000; Total award amount: $1,180,962
The goal of this project is to clone and characterize another new tumor suppressor gene from the q21 region of chromosome 13.

CONCLUSIONS:

In the second year of this project, we further defined a tumor suppressor locus at 13q21, and demonstrated that this tumor suppressor gene is responsible for aggressive behavior of prostate cancer. Such a gene is very important to our battle against prostate cancer, because currently it is more urgent to find molecular markers that can differentiate clinically aggressive prostate cancers from those latent ones. We also clarified the role of the PTEN tumor suppressor gene in prostate cancer. PTEN is so far the most frequently mutated gene in prostate cancer, but we found that PTEN is specifically involved in high grade primary tumors or metastases of prostate cancer.

REFERENCES:

See the two papers included in the appendix for the list of references.

[CANCER RESEARCH 60, 3880–3883, July 15, 2000]

Deletion at 13q21 Is Associated with Aggressive Prostate Cancers[1]

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ABSTRACT

Previous cytogenetic and molecular genetic analyses suggest that the q21 band of chromosome 13 harbors a tumor suppressor gene(s) involved in prostatic carcinogenesis. The precise genetic location, however, has not been defined. In this study, we examined prostate cancer specimens and cell lines/xenograft for genetic deletions at 13q21, using the methods of tissue microdissection and duplex PCR. Deletions at 13q21 were detected in 13 of 147 (9%) prostate cancer samples. Deletion of the same region was also detected in the LNCaP cell line and the PC-82 xenograft of prostate cancer. The overlapping region of deletion in LNCaP and PC-82 spans 3.1 cM or 2.9 cR, which is equivalent to 1–3 Mb. The endothelin receptor B gene, a possible tumor suppressor gene at 13q21, was not located in the region of deletion. Among the 13 prostate neoplasms with deletion at 13q21, 5 were metastases, and 7 were poorly differentiated primary tumors. The only primary tumor that was not poorly differentiated but had deletion occurred in one of the youngest patients (49 years) at diagnosis. These results provide evidence that 13q21 may harbor an unidentified gene(s) whose inactivation occurs in some aggressive carcinomas of the prostate. In addition, this study provides a framework for the cloning and identification of the 13q21 gene(s).

INTRODUCTION

Molecular determinants important in the development and progression of prostate cancer are poorly understood, despite the fact that this neoplasm has become a significant health problem (1). Cytogenetic and molecular genetic analyses have indicated that interstitial deletions on chromosomes 8p, 13q, 10q, 6q, 7q, 17q, and 18q occur frequently in human prostate cancer, suggesting the existence of tumor suppressor genes on these chromosomal arms (2, 3). At present, however, only 10q has been identified as having a tumor suppressor gene (*i.e., PTEN*) that has been implicated in prostate cancer (4, 5). The target genes from the remaining chromosomes have yet to be mapped and identified.

Deletion of portions of chromosome 13 has been detected by various genetic approaches in human prostate cancer. In a cytogenetic banding study, nonrandom loss of chromosome 13 was observed in a xenografted cell line (6). CGH[3] demonstrated that loss of 13q is the second most frequent chromosomal alteration, having occurred in 32% of primary tumors, 56–75% of recurrent and metastatic tumors, and each of the four commonly used prostate cancer cell lines derived from metastatic prostate cancer (7–11). One CGH study suggested a deletion region at the q21 band of chromosome 13 (10). In our LOH assay, we identified a distinct region of LOH in a 7-cM DNA segment involving markers D13S269 and D13S162 at 13q21 (12). These studies suggested that a tumor suppressor gene is located at 13q21; however, the DNA segment containing this gene was still too large for its identification.

To fine map the region of deletion and to evaluate the clinical significance of 13q21 deletion in prostate cancer, we analyzed a number of STS markers at 13q21 for genetic deletions in prostate cancer, using the approaches of tissue microdissection and duplex PCR. The minimal region of the deletion was confined to a DNA fragment of 3.1 cM or 2.9 cR (1–3 Mb), and the deletion at 13q21 appeared to be associated with tumor aggressiveness.

MATERIALS AND METHODS

Tumor Specimens, Cell Lines, and Xenograft. A total of 147 prostate cancer tissues from 125 patients were examined for deletion in this study. Of them, 103 were primary tumors, 6 were lymph node metastases obtained at surgery, and 38 were either primary tumors (12 specimens) or metastases (26 specimens) from various organ sites obtained at autopsy from 16 patients who died of prostate cancer. Among the 103 primary tumors from surgery, one was a well-differentiated tumor (Gleason score, 4), 19 were moderately differentiated cancers (Gleason score, 5 or 6), 40 were moderately poorly differentiated tumors (Gleason score, 7), and 43 were poorly differentiated neoplasms with Gleason scores of 8–10. Each of the primary tumors from autopsy was a high-grade cancer. Patient age ranged from 42–88 years. Tumor tissues were zinc formalin-fixed and paraffin-embedded, and the cells for DNA isolation were collected from 7-μm H&E-stained sections using a previously described protocol for preparation of histological sections on glass slides before microdissection (13), which ensured a minimum of 70% neoplastic cells. Nonneoplastic cells from lymph nodes or seminal vesicles in most of the cases or from normal prostate stroma or urothelium in the remainder of the cases were obtained from paraffin blocks that contained no neoplastic cells.

Prostate cancer cell lines LNCaP, PC-3, DU 145, and TSU-Pr1 were purchased from American Type Culture Collection (Manassas, VA) and propagated following the manufacturer's instructions. The PC-82 prostate cancer xenograft was described previously (14).

DNA Preparation. For most of the tumor specimens, DNA was isolated from microdissected cells by adding proteinase K solution and incubating at 55°C overnight, followed by boiling the solution for 10 min to inactivate proteinase K, as described previously (12). One μl of DNA sample was used in each PCR. For tumor specimens including all of the autopsy cases where greater amount of tissues were available, phenol/chloroform extraction and ethanol precipitation were performed after the proteinase K treatment. For these samples, 5–50 ng of DNA were used for each PCR. For the cell lines and the PC-82 xenograft, genomic DNA was isolated by using the Wizard Genomic DNA Purification Kit (Promega, Madison, WI).

STS Markers. We initially analyzed the two mapped genetic markers, *i.e.*, D13S269 and D13S162, that are located in the 13q21 LOH region in our previous study (12). After deletion for either of these markers was detected, additional markers that flank the deleted ones were analyzed to further define the segment with deletion. Selection of additional markers was based on the latest version of the integrated human genomic map (15),[4] which is available on line from the Whitehead Institute/Massachusetts Institute of Technology Center for Genome Research, Human Genetic Mapping Project and from the high-resolution yeast artificial chromosome-cosmid-STS map of human chromosome 13 (16). Primer sequences of these markers are available from the Genome Database.[5] The endothelin receptor B (*EDNRB*) gene, which is close to but telomeric to marker D13S162 at 13q21 (17), was also examined using a STS marker derived from its fourth exon (18). Primer sequences for exon 4 of the *EDNRB* gene are 5′-ATCCCTATAGTTTTACAAGACAGC-3′ (for-

Received 12/8/99; accepted 5/10/00.

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[1] Supported in part by Grant DAMD 17-98-1-8636 from the United States Army Prostate Cancer Research Program and by NIH Grant 1 R01 CA87921 from the National Cancer Institute.

[2] To whom requests for reprints should be addressed, at Department of Pathology, University of Virginia Health System, P. O. Box 800214, Charlottesville, VA 22908-0214. Phone: (804) 924-9011; Fax: (804) 924-9206; E-mail: jd4q@virginia.edu.

[3] The abbreviations used are: CGH, comparative genomic hybridization; LOH, loss of heterozygosity; STS, sequence tagged site.

[4] Supplementary data from the Whitehead Institute/Massachusetts Institute of Technology Center for Genome Research, Human Genetic Mapping Project (http://www-genome.wi.mit.edu).

