ATM activation is accompanied with earlier stages of prostate tumorigenesis

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Abstract

The ATM (ataxia telangiectasia mutated) kinase plays an essential role in maintaining genome integrity by coordinating cell cycle arrest, apoptosis, and DNA damage repair. Phosphorylation of ATM at serine 1981 (ATMpSer1981) by DNA damage activates ATM, which subsequently phosphorylates H2AX Ser139 (γH2AX), Chk2 Thr68 (Chk2pThr68), and p53 Ser15 (p53pSer15). To determine the role of the ATM pathway in prostate cancer tumorigenesis, we have analyzed 35 primary prostate cancer specimens for ATMpSer1981, Chk2pThr68, γH2AX, and p53pSer15 by immunohistochemistry (IHC) in normal glands, prostatic intraepithelial neoplasias (PINs), and carcinomas. Increases in the intensities of ATMpSer1981, Chk2pThr68, and γH2AX and in the percentage of cells that are positive for ATMpSer1981, Chk2pThr68, or γH2AX were observed in PINs (p < 0.001) compared to normal prostatic glands and carcinoma. However, this pattern of immunostaining was not seen for p53pSer15. Thus, ATM and Chk2 are specifically activated in PINs. As PINs are generally regarded as precursors of prostatic carcinoma, our results suggest that ATM and Chk2 activation at earlier stages of prostate tumorigenesis suppresses tumor progression, with attenuation of ATM activation leading to cancer progression.

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Keywords: ATM; Chk2; p53; γH2AX; Prostate tumorigenesis

1. Introduction

Prostate cancer is the second leading cause of cancer-related deaths in males in the developed world [1] and affects one in nine men over the age of 65 in the United States [2]. Evidence indicates that the cancer is initiated from prostatic epithelial cells and progresses into local lesions of PINs before becoming malignant carcinoma [3]. Many factors have been reported to promote prostate tumorigenesis, including the activation of growth promoting signals (HER-2/neu, ErbB-2, and Akt) [4–8] and the inactivation of tumor suppressors, Nkx3.1 [9,10], KLF6 [11], p53 [12], p27Kip1 [13–15], and PTEN [16–18].

One of the mechanisms of tumor suppression is the DNA damage response, which maintains genome stability. Genomic instability due to the disruption or attenuation of the DNA damage response is the leading cause of tumorigenesis [19–22]. The ATM (ataxia telangiectasia mutated) kinase plays a central role in the coordination of the DNA damage response [23,24]. ATM activation exists as an inactive dimer. The generation of active ATM monomer is initiated by DNA DSBs (double-stranded breaks)-induced auto-phosphorylation of Ser1981 of human ATM [25]. ATM activation is facilitated by interaction with the MRE11–RAD50–NBS1 complex [26]. Activated ATM sets a motion of downstream events, including phosphorylation of Ser139 of histone H2AX (γH2AX) [24] and Brca1 to promote DNA
damage repair. Furthermore, activation of ATM results in the phosphorylation/activation of Chk2 and phosphorylation/stabilization of p53 [24], leading to cell cycle arrest to repair DNA damage, or apoptosis if damage is too severe to be repaired. Thus, inactivation of ATM or components of the ATM pathway, including MRE11, NBS1, CHK2, and p53 are frequently observed in human cancers.

One of the functions of tumor suppressors is to sense oncogenic events in order to stop tumor initiation and progression. Consequently, this places a selective pressure for the inactivation of this “tumor surveillance” system in order to promote tumor progression. p14ARF functions in tumor surveillance [27]. Upon sensing hyper-proliferative signals, p14ARF suppresses tumor-igenesis through p53-dependent and -independent pathways [27]. Interestingly, we have recently shown that ATM contributes to this p14ARF-mediated tumor suppression [28], indicating a role for ATM in tumor surveillance.

