ATM Protein Overexpression in Prostate Tumors
Possible Role in Telomere Maintenance

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Abstract

It has been postulated that telomere dysfunction and telomerase activation have important roles in prostate tumorigenesis. Since the ataxia-telangiectasia mutated gene product (ATM protein) is involved in maintaining telomere length and integrity, we hypothesized that its expression might be altered in prostate tumors and, thus, examined its profile in 49 tumor samples. The majority (32/49) had ATM protein levels higher than those observed in normal tissues, with only 5 of 49 tissue samples showing reduced or absent ATM levels. Three of these were from the group of 6 young-onset or sibling-pair tumors. There was a trend toward higher ATM expression in tumors with a higher Gleason score (23/32 [72%] for grade 8-10 vs 9/17 [53%] for grades 5-7), although this difference was not statistically significant. These findings support our hypothesis that the presence of the ATM protein at the same or a higher level than that in normal prostate cells might have an important role in the maintenance of the shortened telomeres commonly found in prostate cancer cells.

The molecular basis of prostate cancer includes heritable and somatic genetic changes with a marked genetic instability and strong age dependence.1,2 An important mechanism leading to genetic instability in eukaryotic cells is telomere dysfunction. These specialized DNA-protein structures terminate the ends of linear eukaryotic chromosomes and enable cells to distinguish true chromosome ends from double-strand breaks within the genome. Uncapped chromosome ends are at risk of degradation, recombination, or fusion by cellular DNA repair systems, resulting in genetic information becoming lost, rearranged, or unstable (reviewed by Kim et al3).

Several proteins have been identified that associate with telomeres, regulating their structure or length.3,4 The TRF1 and TRF2 proteins bind directly to the telomeric DNA, with POT1 binding specifically to the single-stranded 3’ overhang. Other proteins such as TIN2, PinX1, Tankyrase, and hRAP1 also localize specifically to telomeres, but indirectly by binding to TRF1 or TRF2. In addition, proteins such as Ku and the meiotic recombination 11 homologue–Rad50–Nijmegen breakage syndrome (Mre11-Rad50-NBS1) complex that are involved in double-strand DNA repair also are localized at telomeres. Evidence is accumulating that ataxia-telangiectasia mutated (ATM), the protein altered in individuals with the recessive cancer-prone syndrome ataxia-telangiectasia (AT), is linked functionally to maintaining telomere length and integrity. AT cells have abnormally shortened telomeres (reviewed by Pandita5), and mice that are doubly null for ATM and the telomerase RNA component (Terc) show increased telomere erosion and genomic instability.6 The TRF1 protein forms stable complexes with ATM in cells and is a substrate for ATM-dependent phosphorylation.7
Telomere shortening and telomerase activation both occur in prostate tumors, with telomere shortening found as an early somatic DNA alteration, whereas telomerase activation is a prevalent marker in malignant prostate tissues. Thus, we hypothesized that because the ATM protein has a major role in such processes, its expression is likely to be altered in prostate tissue and might be associated with grade. Therefore, we undertook a pilot study to test this hypothesis by examining the expression profile of the ATM protein in 49 malignant prostatic tissue samples.

### Materials and Methods

#### Tissue Samples

The procedures followed were approved by the locoregional ethics committee of the Royal Marsden NHS Trust, London, England, and International Agency for Research on Cancer, Lyon, France, in accord with the ethical standards of the Helsinki Declaration of 1975. Specimens were obtained from 49 prostate cancer cases; patient ages ranged from 49 to 76 years. The 49 tumor tissue samples analyzed were in 3 groups:
- biopsy (n = 36) or radical (n = 1) prostatectomy specimens from patients with organ-confined prostate cancer and defined as early disease (T1-T2 N0 M0) or transurethral resection of the prostate specimens (n = 12) from patients with locally advanced or metastatic prostate cancer (T3-T4 N0-N1 M0-M1). Clinical staging was by digital rectal examination, serum prostate-specific antigen level, bone scan, and computed tomography or magnetic resonance imaging of the pelvis in patients undergoing radical treatment. In addition, tissue sections from 6 patients who were members of an early-onset cohort (disease diagnosed when they were younger than 60 years) or sibling cohort (brother pairs affected with prostate cancer, one when younger than 65 years) were analyzed.

The average age ± SD at first diagnosis of the tumor in the 49 cases was 66.8 ± 7.0 years; the 6 patients in the early-onset–sibling group were slightly younger (55.6 ± 7.4 years). The Gleason scores were assessed at the time of the scoring of the ATM protein expression (Table 1); the majority were high-grade, poorly differentiated tumors (32/49 [65%]).

#### Immunohistochemical Analysis of ATM Expression

The immunostaining analysis of ATM expression was performed as previously described, with epitope unmasking and antibody dilutions optimized. Formalin-fixed and paraffin wax–embedded tissue sections were used for this analysis.