[5] http://gdbwww.gdb.org.

ward) and 5′-ATTTTCTTACCTGCTTTAG GTG-3′ (reverse). PCR primers were either purchased from Research Genetics (Huntsville, AL) or synthesized by Life Technologies, Inc. (Gaithersburg, MD).

In addition to 13q21 markers, each PCR contained one internal control STS marker, *i.e.*, one of the exons from the KAI1 gene whose deletion has not been found in prostate cancer (19, 20). Internal controls were necessary for reliable detection of chromosomal deletions. Depending on the size of PCR products of a 13q21 marker, exon 5, 7, or 8 of the KAI1 gene was used. The primer sequences of these KAI1 exons have been described previously (19). Sizes of PCR products, annealing temperatures, KAI1 control exons, genetic and physical maps, and deletion status in LNCaP and PC-82 cells for the 13q21 markers are listed in Table 1.

Deletion Analysis. The duplex PCR approach was used for the deletion detection. Each PCR, which was in a volume of 10 μl, contained 1 μl of genomic DNA, 1× PCR buffer [20 mM Tris-HCl (pH 8.8), 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$, 0.1% Triton X-100, and 0.1 mg/ml BSA], 0.4 μM of each primer, 20 μM of each deoxynucleotide triphosphate, 1 μCi of [α-^{33}P]dATP (3000 Ci/mmol; ICN, Irvine, CA), 0.6 unit of platinum Taq antibodies (Life Technologies, Inc.), and 0.6 unit of Taq DNA polymerase. After an initial denaturation at 95°C for 5 min, 30 cycles, each consisting of denaturation at 94°C for 45 s, annealing for 45 s, and extension at 72°C for 1 min, were performed.

PCR products were separated in 5% nondenaturing polyacrylamide gels (size, 20 × 20 cm) at 200 V for 1–2 h. The gels were dried and exposed to Kodak Biomax MR film at room temperature overnight. Absence of a PCR product for a 13q21 marker in a tumor defined a deletion. However, the microdissected tumor samples were sometimes contaminated with nonneoplastic cells sufficient to give rise to PCR products. When this occurred, signal intensities for PCR products were quantitatively measured by scanning and analyzing PCR bands from a film using ScanDNASIS software (Hitachi Software, San Bruno, CA); the ratio of signal intensity of a 13q21 marker to that of the internal control marker was calculated for each DNA sample, and a deletion was considered to be present when such a ratio in a tumor was less than half of that in its matched nonneoplastic cells.

All experiments were repeated one to three times, and the deletions were detected in each of the experiments.

RESULTS

Based on the multiplex PCR method used for the detection of homozygous deletion in our previous study (21), we first adjusted the experimental procedures to maximize the sensitivity and consistency in detecting genetic deletion. Compared with regular PCR and agarose gel electrophoresis, we found that the procedure of radioactive PCR with [α-^{33}P]dATP, use of the hot-start approach by adding Taq antibodies, separation of PCR products with nondenaturing PAGE, and exposure of gels to Kodak Biomax MR film was more consistent, quantitative, and sensitive in demonstrating genetic deletions in tumor samples.

We first analyzed the two markers (*i.e.*, D13S269 and D13S162) that were located in the LOH region at 13q21, based on our previous study (12), in each of the 147 tumor specimens using the improved method of duplex PCR assay (Table 1). Some tumors showed absent or reduced band intensities for D13S269 and/or D13S162 compared with that of the internal control marker and with that of a normal control. We then repeated the PCR for these tumors, along with their matched nonneoplastic cells. As shown in Fig. 1 for some specimens, deletions at D13S269 and D13S162, which could be either homozygous deletions or hemizygous deletions, were repeatedly detected in 13 tumor specimens. Whereas 11 of 13 tumors lost both D13S269 and D13S162, 2 tumors lost D13S269 only (Table 2).

In total, 13 of 147 (9%) prostate cancer samples showed deletion at 13q21. Patient age at diagnosis and Gleason score for these neoplasms are shown in Table 2. Among these tumors, five were metastases, and seven were poorly differentiated primary tumors. The only primary tumor that was not poorly differentiated but had a deletion (case 233) occurred in one of the youngest patients (49 years) at diagnosis.

To determine whether deletion at 13q21 also occurred in prostate cancer cell lines and xenograft, we analyzed D13S269 and D13S162 for deletion using the same duplex PCR method. As shown in Table 1 and Fig. 2, whereas three cell lines did not show any deletions, the LNCaP cell line and the PC-82 xenograft showed a significant signal reduction at D13S269 and D13S162. Measurement of signal intensities using the ScanDNASIS program indicated that the signal ratio of the deleted marker to the internal control in LNCaP cells was only about one-fourth of that in the normal placenta control DNA. Deletion analysis of cell lines and xenograft was also repeated using regular PCR and agarose gel electrophoresis, and deletion of D13S269 and D13S162 was demonstrated in each experiment (data not shown).

Table 1 *Genetic and radiation hybrid (RH) maps, sizes of PCR products, annealing temperatures, internal KAI1 control markers, and deletion status in LNCaP and PC-82 cells for the 13q21 markers*

Marker names[a]	Genetic map (cM)	RH map (cR)	PCR product (bp)	Annealing temperature (°C)	KAI1 exons	LNCaP	PC-82
D13S1273	49.4		115	55	7	+	+
D13S1260	50.7		141	57	7	+	+
D13S1317	51.9		223	57	8	+	+
D13S275	52.7		239	55	7	−	+
D13S1310	53.5		143	55	7	−	+
D13S1318	54.0		259	57	7	−	+
WI-5860		191.5	224	57	8	−	+
WI-3660			132	61	7	−	+
D13S1090E[b]	56.6		107	61	7	−	+
D13S152	56.6		138	55	7	−	−
D13S745			189	57	7	−	−
D13S791			294	61	7	−	−
D13S1249	57.3		220	60	8	−	−
D13S166	57.3		115–125	57	7	−	−
D13S156	57.3	193.2	272–286	60	7	−	−
D13S269	58.3		142	60	7	−	−
H65656	59.7	194.4	~150	61	7	−	−
D13S162	59.7	194.4	182–202	55	7	−	−
BS610/611[b]	59.7	194.4	95	57	7	+	−
D13S1562			148	60	7	+	−
WI-16413			132	60	7	+	−
D13S160	62.7	207.7	229–241	57	8	+	+
EDNRB			170	60	5	+	
D13S170	65.4		113	57	7	+	+

[a] The markers are listed in order from centromere to telomere according to the published genomic maps (15, 16).[4]

[b] Markers D13S1090E and D13S152 are located in one BAC clone and are thus considered to be at the same genetic location (data not shown). Similarly, markers BS610/611, H65656, and D13S162 are in another BAC clone. Primers sequences for marker BS610/611 are 5′-TATTCATCCAGCCCCCTCAATG-3′ and 5′-AGATGTGCAGGAGATGAATGGC-3′.

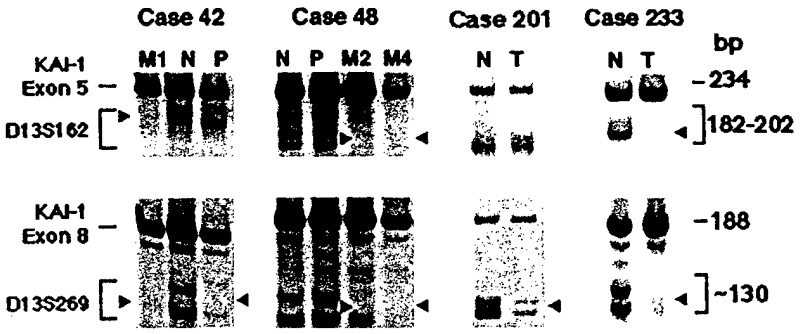

Fig. 1. Detection of deletion at 13q21 in prostate cancer by duplex PCR assay. Case number and tissue type are indicated at the *top*, STS markers are indicated at the *left*, and the size of the PCR products is indicated at the *right*. Each *arrow* denotes a deletion at a marker in a neoplasm. *Lanes P* and *M*, primary tumor and metastasis obtained from autopsy specimens; *Lane T*, primary tumor obtained at surgery; *Lane N*, matched nonneoplastic cells in each case. Due to the polymorphic feature of microsatellite markers, two bands that represent two alleles are seen for both markers in most cases. Case 42 (*Lane P*) appears to have LOH instead of deletion at D13S162.

