Overexpression, Amplification, and Androgen Regulation of TPD52 in Prostate Cancer

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ABSTRACT

Gains in the long arm of chromosome 8 (8q) are believed to be associated with poor outcome and the development of hormone-refractory prostate cancer. Based on a meta-analysis of gene expression microarray data from multiple prostate cancer studies (D. R. Rhodes et al., Cancer Res 2002;62:4427–33), a candidate oncogene, Tumor Protein D52 (TPD52), was identified in the 8q21 amplicon. TPD52 is a coiled-coil motif-bearing protein, potentially involved in vesicle trafficking. Both mRNA and protein levels of TPD52 were highly elevated in prostate cancer tissues. Array comparative genomic hybridization and amplification analysis using single nucleotide polymorphism arrays demonstrated increased DNA copy number in the region encompassing TPD52. Fluorescence in situ hybridization on tissue microarrays confirmed TPD52 amplification in prostate cancer epithelia. Furthermore, our studies suggest that TPD52 protein levels may be regulated by androgens, consistent with the presence of androgen response elements in the upstream promoter of TPD52. In summary, these findings suggest that dysregulation of TPD52 by genomic amplification and androgen induction may play a role in prostate cancer progression.

INTRODUCTION

Genetic alterations are believed to accumulate in the course of neoplastic progression. In prostate cancer, progression has been associated with early and late molecular events. Loss of the short arm of chromosome 8 (8p) is considered an early event and is seen in prostatic intraepithelial neoplasia and nearly all prostate cancers (1–5). Later events such as gains of the long arm of chromosome 8 (8q) have been implicated in the progression to more aggressive prostate cancer (1, 2, 6–9). The critical sites of 8q gain include 8q21 (10–12), 8q23–24 (13–17). Elongin C was proposed as a putative prostate cancer oncogene amplified at 8q21(12). The c-myc oncogene located at 8q24 has also been found to be variably amplified in prostate cancer (7, 15, 16, 18, 19).

Here we present data supporting the role of TPD52 as a candidate oncogene. TPD52, located on 8q21(20), has previously been demonstrated to be amplified and overexpressed in breast cancer (21, 22). We demonstrate through cDNA expression array analysis and immunohistochemistry that TPD52 is overexpressed in prostate cancer.

Received 12/11/03; revised 3/5/04; accepted 3/17/04.

Grant support: Specialized Program of Research Excellence for Prostate Cancer National Cancer Institute Grants P50CA90381 (M. A. Rubin) and P50CA69568 (K. J. Pienta), National Cancer Institute Grants CA 70773 (A. M. Chinnaian and M. A. Rubin), Department of Defense grant DAMD17-03-2-0033 (M.A. Rubin and A.M. Chinnaian) and R01AG21404 (M. A. Rubin), and American Cancer Society Grant RSG-02-179-MGO (A. M. Chinnaian Department of Defense (M.A. Rubin and A.M. Chinnaian) and M. A. Rubin).

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Array comparative genomic hybridization (aCGH), amplification analysis using single nucleotide polymorphism (SNP) chips, and fluorescence in situ hybridization (FISH) all implicate amplification of the chromosomal region containing TPD52. A survey of gene expression studies provides evidence that TPD52 is overexpressed in several other common human malignancies.

MATERIALS AND METHODS

Patient Population and Tissue Collection. Prostate tissue samples were taken from the radical prostatectomy series and the rapid autopsy program at the University of Michigan Prostate Cancer Specialized Program of Research Excellence Tissue Core with institutional review board approval. Clinically localized prostate cancer samples used for this study were taken from a cohort of men who underwent radical retroperitoneal prostatectomy as a monotherapy (i.e., no hormonal or radiation therapy) for clinically localized prostate cancer between January 1995 and December 2001. Tumors were staged using the tumor-node-metastasis (TNM) system (23) and graded according to the system originally described by Gleason (24, 25). The snap-frozen samples used for immunoblot, aCGH, and SNP analysis were all evaluated histologically by the study pathologist (M. A. R.). All samples were trimmed to ensure that >95% of the sample used represented the desired lesion. Hormone-refractory metastatic prostate cancer samples from 15 autopsy cases performed from 1997 to 2000 were also collected from the rapid (“warm”) autopsy program (26). The patients’ ages ranged from 40 to 84 years, with a median age of 67.5 years. Hormone-naïve metastatic prostate cancers were collected at the University of Ulm Hospital as part of an ongoing institutional review board-approved research program to study the molecular signature of metastatic prostate cancer.

