Award Number: DAMD17-98-1-8472

TITLE: Isolation of Novel Prostate Cancer Tumor Suppressor Genes in African American and Caucasian Men thru Laser Microdissection and Representational Difference Analysis

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REPORT DATE: September 1999

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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Isolation of Novel Prostate Cancer Tumor Suppressor Genes in African American and Caucasian Men thru Laser Microdissection and Representational Difference Analysis

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Significant progress has been made for all three specific aims. Specific Aim 1: Obtaining metastatic prostate cancer DNA of quality sufficient for Representational Difference Analysis (RDA), has been achieved using alternate means. Specific Aim 2: Employ RDA to identify DNA regions which have been homozgyously deleted, has been achieved with identification of a novel region of homozgyous deletion on chromosome 12p (Cancer Research, 58, 5652-5655, 1998). Specific Aim 3: To prioritize regions of homozgyous deletion based on frequency of deletion of this region in metastatic tumors, and to identify candidate genes within these regions, has been achieved, with previously unidentified deletion of the identified chromosome 12p region in 50% of metastatic prostate cancers studied (Genes, Chromosomes, and Cancer 25:270-276, 1999). Examination of genes within this region suggests that p27 or TEL to be likely candidates. These results validate this approach and additional studies are underway to identify additional genomic regions of interest in metastatic prostate cancer.

Prostate Cancer

NSN 7540-01-280-5500
FOREWORD

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For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46.

N/A In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

N/A In the conduct of research utilizing recombinant DNA, the investigator(s) adhered to the NIH Guidelines for Research Involving Recombinant DNA Molecules.

N/A In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.

[Signature] 8/16/99

PI - Signature  Date
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September 1999 ANNUAL REPORT

Title  Isolation of Novel Prostate Cancer Tumor Suppressor Genes in African-American and Caucasian Men through Laser Microdissection and Representational Difference Analysis

P.I.  Bova, G. Steven

Introduction

Prostate cancer will metastasize and kill approximately 40,000 American men in 1999\(^1\). Aggressive metastatic prostate cancer is common in Americans of African and Caucasian descent, but is more common in African-Americans. \(^2\) Although research in colon, breast and other cancers suggests that the molecular mechanisms of cancer lethality are amenable to scientific comprehension\(^3,4\), the molecular basis of prostate cancer metastasis and subsequent death is poorly understood. The two major goals of this study are to put currently suspected molecular defects in prostate cancer on a knowledge map, and to identify novel molecular alterations in prostate cancer and add these to the map. The study funded by the DOD Prostate Cancer Research Program (USAMRMC) is aimed at identifying such novel molecular alterations in lethal metastatic prostate cancer by innovative application of state of the art molecular techniques. Special effort is made to identify prostate cancer alterations that are important in Americans of both African and Caucasian descent. In Specific Aim 1, we proposed to obtain ultrapure samples of high quality metastatic prostate cancer DNA through immunostain-targeted Laser Capture Microdissection (LCM) from metastatic prostate cancer in 1-3 African-Americans and 1-3 Caucasian-Americans. **Specific Aim 2** is to employ Representational Difference Analysis (RDA) to identify regions which have been homozygously deleted in these metastatic prostate cancers which are less than 1500 kb in size. **Specific Aim 3** is to prioritize regions of homozygous deletion based on frequency of deletion of this region in a panel of 41 metastatic tumors, and to identify candidate genes within the highest priority regions. Genes which are altered by deletion, mutation, and/or methylation in 20% or more of a panel of 100 other well-characterized prostate cancers will be high priority candidates for further examination and potential therapeutic targeting.

Body

**Progress on Specific Aim 1:** We have found obtaining sufficient quantities of DNA from LCM to be excessively time consuming using the current technology. We are now working with the laser microdissection equipment manufacturer to increase the speed and efficiency of the LCM process, so that high quantities can be obtained efficiently. Nonetheless, we have found that careful cryostat dissection provides samples of sufficient quantity and quality for RDA, as evidenced by our success in Specific Aims 2 and 3. A detailed description of the samples used is contained in the published account of this work\(^5,6\). We continue to work to obtain the purest samples possible for RDA, through LCM or through other microdissection technologies.

**Progress on Specific Aim 2:** Using representational difference analysis, we have identified and confirmed the presence of a homozygously deleted region on chromosome 12p in prostate cancer, as detailed in our recent publication \(^5\). This is the first homozygous deletion identified in this region in prostate cancer. This is the first successful application of RDA technology in prostate cancer that we are aware of. These results support our hypothesis that RDA can be performed in prostate cancer DNA samples to identify regions of homozygous deletion. The time required for the RDA process and subsequent extensive work required in Specific Aim 3 to further characterize this homozygous deletion has been more time consuming than we anticipated. If time and resources allow, we will again
perform RDA in additional samples in an effort to identify other regions of homozygous deletion in prostate cancer using this proven technology.

**Progress on Specific Aim 3:** Examination of the homozygously deleted region in a series of primary and metastatic prostate cancers has demonstrated previously undetected loss of heterozygosity in this region in 50% of metastatic prostate cancers, as detailed in our recent publication\(^6\). There appears to be no difference in the rate of loss of heterozygosity in this region in Caucasians and African-Americans, suggesting that both populations are affected by a putative important molecular alteration in this region. This finding supports our hypothesis that homozygous deletions are markers of important genomic instability in prostate cancer. Studies are now underway to identify the gene or genes in this region which may play a role in prostate cancer tumorigenesis. Our initial data suggest that the genes p27 and TEL to be the most likely candidates.

Our current plan is to continue to study genes within the identified 12p region until a strong candidate or candidates are identified. We are examining the expression of genes in the region, and are also sequencing genes within this region. If time and resources permit, we will commence another round of the very laborious and time-consuming RDA process to try to identify other regions of homozygous deletion.