To determine the size of the region with deletion, we selected more STS markers (Table 1) that flank D13S269 and/or D13S162 at 13q21 and examined them in LNCaP and PC-82 tumor cells that showed deletions at D13S269 and D13S162 and had a sufficient quantity of pure tumor DNA for analysis (Table 1; Fig. 2). The deletion region in LNCaP was different from but overlapped that in PC-82, and the common region of deletion was defined as a DNA segment of 3.1 cM within markers D13S1090E and BS610/611 or 2.9 cR within markers WI-5860 and BS610/611 (Table 1). According to the current genomic maps (15, 16), the size of this common region of deletion was estimated to be 1–3 Mb. The endothelin receptor B (*EDNRB*) gene, which is located at least 3 cM telomeric to the common region of deletion at 13q21 and has been suggested as a tumor suppressor gene, was also analyzed in five tumors and in all of the cell lines but showed no deletion (data not shown).

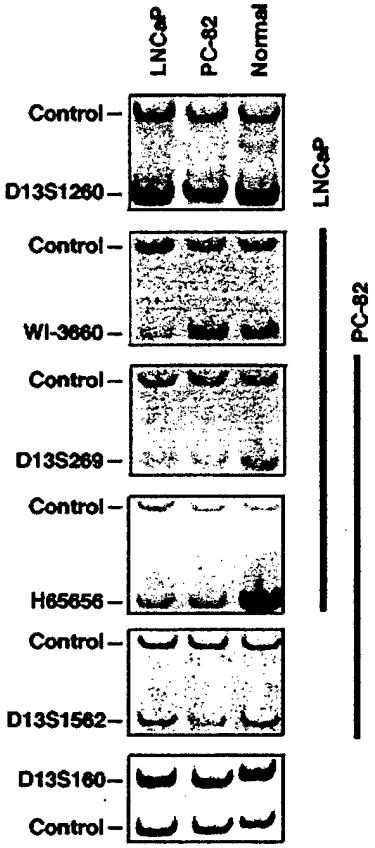

Fig. 2. Deletions of 13q21 markers in LNCaP cell line and PC-82 xenograft of prostate cancer. Sample names are indicated at the *top*, and marker names are indicated at the *left*. Markers deleted are indicated by *vertical bars* at the *right*. Normal DNA was from a normal human placenta.

DISCUSSION

In this study, we first improved the multiplex PCR procedure used in our previous study (21) for the detection of genetic deletion in human tumors. Use of radioactive PCR with a hot-start approach, PAGE, and exposure to Kodak Biomax MR film made deletion detection more reliable when compared with regular PCR and agarose gel electrophoresis. As shown in Fig. 1, some tumor samples had no signal or a very faint signal at the deleted markers D13S269 and D13S162 compared with their matched nonneoplastic cells. Detection of deletion in the LNCaP cell line but not in any other cell lines further indicated the feasibility of our methods because a high-resolution cytogenetic banding study showed that each LNCaP cell has an interstitial deletion at 13q21 in some but not all of its copies of chromosome 13 [it has a near tetraploid karyotype (22)].

Genetic deletion at 13q21 in prostate cancer was first demonstrated by a cytogenetic banding study in the LNCaP cell line (22). In CGH analyses, deletion of 13q was detected in human prostate cancer tissues and in each of the four commonly used prostate cancer cell lines (7–11), and a common region of deletion was suggested to be located at 13q21 (10). Recently, our LOH study further showed that the deletion at 13q21 involved markers D13S313, D13S269, D13S162, and D13S1306 in a DNA interval of 7 cM (12). Using the methods of tissue microdissection and an improved duplex PCR assay, we found that genetic deletions at 13q21 occurred in 13 of 147 (9%) prostate cancer specimens and 2 of 5 (40%) prostate cancer cell lines and xenograft. Consistent with previous studies, our data provide additional evidence for the existence of a tumor suppressor gene(s) at 13q21.

Although previous studies identified a region of deletion at 13q21 in prostate cancer, the size of the region with deletion was still too large for gene identification, and the precise location of the target gene remained to be defined. Using densely mapped genetic markers and the LNCaP cell line and PC-82 xenograft of prostate cancer, we defined the common region of deletion to be in a 3.1-cM segment within markers D13S1090E

Table 2 *Prostate cancers showing deletions at D13S269 and D13S162 and patient age at diagnosis and Gleason score (G.S.). "M" indicates a metastasis*

Presence or absence of a marker in a tumor is indicated by + or −, respectively.

Case no.	Age (yrs)	G.S.	D13S269	D13S162
42-P	68	9	−	+
42-M1	68	M	−	−
48-M2	71	M	−	−
48-M4	71	M	−	−
53-P	67	9	−	−
53-M3	67	M	−	−
104-T	75	9	−	−
110-T	65	9	−	−
122-M	68	M	−	−
200-T	71	8	−	−
201-T	80	10	−	+
228-T	69	9	−	−
233-T	49	7	−	−

and BS610/611 and a 2.9-cR segment between WI-5860 and BS610/611 (Table 1). Considering that, on average, 1 cM is equivalent to 1 Mb and 1 Mb is equivalent to 3.7 cR for the Genebridge4 Radiation Hybrid Panel,[6] our findings indicate that the minimal region of deletion containing the target gene should be in the size range of 1–3 Mb.

Twelve of the 13 prostate cancer samples with homozygous deletion at 13q21 were either poorly differentiated primary tumors or metastases. The only primary tumor that was not poorly differentiated but had a deletion occurred in one of the youngest patients (49 years) at diagnosis (Table 2). The LNCaP cell line and the PC-82 xenograft, which were noted above to have a deletion at the same region of 13q21, were also derived from metastases of prostate cancer (23). These data indicate that deletion at 13q21 occurs in biologically aggressive prostate cancers. Consistently, a previous study found that patients whose prostate cancers showed LOH at 13q were diagnosed at a significantly younger age than those whose tumors lacked LOH at 13q (12).

The endothelin receptor B (*EDNRB*) gene is located at 13q21, telomeric to marker D13S162 but centromeric to marker D13S160 (17). Considering that promoter methylation of the *EDNRB* gene occurs frequently in prostate cancer and that this gene has been suggested to act as a tumor suppressor (24), we analyzed its deletion status in five tumors that showed deletion at D13S269 and D13S162 and in all of the prostate cancer cell lines. No deletion at the *EDNRB* gene was detected. Based on the current genomic map, *EDNRB* is at least 3 cM telomeric to the common region of deletion. Therefore, *EDNRB* is not the target gene for the 13q21 deletion region in prostate cancer. Currently, there is no gene that is located in the region of deletion that has been identified as a tumor suppressor. This study provides a framework for the identification of this gene.

Genetic deletion involving 13q21 has also been detected by CGH in malignant fibrous histiocytomas (25, 26) and other sarcomas (27, 28), and gliomas (29, 30). These studies suggest that different types of tumors may share the same genetic alteration at 13q21 during carcinogenesis or progression.