We provide evidence for the first time that ATM is specifically activated in the process of prostate tumorigenesis. ATM activation was detected in prostatic glands, PINs, and prostatic carcinoma. ATM is activated to significantly higher levels in PINs than in prostatic glands and carcinomas. This pattern of ATM activation in relation to prostate cancer progression (initiation-PIN-carcinoma) strongly suggests that ATM senses prostatic oncogenic events and that attenuation of ATM activation promotes prostate cancer progression. Consistent with this scenario, we found a similar pattern for Chk2 activation, a substrate of ATM. Comparable findings were also previously reported in lung and bladder tumors [29,30]. Thus, our results indicate the involvement of ATM in tumor surveillance.

2. Materials and methods

2.1. Collection of primary prostate cancer specimens and pathological examination

Prostate cancer specimens were collected at St. Joseph’s Hospital in Hamilton, Ontario, Canada under the approval from the local Ethics Board and consent from patients. Cancers were examined and graded according to the Gleason system by the pathologists (Drs. Sam Salama, Jean-Claude Cutz) at St Joseph’s Hospital [31]. Normal, high grade PINs, and carcinoma areas were examined in each patient on the same slide. Gleason scores, age of onset of cancer among the following family members: brother, father, maternal and paternal uncles, maternal and paternal grandfathers. Patients who could not recall this information are listed as “n/a”.

We located morphologically normal glands, PINs, and carcinoma on the same slide from each case to determine the signal intensity of each specific protein. Approximately 1000 cells were analyzed for each case.

The intensity of staining was double-blind graded on a scale of 1–3 (1=weak staining, 2=moderate staining, and 3=strong staining). We used the same slide from each case to determine the signal intensity of each specific protein. Approximately 1000 cells were analyzed for each case.

### Table 1

Characteristics of patients used in immunohistochemistry analysis

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<td>35</td>
<td>53</td>
<td>6</td>
<td>N</td>
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Mean±SD: 60.8±6.2, 6.8±0.6

Age of onset (Mean±Standard Deviation, SD), Gleason scores (Mean±Standard Deviation, SD), and familial history are indicated. n/a=Unknown, N=No, Y,Yes.

2.2. Immunohistochemistry staining of ATMpSer1981, Chk2Thr68, p53Ser15, and γH2AX

Slides were prepared from paraffin-embedded prostate cancer specimens, which were deparaffinized in xylene, cleared in a graded ethanol series, and heat-treated for 20 min in Antigen Unmasking Solution (Vector Laboratories) in a food steamer. Primary antibodies specific for Ser1981 phosphorylated ATM (Rockland Inc, 1:100), ATM (Calbiochem, 1:75), Chk2pThr68 (Cell Signaling Technology, 1:100), p53pSer15 (Cell Signaling Technology, 1:75), p53 (Santa Cruz Biotechnology, DO-1, 1:100), and γH2AX (Cell Signaling Technology, 1:100) were incubated with the sections overnight at 4 °C. Negative controls were incubated with a non-specific anti-rabbit IgG. Biotinylated goat anti-rabbit IgG and Vector ABC reagent (Vector Laboratories) were then sequentially added according to the manufacturer’s instructions. Washes were performed with Tris-buffered saline with 0.1% Tween-20 (TBST) for Chk2Thr68, p53pSer15, and γH2AX. Phosphate buffered saline (PBS) was used in washes performed for ATM and Ser1981 phosphorylated ATM. Chromogen reaction was carried out with diaminobenzidine, and counterstaining was done with hematoxylin.


2.3. Evaluation of immunohistochemistry staining

We located morphologically normal glands, PINs, and carcinoma on the same slide from each case to determine the signal intensity of each specific protein. Approximately 1000 cells were analyzed for each case.

The intensity of staining was double-blind graded on a scale of 1–3 (1=weak staining, 2=moderate staining, and 3=strong staining). We used the intensity of staining in PINs as the basis for strong staining (see Fig. 1).
semiquantitative score on a 10% increment scale ranging from 0 to 100% was used to assess the percentage of stained cells in each cell type. The final composite staining score for immunostaining was based on the intensity of staining (1, 2, or 3) multiplied by the percentage of immunopositive cells (0–100).