After dewaxing, endogenous peroxidases were inactivated by incubation in a 0.3% hydrogen peroxide–methanol solution for 30 minutes, and the slides were rehydrated in distilled water. Each section then was treated with antigen unmasking solution (Vector Laboratories, Biosys, Compiegne, France) 3 times for 5 minutes each in a microwave oven, according to the supplier’s specifications. After blocking nonspecific protein binding with phosphate-buffered saline (PBS) containing 5% (wt/vol) skimmed milk and 0.1% (wt/vol) bovine serum albumin (BSA) for 45 minutes, slides were incubated overnight at 4°C with the primary monoclonal ATM antibody at a dilution of 1:100 (ATML2 raised against amino acids 2581-2599). The antibody was diluted in PBS containing 0.1% (wt/vol) BSA. After PBS washings, slides were incubated for 45 minutes with the secondary antibody: an antimouse antibody (1:200 dilution in PBS–0.1% [wt/vol] BSA; Vectastain ABC Kit, Vector Laboratories). Peroxidase binding was revealed by development of the sections in 3,3'-diaminobenzidine following streptavidin signal amplification (Vectastain ABC Kit). Hematoxylin counterstaining was performed before the dehydration and mounting of slides. Normal prostate sections were included in each batch of prostate tumor sections analyzed.

#### Control Samples

The specificity of the ATM antibody has been assessed previously using 2 formalin-fixed, paraffin-embedded spleen.
samples from patients with clinically diagnosed AT.10 In sections used as negative control samples, the primary antibody was omitted.

**Immunohistochemical Scoring**

To compare the relative levels of ATM expression, a score corresponding to the product of the nuclear staining intensity and the percentage of tumor cells staining positive was calculated for the protein in each tissue section.11 Nuclear staining intensity was scored as follows when compared with the immunoreactivity seen in normal prostatic tissue samples for the same antibody: null, 0; low, 1; moderate, 3; and high, 5. The percentage of tumor cells with positive nuclear staining was categorized as follows: less than 25%, 1; 25% to 50%, 2; 50% to 75%, 3; and more than 75%, 4. The resulting scores (from 0 to a maximum of 20) permitted the classification of the expression of each protein as negative (score, 0), low (score, 1-5), normal (score, 6-12), or high (score, >12). Each slide was assessed by at least 2 investigators (S.A., C.S.F., P.T.). Differences in ATM expression levels between groups of tumors or with different Gleason scores were assessed by using the Fisher exact test.

**Results**

**ATM Expression in Normal Prostate Sections**

ATM expression was examined in 21 histologically normal prostate sections obtained from benign prostate hyperplasia (transitional zone) and in 4 sections from cystectomy specimens (peripheral zone). By using the experimental protocol described in the “Materials and Methods” section, ATM protein was found to be expressed at a moderate level in 50% of the epithelial and basal cells of the hyperplastic glands of the transitional zone and the peripheral zone (data not shown). Thus, normal ATM expression was classified as a score of 6 to 12, reflecting the considerable heterogeneity seen between different glands within the same prostate. Similar levels of expression were noted whenever there was normal prostatic tissue surrounding tumor tissue.

**Tumor Tissue Samples**

Reduced ATM levels were found in only 5 (10%) of the 49 tumor tissue samples analyzed, with 1 sample having no detectable ATM expression and 4 having a low ATM expression (score, 1-5). In the tumor sample with no detectable ATM expression, the lymphocytes present within the section served as an internal positive control for the immunoreactivity of the antibody. When the expression profiles were analyzed according to tumor diagnosis, the tumor with no detectable ATM staining was 1 (4%) of 25 early disease tissue samples was found in 1 (6%) of 18 locally advanced or metastatic tumors and in 3 (50%) of 6 samples from the early-onset and sibling group. A level of expression comparable with that seen in normal tissue sections was found in 12 tumors (24%) (score, 6-12). The remaining 32 tissue samples (65%) all had higher levels of ATM expression than that observed in normal tissue samples. There was a trend toward higher ATM expression in the samples with a high Gleason score (23/32 [72%] for grade 8-10 vs 9/17 [53%] for grades 5-7), although this difference was not statistically significant (P = .219).

**Discussion**

We identified that ATM protein expression levels in prostate cancers are maintained at the same or higher levels than those occurring in normal prostate tissues. The small group of tumors (n = 6) from the early-onset and sibling-pairs cases showed a different profile of ATM expression from the other 43 tumors examined in this study set, with 50% (3/6) of the former group showing a reduced ATM expression level compared with 5% (2/43) of the early disease tumors and locally advanced or metastatic tumors (P = .009). This difference, albeit based on a small number of samples examined, suggests that the molecular basis of the genetic alterations in these tumors might have a phenotype different from that of sporadic prostate tumors. The increased expression identified in the majority of tumors in the present study also is in contrast with the ATM expression profile found in nonfamilial breast tumors, in which a reduction in expression occurred in a large majority of tumors compared with the levels in normal tissues.10-12

It has been postulated that telomere dysfunction and telomerase activation have important roles in different stages of prostate tumorigenesis. Telomere shortening in human somatic cells can have 3 cellular outcomes: genomic instability, senescence, or death. Genomic instability clearly predisposes cells to neoplastic transformation, while cellular senescence and death suppress tumor formation by limiting the number of times that cells can divide. Telomere shortening has been found in the proliferating intermediate luminal cells in the prostate, frequently in the absence of telomerase activity.8