Our laboratory has also been extensively occupied with the construction of a web-based database for cancer research, which will facilitate integration of data from these studies. A preliminary description of the database is located at: http://pelicanlab.jhmi.edu

In an oversight on our part, the USAMRMC was not appropriately acknowledged for its support of this work in the two published articles mentioned above, partly because we were confused on how to appropriately acknowledge this support (DOD, prostate cancer initiative?, USAMRMC?). We apologize for this. Two manuscripts in preparation will reference support from the USAMRMC unless we are advised otherwise.

**Key Research Accomplishments**

- Identification of a small focused region of homozygous deletion on chromosome 12p in metastatic prostate cancer.
- Publication of this finding in Cancer Research.
- Presentation of these findings at the Cold Spring Harbor biennial tumor suppressor genes meeting, at the AACR meeting, and at the American Urological Association meeting.
- Identification of loss of heterozygosity in this region in 50% of tumors
- Publication of this finding in Genes, Chromosomes, and Cancer.
- Presentation of this work at the American Urological Association meeting
- Support for our initial hypothesis.
- Continuing work in the 12p region: mutational analysis of genes in the region, expression analysis, analysis of genes contained in the region.

**Reportable Outcomes**

- manuscripts, abstracts, presentations: as detailed above
- patents and licenses applied for/issued: none
- degrees obtained that are supported by this award: none
Conclusions

Our hypothesis that homozygous deletions can be identified in metastatic prostate cancers, and that they can be harbingers of other important genomic changes within a region, is supported by our published findings. Within the chromosome 12p region that we have identified may be a gene (and gene product) that is frequently mutated or otherwise dysfunctional in prostate cancer. Understanding the function of this gene and gene product, and modifying the function of this gene and gene product could help improve prevention and treatment of prostate cancer. We will continue our studies to test these hypotheses.

Reference List


Identification of 12p as a Region of Frequent Deletion in Advanced Prostate Cancer

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Abstract

The identification of homozygous deletions in malignant tissue has been a powerful tool for the localization of tumor suppressor genes. Representational difference analysis (RDA) uses selective hybridization and the PCR to isolate regions of chromosomal loss and has facilitated the identification of tumor suppressor genes such as BRCA2 and PTEN. Twenty RDA clones were generated by comparing genomic DNA from a prostate cancer xenograft to the same patient's normal kidney DNA. Southern blot analysis of the tester and driver and of normal and xenograft DNA, using the differential products as probes, showed the homozygous deletion in 16 of 20 RDA clones. The sequence of one of the differential products overlapped HSU99862, a genomic GenBank sequence on chromosome 12p12-13. Multiplex PCR of the xenograft DNA using polymorphic repeats mapped the deletion to a 1.5-2C region on 12p. Genomic DNA isolated from a panel of cryostat microdissected metastatic prostate adenocarcinomas/normal pairs was screened for loss of heterozygosity using the same polymorphic repeats. Loss of heterozygosity was demonstrated in 9 (47%) of 19 patients. This region may contain, or lie in close proximity to, tumor suppressor genes important in the progression and/or initiation of prostate cancer.

Introduction

The deletion of genomic DNA is a common feature of neoplastic cells. Loss of these regions can inactivate tumor suppressor genes important in the initiation and progression of malignancy. LOH analysis has been useful in identifying regions of interest within the genome but has been of limited use in the cloning of novel candidate tumor suppressor genes. This has been true for malignancies in general and specifically for prostate cancer. Losses of 6q, 8p, 8q, 10p, 10q, 13q, 16q, 17p, 18q, and 19p have all been implicated in prostate cancer (1-6). However, to date no LOH study has led to cloning a gene important in prostate carcinoma, in part because of the many limitations of allelic loss studies (7).

In contrast, identification of homozygous deletions in malignant tissue has been strongly associated with the presence of suppressor gene loci (8). RB (9) and PTEN (10) are two examples of tumor suppressor genes that were identified at least partly through the recognition of homozygous deletion of these genes in various cancerous tissues or cell lines. Three homozygous deletions have been described in prostate cancer. Morton et al. (11) described a homozygous deletion in the prostate cancer cell line PC-3 of the α-catenin gene. This protein helps mediate cell-cell adhesion through the E-cadherin protein complex. Abnormalities in this pathway have been implicated in high grade and stage disease (12). The recently cloned PTEN gene is homozygously deleted in two prostate cancer cell lines (10) and is frequently altered in advanced prostate cancer (13). A third homozygous deletion at 8p22 was described by Bova et al. (1) in a region frequently demonstrating LOH. A gene, N33, was identified within the deletion but found to be normally expressed and not mutated in prostate cancer cell lines (14). At this time, this gene does not appear to play a role in prostate cancer.

RDA (15) is a method for isolating DNA fragments that are present in only one of two nearly identical genomes. The DNA is size-selected to simplify the genome and then undergoes subtractive hybridization followed by PCR amplification of genomic fragments found differentially in the two genomes. By comparing normal to malignant DNA from the same patient, homozygous deletions can be identified. Use of this technique has facilitated the identification of two tumor suppressor genes to date, BRCA2 (16) and PTEN (10). We report the use of RDA in a prostate cancer xenograft to identify a novel homozygous deletion.

Materials and Methods

Autopsy DNA. Prostate cancer tissue was obtained from 19 patients with metastatic prostate cancer undergoing autopsy between January 1995 and June 1997. All of the patients had undergone androgen-deprivation therapy and died from complications of metastatic disease. DNA was extracted from normal and malignant tissue samples as described previously (1). Institutional Review Board-approved informed consent was obtained from patients and family.

Immunohistochemistry. PSA immunohistochemistry on xenograft tissue sections was performed using standard automated techniques (Ventana Medical Systems, Lake Success, NY) with Chemicon (Temecula, CA) rabbit antihuman PSA polyclonal antiserum at 1:500 dilution as the primary antibody, and biotinylated goat antirabbit monoclonal secondary antibody (Ventana Medical Systems). Staining was performed with peroxidase-based chromogen (diaminobenzidine) detection. Slides were examined using standard light microscopy with cytoplasmic and intraglandular brown pigment deposition indicative of the presence of PSA. Intratumoral connective tissue cells and tumor nuclei served as internal negative controls.