Quantitative Real-Time PCR. Quantitative real-time PCR for TPD52 expression was performed using SYBR Green essentially as described previously (27). Briefly, total RNA isolated from 11 benign prostate samples, 33 clinically localized prostate cancer samples, and 15 metastatic prostate cancer samples was reverse transcribed into first-strand cDNA. The quantity of cDNA in each sample was calculated by interpolating its Ct value versus a standard curve of Ct values obtained from serially diluted cDNA from commercially available pooled normal prostate samples (Clontech) and one of the prostate tissue (27–30), to be an accurate housekeeping gene in a variety of tissues. Through validation with over 40 genes identified as being differentially expressed in prostate cancer, we found that the addition of HMBS as a second internal control improves our correlation to cDNA microarray data, consistent with other reports (31). No reverse transcription controls were included when the 3′-untranslated region primers were used. Primer sequences (5′ to 3′) are as follows: TPD52_cds-sense, GGTCGCTCTTTGCTGGCTTGCC; TPD52_cds-antisense, TCCATGATTTAAAAGTTGGGGAGTT; TPD52_3′UTR-sense, GATCCGTGCTGCTAATCTTT; and TPD52_3′UTR-antisense, CACTTGCCAACCCCATTTCTATC. GAPDH and HMBS primers were used as described previously (2).
Cell Culture and Androgen Treatment. LNCaP cells (American Type Culture Collection, Manassas, VA) were grown in RPMI 1640 without phenol red (Sigma, St. Louis, MO) supplemented with 10% fetal bovine serum (BioWhittaker). Dexamethasone, 50 units/ml penicillin, and 50 μg/ml streptomycin. Cells were maintained in a 5% CO2, 95% air-humidified atmosphere at 37°C and cultured in phenol red-free medium with 5% stripped fetal bovine serum (BioWhittaker) 48 h before experiments. The cells were plated on 100-mm dishes with 50% density. Forty-eight h later, the cells were treated with either vehicle control or 1 μM synthetic androgen R1881 (New England Nuclear).

**Immunohistochemistry.** Sections of 4-μm-thick, paraffin-embedded tissue microarrays (TMAs) were dewaxed and rehydrated using xylene and ethanol, respectively. After immersion in 10 mM citrate buffer (pH 6.0), the slides underwent microwave pretreatment for 10 min for optimal antigen retrieval. The affinity-purified TPD52 rabbit polyclonal antibody (22) was applied at a 1:1,000 dilution in blocking buffer overnight at 4°C. After washing three times with PBS-T buffer, the membrane was incubated with horse-radish peroxidase-linked donkey anti-rabbit IgG antibody (Amersham Pharmacia Biotech) at a 1:5,000 dilution for 1 h at room temperature. The signals were visualized with the enhanced chemiluminescence detection system (Amersham Pharmacia Biotech) and autoradiography. To monitor equal loading, the TPD52 antibody-probed membrane was stripped with Western Re-Proto buffer (Geno-tech, St. Louis, MO) blocked in TBS-T with 5% nonfat dry milk, and incubated with rabbit anti-GAPDH antibody (1:25,000 dilution; Abcam, Cambridge, MA) and anti-heterochromatin protein β (HP1β; 1:1,000 dilution; Upstate, Charlotte, VA) for 2 h. In prostate cancer, many epithelial cell proteins show differential expression. Changes in keratin expression during the development of benign prostatic hyperplasia and prostate cancer have been reported (32, 33).

Hence we did not use keratins as controls.

**Immunoblot Analyses.** Tissues were homogenized in NP40 lysis buffer containing 50 mM Tris-HCl (pH 7.4), 1% NP40 (Sigma), and complete proteinase inhibitor mixture (Roche). Fifteen μg of protein extracts were mixed with SDS sample buffer and electrophoresed to a 10% SDS-polyacrylamide gel under reducing conditions. The separated proteins were transferred onto nitrocellulose membranes (Amersham Pharmacia Biotech, Piscataway, NJ). The membrane was incubated for 1 h in blocking buffer (Tris-buffered saline with 0.1% Tween (TBS-T) and 5% nonfat dry milk). The affinity-purified TPD52 rabbit polyclonal antibody (22) was applied at a 1:1,000 dilution in blocking buffer overnight at 4°C. After washing three times with PBS-T buffer, the membrane was incubated with horse-radish peroxidase-linked donkey anti-rabbit IgG antibody (Amersham Pharmacia Biotech) at a 1:5,000 dilution for 1 h at room temperature. The signals were visualized with the enhanced chemiluminescence detection system (Amersham Pharmacia Biotech) and autoradiography.

**Tissue Microarray Construction, Digital Image Capture, and Analysis.** As described previously, high-density (TMAs) composed of samples from a wide range of prostate tissues were assembled using a manual tissue arrayer [Beecher Instruments, Silver Spring, MD (34, 40–42)]. Three to four 0.6-mm-thick H&E-stained sections as well as all microdissected archival material (43). Genomic gain was defined a log2 ratio greater than or equal to the tumor background threshold (43). Gene copy number along the genome is proportional to the ratio of fluorescent intensities. Each array consists of 2460 BACs spotted in triplicate on chromium slides that provides a resolution of approximately 1.4 Mb. All BACs have been mapped on the University of California, Southern California genome assembly12 and can be computationally linked to the underlying and annotated genome sequence. Seven consecutive clones were identified at 8q21 that were used to help determine the amplicon size.