Activation of telomerase is found in many tumors and occurs in prostate cancer, although generally not in benign prostatic hyperplasia or healthy prostate tissues. This activation has been suggested to facilitate tumor progression by increasing the proliferation of cells, particularly those that have shortened telomeres, and stabilizing potential genomic...
Immunostaining of normal prostate tissue and prostate carcinoma samples. The immunostaining was defined as null, low, normal, or high based on the intensity and the percentage of cancer cells expressing the ataxia-telangiectasia mutated (ATM) protein compared with the immunoreactivity seen in normal prostate sections. A, ATM expression in hyperplastic glands of the transitional zone (×400). B, Prostate adenocarcinoma with low ATM staining. Few of the nuclei are prominently stained (×400). C, Prostate carcinoma with normal ATM expression with respect to the number of nuclei stained and the intensity of nuclear staining (×400). D, Prostate carcinoma with high ATM staining. Virtually all nuclei are stained intensely (×400).

Table 2
Combined Gleason* and ATM Scores

<table>
<thead>
<tr>
<th></th>
<th>All Samples (N = 49)</th>
<th>Early Disease (n = 25)</th>
<th>Locally Advanced or Metastatic Cancer (n = 18)</th>
<th>Early-Onset or Sibling Cohort (n = 6)</th>
</tr>
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<tbody>
<tr>
<td>Gleason score 8-10</td>
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<tr>
<td>ATM null (%)</td>
<td>32</td>
<td>17</td>
<td>13</td>
<td>2</td>
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<tr>
<td>Low (%)</td>
<td>2 (6)</td>
<td>1 (6)</td>
<td>0 (0)</td>
<td>0 (0)</td>
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<tr>
<td>Normal (%)</td>
<td>6 (19)</td>
<td>2 (12)</td>
<td>3 (23)</td>
<td>1 (50)</td>
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<tr>
<td>High (%)</td>
<td>23 (72)</td>
<td>14 (92)</td>
<td>9 (69)</td>
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<td>Gleason score 5-6</td>
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<tr>
<td>ATM null (%)</td>
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<td>4</td>
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<tr>
<td>Low (%)</td>
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<td>2 (50)</td>
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<tr>
<td>Normal (%)</td>
<td>6 (35)</td>
<td>4 (50)</td>
<td>1 (20)</td>
<td>1 (25)</td>
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<td>High (%)</td>
<td>9 (53)</td>
<td>4 (50)</td>
<td>4 (80)</td>
<td>1 (25)</td>
</tr>
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</table>

ATM, ataxia-telangiectasia mutated.

* No Gleason score 7 tumors were analyzed. See the text for an explanation of immunohistochemical scoring.
instability, thus leading to immortalization (see Meeker et al and Hackett and Greider). The primary function of ATM in telomere maintenance is believed to involve its direct action on telomeres or telomere proteins rather than through regulation of telomerase activity. The 2 alternatively spliced isoforms of the PIN2/TRF1 gene (Pin2 and TRF1), differing in an internal deletion of 20 amino acids, form stable complexes with ATM and are phosphorylated on Ser219 in vitro and in vivo by ATM kinase activity. Phosphorylation of Pin2/TRF has been shown to inhibit its physiologic functions and negatively regulate its function in the DNA damage response. Up-regulation of PIN2/TRF1 has been shown to inhibit its physiologic functions and negatively regulate its function in the DNA damage response.7 Up-regulation of PIN2/TRF1 accelerates telomerase shortening, as found in AT cells in which the lack of ATM to suppress Pin2/TRF1 function might contribute to accelerated telomere loss.

Therefore, we hypothesized that the presence of the ATM protein, at the same or a higher level than that commonly occurring in normal prostate cells, might have an important role in maintaining the shortened telomeres found in prostate cancer cells. This observation is in agreement with the model for involvement of telomere shortening in prostate tumorigenesis proposed by Meeker et al. We now postulate that normal or increased ATM expression is maintained from the time period in which critical telomere shortening is reached and telomerase activation found and, perhaps, from the early neoplastic stages of prostate tumorigenesis (including prostatic intraepithelial neoplasia). However, we have examined only tissues with invasive and metastatic cancer and have not addressed the stage of neoplasia at which ATM expression becomes modulated. This hypothesis will be tested by examining morphologically identifiable earlier lesions for the expression profiles of ATM together with those of other proteins (eg, the Mre11-Rad50-NBS1 complex) also implicated in telomere maintenance.

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Figure 1 Model for involvement of telomere shortening in human prostate tumorigenesis. Based on our observations, we hypothesize that normal or increased ataxia-telangiectasia mutated (ATM) expression occurs during the period when telomere shortening and telomerase activation are found and would enhance the stabilization of shortened telomeres, thus stabilizing the genomic instability leading to immortalization and tumorigenesis. GSTP1, glutathione S-transferase–π; PIN, prostatic intraepithelial neoplasia. From Meeker et al. Used by permission.
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References