Tumor DNA Isolation and Extraction. A prostate cancer metastasis of the rib was harvested at autopsy from a 55-year-old male with metastatic, androgen-deprived prostate carcinoma. The tissue was minced and placed s.c. in male severe combined immunodeficient mice. After two passages, the xenograft was harvested. Genomic DNA from this xenograft was extracted as described previously (1).

RDA. RDA was performed as described previously (15) with two exceptions, both described by Schutte et al. (16): (a) the hybridization times were increased to 40 h to account for the increased genomic complexity of the xenograft; and (b) during the PCR amplification of the hybridized DNA, the Taq polymerase is added after a 3-min incubation at 85°C. This step reduces priming mediated by duplexes of near-identical repetitive elements. After three rounds of hybridization and PCR amplification, differential products could be visualized by gel electrophoresis.

Cloning. The differential products were ligated into pBluescript II plasmid vector (Stratagene, Menasha, WI) using T4 ligase and transformed into competent bacteria according to the manufacturer's instructions. Clones were grown on Luria-Bertani medium/ampicillin plates. Transformed colonies were selected and grown in 10 ml of Luria-Bertani media, and the plasmids (DPCX-
1–20) were extracted using Wizard Plus Miniprep (Promega, Madison, WI). One μg of each DPCX plasmid was digested with 1 unit of BamHI at 37°C for 1 h, and the digest was resolved on a 1.5% low-melting-point agarose gel. The inserts were extracted from the gel using QIAquick Gel Extraction Kit according to the manufacturer’s instructions (Qiagen, Santa Clarita, CA).

**Southern Blot Analysis.** Five μg of tester and driver or xenograft and kidney DNA were ran in parallel lanes on a 2% agarose gel, transferred to Hybond-N membrane (Amersham, Arlington Heights, IL) in 0.4 M NaOH-0.6 m NaCl, and then covalently linked to the membrane with UV radiation (Stratagene). The DPCX inserts were labeled with [α-32P]dCTP using the Multiprime DNA labeling system (Amersham) according to the manufacturer’s instructions. Probes were boiled with 0.5 ml of 2-mg/ml sonicated salmon sperm DNA, cooled on ice, and then hybridized overnight at 64°C in Rapidhyb buffer (Amersham). The blots were then washed in 15 mm NaCl, 1 mm NaH2PO4, 0.1 mm EDTA, and 0.5% SDS (pH 7.4) at 64°C and then autoradiographed. Blots were reprobed with inserts that were not deleted to demonstrate that equal quantities of DNA were present on each blot.

**Sequencing.** Plasmids that contained confirmed homozygous deletions were then sequenced using the fluorescent dideoxy terminator method of sequencing on a Perkin-Elmer Applied Biosystems Division (Foster City, CA) 377 automated DNA sequencer, following standard protocols (17).

**Multiplex PCR Amplification.** Primers were designed for each DPCX fragment and purchased from Biosynthesis (Lewisville, TX) as follows: (a) DPCX-2, 5'-CTAAAGGAGGATGGCTAATC-3' and 5'-CCCCTAAAACACTGGCTCATAG-3'; (b) DPCX-5, 5'-AAAATCTGGGAGGCAAGC-3' and 5'-GGCGAGAGGGCAAAACG-3'; (c) DPCX-6, 5'-AAATCTGGGAGGCAAGC-3' and 5'-GGCGAGAGGGCAAAACG-3'; (d) DPCX-7, 5'-GGCGAGAGGGCAAAACG-3' and 5'-GGCGAGAGGGCAAAACG-3'; (e) DPCX-13U, 5'-CTCTTACCGCTGATATCC-3' and 5'-GCAACGCGTGGTCGTC-3'; (f) DPCX-17, 5'-ACCGGCGAATTCTCGCTGAA-3' and 5'-GAATCTGGGAGGAAAG-3'; and (g) DPCX-18, 5'-CTCTTACCGCTGATATCC-3' and 5'-GCAATGAGGCGCGTG-3'.

Primers in the region of DPCX-2 (D1251695, D12577, D12589, D12597, D12598, D125358, and D1251581) and control primers (D135328 and D85549) were purchased from Research Genetics (Huntsville, AL). PCR amplification using these primers was used to create a map of the homozygous deletion and of the region of maximum LOH using the genomic map available from Genethon (18). Six μl of F primer (20 μm) was end-labeled with 3 μl [γ-32P]ATP (ICN Radiochemicals, Irvine, CA) using 20 units of T4 kinase (Life Technologies). Reaction mixture was incubated at 37°C for 1 h. The enzyme was inactivated by incubation at 65°C for 20 min.

Genomic DNA (50 ng) underwent PCR amplification in 10 mm Tris-HCl, 50 mM KCl (pH 8.3), 1.5–3.5 mM MgCl2, 1 unit Taq DNA polymerase, 15 mm dNTP, and 80 μm of labeled F primer and unlabeled R primer. Amplified PCR fragments were denatured at 94°C for 5 min and cooled on ice. Then 2 μl of each sample was separated by polyacrylamide uniaxial gel electrophoresis and autoradiographed. Allelic imbalances were considered to be allelic losses if by visual estimation a 50% or greater signal reduction was seen for a tumor allele as compared with the normalized retained noncancerous counterpart. To confirm homozygous deletions, reactions were repeated under identical conditions except multiplexed with markers (γ-32P-labeled D135328 or D85549) known to be rarely homozygously deleted. These markers served as an additional control for variations in DNA template concentration.

**Results**

Histological analysis of the second-passage xenograft demonstrated gland formation morphology consistent with metastatic prostate adenocarcinoma. Intraluminal and intracytoplasmic expression of PSA was demonstrated by immunohistochemistry, again consistent with prostatic origin. After three rounds of RDA, differential products were visualized on an agarose gel and cloned into pBluescript II. Southern blot analysis of the tester versus driver blots revealed that 16 of the 20 clones were present in the tester but not in the driver DNA. Similar analysis of the remaining 16 probes on xenograft/normal DNA blots confirmed the homozygous deletion of 13 probes (Fig. 1), whereas 3 probes—DPCX-1, -3, and -13—were only hemizygoously deleted. To demonstrate that the decrease in signal intensity was not an artifact of DNA concentrations, DPCX-9 (an RDA clone which was not deleted as demonstrated by Southern blot analysis) was used as a probe (Fig. 1). Sequencing revealed that DPCX-2 was identical to DPCX-4, -10, -15, and -19 and that DPCX-17 was identical to DPCX-14U. Thus, eight independent fragments were cloned from areas of homozygous deletion.

