Genomic and Clinicopathologic Characterization of *ATM*-deficient Prostate Cancer

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ABSTRACT

Purpose: The *ATM* (ataxia telangiectasia mutated) gene is mutated in a subset of prostate cancers, and *ATM* mutation may confer specific therapeutic vulnerabilities, although ATM-deficient prostate cancers have not been well-characterized.

Experimental Design: We genetically validated a clinical grade IHC assay to detect ATM protein loss and examined the frequency of ATM loss among tumors with pathogenic germline *ATM* mutations and genetically unselected primary prostate carcinomas using tissue microarrays (TMAs). Immunostaining results were correlated with targeted somatic genomic sequencing and clinical outcomes.

Results: ATM protein loss was found in 13% (7/52) of primary Gleason pattern 5 cancers with available sequencing data and was 100% sensitive for biallelic *ATM* inactivation. In a separate cohort with pathogenic germline *ATM* mutations, 74% (14/19) had ATM protein loss of which 70% (7/10) of evaluable cases had genomic evidence of biallelic inactivation, compared with zero of four of cases with intact ATM expression. By TMA screening, ATM loss was identified in 3% (25/831) of evaluable primary tumors, more commonly in grade group 5 (17/181; 9%) compared with all other grades (8/650; 1%; $P < 0.0001$). Of those with available sequencing, 80% (4/5) with homogeneous ATM protein loss and 50% (6/12) with heterogeneous ATM protein loss had detectable pathogenic *ATM* alterations. In surgically treated patients, ATM loss was not significantly associated with clinical outcomes in random-effects Cox models after adjusting for clinicopathologic variables.

Conclusions: ATM loss is enriched among high-grade prostate cancers. Optimal evaluation of ATM status requires both genomic and IHC studies and will guide development of molecularly targeted therapies.

Introduction

The ataxia telangiectasia mutated (*ATM*) gene encodes a PI3K-related serine/threonine kinase involved in the maintenance of genomic integrity. ATM plays a central role in the sensing and cellular response to DNA damage, and acts as a key signal transducer in the double-strand break repair process (1). Biallelic germline mutation of the *ATM* gene leads to ataxia telangiectasia syndrome, characterized by neurodegeneration, immune deficiencies, and increased cancer susceptibility (2). Heterozygous deleterious germline mutations in *ATM* occur in around 0.5%–1% of the population and are also associated with tumor predisposition, including breast, pancreatic, lung, thyroid, and prostate cancers (2). In prostate cancer, tumoral *ATM* mutations may be either germline or somatic in origin, and are present in 5%–8% of castration-resistant tumors overall, an enrichment of approximately twofold over the frequency in localized prostate cancers (3–5). This enrichment suggests an association of *ATM* mutations with aggressive disease. Indeed, germline *ATM* mutations are significantly more common among patients with lethal compared with indolent prostate cancer (6) and more common among patients with high-grade disease (7), although whether this association persists for somatic *ATM* alterations is unknown.

Beyond acting as a potential prognostic biomarker in prostate cancer, *ATM* status may also be predictive of response to novel targeted therapies. Initial trials of the PARP inhibitor, olaparib, in metastatic castration-resistant prostate cancer (mCRPC) showed an impressive response rate among patients with mutations in the homologous recombination repair pathway, apparently including patients with *ATM* deficiency (8, 9). However, retrospective series and recent findings from prospective phase II and phase III trials examining a variety of PARP inhibitors (olaparib, rucaparib, niraparib, and talazoparib) in patients with mCRPC have shown that tumor responses, as well as progression-free survival estimates, are relatively modest in patients with the *ATM* mutation compared with patients with the *BRCA1/2* mutation who appear to derive the majority of the benefit (10–14). This suggests that PARP inhibitor treatment may not induce synthetic lethality, or perhaps not to the same extent, in *ATM*-mutated prostate cancers compared with *BRCA1/2*-mutated cases (15, 16). However, the recent emergence of potent ATR (ataxia telangiectasia and Rad3-related protein) inhibitors has renewed interest in interrogating *ATM* status in prostate cancer and other malignancies (16–19), because initial phase I trials have shown favorable responses to ATR inhibitors that may be specific to ATM-deficient tumors (20). Preliminary studies also suggest a potential sensitivity of *ATM*-mutated prostate cancers to anti-PD-1 immunotherapy (21, 22), radium-223 treatment (23), and bipolar androgen therapy (24, 25).