**Copy Number of TPD52 Was Determined by SNP Arrays.** Copy numbers were determined by analysis of SNP arrays (46). The protocol used to determine copy number from SNP arrays is reported separately (47). Briefly, DNA was digested with XbaI, ligated to a single primer, and subjected to single primer extracted from a 2-mm core of each metastasis using a Qiagen Mini-Prep kit (Valencia, CA). It was then subjected to PCR amplification under conditions favoring the generation of 200-bp amplicons. These amplicons were then fragmented, fluorescently labeled, and hybridized to an Affymetrix SNP array containing over 400,000 probes interrogating over 116,500 SNP loci (48). Using the informatics platform dChip (49), signal intensities at each probe locus were analyzed compared with a composite reference, representing germ-line DNA from 19 individuals, to determine copy number at each SNP locus using methods described previously (47). The region displayed results represent obtained from over 60 SNPs at 8q21 with 3 SNPs within the region spanned by TPD52.

**Quantitative Real-Time PCR to Validate Copy Number.** Quantitative real-time PCR was performed on a PRISM 7700 sequence detector (Applied Biosystems) and Opticon Chromo 4 (MJ Research) using a Quantitect SYBR Green kit (Qiagen). We have quantified each tumor DNA by comparing the target locus to the reference Line-1, a repetitive element for which copy numbers per haploid genome are similar among all human normal and neoplastic cells (50). Quantification is based on standard curves from a serial dilution of human normal genomic DNA. The relative target copy number level was also measured to normal human genomic DNA as a calibrator.

Copy number change of target gene relative to the Line-1 and the calibrator was determined by using the formula (Ttarget/Tline1)/(Ctarget/Cline1)12, where Ttarget and Tline1 are quantity from tumor DNA by using target and Line-1, and Ctarget and Cline1 are quantity from calibrator by using target and Line-1. PCRs for each primer set were performed in at least triplicate, and means were reported. Conditions for quantitative PCR reaction were as follows: one cycle of 50°C for 15 min; one cycle of 94°C for 2 min; and 40 cycles of 94°C for 20 s, 56°C for 20 s, and 72°C for 20 s. At the end of the PCR reaction, samples were subjected to a melting analysis to confirm specificity of the amplicon. Primers were designed by using Primer 313 to span a 100–150-bp nonrepetitive region and synthesized by Invitrogen. Each primer set was subsequently compared with the human genome using the BLAST algorithm to determine its uniqueness. All primer sets were further confirmed to generate a single desired size fragment that was evaluated by gel electrophoresis. For verification, the presence or absence of PCR products was also evaluated by agarose gel electrophoresis. Primer sequences for each target used in this study are published as the supporting information. Primer sequences (designed to include both intronic and exonic elements in the amplicon, to avoid amplification of mRNA) were as follows: LINE-1, AAGACCGCTCAAATCTACGGT (forward) and TGTCTTGAATGCGTGTTCAAGG (reverse); exon 2, ATGGTT-TAAATCCCCAACA (forward) and TCCCTCGAGGGCTCTGCTT (reverse); exon 4, GAGGTGCCATTTTCTTTTG (forward) and GACGTGA-GCAACACGACAA (reverse); and exon 6, TGGTGGGTATTGTTGGA-GAG (forward) and GCATTGGTAGACAGAAAC (reverse).

**Fluorescent in Situ Hybridization for TPD52/Elongin c.** TMA sections were pretreated with a 50 mM Tris and 100 mM EDTA solution at 199°F for 15 min and digested with Digest All 2 (Zymed, South San Francisco, CA) for 3 min. The TMAs and BAC FISH probes were then denaturated at 94°C for 3 min and hybridized overnight at 37°C. The BAC FISH probes were digoxigenin-labeled BAC RP11-367E12 probe for the Elongin c gene (TCEB1) and the biotin-labeled BAC probes RP11-941H19 and RP11-92K15 for the TPD52 gene. Posthybridization washing was with 0.5× SSC for 5 min, and the fluorescence detection was carried out using anti-digoxigenin-FITC and streptavidin–Alexa-594 conjugates (Molecular Probes, Eugene, OR). Slides were then counterstained and mounted in 4′,6-diamidino-2-phenylindole–Vectorshield (Vector Laboratories, Burlingame, CA). FISH signals were ana-
alyzed using the Oncor Imaging System, and images were captured using a charge-coupled device camera (Photometrics, Tucson, AZ).

Statistical Analysis. Pertinent clinical parameters, pathology results, and TPD52 expression with recurrence-free survival was first evaluated by bivariate (univariate) analysis. The relationship between preoperative variables and recurrence-free survival was then examined using Cox proportional-hazard regression. TPD52 protein expression was evaluated as a mean score based on all TMA cores from a single patient. Expression was graphically represented using error bars with 95% confidence intervals. Differences between tissue types (e.g., benign versus localized prostate cancer) were evaluated using ANOVA with a post hoc Scheffe analysis to take multiple tissue types into account. The association of clinical parameters, pathology results, and TPD52 expression with recurrence-free survival was first evaluated by bivariate (univariate) analysis. The relationship between preoperative variables and recurrence-free survival was then examined using Cox proportional-hazard regression models. All decisions were made using a 0.05 significance level, and all analyses were run using the SPSS software (SPSS Systems, Chicago, IL).