REFERENCES

1. Landis, S. H., Murray, T., Bolden, S., and Wingo, P. A. Cancer statistics, 1999. CA Cancer J. Clin., *49:* 8–31, 1999.
2. Isaacs, W. B., and Bova, G. S. Prostate cancer. *In:* B. Vogelstein and K. W. Kinzler (eds.), The Genetic Basis of Human Cancer, pp. 653–660. New York: McGraw-Hill, Inc., 1997.
3. Brothman, A. R., Maxwell, T. M., Cui, J., Deubler, D. A., and Zhu, X. L. Chromosomal clues to the development of prostate tumors. Prostate, *38:* 303–312, 1999.
4. Li, J., Yen, C., Liaw, D., Podsypanina, K., Bose, S., Wang, S. I., Puc, J., Miliaresis, C., Rodgers, L., McCombie, R., Bigner, S. H., Giovanella, B. C., Ittmann, M., Tycko, B., Hibshoosh, H., Wigler, M. H., and Parsons, R. *PTEN*, a putative protein tyrosine phosphatase gene mutated in human brain, breast, and prostate cancer. Science (Washington DC), *275:* 1943–1947, 1997.
5. Vlietstra, R. J., van Alewijk, D. C., Hermans, K. G., van Steenbrugge, G. J., and Trapman, J. Frequent inactivation of PTEN in prostate cancer cell lines and xenografts. Cancer Res., *58:* 2720–2723, 1998.
6. Pittman, S., Russell, P. J., Jelbart, M. E., Wass, J., and Raghavan, D. Flow cytometric and karyotypic analysis of a primary small cell carcinoma of the prostate: a xenografted cell line. Cancer Genet. Cytogenet., *26:* 165–169, 1987.
7. Cher, M. L., MacGrogan, D., Bookstein, R., Brown, J. A., Jenkins, R. B., and Jensen, R. H. Comparative genomic hybridization, allelic imbalance, and fluorescence *in situ* hybridization on chromosome 8 in prostate cancer. Genes Chromosomes Cancer, *11:* 153–162, 1994.
8. Cher, M. L., Bova, G. S., Moore, D. H., Small, E. J., Carroll, P. R., Pin, S. S., Epstein, J. I., Isaacs, W. B., and Jensen, R. H. Genetic alterations in untreated metastases and androgen-independent prostate cancer detected by comparative genomic hybridization and allelotyping. Cancer Res., *56:* 3091–3102, 1996.
9. Visakorpi, T., Kallioniemi, A. H., Syvanen, A. C., Hyytinen, E. R., Karhu, R., Tammela, T., Isola, J. J., and Kallioniemi, O. P. Genetic changes in primary and recurrent prostate cancer by comparative genomic hybridization. Cancer Res., *55:* 342–347, 1995.
10. Nupponen, N. N., Hyytinen, E. R., Kallioniemi, A. H., and Visakorpi, T. Genetic alterations in prostate cancer cell lines detected by comparative genomic hybridization. Cancer Genet. Cytogenet., *101:* 53–57, 1998.
11. Nupponen, N. N., Kakkola, L., Koivisto, P., and Visakorpi, T. Genetic alterations in hormone-refractory recurrent prostate carcinomas. Am. J. Pathol., *153:* 141–148, 1998.
12. Hyytinen, E. R., Frierson, H. F., Boyd, J. C., Chung, L. W. K., and Dong, J. T. Three distinct regions of allelic loss at 13q14, 13q21–22, and 13q33 in prostate cancer. Genes Chromosomes Cancer, *25:* 108–114, 1999.
13. Moskaluk, C. A., and Kern, S. E. Microdissection and polymerase chain reaction amplification of genomic DNA from histological tissue sections. Am. J. Pathol., *150:* 1547–1552, 1997.
14. Gao, J., and Isaacs, J. T. Development of an androgen receptor-null model for identifying the initiation site for androgen stimulation of proliferation and suppression of programmed (apoptotic) death of PC-82 human prostate cancer cells. Cancer Res., *58:* 3299–3306, 1998.
15. Hudson, T. J., Stein, L. D., Gerety, S. S., Ma, J., Castle, A. B., Silva, J., Slonim, D. K., Baptista, R., Kruglyak, L., Xu, S. H., Hu, X., Colbert, A. M. E., Rosenberg, C., Reeve-Daly, M. P., Rozen, S., Hui, L., Wu, X., Vestergaard, C., Wilson, K. M., Bae, J. S., Maitra, S., Ganiatsas, S., Evans, C. A., DeAngelis, M. M., Ingalls, K. A., Nahf, R. W., Horton, L. T., Anderson, M. O., Collymore, A. J., Ye, W., Kouyoumjian, V., Zemsteva, I. S., Tam, J., Devine, R., Courtney, D. F., Renaud, T. M., Nguyen, H., O'Connor, T. J., Fizames, C., Faure, S., Gyapay, G., Dib, C., Morissette, J., Orlin, J. B., Birren, B. W., Goodman, N., Weissenbach, J., Hawkins, T. L., Foote, S., Page, D. C., and Lander, E. S. An STS-based map of the human genome. Science (Washington DC), *270:* 1945–1954, 1995.
16. Cayanis, E., Russo, J. J., Kalachikov, S., Ye, X., Park, S. H., Sunjevaric, I., Bonaldo, M. F., Lawton, L., Venkatraj, V. S., Schon, E., Soares, M. B., Rothstein, R., Warburton, D., Edelman, I. S., Zhang, P., Efstratiadis, A., and Fischer, S. G. High-resolution YAC-cosmid-STS map of human chromosome 13. Genomics, *47:* 26–43, 1998.
17. Deloukas, P., Schuler, G. D., Gyapay, G., Beasley, E. M., Soderlund, C., Rodriguez-Tome, P., Hui, L., Matise, T. C., McKusick, K. B., Beckmann, J. S., Bentolila, S., Bihoreau, M., Birren, B. B., Browne, J., Butler, A., Castle, A. B., Chiannilkulchai, N., Clee, C., Day, P. J., Dehejia, A., Dibling, T., Drouot, N., Duprat, S., Fizames, C., Fox, S., Gelling, S., Green, L., Harrison, P., Hocking, R., Holloway, E., Hunt. S., Keil, S., Lijnzaad, P., Louis-Dit-Sully, C., Ma, J., Mendis, A., Miller, J., Morissette, J., Muselet, D., Nusbaum, H. C., Peck, A., Rozen, S., Simon, D., Slonim, D. K., Staples, R., Stein, L. D., Stewart, E. A., Suchard, M. A., Thangarajah, T., Vega-Czarny, N., Webber, C., Wu, X., Hudson, J., Auffray, C., Nomura, N., Sikela, J. M., Polymeropoulos, M. H., James, M. R., Lander, E. S., Hudson, T. J., Myers, R. M., Cox, D. R., Weissenbach, J., Boguski, M. S., and Bentley, D. R. A physical map of 30,000 human genes. Science (Washington DC), *282:* 744–746, 1998.
18. Puffenberger, E. G., Hosoda, K., Washington, S. S., Nakao, K., deWit, D., Yanagisawa, M., and Chakravart, A. A missense mutation of the endothelin-B receptor gene in multigenic Hirschsprung's disease. Cell, *79:* 1257–1266, 1994.
19. Dong, J. T., Isaacs, W. B., Barrett, J. C., and Isaacs, J. T. Genomic organization of the human KAI1 metastasis-suppressor gene. Genomics, *41:* 25–32, 1997.
20. Dong, J. T., Suzuki, H., Pin, S. S., Bova, G. S., Schalken, J. A., Isaacs, W. B., Barrett, J. C., and Isaacs, J. T. Down-regulation of the KAI1 metastasis suppressor gene during the progression of human prostatic cancer infrequently involves gene mutation or allelic loss. Cancer Res., *56:* 4387–4390, 1996.
21. Dong, J. T., Sipė, T. W., Hyytinen, E. R., Li, C. L., Heise, C., McClintock, D. E., Grant, C. D., Chung, L. W., and Frierson, H. F., Jr. PTEN/MMAC1 is infrequently mutated in pT2 and pT3 carcinomas of the prostate. Oncogene, *17:* 1979–1982, 1998.
22. Gibas, Z., Becher, R., Kawinski, E., Horoszewicz, J., and Sandberg, A. A. A high-resolution study of chromosome changes in a human prostatic carcinoma cell line (LNCaP). Cancer Genet. Cytogenet., *11:* 399–404, 1984.
23. Horoszewicz, J. S., Leong, S. S., Chu, T. M., Wajsman, Z. L., Friedman, M., Papsidero, L., Kim, U., Chai, L. S., Kakati, S., Arya, S. K., and Sandberg, A. A. The LNCaP cell line: a new model for studies on human prostatic carcinoma. *In:* G. P. Murphy (ed.), Models for Prostate Cancer, pp. 115–132. New York: Alan R, Liss, Inc., 1980.
24. Nelson, J. B., Lee, W. H., Nguyen, S. H., Jarrard, D. F., Brooks, J. D., Magnuson, S. R., Opgenorth, T. J., Nelson, W. G., and Bova, G. S. Methylation of the 5′ CpG island of the endothelin B receptor gene is common in human prostate cancer. Cancer Res., *57:* 35–37, 1997.
25. Larramendy, M. L., Tarkkanen, M., Blomqvist, C., Virolainen, M., Wiklund, T., Asko-Seljavaara, S., Elomaa, I., and Knuutila, S. Comparative genomic hybridization of malignant fibrous histiocytoma reveals a novel prognostic marker. Am. J. Pathol., *151:* 1153–1161, 1997.
26. Mairal, A., Terrier, P., Chibon, F., Sastre, X., Lecesne, A., and Aurias, A. Loss of chromosome 13 is the most frequent genomic imbalance in malignant fibrous histiocytomas. A comparative genomic hybridization analysis of a series of 30 cases. Cancer Genet. Cytogenet., *111:* 134–138, 1999.
27. Szymanska, J., Tarkkanen, M., Wiklund, T., Virolainen, M., Blomqvist, C., Asko-Seljavaara, S., Tukiainen, E., Elomaa, I., and Knuutila, S. Gains and losses of DNA sequences in liposarcomas evaluated by comparative genomic hybridization. Genes Chromosomes Cancer, *15:* 89–94, 1996.
28. Szymanska, J., Serra, M., Skytting, B., Larsson, O., Virolainen, M., Akerman, M., Tarkkanen, M., Huuhtanen, R., Picci, P., Bacchini, P., Asko-Seljavaara, S., Elomaa, I., and Knuutila, S. Genetic imbalances in 67 synovial sarcomas evaluated by comparative genomic hybridization. Genes Chromosomes Cancer, *23:* 213–219, 1998.
29. Kim, D. H., Mohapatra, G., Bollen, A., Waldman, F. M., and Feuerstein, B. G. Chromosomal abnormalities in glioblastoma multiforme tumors and glioma cell lines detected by comparative genomic hybridization. Int. J. Cancer, *60:* 812–819, 1995.
30. Nishizaki, T., Ozaki, S., Harada, K., Ito, H., Arai, H., Beppu, T., and Sasaki, K. Investigation of genetic alterations associated with the grade of astrocytic tumor by comparative genomic hybridization. Genes Chromosomes Cancer, *21:* 340–346, 1998.