2.4. Statistical analysis

Statistical analysis was performed using GraphPad Prism 4.0 software for Windows. The composite staining scores for ATMpSer1981, Chk2pThr68, γH2AX, and p53pSer15 in normal glands, PINs, and carcinoma were compared using the Mann–Whitney non-parametric test. A p<0.05 was considered statistically significant. All p-values are two-tailed.

3. Results

3.1. Specific activation of ATM in prostate cancer tumorigenesis

Cancer results from genome instability and the DNA damage response is the mechanism that maintains genomic integrity. ATM kinase plays an essential role in the coordination of the DNA damage response. The role of ATM in the suppression of prostate tumorigenesis is unknown. To address this issue, we have examined ATM activation during the process of prostate tumorigenesis. Phosphorylation of Ser1981 of ATM disrupts the ATM dimer, resulting in the generation of active ATM monomer [22]. Detection of ATMpSer1981 using a specific antibody (Rockland Inc) is the most widely used assay for assessing ATM activation in vivo. Using this system, we could detect ATMpSer1981 signals in PIN, since samples incubated with control Rabbit IgG (Fig 1A) showed no detectable signals. Furthermore, the ATMpSer1981 signals could be competed-out by a ATM peptide with Ser1981 phosphorylated [1974-SLAFEEG-pS(1981)-QSTTISS-1988] (Fig 1B) but not by the corresponding peptide without Ser1981 phosphorylated [1974-SLAFEEG-S(1981)-QSTTISS-1988] (data not shown). Consistent with ATM’s functions in DNA damage recognition [32], we were able to demonstrate that ATMpSer1981 signals are localized exclusively in the nucleus (Fig. 1). Taken together, these data reveal that the ATMpSer1981 signals detected are specific for the Ser1981 phosphorylated ATM.

3.2. Higher levels of ATM activation in earlier stages of prostatic tumorigenesis

The fact that ATM activation was observed in primary prostate cancer specimens prompted us to determine its activation in the context of prostate cancer development. It is believed that high grade PINs are precursors of prostatic carcinoma [3]. We thus examined ATM activation in morphologically normal prostatic glands, PINs, and carcinomas derived from the same individual prostate cancer specimens. ATM activation was detected exclusively in the nuclei of morphologically normal prostatic glands, PINs, and carcinomas (Fig. 2). The levels of ATM activation are relatively lower in normal prostatic glands and carcinoma when compared to ATM activation in PINs (Fig. 2). This did not result from differences in cellularity among these tissues, as a close examination of
regions with comparable cell density also showed higher levels of ATM activation in PINs than in both normal prostatic glands and carcinomas (Fig. 2, far right column). Furthermore, this analysis was carried out using the same specimen/slide (Fig. 2).

To further confirm that ATM was specifically activated in PINs, we have analyzed approximately 1000 cells for each cell type (normal, PIN, and carcinoma) in tissues from individual patients. In all 35 primary prostate cancer specimens, we could identify morphologically normal prostatic glands, PINs, and carcinomas (data not shown). Following the intra-patient comparison (using normal glands, PINs, and carcinomas within the same patients/slides), we have determined the levels of ATM activation in normal glands, PINs, and carcinomas based on a global composite scoring system (by multiplying the intensity of staining with the percentage of immunopositive cells; see Materials and methods for details). We were able to show elevated ATM activation in PINs when compared its activation in carcinomas and normal glands (Table 2). Thus, the differences in ATMpSer1981 observed among normal prostatic glands, PINs, and carcinomas did not result from immunohistochemistry procedures that were performed separately. Mann–Whitney test revealed significant differences ($p<0.001$) in ATM

![Fig. 2. ATM activation in normal prostatic gland, PIN, and carcinoma. Morphologically normal prostatic gland, PIN, and carcinoma from the same patient (or slide) were H&E stained and immunohistochemically stained for Ser1981 phosphorylated ATM using an anti-pSer1981-ATM antibody. The inset areas are enlarged (right column).](image)