RESULTS

Meta-Analysis of DNA Microarray Studies Demonstrates Overexpression of TPD52 Transcript in Prostate Cancer. Meta-analysis of prostate cancer profiling studies (51) has identified several markers of prostate cancer including hepsin, AMACR, and fatty acid synthase (34, 52–56). TPD52 was a gene identified by this meta-analysis (Fig. 1A; Ref. 51) and also found in a region often amplified in prostate cancer, chromosome 8q21 (7, 20). Based on the four prostate expression array data sets, overexpression of TPD52 was observed in prostate cancer samples (n = 63) when compared with histologically benign prostate tissue (n = 32) with a false discovery rate (Fig. 1A, FDR) of 0.05. Overexpression of the TPD52 transcript in prostate cancer was validated using quantitative reverse transcription-PCR (Fig. 1B). Box plot representations of the quantitative reverse transcription-PCR data demonstrate a significant increase in the mRNA level of TPD52 in clinically localized prostate cancer (P = 1e−5; Fig. 1C).

Overexpression of TPD52 Protein in Prostate Cancer. To determine whether TPD52 overexpression at the transcript level corresponded with overexpression at the protein level, prostate tissue extracts were prepared from benign prostate tissue, prostate cancer, and metastatic prostate tumors, and immunoblot analysis was performed using an affinity-purified antibody specific for TPD52 (22, 57). Consistent with the TPD52 mRNA results, TPD52 protein levels were elevated in clinically localized prostate cancer and metastatic prostate cancer compared with benign prostate tissue samples. No changes in expression were appreciated with the two control genes (HP1B and GAPDH) between the different tissue types (Fig. 2A).

Immunohistochemistry Using High-Density Tissue Microarrays Confirmed TPD52 Protein Overexpression in Prostate Cancer. To validate that overexpression of the TPD52 transcript was associated with overexpression at the protein level, we performed immunohistochemistry using the affinity-purified polyclonal antibody against TPD52. Using high-density prostate cancer TMAs, we were able to characterize TPD52 protein expression in a wide range of prostate samples. TPD52 protein expression was cytoplasmic, consistent with a previous report in breast cancer (22). Weak to moderate TPD52 expression was seen in benign prostate tissue (Fig. 2B, 1 and 2). Strong protein expression was consistently seen in clinically localized prostate cancer samples (Fig. 2B, 3 and 4) and metastatic prostate cancer (Fig. 2B, 5). TPD52 expression was confined to the cytoplasm and was not observed in the nucleus (Fig. 2B, 6). Protein expression was strongest in clinically localized and metastatic prostate cancer. The mean TPD52 protein expression levels are presented in Table I and Fig. 2C. There was no significant difference seen between clinically localized prostate cancer and hormone-refractory prostate cancer. In a subset of 54 cases of clinically localized prostate cancer (i.e., clinical stage T1a-c or T2), we looked for associations with PSA failure after radical prostatectomy for clinically localized prostate cancer. In this cohort, 37% (7 of 19) failures were seen in TPD52 high expressors in contrast to 20% (7 of 35) failures in moderate expressors. These differences demonstrated a trend toward higher PSA failure rates in men with high-TPD52-expressing tumors (log rank P = 0.12). No significant associations were observed between TPD52 protein expression and Gleason score, tumor stage, or surgical margin status.

Androgen Regulation of TPD52 Protein. Sequence analysis demonstrated the presence of androgen-responsive elements in the putative promoter region of TPD52 (Ref. 58; Fig. 2D). The hormone-responsive prostate cancer cell line LNCaP was treated with the
Fig. 2. A–E, overexpression and androgen regulation of TPD52 protein in prostate cancer. Prostate whole tissue lysates were prepared from benign prostate tissue, cancer, and metastatic tumors. Using an affinity-purified antibody specific for TPD52 peptide, immunoblot analysis demonstrated an up-regulation of TPD52 in prostate cancer and metastatic tissue lysates compared with benign prostate tissue samples (A). TPD52 protein expression was evaluated using high-density tissue microarrays. The tissue microarray analysis reveals strong cytoplasmic TPD52 protein expression in neoplastic prostate tissues [i.e., high-grade prostatic intraepithelial neoplasia, localized prostate cancer, and metastatic prostate cancer (B and C)]. Weak to moderate TPD52 expression was seen in benign prostate tissue (B, 1 and 2; ×200). Strong protein expression was consistently seen in localized prostate cancer samples (B, 3 and 4; ×200) and metastatic prostate cancer (B, 5; ×200). TPD52 expression was confirmed to the cytoplasm and was not observed in the nucleus (B, 6; ×600). Protein expression was strongest in localized and metastatic prostate cancer. The mean TPD52 protein expression levels are presented in C using error bars with 95% confidence intervals (Benign, benign prostate tissue; PCA, atrophic prostate glands also referred to as proliferative inflammatory atrophy; PIN, high-grade prostatic intraepithelial neoplasia; PCa, prostate cancer; METS, hormone-refractory metastatic prostate cancer).