[6] http://carbon.wi.mit.edu:8000/ftp/distribution/human_STS_releases/july97/07-97.INTRO.html.

Mutations of *PTEN/MMAC1* in Primary Prostate Cancers from Chinese Patients[1]

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ABSTRACT

***PTEN/MMAC1* is a putative tumor suppressor gene located on 10q23, one of the most frequently deleted chromosomal regions in human prostate cancer. Although mutations of *PTEN* have often been detected in metastases of prostate cancer, localized tumors have shown lower rates of mutation, which have varied from 0 to 20% among different studies. It is unknown whether the rate of *PTEN* mutations is different in prostate cancer from Asian men compared with Western men. To further clarify the role of *PTEN* in prostate cancer and to examine the gene for mutations in Asian men, we analyzed 32 cases of primary prostate cancers from Chinese patients, each of whom was not diagnosed by screening with serum prostate-specific antigen, for *PTEN* mutations using the methods of tissue microdissection, single-strand conformational polymorphism, and direct DNA sequencing. Seventy % of the tumors were Gleason scores 8–10, whereas the remainder were Gleason score 7. Six metastases of prostate cancer from American patients were also analyzed. Five of 32 (16%) primary prostate cancers from Chinese men and two of six metastases from American men showed mutations in a total of 10 codons of *PTEN*, which involved exons 1, 2, 5, 8, and 9. Two of the mutations were truncation type, whereas the rest were missense mutations. The mutation frequency in these cases from Asian patients was higher than that in our previous study of cases in radical prostatectomy specimens from American men, in which the 40 primary tumors were lower grade and had been detected by serum prostate-specific antigen test. We conclude that mutation of *PTEN* occurs more often in primary prostate cancers of Chinese men, whose tumors are high grade and reflective of an unscreened population.**

INTRODUCTION

A candidate tumor suppressor gene designated *PTEN*, *MMAC1*, or *TEP-1* (referred to as *PTEN* hereafter) was identified (1–3) from the q23.3 region of chromosome 10, one of the most frequently deleted regions in prostate cancer (4). The *PTEN* gene has nine exons that encode a 403-amino acid protein of a dual-specific phosphatase with putative actin-binding and tyrosine phosphatase domains. Introduction of PTEN into cancer cells that lack *PTEN* function ~~negatively regulates~~ cell migration and ~~survival~~ inducing cell cycle arrest and apoptosis via negative regulation of the phosphatidylinositol 3′-kinase/protein kinase B/Akt signaling pathway (5–7). Mutation and down-regulation of the *PTEN* gene have been detected in various human cancers including that of the prostate (8–10). In addition, germ-line mutations in *PTEN* are associated with Cowden disease (11), in which patients are at increased risk for certain cancers.

Thus far, *PTEN* appears to be the most frequently mutated gene in metastases of prostate cancer, occurring in at least 1 metastatic site in 12 of 19 (63%) patients who had multiple metastases (12) and in 9 of 15 (60%) cell lines and xenografts primarily derived from metastases of prostate cancer (13). These results indicate a role for *PTEN* in the progression of prostate cancer. Mutations of *PTEN* in localized prostate cancers have been found at lower frequencies including 1 of 28 (4%; Ref. 14), 1 of 25 (4%; Ref. 15), 1 of 40 (2.5%; Ref. 16), 0 of 45 (17), and 1 of 22 (5%; Ref. 18). Somewhat higher rates of mutations have been observed in other studies including 10 of 80 [12.5%; 10 of 23 (43%) in cases with loss of heterozygosity at *PTEN*; Ref. 19], 5 of 37 (13.5%; Ref. 20), 8 of 60 (13%; Ref. 21), and 1 of 10 (10%; Ref. 9). In hereditary prostate cancer, the role of *PTEN* has not been detected (22, 23).

The incidence of prostate cancer is lower in Asian men compared with Western men, but the specific genetic or environmental factors that are important are unknown (24, 25). Obviously, more cancers are detected in Western men because of screening with serum PSA[3] test. The frequency of PTEN mutations in prostate cancer from Asian men has been little studied. One study of 45 primary prostate cancers from Japanese patients did not detect any *PTEN* mutation (17). In this study, we analyzed primary prostate cancers from 32 Chinese patients, who were not diagnosed using the PSA test. Rather, they were diagnosed after showing clinical symptoms. We also analyzed six metastases from American patients who died of prostate

Received 8/23/00; revised 11/27/00; accepted 11/28/00.
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[1] This study was supported in part by Grant DAMD 17-98-1-8636 from the United States Army Prostate Cancer Research Program and by Grants 1R01CA85560 and 1R01CA87921 from the National Cancer Institute, NIH.
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[3] The abbreviations used are: PSA, prostate-specific antigen; SSCP, single-strand conformational polymorphism.

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cancer to document additional PTEN mutations in fatal prostatic disease.