### Table 2

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Composite staining score $^a$</th>
<th>Chk2pThr68 $^b$</th>
<th>γH2AX $^c$</th>
<th>p53pSer15 $^d$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>Median</td>
<td>Mean±SD</td>
<td>Median</td>
<td>Mean±SD</td>
</tr>
<tr>
<td>PIN $^b$</td>
<td>180.0</td>
<td>177.7±56.3</td>
<td>160.0</td>
<td>156.7±45.1</td>
</tr>
<tr>
<td>Carcinoma</td>
<td>40.0</td>
<td>48.9±24.2</td>
<td>60.0</td>
<td>61.4±30.4</td>
</tr>
</tbody>
</table>

$^a$ Composite score=Staining Intensity (1, 2, or 3)×Percentage of Immunopositive Cells (0–100). Maximum score is 300.

$^b$ PIN, prostatic intraepithelial neoplasia (high-grade).

$^c$ Composite staining score was significantly higher in PIN than normal ($p<0.001$) and carcinoma ($p<0.001$) (Mann–Whitney).

$^d$ There were no significant differences between normal and carcinoma ($p=0.08$) (Mann–Whitney).

$^e$ Composite staining score was significantly higher in carcinoma than normal ($p=0.01$) (Mann–Whitney).

$^f$ Composite staining score was significantly higher in carcinoma than normal ($p=0.03$) (Mann–Whitney).

$^g$ There were no significant differences between normal, PIN, and carcinoma ($p>0.05$) (Mann–Whitney).
activation (ATMpSer1981) between PIN and normal glands as well as between PIN and carcinoma (Table 2). While ATM activation seems elevated in carcinoma when compared to normal glands (mean score of 38.6±20.3 and median of 30 for normal glands versus a mean score of 48.9±24.2 and median of 40.0 for carcinoma) (Table 2), the difference is not statistically significant (p=0.08) (Table 2). Additionally, the increases in ATM activation in PINs is not a result of enhanced ATM expression in PINs, as normal prostatic glands, PINs, and carcinoma expressed comparable levels of the ATM protein (Fig. 3, see the total ATM column). Taken together, the above data demonstrate specific ATM activation in PINs. As the Gleason scores for our specimens are clustered between 6 and 8 (Table 1), we could not analyze a correlation between ATM activation and Gleason scores. Nevertheless, as PINs are generally regarded as precursors of prostate carcinoma, the above observations indicate that ATM is specifically activated in earlier stages of prostate tumorigenesis.

### 3.3. Activation of Chk2 in earlier stages of prostatic tumorigenesis

To further investigate ATM activation in PINs, we examined whether ATM activation leads to phosphorylation of known ATM substrates. Two key ATM substrates are Chk2 and p53. ATM phosphorylates Chk2 Thr68 and p53 Ser15, resulting in their activation [24]. We were able to demonstrate increases in Chk2pThr68 in PINs when compared to normal prostatic glands (Fig. 3), with levels of Chk2pThr68 declining in carcinoma (Fig. 3). Consistent with the nuclear localization of Chk2pThr68, Chk2pThr68 was detected in the nucleus (Fig. 3). Mann–Whitney tests confirmed enhanced Chk2 activation (Chk2pThr68) in PINs (p<0.001) than in normal prostatic glands and carcinoma (p<0.001) (Table 2). While Chk2 activation is reduced in carcinomas when compared to PINs (Table 2), its activation is still elevated in carcinoma versus normal glands (Table 2, p=0.01) (see Discussion for details).