Therefore, if one calculates copy gain as a log 2 ratio of 0.24, the ratios for the clinically localized and hormone-refractory metastatic tumors demonstrated a genomic gain at the BAC containing clone RP11-214E11. This area of chromosome 8q21 has also been linked to prostate cancer progression (7, 59). Therefore, we set out to determine the extent of chromosome 8q21 amplification in metastatic prostate cancer samples using two independent techniques. Using an aCGH platform containing approximately 2400 BAC clones with an average genome-wide resolution of 1.4 Mb (44, 45), we examined the data for 7 consecutive BACs at 8q21, with one containing TPD52 (Table 2). Although copy number cannot be accurately determined from this analysis, the BAC containing TPD52 (clone RP11-214E11) demonstrated a significant copy number increase based on the log2 ratios for the clinically localized and hormone-refractory metastatic prostate cancers with mean log2 ratios of 0.24 and 0.30, respectively. Therefore, if one calculates copy gain as a log2 ratio of ≥0.24, the cases with amplification are presented in Table 2. Approximately 45% (25 of 56) of primary prostate cancers and 63% (5 of 8) metastatic tumors demonstrated a genomic gain at the BAC containing TPD52.

synthetic androgen R1881 over different time points. Up-regulation of PSA served as a positive control for androgen action (D and E).

Amplification of TPD52 as Determined by aCGH and SNP Arrays. TPD52 is located in a known area of amplification on chromosome 8q21 (21, 22). This area of chromosome 8q has also been linked to prostate cancer progression (7, 59). Therefore, we set out to determine the extent of chromosome 8q21 amplification in metastatic prostate cancer samples using two independent techniques. Using an aCGH platform containing approximately 2400 BAC clones with an average genome-wide resolution of 1.4 Mb (44, 45), we examined the data for 7 consecutive BACs at 8q21, with one containing TPD52 (Table 2). Although copy number cannot be accurately determined from this analysis, the BAC containing TPD52 (clone RP11-214E11) demonstrated a significant copy number increase based on the log2 ratios for the clinically localized and hormone-refractory metastatic prostate cancers with mean log2 ratios of 0.24 and 0.30, respectively. Therefore, if one calculates copy gain as a log2 ratio of ≥0.24, the cases with amplification are presented in Table 2. Approximately 45% (25 of 56) of primary prostate cancers and 63% (5 of 8) metastatic tumors demonstrated a genomic gain at the BAC containing TPD52.

Table 1. TPD52 protein expression in prostate cancer as determined by immunohistochemistry using TMAs.

<table>
<thead>
<tr>
<th>Tissue type</th>
<th>TMA samples</th>
<th>Mean staining intensity</th>
<th>SE</th>
<th>Low</th>
<th>High</th>
</tr>
</thead>
<tbody>
<tr>
<td>Benign</td>
<td>101</td>
<td>2.45</td>
<td>9.70E-02</td>
<td>2.25</td>
<td>2.64</td>
</tr>
<tr>
<td>PIA</td>
<td>31</td>
<td>2.32</td>
<td>0.13</td>
<td>2.07</td>
<td>2.58</td>
</tr>
<tr>
<td>PIN</td>
<td>91</td>
<td>3.34</td>
<td>8.00E-02</td>
<td>3.18</td>
<td>3.5</td>
</tr>
<tr>
<td>PCA</td>
<td>283</td>
<td>3.61</td>
<td>4.33E-02</td>
<td>3.52</td>
<td>3.69</td>
</tr>
<tr>
<td>METS</td>
<td>384</td>
<td>3.61</td>
<td>4.10E-02</td>
<td>3.53</td>
<td>3.69</td>
</tr>
</tbody>
</table>

* TMA, tissue microarray; CI, confidence interval; Benign, benign prostate tissue; PIA, proliferative inflammatory atrophy; PIN, high-grade prostatic intraepithelial neoplasia; PCA, prostate cancer; METS, hormone-refractory metastatic prostate cancer.

* Staining intensity is score from negative (score = 1) to strong (score = 4).