MATERIALS AND METHODS

Tumor Samples. Thirty-two formalin-fixed, paraffin-embedded prostate cancer specimens from radical prostatectomy from previously untreated Chinese patients were used in this study. These patients went to physicians after showing various symptoms of prostate cancer, *e.g.*, difficulty in voiding, urodynia, urgent and frequent urination, and hematuria. None of them were involved in PSA screening. Their prostates were examined by one or more of the following means: rectal ultrasound detection, digital rectal examination, computed tomography, and magnetic resonance imaging. Biopsy was performed for the patients who were suspected to have prostate cancer, and only those whose cancers were at stages B–C underwent radical prostatectomy. The prostatectomies were performed by four surgeons over a period of 5 years. All specimens were from archived paraffin blocks that had been used in routine diagnosis of cancer, and none of them were collected specifically for this study. In addition, DNA was available from six distant metastases from American patients who died of prostate cancer. The clinicopathological characteristics of the tumors are listed in Table 1. The exact tumor stage for the Chinese patients was not available. Tumor cells for DNA isolation were collected from 7 μm H&E-stained sections by microdissection using a protocol described previously (26), which typically ensured a minimum of 70% neoplastic cells for each sample. Nonneoplastic cells collected were present on the same slides as cancer cells and included stromal cells, lymphocytes, and urothelium; in most cases, they did not include nonneoplastic prostatic epithelium. For the cases of metastases, nonneoplastic cells were collected from lymph nodes or seminal vesicles. Use of the human specimens in this study was approved by the institutional human investigation committee.

PCR-SSCP Analysis. Each of the primary prostate cancers was first screened for mutation by using the PCR-SSCP approach. Primers used for each *PTEN* exon were the same as described previously (16). PCRs for the SSCP contained 5–10 ng of genomic DNA, 1× PCR buffer [20 mM Tris-HCl (pH 8.8), 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$, 0.1% Triton X-100, and 0.1 mg/ml BSA], 1 μM of each primer, 3 μM of each deoxynucleotide triphosphate, 1 μCi of [α-^{32}P]dCTP (3000 Ci/mmol), 0.6 unit of Taq DNA polymerase, and 0.1 unit of Pfu DNA polymerase and was incubated at 95°C for 5 min followed by 30 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min. The ^{32}P-labeled PCR products were electrophoresed at 5 W overnight at room temperature in a 6% nondenaturing polyacrylamide gel in 1× TPE buffer (pH 6.8) as described previously (27). PCR products were also analyzed in a 0.25× MDE gel (FMC BioProducts, Rockland, ME) containing 10% glycerol, which was also run at 5 W overnight at room temperature. After electrophoresis, the gels were dried and exposed to X-ray film for 1–2 days. Samples showing a bandshift for a specific exon were reamplified for both tumor DNA and matched nonneoplastic cells to confirm the shift, using the same conditions.

DNA Sequencing. For the samples which repeatedly showed a bandshift in the SSCP analysis, shifted bands were cut and immersed in 20 μl of H_2O, following the protocol described by Kukita *et al.* (27). Two μl of the released DNA were amplified by PCR using the same primers, as in SSCP analysis, in a 50-μl of reaction. The PCR conditions were the same except that 200 μM of each deoxynucleotide triphosphate and no [^{32}P]dCTP were used. These PCR products were purified by using the QIAquick PCR purification kit (Qiagen, Valencia, CA), and were sequenced by using the ThermoSequenase Cycle Sequencing kit (USB) following the manufacturer's instructions. Sequencing data were collected and analyzed by using the ScanDNASIS and MacDNASIS software (Hitachi Software, San Bruno, CA).

For the six metastases of prostate cancer, which tended to be more homogeneous in neoplastic cells, their DNAs were amplified by PCR for each of the *PTEN* exons, and the resultant PCR products were purified and directly sequenced by the same

Table 1 Clinicopathological characteristics of prostate cancer specimens analyzed and mutation status of *PTEN* in each case

Case no.	Patient Age (yr)	Gleason score	*PTEN* mutation
80	NA[a]	7	No
82	52	9	No
83	64	8	No
84	51	10	No
85	NA	10	No
86	61	8	No
89	61	8	Exon 5 (T418C, polymorphism)
90	63	8	No
91	NA	9	Exon 2 (A1197G, R55G)
92	NA	8	Exon 5 (I101A, H35V)
95	60	10	No
96	65	8	No
98	NA	7	No
99	83	7	No
100	75	9	No
101	NA	9	No
102	NA	7	No
103	NA	9	No
104	75	9	No
105	74	9	No
107	83	NA	No
108	71	NA	No
109	NA	10	Exon 5 (Q150G)
110	65	9	No
111	70	7	No
113	67	7	Exon 1 (G20Stop)
114	59	8	Exon 8 (H272Y)
116	66	7	No
117	NA	8	Exon 9 (A1086G, polymorphism)
119	56	9	No
120	NA	7	No
121	66	7	No
42[b]	77	Lymph node	No
46[b]	75	Liver	No
47[b]	70	Lymph node	Exon 9 (A1031G, K344R; C1043T, T348I; A1144T, T382S)
48[b]	73	Lymph node	No
49[b]	75	Lymph node	Exon 5 (C328T, Q110Stop)
51[b]	66	Liver	No

[a] NA, not available.
[b] Lymph node and liver were the organ sites of metastases.

[30 mM Tris, 20 mM PIPES (1,4-piperazinediethanesulfonic acid) and 1 mM Na_2EDTA, pH 6.8]

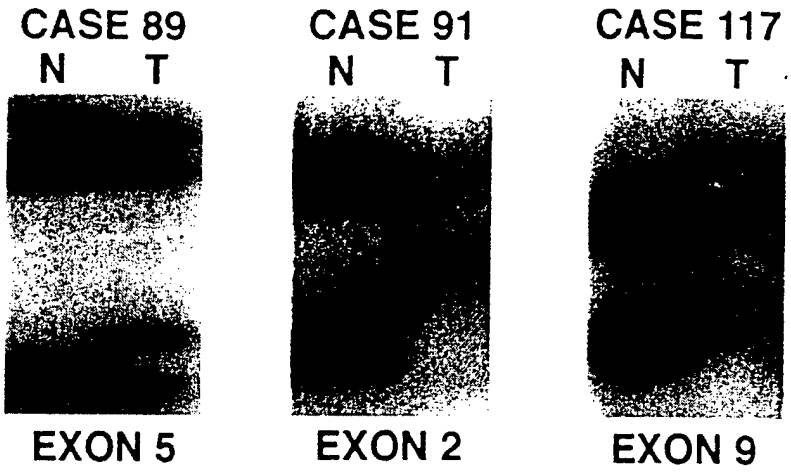

Fig. 1 Examples of SSCP analysis of *PTEN* in prostate cancer specimens. *N* and *T*, nonneoplastic and tumor cells, respectively. For each example, the case number is indicated at the *top*, and exon number is at the *bottom*. Each case has shifted bands in their tumor cells compared with nonneoplastic cells.

procedure as described above. For an exon showing a mutation, the PCR sequencing procedure was repeated to confirm the mutations. Once confirmed, matched normal DNA for a specific exon was also amplified by PCR and sequenced to determine whether a mutation was somatic or germ line.

Statistical Analysis. The difference in the frequency of *PTEN* mutations between primary tumors in the current study and that of our previous study (16) was analyzed statistically by the use of Fisher's exact test (two-tailed; Ref. 28).

RESULTS

Seventy % of the 32 primary prostate cancers from Chinese men were Gleason scores 8–10, whereas 30% were Gleason score 7. PCR-SSCP and direct DNA sequencing analyses of these samples revealed *PTEN* sequence alterations in 7 cases. Examples of bandshifts for tumors in SSCP assay, which indicated the existence of sequence alterations in the *PTEN* gene, are shown in Fig. 1, and examples of DNA sequencing ladders that identify *PTEN* mutations are shown in Fig. 2. Tumor cases and their *PTEN* mutation status are listed in Table 1. Although 2 of the 7 cases had alterations that did not change the PTEN polypeptide, five cases (16%) had mutations that could potentially change PTEN function (Table 1). Case 113 had a nonsense mutation at codon 20 that would truncate the majority of the PTEN protein. Case 92 had two missense mutations in its exon 5, which changed codons 101 and 135 from isoleucine to alanine and valine, respectively. Cases 91, 109, and 114 showed missense mutations that changed codons 55, 150, and 272 from arginine, glutamine, and histidine to glycine, glycine, and tyrosine, respectively.