Surprisingly, in all 35 patients examined, there were no statistically significant differences in p53pSer15 among normal prostatic glands, PINs, and carcinoma (Fig. 4, Table 2, p>0.05). p53 is frequently mutated in human cancers and mutant p53 is commonly more stable than wild type p53. It is thus possible that the specific conformation of mutant p53 may prevent its Ser15 from being phosphorylated by ATM. To examine such a possibility, we have determined p53 protein levels in the corresponding patients by IHC. No differences in p53 protein expression were observed among morphologically normal prostatic glands, PINs, and carcinomas (p>0.05) (Fig. 4). This pattern is consistent with that of p53pSer15 observed in those tissues (Fig. 4). These observations thus suggest that inability to detect changes in p53pSer15 in prostate cancer development may not be caused by mutations in p53 protein. Taken together, the above results indicate that ATM may not phosphorylate p53 Ser15 in prostate tumorigenesis (see Discussion for details).

As the pattern of Chk2pThr68 closely follows that of ATMpSer1981 and the coexistence of ATMpSer1981 and Chk2pThr68 in the same tissues (Fig 3), Chk2 may be a bone fide ATM target and may play an important role in ATM-mediated tumor surveillance in prostate tumorigenesis. This agrees with the concept that the progression from PIN to carcinoma results from attenuation of ATM activation.

### 3.4. Specific increases in γH2AX in PINs

We have further determined the possible mechanisms regulating ATM activation in PINs. Primary ATM-activating signals are DSBs. The localization of γH2AX to DSBs is generally believed to be a very early event in the recruitment of other components of DNA repair machinery onto DNA damage-induced foci [24], and thus formation of γH2AX is widely used as an indicator of DNA damage. Accordingly, we examined the existence of γH2AX in normal prostatic glands, PINs, and carcinomas. In comparison to normal prostatic glands and carcinoma, γH2AX was increased in PINs, which localizes...
exclusively in the nucleus (Fig. 3). We were able to show a significant increase in the levels of γH2AX staining in PINs compared to normal ($p<0.001$) and carcinoma ($p<0.001$) tissues (Table 2). Although the levels of γH2AX staining are significantly lower in carcinomas than in PINs, the carcinoma-associated γH2AX staining is elevated when compared to normal glands (Table 2, $p=0.03$). Taken together, the above observations not only support the possibility that the presence of DNA damage in the evolution of prostate cancer activates ATM, but is also consistent with the concept of specific ATM activation in PINs, as ATM phosphorylates H2AX Ser139 to produce γH2AX.

4. Discussion

While the DNA damage response plays a major role in tumor suppression, how this response actively participates in the suppression of prostate tumorigenesis remains elusive. We provide evidence that ATM is activated in earlier stages of prostate tumorigenesis, with activation levels of ATM decreasing as PINs progress into carcinoma. This pattern of ATM activation reveals that earlier oncogenic events result in ATM activation, which is attenuated by events occurring in the later stages of prostate tumorigenesis. It is thus tempting to speculate that the activation of ATM at the earlier stages of prostate tumorigenesis prevents tumor progression and its desensitization to oncogenic signals promotes tumor development. This would suggest an intriguing possibility that ATM plays a role in tumor surveillance, which is supported by our earlier study showing that ATM contributes to p14ARF-mediated tumor surveillance [28]. As ATM sits very upstream in the coordination of the DNA damage response pathway, employing ATM in the surveillance of oncogenic abnormalities may be a very effective method of tumor suppression.

Our observed ATM activation in morphologically normal prostatic glands of prostate cancer tissues may not contradict with our conclusion involving ATM in prostatic tumor surveillance. These glands may harbor very early oncogenic events, as these coexist with PINs and prostatic carcinomas. The fact that ATM is activated at lower levels in these morphologically normal glands than in PINs may actually support this possibility, as one would envisage that ATM activation increases once oncogenic insults become severe.

The concept of ATM involvement in tumor surveillance is further supported by two recent publications which demonstrated increases in ATM activation in lung and bladder pre-cancerous lesions, and decreases in ATM function in their respective cancers [29,30]. It has been suggested that abnormal cell cycle progression via over-expression of cyclin E, Cdc25A, and E2F1 produces “DNA replication stress” that leads to activation of the DNA damage response, including ATM activation and phosphorylation of its downstream targets, p53 Ser15 (p53pSer15), γH2AX, and Chk2 [29,30].