Multiplex PCR amplification, using D135328 as a control, confirmed that DPCX-2, -5, -6, -7, -13U, -17, and -18 were homozygously deleted in the xenograft compared with normal DNA from the same patient (Fig. 2). DNA extracted from a liver metastasis from the same patient demonstrated decreased PCR amplification compared with noncancerous DNA (Fig. 2), which was consistent with the presence of a homozygous deletion partially obscured by residual noncancerous DNA present in the sample.

The sequences for DPCX-2 and DPCX-16 both mapped to different regions of HUSUS9962, a genomic GenBank sequence on chromosome 12p12–13. PCR of DNA from the Corvall National Institute of General Medical Sciences human/rodent somatic cell hybrid library (Camden, 4). G. S. Bova and W. B. Isaacs, unpublished data.
prostate cancer tissue and noncancerous DNA obtained at autopsy from 19 patients. Nine (47%) of the nineteen demonstrated LOH in this region (Fig. 3C). The overlapping region of LOH was similar to the homozygous deletion, and the frequency of allelic loss was highest in markers homozygously deleted in the xenograft (Table 1).

Discussion

An approach to identifying candidate tumor suppressor genes is to identify homozygous deletions of genomic DNA within tumors. RDA has recently proven to be useful in facilitating identification of these regions. The PTEN gene was recently isolated by performing RDA on human breast cancer cells. A homozygously deleted region was identified at 10q23. Subsequent analysis revealed this deletion contained the PTEN gene and that it was frequently deleted in breast, brain, and prostate cancer (10). A similar experimental design was used by Schutte et al. in which the analysis of a pancreatic cancer xenograft demonstrated a homozygous deletion at 13q12.3 in the region of BRCA-2 (16).

Data presented here suggest the presence of a previously unknown prostate cancer tumor suppressor locus. The region was identified by RDA and confirmed by Southern analysis and multiplex PCR. Using the National Center for Biotechnology Information data base, the Coriell National Institute of General Medical Sciences humane-roden somatic cell hybrid panel, and polymorphic primers located in the area of the deletion, we have mapped the deletion of a 1–5-cM region of 12p. Multiplex PCR amplification of DNA isolated from a liver metastasis from the same patient demonstrated decreased PCR amplification compared to the noncancerous DNA (Fig. 2), which is consistent with a homozygous or hemizygous deletion in this region. The small amount of amplification may be attributable to normal cells within the metastatic tumor deposit.

To further support our conclusion that this region contains a genes important in prostate cancer, we examined purified DNA samples obtained from metastatic deposits from 19 patients with androgen-deprived prostate cancer. Forty-seven percent of the metastatic lesions demonstrated LOH in this region.

Previous studies have not detected 12p abnormalities in prostate cancer. Comparative genomic hybridization has been used in two large studies to examine genetic changes in prostate cancer. Visakorpi et al. (6) examined 31 primary and 9 locally recurrent prostate carcinomas. The regions of most frequent copy number loss were 8p(32%), 13q (32%), 6q (22%), 16q (19%), 18q (19%), and 9p (16%). CHER et al. (5) were the first to use comparative genomic hybridization to study metastatic prostate cancers. In their study, copy loss was noted in 8p(80%), 13q (75%), 16q (55%), 17p (50%), and 10q (50%).

Several groups have examined localized and metastatic prostate cancer for LOH. Carter et al. (2) used Southern blot analysis to examine 17 polymorphic markers on 11 different chromosomal arms in 28 localized and 4 metastatic lesions. Twenty-nine and 31% demonstrated LOH at 10q and 16q, respectively. In this study, 12p was not

Table 1  Informativeness of microsatellite markers in all of the 19 patients studied and high-resolution deletion mapping of chromosome 12p 12–13 in 9 cases demonstrating LOH

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<th>A3</th>
<th>A8</th>
<th>A13</th>
<th>A17</th>
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<td>4/11 (36%)</td>
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<td>L</td>
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a N. noninformative; LOH: R: retained.

b Marker was homozygously deleted in xenograft.

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examined. A second approach, by Kunimi et al. (4), also used Southern blotting to examine 10 primary tumors, 4 cases of lymph node metastasis, and 4 cases of brain metastasis. 8p, 10p, 10q, 16q, and 18q were found to demonstrate LOH in 50, 55, 30, 60, and 43%, respectively. In this study, 12p was examined at one polymorphic marker (D12S16), the exact location of which is unknown. One brain metastasis demonstrated LOH at this marker. A third study, by Sakr et al. (3), examined 19 primary tumors and lymph node metastases for evidence of LOH by PCR amplification of polymorphic repeats. Markers on 8p, 10q, and 16q demonstrated LOH in 25, 12, and 8%, respectively, of metastatic samples and 29, 18, and 42%, respectively, of primary tumors. Although no LOH was detected on 12p, only one marker was examined—F38VWF—which is approximately 9 cm telomeric from our region of interest.

Our data demonstrating 47% LOH at 12p12–13 in metastatic prostate cancer would make this region one of the most frequently altered genomic regions in prostate cancer. Data from the published comparative genomic hybridization studies did not detect significant copy-number loss or gain at 12p despite examining the entire genome (5, 6). However, this methodology lacks the resolution of microsatellite analysis. Only two of the previous LOH studies have examined 12p. Neither examined a marker near our region of interest. Although 12p has not previously been implicated in prostate cancer, Berube et al. (19) did find that the microcell transfer of a portion of 12q suppressed tumorigenicity in nude mice and that the loss of the chromosomal fragment restored the malignant phenotype.