We also analyzed six metastases of prostate cancer from American men, using the methods of PCR amplification and direct DNA sequencing. Two cases showed *PTEN* mutations. Case 49 had a nonsense mutation at codon 110 in exon 5 that would truncate the PTEN protein, and case 47 had three missense mutations in exon 9 of *PTEN*, changing codon 344 from lysine to arginine, codon 348 from threonine to isoleucine, and codon 382 from threonine to serine.

Each of the above mutations was somatic, as the matched nonneoplastic cells showed no mutations. The difference in the frequency of 16% for *PTEN* mutation in the cancers from Chinese patients compared with the frequency of 2.5% in our prior analysis of 40 resected primary tumors detected in American men after PSA test and biopsy (16) showed a trend in significance ($P = 0.08$).

DISCUSSION

The *PTEN* gene was isolated from the q23 region of chromosome 10, one of the most frequently deleted regions in prostate cancer (4, 29, 30). Mutations of the gene have been detected in various human cancers including that of the prostate (9, 12, 13, 19–21), implicating *PTEN* in the development and/or progression of prostate cancer. It is thus far the most frequently mutated gene in prostate cancer. Our finding of *PTEN* mutations in 5 of 32 primary, high-grade prostate cancer specimens confirms that *PTEN* is a major gene, if not the target gene, for the 10q23 region of deletion in a subset of prostate cancers.

Mutation frequencies of *PTEN* in prostate cancer differ among studies, largely because of differences in tumor grade and stage in the study populations. Mutations up to 60% have been detected in studies of prostate cancer cell lines and xenografts from metastases (13), whereas in some studies of localized disease, few or no mutations have been detected (16, 17). In this study, we detected *PTEN* mutations in 5 of 32 (16%) primary prostate cancers from Chinese patients who were diagnosed with clinical symptoms but without the aid of the serum PSA screening test. This frequency was higher than that (1 of 40 or 2.5%) detected in primary prostate cancers from American patients who were diagnosed by PSA test in our previous study (16). The majority of tumors from the Chinese patients were high grade (Gleason scores 8–10), whereas the majority of tumors in the American patients were lower grade (Gleason scores 5–7), indicating that PTEN mutations occur more often in tumors with high Gleason scores, even in those that are primary lesions. This conclusion is consistent with published studies of primary prostate cancers (15, 17, 20). In one study of 37 primary tumors with 20 (54%) high-grade and 17 (46%) lower grade lesions, five cases, four of which were high-grade tumors, had PTEN mutations (20). In another study of 45 primary tumors that were mainly low-grade cancers [30 (67%) lower grade

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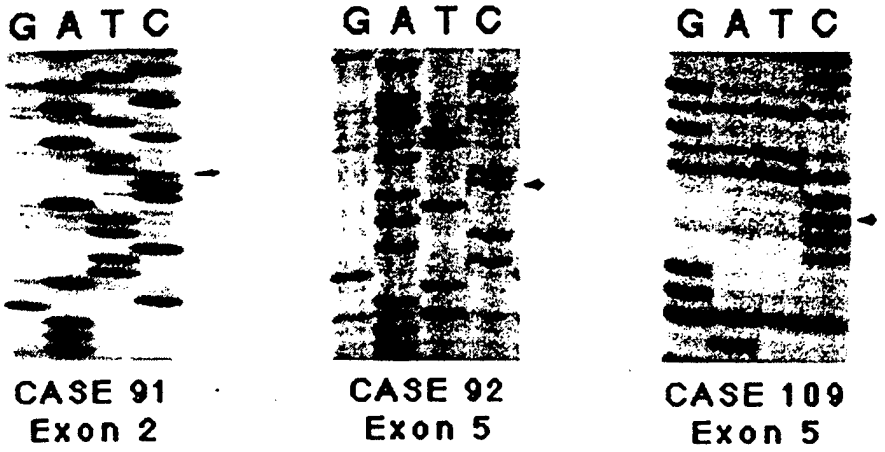

Fig. 2 Examples of sequencing analysis of shifted bands from SSCP experiments. *Arrow,* mutated nucleotide; *bottom,* case number and exon number.

cases and 15 (33%) high-grade cases], no PTEN mutations were found (17). Summarizing five studies in which both tumor grade and PTEN mutations were available (15–17, 20), we found that 9 of 67 (13.4%) high-grade tumors showed PTEN mutations, whereas only 3 of 117 (2.6%) lower grade cases showed mutations. The former rate is significantly higher than the latter ($P = 0.01$) using the χ^2 analysis-of-contingency table (28). Consistent with mutation studies, loss of PTEN expression has also been shown to correlate with high grade of primary prostate cancer (9, 10).

It has been reported that prostate cancer incidence is lower in Asian men than in Western men (24, 25). Although one study of Japanese patients did not detect any *PTEN* mutations in 45 primary tumors that were mainly low-grade cancers (17), we found more frequent *PTEN* mutations in a group of Chinese patients that had mainly high-grade tumors in this study; the latter is consistent with studies in Western men (20). These results suggest that *PTEN* is likely not a genetic factor contributing to the racial difference in prostate cancer incidence. This conclusion is further supported by the fact that all of the *PTEN* mutations were detected in prostate cancer cells only and not in their matched nonneoplastic cells. Also, no *PTEN* mutation has been detected in familial prostate cancers (22, 23). The differences in PTEN mutation rates in our study compared with that of Orikasa *et al.* (17) may be attributable to differences in the distribution of tumor grades between the study samples.

We detected multiple mutations for *PTEN* in two tumors, *i.e.,* case 92 had two missense mutations in exon 5 and case 47 had three missense mutations in exon 9 (Table 1). The heterogeneous nature of prostate cancer is well known (31); therefore, it is likely that multiple mutations of *PTEN* in one tumor may come from different subclones of tumor cells. In an analysis of metastases involving multiple organ sites in patients who died of prostate cancer, Suzuki *et al.* (12) found that different metastases within the same patient had different PTEN mutation status, indicating a complex genetic relationship between various subclonal lineages of prostate cancer cells. Mutation of exon 5 appears to be more frequent than that of other exons in both Cowden disease and various somatic cancers (8).

In summary, *PTEN* mutations were seen more often in primary prostate cancers from Chinese men compared with localized tumors from American patients. This difference is likely attributable to the presence of an excess of high-grade cancers in the Chinese patients. Whether primary prostate tumors with *PTEN* mutations have a greater proclivity to metastasize than those of similar grade and stage without mutations remains to be determined.

REFERENCES

1. Li, J., Yen, C., Liaw, D., Podsypanina, K., Bose, S., Wang, S. I., Puc, J., Miliaresis, C., Rodgers, L., McCombie, R., Bigner, S. H., Giovanella, B. C., Ittmann, M., Tycko, B., Hibshoosh, H., Wigler, M. H., and Parsons, R. *PTEN,* a putative protein tyrosine phosphatase gene mutated in human brain, breast, and prostate cancer. Science (Washington DC), *275:* 1943–1947, 1997.

2. Steck, P. A., Pershouse, M. A., Jasser, S. A., Yung, W. K. A., Lin, H., Ligon, A. H., Langford, L. A., Baumgard, M. L., Hattier, T., Davis, T., Frye, C., Hu, R., Swedlund, B., Teng, D. H. F., and Tavtigian, S. V. Identification of a candidate tumour suppressor gene, *MMAC1,* at chromosome 10q23.3 that is mutated in multiple advanced cancers. Nat. Genet., *15:* 356–362, 1997.