Table 2. BACs located near TPD52 locus mapping to 8q21.13

<table>
<thead>
<tr>
<th>Clone</th>
<th>KB August 2001</th>
<th>PCs (N)</th>
<th>Est. amp</th>
<th>S.E.</th>
<th>MetS (N)</th>
<th>Est. amp</th>
<th>S.E.</th>
</tr>
</thead>
<tbody>
<tr>
<td>RP11-90B7</td>
<td>89791</td>
<td>−0.04 (62)</td>
<td>5/62</td>
<td>0.021</td>
<td>0.15 (8)</td>
<td>3/8</td>
<td>0.20</td>
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<tr>
<td>RP11-115D10</td>
<td>90647</td>
<td>0.16 (62)</td>
<td>18/62</td>
<td>0.018</td>
<td>N/A (2)</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>RP11-195P5</td>
<td>90833</td>
<td>−0.063(54)</td>
<td>4/54</td>
<td>0.019</td>
<td>−0.038(6)</td>
<td>1/6</td>
<td>0.21</td>
</tr>
<tr>
<td>RP11-214E11</td>
<td>91519</td>
<td>0.24 (56)</td>
<td>25/56</td>
<td>0.028</td>
<td>0.30 (8)</td>
<td>5/8</td>
<td>0.21</td>
</tr>
<tr>
<td>RP11-99E16</td>
<td>91964</td>
<td>0.12 (60)</td>
<td>9/60</td>
<td>0.023</td>
<td>0.11 (9)</td>
<td>5/9</td>
<td>0.18</td>
</tr>
<tr>
<td>RP11-80C11</td>
<td>92687</td>
<td>0.15 (59)</td>
<td>10/59</td>
<td>0.024</td>
<td>0.34 (9)</td>
<td>5/9</td>
<td>0.12</td>
</tr>
<tr>
<td>RP11-257P3</td>
<td>92974</td>
<td>0.18 (57)</td>
<td>19/57</td>
<td>0.024</td>
<td>0.45 (9)</td>
<td>7/9</td>
<td>0.13</td>
</tr>
</tbody>
</table>

* BAC, bacterial artificial chromosome; Est. Amp., estimated number of cases amplified using a log2 ratio of 0.25 at the cutoff point; NA, not available.

* Clones closest to TPD52.
region on the long arm of chromosome 8 containing TPD52 (red probe) but not in histologically benign prostate right panel. The approximate boundaries of TPD52 are denoted by the dashed blue lines identified. Copy numbers along a segment of 8q21 (cytoband reference in combining newly developed SNP chips and novel informatics tools. By comparing the signal intensity at each SNP locus with the intensity of normal controls, amplifications can be post hoc for clinically localized prostate cancer was 2.8. Significant copy number increases were seen between benign and clinically localized prostate cancer (mean difference, 0.8; SE, 0.13; left panel). The highest mean copy amplification was observed in hormone-refractory prostate cancer (mean copy number, 3.3). The mean copy number tissue from the same patient (the figure. The copy number for the SNPs was determined by Hidden-Markov modeling, and the copy number from quantitative real-time PCR was estimated by averaging results of from TPD52. A fourth hormone-refractory metastasis is amplified adjacent to and possibly overlapping part of the region spanned by TPD52. The region displayed represents data obtained – 10% of the probed loci; this is displayed in dashed lines metastases evaluated, with only one exception (WA18-2), had heterozygous loci either within or immediately adjacent to the region spanned by TPD52 (boundaries are denoted by dashed lines). SNP array analysis is regularly unable to identify either allele in 5–10% of the probed loci; this is displayed in black as None. B, a second novel approach was taken combining newly developed SNP chips and novel informatics tools. By comparing the signal intensity at each SNP locus with the intensity of normal controls, amplifications can be identified. Copy numbers along a segment of 8q11 (cytoband reference in black on the left) are displayed for four hormone-naïve and five hormone-refractory metastatic prostate tumors. The approximate boundaries of TPD52 are denoted by the dashed blue lines. Four of five hormone-naïve and three of five hormone-refractory metastases appear to be amplified at TPD52. A fourth hormone-refractory metastasis is amplified adjacent to and possibly overlapping part of the region spanned by TPD52. The region displayed represents data obtained from >60 SNP loci: 3 SNP loci were within the region spanned by TPD52. The results of the SNP copy number estimates are compared with quantitative real-time PCR results below the figure. The copy number for the SNPs was determined by Hidden-Markov modeling, and the copy number from quantitative real-time PCR was estimated by averaging results of amplicons overlying exons 2, 4, and 6 of TPD52 (see “Materials and Methods” for details). C, FISH analysis was performed using tissue microarrays with a BAC probe specific for a region on the long arm of chromosome 8 containing TPD52 (red probe). Amplification was observed in prostate cancer samples (right panel) but not in histologically benign prostate tissue from the same patient (left panel). The highest mean copy amplification was observed in hormone-refractory prostate cancer (mean copy number, 3.3). The mean copy number for clinically localized prostate cancer was 2.8. Significant copy number increases were seen between benign and clinically localized prostate cancer (mean difference, 0.8; SE, 0.13; post hoc Scheffé analysis, P < 0.0001) and between localized prostate cancer and hormone-refractory prostate cancer (mean difference, 0.54; SE, 0.13; post hoc Scheffé analysis, P < 0.001). D, the variation in amplification for TPD52 as determined by FISH is presented graphically using error bars with 95% confidence intervals. Although this amplification was specific for a BAC containing TPD52, a separate probe using a BAC containing elongin C, located at 8q21, demonstrated amplification (green probe). There was no significant difference in amplification between these two BAC probes (data not shown).
the probed loci; this is displayed in black as None. We were also able to estimate amplification using the SNP arrays by measuring the intensity of expression of each SNP (Fig. 3B). Copy numbers along a segment of 8q21 (cytoband reference in black on the left) are displayed for four hormone-naïve and four hormone-refractory metastatic prostate tumors. The approximate boundaries of TPD52 are denoted by the dashed blue lines. Three of four hormone-naïve and three of five hormone-refractory metastases are amplified at TPD52. A fourth hormone-refractory metastasis is amplified adjacent to (and possibly overlapping part of) the region spanned by TPD52. The region displayed represents data obtained from >60 SNP loci; 3 SNP loci were within the region spanned by TPD52. Similar to the aCGH data, a copy number increase was seen at TPD52. In addition, the SNP analysis was able to define a region of amplification that was seen in the hormone-refractory tumors and, to a lesser degree, in the hormone-naïve metastatic tumors. This region includes TPD52 and is consistent with previous work that suggests that 8q21 gain is associated with worse clinical outcome (7, 59).