Abnormalities in 12p have been implicated in other tumor types. Sato et al. (20) demonstrated that 38% of ovarian cancers had allelic loss on 12p. The marker used in this study (D12S16) is not well localized on currently available genetic maps. 12p12.3 has been strongly implicated in acute lymphoblastic leukemia. Baccichet and Sinnett (21) demonstrated allelic loss of this region in 47% of the cases that they studied. The region of maximal LOH, D12S39 to D12S358, is identical to our region of interest. Lastly, abnormalities of 12p have been implicated in lung cancer. Takeuchi et al. (22) demonstrated LOH on 12p in 33% of non-small cell lung cancers. The region of maximal LOH was D12S269 to D12S308, which is approximately 5 cm closer to the centromere than the region from which we have identified.

Genes that are believed to lie within our region of interest according to the National Center for Biotechnology Information human genome map include mesenchymal glutamine S-transferase, NKG2-A and NKG2-B type II integral membrane proteins, translational initiation factor 2 gamma subunit, BET-related protein, human globular epithelial protein 1 (GLEPP1), and human epidermal growth factor receptor kinase substrate. It is possible that no genes of importance to tumor suppression lie within the region of 12p identified here. Although the fact that 47% of our tumors demonstrated LOH at 12p12–13 provides strong supportive evidence of a tumor suppressor gene at this site, we note that another site of a homozygous deletion (8p22) also frequently demonstrates LOH, and no prostate tumor suppressor gene has been identified at that site to date (14). It is also important to recognize that the source of this xenograft and the metastatic foci examined for LOH were metastatic androgen-deprived tumors. These tumor foci likely underwent multiple damaging genetic events before and after metastasis, only some of which are critical to the tumor phenotype. However, the fact that the deletion was found in DNA isolated both from the xenograft and from a liver metastasis from the same patient is consistent with the hypothesis that the deletion of this region was present before metastasis. Taken together, the data are strongly suggestive that inactivation of a tumor suppressor gene located within this region could be responsible for initiation, metastasis, or progression to androgen independence in prostate cancer.

Conclusion. In conclusion, by using RDA we have identified a novel homozygous deletion at 12p12–13 in a metastatic prostate cancer xenograft. A high percentage of cryostat microdissected metastatic prostate tumors demonstrate LOH in this region. This region has not been previously implicated in prostate cancer but has been implicated in other malignancies. This region may contain, or lie in close proximity to, tumor suppressor genes important in the progression and/or initiation of metastatic prostate cancer.

Acknowledgments
We thank Mario Eisenberger, Michael Carducci, Pendleton Powers, and Victoria Sinibaldi for clinical assistance and John T. Isaacs, John Lamb, Charles M. Ewing, and John Robinson for technical assistance.

References
Deletion Mapping at 12p12–13 in Metastatic Prostate Cancer

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The identification of homozygous deletions in malignant tissue is a powerful tool for the localization of tumor suppressor genes. Representational difference analysis (RDA) uses selective hybridization and the polymerase chain reaction (PCR) to isolate regions of chromosomal loss and has facilitated the identification of tumor suppressor genes, such as BRCA2 and PTEN. We have recently identified a 1–5-cM homozygous deletion on 12p12–13 in a prostate cancer xenograft and found that 47% of patients who died of prostate carcinoma demonstrate focal loss of heterozygosity (LOH) in this region in metastatic deposits. We have now characterized the region of interest by assembling a yeast artificial chromosome (YAC) contig spanning the homozygous deletion and identifying which known genes and expressed sequence tags (ESTs) lie within the homozygous deletion. A rib metastasis was harvested at autopsy and placed subcutaneously in a male SCID mouse. Genomic DNA from this xenograft and from the patient's normal renal tissue was extracted. Multiplex PCR, with the xenograft and normal DNA used as template, was performed using primers for loci on the Whitehead contig 12.1 believed to be near our region of interest. We found that our deletion lay in a 1–2-Mb interval between WI-664 and D12S358. We then used the same primers to construct a YAC contig across the homozygous region. PCR amplification of YAC DNA, using primers for the genomic sequences of known genes and ESTs reported to lie on 12p12–13, was used to identify candidate genes that lay within the deletion. Duplex PCR, with control primers known not to be deleted in the xenograft, was used to confirm that both the CDKN1B and ETV6 genes were homozygously deleted in the xenograft. Mutations in either or both of these genes may play an important role in metastatic prostate carcinoma. Genes Chromosomes Cancer 25:270–276, 1999. © 1999 Wiley-Liss, Inc.

INTRODUCTION

The deletion of genomic DNA is a common feature of neoplastic cells. Loss of these regions can inactivate tumor suppressor genes important in the initiation and progression of malignancy. Identification of homozygous deletions in malignant tissue has been strongly associated with the presence of suppressor gene loci (Marshall, 1991). RB1 (Friend et al., 1986) and PTEN (Li et al., 1997) are two examples of tumor suppressor genes that were identified at least partly through the recognition of homozygous deletion of these genes in various cancers or cell lines.

We have recently identified a homozygous deletion in a prostate cancer xenograft by using representational difference analysis (RDA) (Kibel et al., 1998). The deletion was mapped to a 1–5-cM region of chromosome arm 12p bounded by the polymorphic markers D12S77 and D12S358. In addition, we determined that 9/19 (47%) of cryostat-microdissected metastatic prostate tumors obtained from different patients demonstrate focal loss of heterozygosity (LOH) in this region. The region of maximum LOH overlapped the homozygous deletion (Kibel et al., 1998).

Of the three previously described homozygous deletions in prostate cancer, two have been found to contain genes important in prostate cancer. Morton et al. (1993) described a homozygous deletion in the human prostate cancer cell line PC-3 of the α-catenin gene. This protein helps mediate cell-cell adhesion through the E-cadherin protein complex. Abnormalities in this pathway have been implicated in high-grade and high stage disease (Paul et al., 1997). The recently cloned PTE.V gene is homozygously deleted in two prostate cancer cell lines (Li et al., 1997) and is frequently altered in advanced prostate cancer (Suzuki et al., 1998). Only the third homozygous deletion, at 8p22, described by Bova et al. (1993), has not yet been linked to a proved tumor suppressor locus (Bookstein et al., 1997).