Consistent with these reports, we also found the enhanced Chk2pThr68 and γH2AX in PINs. Chk2pThr68 and γH2AX reach the highest levels in PINs and then decrease in carcinoma (Table 2). In comparison to normal glands, the levels of Chk2pThr68 and γH2AX are still elevated in carcinoma (Table 2). This pattern of Chk2pThr68 and γH2AX is consistent with their involvement in the surveillance of prostate tumorigenesis via ATM. However, as ATM activation is not statistically higher in carcinoma than in normal glands (Table 2), the fact that both Chk2pThr68 and γH2AX remain elevated in carcinoma over normal glands (Table 2) may be attributable to (1) the possible longer half-life of Chk2pThr68 and γH2AX than the half-life of ATM activation or (2) additional signals in carcinoma other than those of tumor surveillance in pre-cancerous lesions (PINs) resulting in Chk2pThr68 and γH2AX. The latter possibility is supported by the presence of DNA damage-insults in carcinoma.

While increases of Chk2pThr68 and γH2AX in PINs support the involvement of ATM in prostate tumor surveillance, this pattern is not observed for p53 Ser15, a substrate of ATM. p53pSer15 is not predominantly increased in PINs over normal prostatic glands and carcinomas (Table 2). This might not result from a specific anti-p53pSer15 antibody used. We have
purchased anti-p53pSer15 antibodies from a well-trusted company (Cell Signaling Technology, #9284) and this antibody has been demonstrated to recognize p53pSer15 in IHC and other applications [30]. We were also able to detect p53pSer15 in etoposide-treated culture cells and in primary breast cancer specimens by IHC (data not shown). Due to our relatively small sample size, we may not be able to exclude the possibility that ATM also phosphorylates p53pSer15 in prostate cancer tumorigenesis. However, the fact that we do see statistically significant differences in Chk2pThr68 in PINs versus normal prostatic glands and carcinoma in our limited size of primary prostate cancer specimens may suggest that ATM phosphorylates Chk2 more efficiently than the phosphorylation of p53 Ser15 in prostate cancer tumorigenesis.

The fact that we could detect activation of the ATM in PINs, and that only a small portion of carcinoma shows activated ATM suggests that attenuation of ATM activation may take place during the progression of PIN lesions to carcinoma. Thus, reduction of ATM-mediated tumor surveillance may be required for prostate cancer progression. However, as the activation levels of ATM and its substrate Chk2 in prostate carcinoma are not lower than those in morphologically normal prostatic glands, the ATM pathway in carcinoma may not be defective or inactivated. Instead, the activation of ATM may be desensitized or attenuated in response to oncogenic signals to a level that is sufficient to allow for prostate cancer evolution.

Factors leading to ATM activation may include DNA damage that might be induced though the hypothetical “DNA replication stress” mediated by oncogenic signals [29,30] consistent with the presence of γH2AX in PINs. Thus, ATM may facilitate the production of γH2AX. Additionally, as Bcl-2 amplification commonly occurs in prostate cancer development [33–36] and has recently been reported to induce genome instability by the suppression of DNA mismatch repair [37], it is thus possible that abnormal Bcl-2 signals may also attribute to ATM activation in prostate tumorigenesis.

Regardless of what may be the primary causes of ATM activation, the concept of ATM-mediated tumor surveillance in prostate cancer tumorigenesis is consistent with reports showing that ATM polymorphisms, as well as mutations/polymorphisms of ATM substrates Brca1 and Chk2, are risk factors for prostate cancer [38–44].

ATM may play multiple roles in prostate tumorigenesis. While we detected ATM activation without statistically significant changes in the ATM protein during earlier stages of prostate tumorigenesis, ATM protein has been reported to increase in advanced prostate tumors with Gleason scores 8–10 [45]. Thus, it has been suggested that increases in ATM protein may function in maintaining the shortened telomeres observed in advanced prostate tumors [45].

Acknowledgments

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References