**FISH for TPD52 Demonstrates an Amplion at 8q21.** FISH analysis was performed using TMA probes containing TPD52 (red probe). As seen in Fig. 3C, a copy number increase was observed in prostate cancer samples (right panel) but not in histologically benign prostate tissue (left panel). The highest mean copy increase was observed in hormone-refractory prostate cancer (mean copy number, 3.3). The mean copy number for clinically localized prostate cancer was 2.8. Interestingly, no significant association was seen between Gleason score and copy number. However, the majority of all cases were Gleason score 6 or 7. Table 3 summarizes this data. Significant copy number increases were seen between benign and clinically localized prostate cancer (mean difference, 0.8; SE, 0.13; post hoc Scheffé analysis, P < 0.0001) and between localized prostate cancer and hormone-refractory prostate cancer (mean difference, 0.54; SE, 0.13; post hoc Scheffé analysis, P < 0.001). The variation in increased TPD52 copy number as determined by FISH is presented graphically in Fig. 3D using error bars with 95% confidence intervals. Parallel FISH studies using an Elongin C-containing BAC (green) also located at 8q21 but 60 Mb away confirmed amplification of this region. The FISH analyses did not reveal differences in the incidence of amplification, between the TPD52 and Elongin C probes (data not shown).

**TPD52 Expression in Other Cancers.** Because the expression of TPD52 was identified in breast and now prostate cancer, we also sought to identify other tumors that may preferentially overexpress TPD52. Using ONCOMINE, a new informatics tool developed by our group, we were able to interrogate other expression array data sets that contained information on TPD52 expression in common human cancers. ONCOMINE contains data from 65 cancer microarray data sets spanning most major types and many subtypes of cancer (60). After the microarray data were normalized and analyzed for differential expression as described previously (60), we searched for cancer types or subtypes other than breast and prostate cancer in which TPD52 was differentially expressed. As shown in Fig. 4, TPD52 is differentially overexpressed in a number of cancer types relative to the normal tissues from which they arose. Confirming previous work, TPD52 was found to be overexpressed in breast cancer, and interestingly, the ONCOMINE analysis revealed that a study comparing estrogen receptor-positive breast cancer with estrogen receptor-negative breast cancer found TPD52 to be preferentially overexpressed in the estrogen receptor-positive cases.

**DISCUSSION**

Chromosome 8q gain is known to be associated with poor outcome in men with clinically localized prostate cancer (7, 59). The current study identifies a potential oncogene associated with prostate cancer progression. Several pieces of evidence support this observation. First, multiple expression array studies have identified TPD52 as overexpressed at the transcript level (51). This observation was confirmed using quantitative reverse transcription-PCR. TPD52 protein overexpression was also observed in prostate cancer using high-density TMA from a large range of patient samples. By two separate chip-based genomic approaches, amplification of 8q21.13 in the region of TPD52 was observed, with the greatest increase in copy number occurring in metastatic prostate cancer samples. By contrast, a BAC clone at 8q21.11, located approximately 60 Mb centromeric to TPD52, did not demonstrate consistent amplification. The c-myc oncogene located at 8q24 has also been found to be variably amplified in prostate cancer (7, 15, 16, 18, 19). One study looking at hormone-refractory prostate tumors demonstrated only 11% amplification for c-myc, but this amplification was associated with prostate cancer progression (18). Therefore, it is clear that multiple loci of amplification exist on 8q. The current study also demonstrates that by using high-density SNP-arrays, the resolution should allow for a better appreciation of these amplification events, which could not be identified using FISH probes. The FISH data would suggest that, because both TPD52 and Elongin C probes demonstrated amplification, both genes sit on a large amplion. However, the SNP and aCGH data presented in this study help us to better appreciate that along this area of 8q, there are several amplification peaks. Both 8q21.11 and 8q21.13 have copy number increases but by SNP analysis and aCGH are shown to represent two discrete areas of amplification on 8q independent of c-myc. As the resolution of the SNP chips increases, genomic complexity should be better appreciated.