The region on 12p12–13 has not been previously implicated in prostate cancer, but has been implicated in other malignancies, such as acute lympho-

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blastic leukemia (ALL) (Baccichet and Sinnett, 1997) and ovarian (Sato et al., 1991) and non-small-cell lung cancer (Takeuchi et al., 1996). This region may contain or lie in close proximity to tumor suppressor genes important in progression and/or initiation of all of these malignancies. In the work reported here, we mapped the deletion on the Whitehead 12.1 contig and determined the location of known genes and expressed sequence tags (EST) in relation to this region of homozygous deletion.

MATERIALS AND METHODS

Xenograft and Normal DNA

The xenograft was developed from a rib metastasis from a 55-year-old man with a metastatic, androgen-deprived prostate carcinoma and confirmed to be of prostatic origin, as described previously (Kihel et al., 1998). DNA was extracted from normal tissue from the same patient and from the xenograft as described previously (Boya et al., 1993). Internal review board-approved informed consent was obtained from the patient.

Uniplex and Duplex Polymerase Chain Reaction (PCR) Amplification

Primers were designed for DNA sequences known to lie on the Whitehead contig 12.1 in the region of the homozygous deletion and were purchased from Genosys (Woodlands, TX): D12S1674, 5'-GTGATAGAGCGGAGACTCCAT-3' and 5'-CCCTACTTCAACAAAGAGAGGAG-3'; D12S77, 5'-GAAGGGCAACACAGTGGA-3' and 5'-CTTCTTCTCCCTCCTC-3'; WI-91, 5'-GTTGCTGGATTACTGACAGCA-3' and 5'-CTCCATCATAGCGGCTCGC-3'; WI-664, 5'-CTTGGTGGGTGTTTGGGGAAG-3' and 5'-TTCATTAGA-CTCGCCCCGAGG-3'; D12S89, 5'-ATTTGAGACGACGTTGTTT-3' and 5'-CATTATGGGGATAGGGGT-3'; WI-4385, 5'-AAGTGCAGTGCTGA-3' and 5'-CTGGTTCTCCCTCG-3'; D12S89, 5'-CTGTAGTAGTAAGGCTCTCC-3' and 5'-CTTGGCTTGCTG-3'; D12S739, 5'-GCCCTTTGAGGAAGCT-3' and 5'-GCCAGATGAGTTCCG-3'; and WI-2685, 5'-CAGAGGCTGCAAGGAGCCATCTC-3' and 5'-GCACATATGACGAGGAG-3'.

PCR amplification with these primers was used to create a map of the homozygous deletion on the Whitehead contig 12.1. Six microliters of forward primer (20 μM) was end-labeled with 3-μl γ-32P adenosine triphosphate (ICN, Irvine, CA) using 20 units of T4 kinase (Life Technologies, Rockville, MD). The reaction mixture was incubated at 37°C for 1 hr, followed by T4 kinase inactivation by incubation at 68°C for 20 min.

Thirty to fifty nanograms of genomic tumor or noncancerous DNA underwent PCR amplification with 80 nmoles of labeled forward primer and unlabeled reverse primer. Amplified PCR fragments were denatured at 94°C for 5 min and cooled on ice. Two microliters of each sample was separated by polyacrylamide urea gel electrophoresis and autoradiographed. In cases where the noncancerous DNA reaction produced product but the tumor sample did not, the reactions using both tumor and noncancerous DNA were repeated under identical conditions with the original primers and with the addition of a second set of γ-32P-labeled primers (D13S28, purchased from Research Genetics, Huntsville, AL) known to be rarely homozygously deleted (data not shown) to serve as an internal reaction control. Amplification of the control fragment, but not the fragment of interest in the tumor sample, coupled with normal amplification of both fragments in the noncancerous DNA sample indicated the presence of a homozygous deletion.

Yeast Artificial Chromosomes (YAC) Contig

YACs reported to lie in the region on the homozygous deletion were obtained from Research Genetics and grown on YPD plates at 30°C for 2 days. DNA from individual colonies was used as template in PCR reactions using 3.6 pmoles of primer for D12S1674, D12S77, WI-91, WI-664, D12S89, WI-4385, D12S391, D12S358, and WI-2685. Individual clones whose DNA was amplified by contiguous genomic markers in the region of the homozygous deletion were selected for further analysis.

Gene/EST Localization

Primers were designed for genomic sequences of genes and ESTs that were reported to lie in the region of the homozygous deletion (www.ncbi.nlm.nih.gov/science96) and purchased from Genosys. Primers for each gene or EST are listed in Table 1. Five nanograms of each YAC-purified DNA underwent PCR amplification with 3.6 pmoles of each primer. Amplified PCR fragments were separated on 7% polyacrylamide gels, stained with ethidium bromide, and photographed.