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Translational Relevance

Given the key role of ATM (ataxia telangiectasia mutated) as a signal transducer of double-stranded DNA breaks, prostate cancers with *ATM* inactivation have largely been assumed to be similar to those with *BRCA1/2* alterations. However, emerging data suggest that ATM-deficient tumors may be biologically distinct and derive less benefit from PARP inhibitors and potentially more benefit from other targeted therapies compared with *BRCA1/2*-mutated prostate cancers. Here, we develop and genetically validate a clinical grade IHC assay to detect ATM loss and use this assay to identify and characterize several cohorts of ATM-deficient prostate cancers. We demonstrate that ATM loss is highly enriched among high-grade prostate cancers and find that loss is frequently focal and a relatively late molecular event. We propose a combined interrogation approach utilizing both ATM IHC and next-generation sequencing assays to identify ATM-deficient tumors which may benefit from future trials of targeted therapies.

Therefore, the availability of an assay to detect ATM deficiency/loss would have great clinical utility in prioritizing such patients for clinical trials exploring these unique therapeutic vulnerabilities.

Before targeted therapies can be deployed for prostate tumors with ATM inactivation, numerous open questions about the clinical, pathologic, and genomic characteristics of ATM-deficient tumors will need to be resolved. For example, it remains unclear what fraction of patients with *ATM* germline or somatic mutations have detectable second-hit alterations and/or protein loss suggesting complete ATM inactivation and implying likely response to targeted therapy. In addition, how frequently are *ATM* alterations subclonal? What is the sensitivity of DNA-sequencing assays for detection of biallelic inactivation, and what assays should be used to screen patients for ATR inhibitor trial eligibility? Given that *ATM* inactivation and aberrations in other DNA damage repair pathway genes generally occur in the primary tumor rather than during metastatic evolution (26), many of these questions can be resolved by studying primary prostate cancers, which are more readily available compared with metastatic samples. Herein, we developed and genetically validated a clinical grade IHC assay to detect ATM protein loss and used it to assess ATM status in primary prostate cancers with known germline *ATM* mutations, as well as to screen for *ATM* inactivation in a cohort of more than 1,000 primary tumors. We comprehensively compared ATM protein status with DNA-sequencing data and characterized the largest group of ATM-deficient primary prostate cancers reported to date.