3. Li, D. M., and Sun, H. TEP1, encoded by a candidate tumor suppressor locus, is a novel protein tyrosine phosphatase regulated by transforming growth factor β. Cancer Res., *57:* 2124–2129, 1997.

4. Gray, I. C., Phillips, S. M., Lee, S. J., Neoptolemos, J. P., Weissenbach, J., and Spurr, N. K. Loss of the chromosomal region 10q23–25 in prostate cancer. Cancer Res., *55:* 4800–4803, 1995.

5. Davies, M. A., Koul, D., Dhesi, H., Berman, R., McDonnell, T. J., McConkey, D., Yung, W. K., and Steck, P. A. Regulation of Akt/PKB activity, cellular growth, and apoptosis in prostate carcinoma cells by MMAC/PTEN. Cancer Res., *59:* 2551–2556, 1999.

6. Persad, S., Attwell, S., Gray, V., Delcommenne, M., Troussard, A., Sanghera, J., and Dedhar, S. Inhibition of integrin-linked kinase (ILK) suppresses activation of protein kinase B/Akt and induces cell cycle arrest and apoptosis of PTEN-mutant prostate cancer cells. Proc. Natl. Acad. Sci. USA, *97:* 3207–3212, 2000.

7. Wu, X., Senechal, K., Neshat, M. S., Whang, Y. E., and Sawyers, C. L. The PTEN/MMAC1 tumor suppressor phosphatase functions as a negative regulator of the phosphoinositide 3-kinase/Akt pathway. Proc. Natl. Acad. Sci. USA, *95:* 15587–15591, 1998.

8. Ali, I. U., Schriml, L. M., and Dean, M. Mutational spectra of *PTEN/MMAC1* gene: a tumor suppressor with lipid phosphatase activity. J. Natl. Cancer Inst., *91:* 1922–1932, 1999.

9. Whang, Y. E., Wu, X., Suzuki, H., Reiter, R. E., Tran, C., Vessella, R. L., Said, J. W., Isaacs, W. B., and Sawyers, C. L. Inactivation of the tumor suppressor PTEN/MMAC1 in advanced human prostate cancer

through loss of expression. Proc. Natl. Acad. Sci. USA, *95:* 5246–5250, 1998.

10. McMenamin, M. E., Soung, P., Perera, S., Kaplan, I., Loda, M., and Sellers, W. R. Loss of PTEN expression in paraffin-embedded primary prostate cancer correlates with high Gleason score and advanced stage. Cancer Res., *59:* 4291–4296, 1999.

11. Liaw, D., Marsh, D. J., Li, J., Dahia, P. L. M., Wang, S. I., Zheng, Z. M., Bose, S., Call, K. M., Tsou, H. C., Peacocke, M., Eng, C., and Parsons, R. Germline mutations of the *PTEN* gene in Cowden-disease, an inherited breast and thyroid cancer syndrome. Nat. Genet., *16:* 64–67, 1997.

12. Suzuki, H., Freije, D., Nusskern, D. R., Okami, K., Cairns, P., Sidransky, D., Isaacs, W. B., and Bova, G. S. Interfocal heterogeneity of *PTEN/MMAC1* gene alterations in multiple metastatic prostate cancer tissues. Cancer Res., *58:* 204–209, 1998.

13. Vlietstra, R. J., van Alewijk, D. C., Hermans, K. G., van Steenbrugge, G. J., and Trapman, J. Frequent inactivation of PTEN in prostate cancer cell lines and xenografts. Cancer Res., *58:* 2720–2723, 1998.

14. Facher, E. A., and Law, J. C. PTEN and prostate cancer. J. Med. Genet., *35:* 790, 1998.

15. Feilotter, H. E., Nagai, M. A., Boag, A. H., Eng, C., and Mulligan, L. M. Analysis of PTEN and the 10q23 region in primary prostate carcinomas. Oncogene, *16:* 1743–1748, 1998.

16. Dong, J. T., Sipe, T. W., Hyytinen, E. R., Li, C. L., Heise, C., McClintock, D. E., Grant, C. D., Chung, L. W., and Frierson, H. F., Jr. PTEN/MMAC1 is infrequently mutated in pT2 and pT3 carcinomas of the prostate. Oncogene, *17:* 1979–1982, 1998.

17. Orikasa, K., Fukushige, S., Hoshi, S., Orikasa, S., Kondo, K., Miyoshi, Y., Kubota, Y., and Horii, A. Infrequent genetic alterations of the *PTEN* gene in Japanese patients with sporadic prostate cancer. J Hum. Genet., *43:* 228–230, 1998.

18. Pesche, S., Latil, A., Muzeau, F., Cussenot, O., Fournier, G., Longy, M., Eng, C., and Lidereau, R. PTEN/MMAC1/TEP1 involvement in primary prostate cancers. Oncogene, *16:* 2879–2883, 1998.

19. Cairns, P., Okami, K., Halachmi, S., Halachmi, N., Esteller, M., Herman, J. G., Isaacs, W. B., Bova, G. S., and Sidransky, D. Frequent inactivation of PTEN/MMAC1 in primary prostate cancer. Cancer Res., *57:* 4997–5000, 1997.

20. Gray, I. C., Stewart, L. M., Phillips, S. M., Hamilton, J. A., Gray, N. E., Watson, G. J., Spurr, N. K., and Snary, D. Mutation and expression analysis of the putative prostate tumour-suppressor gene *PTEN*. Br. J. Cancer, *78:* 1296–1300, 1998.

21. Wang, S. I., Parsons, R., and Ittmann, M. Homozygous deletion of the *PTEN* tumor suppressor gene in a subset of prostate adenocarcinomas. Clin. Cancer Res., *4:* 811–815, 1998.

22. Cooney, K. A., Tsou, H. C., Petty, E. M., Miesfeldt, S., Ping, X. L., Gruener, A. C., and Peacocke, M. Absence of PTEN germ-line mutations in men with a potential inherited predisposition to prostate cancer. Clin. Cancer Res., *5:* 1387–1391, 1999.

23. Forrest, M. S., Edwards, S. M., Hamoudi, R. A., Dearnaley, D. P., Arden-Jones, A., Dowe, A., Murkin, A., Kelly, J., Teare, M. D., Easton, D. F., Knowles, M. A., Bishop, D. T., and Eeles, R. A. No evidence of germline PTEN mutations in familial prostate cancer. J. Med. Genet., *37:* 210–212, 2000.

24. Gu, F. L., Xia, T. L., and Kong, X. T. Preliminary study of the frequency of benign prostatic hyperplasia and prostatic cancer in China. Urology, *44:* 688–691, 1994.

25. Angwafo, F. F. Migration and prostate cancer: an international perspective. J. Natl. Med. Assoc., *90:* S720–S723, 1998.

26. Moskaluk, C. A., and Kern, S. E. Microdissection and polymerase chain reaction amplification of genomic DNA from histological tissue sections. Am. J. Pathol., *150:* 1547–1552, 1997.

27. Kukita, Y., Tahira, T., Sommer, S. S., and Hayashi, K. SSCP analysis of long DNA fragments in low pH gel. Hum. Mutat, *10:* 400–407, 1997.

28. Glantz, S. A. Primer of Biostatistics, p. 473. New York: McGraw-Hill, 1997.

29. Atkin, N. B., and Baker, M. C. Chromosome study of five cancers of the prostate. Hum. Genet., *70:* 359–364, 1985.

30. Arps, S., Rodewald, A., Schmalenberger, B., Carl, P., Bressel, M., and Kastendieck, H. Cytogenetic survey of 32 cancers of the prostate. Cancer Genet. Cytogenet., *66:* 93–99, 1993.

31. Macintosh, C. A., Stower, M., Reid, N., and Maitland, N. J. Precise microdissection of human prostate cancer reveals genotypic heterogeneity. Cancer Res., *58:* 23–28, 1998.