All genomic sequences that were found to lie in the region of the homozygous deletion underwent PCR using xenograft and normal DNA as template and radiolabeled primers, as described above. Again to confirm homozygous deletions, reactions were repeated under identical conditions with the addi-
<table>
<thead>
<tr>
<th>Genomic locus</th>
<th>Gene at locus</th>
<th>Location (Fig. 1C)</th>
<th>Deleted in xenograft</th>
<th>Primers (5' to 3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>D1251936</td>
<td>Microsomal glutathione S-transferase</td>
<td>Off contig</td>
<td>Not tested</td>
<td>AGCACAGAAATAACAGGAGCCAAAGAG-TGAGCTTGTGCTGTA</td>
</tr>
<tr>
<td>SGC 34934</td>
<td>EST</td>
<td>Off contig</td>
<td>Not tested</td>
<td>GAACATTTGGGAGCAACTTTAATTCTGCA</td>
</tr>
<tr>
<td>SGC 34321</td>
<td>EST</td>
<td>Off contig</td>
<td>Not tested</td>
<td>CACCCCTCCCACACATATTATCTCCTC-CACAGCTTCTCAG</td>
</tr>
<tr>
<td>D1251909</td>
<td>EST</td>
<td>Off contig</td>
<td>Not tested</td>
<td>GAGGTTGCCAGAGTTTTAGTGGAGCATCTCTT</td>
</tr>
<tr>
<td>SsSG317</td>
<td>Cation-dependent mannose-6-phosphate receptor precursor</td>
<td>Off contig</td>
<td>Not tested</td>
<td>GGCAACAAAGAGGAGGAGAG-GAGTCAAAACAAAT</td>
</tr>
<tr>
<td>SHCG 12935</td>
<td>Human epidermal growth factor receptor kinase substrate</td>
<td>Off contig</td>
<td>Not tested</td>
<td>TACATTTGTTCCTCCATGGAGCTA-TGCCCTTTGATAG</td>
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<tr>
<td>D1251900</td>
<td>Placental growth factor</td>
<td>Off contig</td>
<td>Not tested</td>
<td>CTGCTGTTCCCTTCCTCTATATGCCTT-GCCCCCTTCAGAG</td>
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<tr>
<td>A006200</td>
<td>EST</td>
<td>1</td>
<td>Not tested</td>
<td>GACACCGTGCTGCTGCTGAAGCTTTGCTTT-TTGCTGGCTGTTAG</td>
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<tr>
<td>L19161</td>
<td>Translational initiation factor 2 gamma subunit</td>
<td>2</td>
<td>Not tested</td>
<td>TCCCCTGGTTAGGACCTCTAGCAAGCAG-CGTTTGTCCCCTAGC</td>
</tr>
<tr>
<td>A006W05</td>
<td>EST</td>
<td>2</td>
<td>Not tested</td>
<td>TGGTTATTTGCCAATGGCTTGGATGAG-CCCAAGAAGGAGAT</td>
</tr>
</tbody>
</table>
| WI-11901      | EST                                 | 2                  | Not deleted          | TTTTACCCCAATGACAAGGATAGGAGGAA-
| SGC 35438     | NKG2-C type II integral membrane protein | 2                  | Not deleted          | TTGTGTTGTATGAGTCACACCCTGGGCAAGAT-|
| D1251916      | EST                                 | 2                  | Not deleted          | CACCCCAAAATTTTCAAAGGATAGCTG-
| SGC 35334     | Human acidic proline rich protein gene | 2                  | Not tested           | ACCTTTAGTACAAACAGCGGAGATG-
| WI-9218       | Human lectin-like type II integral membrane protein | 2                  | Not deleted          | GTCTTATCAGGACATCACCAGAATAG-
| WI-7055       | NKG2-A type II integral membrane protein | 2                  | Not tested           | GCTCATCTGTTTTGGTGAAGAT-GTCTTGTGCAAGACT-
| A008B32       | ETV6                                | 3                  | Deleted              | TTTTTGAAAGAGGATAGCATTGAGAATAG-
| StsSG4303     | ETV6                                | 3                  | Deleted              | TGGAGGCGTTCAAAAGAAGCTGACAC-
| D1251898      | ETV6                                | 3                  | Deleted              | CAGAGATGCTGAGGAGGAGCCCTGGT-
| A009A32       | EST                                 | 4                  | Not deleted          | CCGCTCCTTTCTAGCTCTAGGAGAGCAG-
| A005C29       | EST                                 | 4                  | Not deleted          | GAACCTAACATTCTGAGGAGCACCCAGG-
| p27 3' exon1  | CDKN1B                              | 4                  | Deleted              | GGCCTCCCGCTTACCTGAGGAGCTGTA-

*Numbers in location column correspond to Figure 1C. PCR was initially run against the YAC contig. If the locus was located in a region possibly homozgyously deleted (region 3 and 4), PCR was repeated using xenograft as template. All PCRs demonstrating evidence of homozgyous deletion were repeated in a multiplex reaction using D135328 or D8549 as a positive control. The ETV6 gene and CDKN1B were found to lie within the homozgyous deletion.

RESULTS

PCR amplification of genomic sequences located in the region of our previously described deletion (Kibel et al, 1998) on the Whitehead contig 12.1 demonstrated that D12589, WI-4385, and D125391 were homozgyously deleted in the xenograft, whereas flanking markers D12577, WI-9128, WI-664, and D125358 were not (Fig. 1B and D). Reactions were repeated using multiplex PCR to provide a positive control in each PCR reaction. This established the boundaries of the deletion at WI-664 and D125358.
The YAC contig constructed around this deletion (Fig. 1A) demonstrates that the entire deletion lies within YACs 952 A2 and 964 C10. PCR amplification of genes and ESTs reported to lie in this region (Table 1) demonstrated that only a minority were located in the region of the deletion (Fig. 1C, regions 3 and 4). The xenograft and normal DNA underwent PCR amplification for all genes and ESTs mapped to YACs 952 A2 and 964 C10, and for several that were believed not to lie within the deletion. Only the CDKN1B and ETV6 genes were found to be homozygously deleted (Fig. 2).

**DISCUSSION**

An approach to identifying candidate tumor suppressor genes is to identify homozygous deletions of genomic DNA within tumors. RDA has been useful in facilitating identification of these regions. The PTEN gene was isolated by performing RDA on human breast cancer cells. A homozygously deleted region was identified at 10q23. Subsequent analysis showed that this deletion contained the PTEN gene and that it was frequently deleted in breast, brain, and prostate cancer (Li et al., 1997). A
similar experimental design was used by Schutte et al. (1995), in which analysis of a pancreatic cancer xenograft demonstrated a homozygous deletion at 13q12.3 in the region of BRCA2.

We have recently used RDA to identify a possible prostate cancer tumor suppressor locus at 12p12–13 (Kibel et al., 1998). The homozygous deletion was identified by RDA and confirmed by Southern analysis and multiplex PCR. Using the National Center for Biotechnology Information database, and polymorphic primers located in the area of the deletion, we mapped the deletion to a 1–5-cM region of 12p. In addition, we demonstrated that the homozygous deletion also existed in a metastasis from the same patient, indicating that the deletion was not simply an artifact of the xenografting process, and that 47% of patients demonstrated LOH at 12p12–13 in their metastatic deposits, indicating that genetic abnormalities at this site are common in prostate cancer (Kibel et al., 1998).