The copy number increase at 8q21.13 cannot be the only explanation for the overexpression of TPD52 at the protein level. This study found that a significantly larger percentage of localized and metastatic prostate tumors express TPD52 by immunohistochemistry than demonstrate an increase in copy number at 8q21.13. Therefore, deregulation of TPD52 cannot be entirely explained by amplification. This study also found that TPD52 was highly expressed in prostatic intraepithelial neoplasia but not to the same degree of intensity as benign prostate epithelium or proliferative inflammatory atrophy, suggesting that TPD52 expression may occur early in the development of cancer.

Although further confirmatory work needs to be performed, androgen response elements were identified upstream of the TPD52 coding region, and cell line experiments provide evidence for the regulation of TPD52 by androgen incubation. These findings suggest that TPD52 expression may be regulated in part by androgens, as suggested by work from DePrimo et al. (61) and Nelson et al. (58). The combination of gene amplification and androgen stimulation likely contributes to the up-regulation of TPD52. At this point, it is unclear what role TPD52 plays in cancer progression. However, the TMA experiments suggest that there was a trend toward PSA failure after radical pros-

**Table 3 TPD52 amplification in prostate cancer progression as determined by FISH**

<table>
<thead>
<tr>
<th>Tissue type (no. of cases)</th>
<th>Mean copy no. (range)</th>
<th>SE</th>
<th>Low</th>
<th>High</th>
</tr>
</thead>
<tbody>
<tr>
<td>Benign (38)</td>
<td>2.0(2–2)</td>
<td>0.00</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>PCA (37)</td>
<td>2.8(2–6)</td>
<td>0.15</td>
<td>2.5</td>
<td>3.1</td>
</tr>
<tr>
<td>Mets (12)</td>
<td>3.3(2–4)</td>
<td>0.05</td>
<td>2.7</td>
<td>4.0</td>
</tr>
</tbody>
</table>

* FISH, fluorescence in situ hybridization; CI, confidence interval; PCA, clinically localized prostate cancer; Mets, hormone-refractory metastatic prostate cancer.

A minimum of 2 tissue microarray samples/case were evaluated.
tatectomy with strong TPD52 expression. One limitation with this immunohistochemical analysis is that the vast majority of clinically localized tumors demonstrated moderate to strong TPD52 expression, making a reproducible threshold difficult to achieve using standard techniques. We have begun the process of trying to determine whether the increased amplification seen is closely associated with protein expression. This work will use a highly sensitive fluorescence-based method that we have recently applied to prostate cancer samples using an automated quantitative imaging system called AQUA (62). This should allow us to help distinguish whether there is any difference in TPD52 protein expression between high-grade prostatic intraepithelial neoplasia and localized prostate cancer. Future work will also concentrate on defining the functional role of TPD52 in prostate cancer progression.

This study is the first to combine SNP arrays and aCGH to help characterize a region of amplification. This process allows for confirmation of these genomic observations using two separate technologies. The similar results seen by use of SNPs by one array-based method and BAC probes by another method suggest that SNP array-based technology, which is commercially available, may make this technology available to a broader group of users. The excellent concordance between these two technologies and the FISH results for TPD52 was very promising.

The use of ONCOMINE, a novel informatics tool, demonstrated the utility of a second novel informatics tool, a module for evaluation of genomic application integrated into dChip (49), used to analyze commercially available SNP arrays. This software was used previously to provide information regarding loss of heterozygosity events in prostate tumors, as reported previously by one of the Lieberfarb et al. (46). It has now been upgraded to also allow for evaluation of amplifications and deletions (47). Therefore, using a single chip-based assay, one can in theory perform genome-wide searches for oncogenes and tumor suppressor genes. One can potentially subclassify tumors at resolutions of approximately 300 kb by identifying areas of loss of heterozygosity, homozygous deletions, and areas of amplifications. SNP array technology should greatly enhance our ability to study genomic aberrations associated with carcinogenesis.

In summary, this study demonstrated overexpression of TPD52 in prostate cancer. This overexpression is likely produced by increased gene copy number in a proportion of cases, and it increases with prostate cancer progression. Androgens may positively regulate this expression, as suggested by the presence of androgen response elements located upstream of TPD52 and regulation of TPD52 protein expression in a prostate cancer cell line study. As demonstrated by a wide survey of expression array data, TPD52 is overexpressed in tumors other than those of the prostate and breast.

Addendum

While this paper was in review, another group reported on PrLZ, a family member of TPD52.
Overexpression, Amplification, and Androgen Regulation of TPD52 in Prostate Cancer

Mark A. Rubin, Sooryanarayana Varambally, Rameen Beroukhim, et al.


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