Our current study further narrows the homozygously deleted region to a 1–2-Mb region of 12p12–13. Because WI-664 has not been mapped on a genomic or radiation hybrid map, a more exact size determination is not possible at this time. However, the entire deletion lies within the two overlapping YACs 952 A2 and 964 C10 (Fig. 1A). In addition, we examined 11 known genes and 9 ESTs and found that only 2 were located within the homozygous deletion: the CDKN1B and ETV6 genes (Table 1). Multiplex PCR was used with a nondeleted locus (D13S328) to serve as an internal positive control of PCR conditions and as an indicator of starting template quality and integrity (Fig. 2). Genes and ESTs were mapped initially on a YAC contig to demonstrate their close proximity to the region of interest.

CDKN1B, also known as p27 or KIP1, is a negative regulator of the cell cycle and functions by binding to G1-specific cyclin-cdk complexes such as cyclin E-cdk2, cyclin A-cdk2, and cyclin D2-ckd4 (Polyak et al., 1994; Toyoshima et al., 1994). Abnormalities in CDKN1B have been found in a variety of malignancies, including breast (Catzenlos et al., 1997), colon (Loda et al., 1997), lung (Yatabe et al., 1998), and prostate (Guo et al., 1997; Cheville et al., 1998; Cordon-Cardo et al., 1998; Cote et al., 1998; Tshihias et al., 1998; Yang et al., 1998) carcinomas.

Six recently published studies have presented strong evidence that CDKN1B plays an important role in prostate cancer. Guo et al. (1997) examined 40 primary prostate tumors and 5 lymph node metastases, using immunohistochemistry, and found that decreased staining correlated with a high proliferative index (as measured by Ki-67 staining) and tumor grade. Importantly, fully 67.5% of the primary tumors and 40% of the metastatic foci were completely negative for CDKN1B. Yang et al. (1998) examined 86 radical prostatectomy specimens from patients with clinically organ-confined disease for CDKN1B expression, again by immunohistochemistry. They found that absent or low expression by immunohistochemistry was a strong independent risk factor for disease-free survival by multivariate analysis. In similar studies, Tshihias et al. (1998) and Cordon-Cardo et al. (1998) report that decreased CDKN1B staining was a strong independent risk factor for treatment failure. In contrast, Cheville et al. (1998) examined 138 radical prostatectomy specimens and found that although decreased CDKN1B staining correlated with a higher Gleason score and pathologic stage, it did not correlate with biochemical failure. Lastly, Cote et al. (1998) used immunohistochemistry to determine the nuclear levels of CDKN1B in 96 pathologic stage C primary prostate tumors. They found that decreased CDKN1B levels were correlated not only with decreased disease-free survival but also with overall survival in this patient population. Interestingly, whereas immunohistochemistry has demonstrated decreased CDKN1B expression in prostate carcinoma, no prior study to date has shown deletions, mutations, or other genetic alterations in this gene in prostate cancer (Kawamata et al., 1995; Ponce-Castaneda et al., 1995). However, both groups examined only localized disease. To our knowledge, there are no previous studies, other
than the one reported here, that examined CDKN1B in metastatic deposits.

ETV6, also known as TEL, is a member of the ETS family of transcription factors, but its exact function is unknown. It was initially identified as a protein fused to platelet-derived growth factor receptor β (Golub et al., 1994). The formation of chimeric ETV6 fusion transcripts appears to be important in several hematologic malignancies (Golub et al., 1995; Papadopoulos et al., 1995; Carroll et al., 1996; Golub, 1997) and the ETV6 gene has been implicated as possible tumor suppressor gene in ALL because LOH has frequently been identified at this site (Bacccheret and Sinekett, 1997). Whereas ETV6 is expressed in a wide variety of tissues (Golub et al., 1994), the only other malignancy in which ETV6 has been implicated is congenital fibrosarcoma through fusion of ETV6 to the NTRK3, neurotrophin-3 receptor, gene. (Knezevic et al., 1998). To our knowledge, ETV6 has not previously been implicated in prostate cancer.

Either of these genes is a strong candidate tumor suppressor gene at this locus, but it should be recognized that other genes may lie within this 1-2 Mb homozygous deletion. It is possible that one of these unknown genes is the responsible prostate tumor suppressor. An alternative hypothesis is that no tumor suppressor lies at this site. Although the fact that 47% of metastatic foci from different patients demonstrated LOH at 12p12-13 provides strong supportive evidence of a tumor suppressor gene at this site, we note that another site of a homozygous deletion (8p22) also frequently demonstrates LOH and no prostatic tumor suppressor gene has been identified at that site to date (Bookstein et al., 1997).

In conclusion, we have identified a novel homozygous deletion in a metastatic prostate cancer xenograft. We have created a YAC contig across this deletion, determined the loci of 20 genes and ESTs, and narrowed the region of interest to a 1-2 Mb region of 12p12-13. The deletion includes both the CDKN1B and ETV6 genes, each of which has previously been implicated in malignancy. Immunohistochemical abnormalities of CDKN1B have previously been linked to prostate carcinoma, but this is the first report of a mutation in this gene in this disease. This report is also the first suggesting that ETV6 inactivation may play a role in metastatic prostate cancer. It is possible that neither of these genes is important in metastatic prostate carcinoma and an as yet unidentified tumor suppressor gene(s) that lies in close proximity to these two genes is truly responsible for the progression and/or initiation of metastasis.

ACKNOWLEDGMENTS

We thank Mario Eisenberger, Michael Carducci, Pendleton Powers, and Vickie Sinibaldi for clinical assistance, and John T. Isaacs, John Lamb, Charles M. Ewing, Dennis Faith, and Debra Nusskern for technical assistance. A.S.K. is an American Foundation of Urologic Disease Scholar.

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