Materials and Methods

Patients and tissue samples

With Johns Hopkins Institutional Review Board (Baltimore, MD) approval, and in accordance with the U.S. Common Rule, three patient sets were included in this study. As this study involved only retrospective analysis of previously collected tissue samples, it was performed under a waiver of consent. (i) The first set included a cohort of all radical prostatectomies from 2004–2014 with primary Gleason pattern 5 and available clinical follow-up data ($n = 77$), of whom 52 had previously described targeted next-generation sequencing data from the UW OncoPlex platform available (27); these cases were used for genetic validation of the ATM IHC assay described below. (ii) The second patient set included 20 primary prostate tumors with available radical prostatectomy tissue with known pathogenic germline mutations in *ATM*. Of these cases, 16 had *ATM* mutations detected during sequencing of benign seminal vesicle or leukocyte DNA performed as a part of previously described studies (6), while the remaining four had *ATM* mutations detected using clinical grade germline sequencing platforms (Invitae, Color Genomics) from saliva samples (12). (iii) The third patient set included seven partially overlapping tissue microarray (TMA) cohorts from radical prostatectomies performed at Johns Hopkins (Baltimore, MD). These cases were used to screen for the frequency of ATM protein loss using the IHC assay. All of these TMA cohorts have been described previously, and were selected on the basis of risk factors. Some focused on high-risk features that enriched for adverse oncologic outcomes, so they do not represent an unbiased survey of the overall radical prostatectomy population at Johns Hopkins (Baltimore, MD). In brief, these consisted of: (i) a set of control patients who lacked a *HOXB13* germline mutation, not selected by outcome ($n = 99$; ref. 28); (ii) a set of high-grade (Gleason score 9/10) tumors at radical prostatectomy from 1998 to 2005, designed for comparison with high-grade urothelial carcinomas, not selected by outcome ($n = 38$; ref. 29); (iii) a set of grade-matched African-American and European-American radical prostatectomy samples from 1995 to 2005, enriched for Gleason score ≥7 pathology, not selected by outcome ($n = 292$; refs. 30, 31); (iv) a set of grade-matched African-American and European-American radical prostatectomy samples from 2006 to 2010, all Gleason score 4+3 = 7 and higher, not selected by outcome ($n = 163$; refs. 30, 31); (v) a set of patients who all developed metastatic disease after radical prostatectomy at Johns Hopkins (Baltimore, MD) from 1995 to 2011, who were evaluated for response to abiraterone or enzalutamide ($n = 34$; ref. 32); (vi) a cohort study of men undergoing radical prostatectomy from 1992 to 2009 with intermediate- and high-risk disease who were followed for development of metastatic disease ($n = 325$; ref. 33); and (vii) a set of men with biochemical recurrence following radical prostatectomy from 1992 to 2009 ($n = 240$; ref. 34).

IHC

ATM protein IHC was performed using the Y170 rabbit monoclonal antibody (Abcam, 32420) on the Ventana Discovery XT Autostaining System (Roche/Ventana Medical Systems). The ATM Y170 clone was used to develop a similar IHC diagnostic assay (35) and was deployed in the phase III GOLD study, which looked at combination of olaparib and paclitaxel in patients with advanced gastric cancer who have progressed following first-line therapy (36). Slides were incubated with primary antibody (1:100 dilution) after antigen retrieval in CC1 buffer, and primary antibody incubation was followed by detection with the OptiView HQ System (Roche/Ventana Medical Systems). Each TMA spot or standard histologic section containing tumor cells was visually dichotomously scored for presence or absence of nuclear ATM signal by a urologic pathologist (T.L. Lotan). The pathologist was blinded to sequencing results when reading the IHC. A spot was considered to show ATM protein loss if any tumor cells in any tumor spot showed loss of nuclear ATM, with intact staining in admixed benign prostate glands and surrounding stromal cells, endothelial cells, or lymphocytes. Spots without internal control staining were considered ambiguous and were not scored. All samples were initially screened for ATM loss by scoring TMA spots; however, for all cases with ATM protein loss on TMA, confirmatory ATM immunostaining was also performed on standard histologic tissue sections.

ERG IHC was performed on the Ventana Platform (Ventana Discovery Ultra, Ventana Medical Systems) using a previously reported genetically validated staining and scoring protocol (37, 38).

In brief, this assay utilized a rabbit anti-human ERG antibody (EPR3864). ERG was scored as positive if any tumor glands showed nuclear ERG expression, negative if no sampled tumor glands showed ERG expression, or ambiguous if endothelial nuclei did not show ERG expression within the evaluated tumor core as an internal positive control.

Cell lines

Isogenic CWR22Rv1 cell lines with and without biallelic *ATM* or *BRCA2* inactivation were derived via CRISPR/Cas9 genome editing as described previously (39). Cells were pelleted and formalin-fixed, paraffin-embedded (FFPE) as described previously (40). *Mycoplasma* testing was conducted every 3 months (latest date October, 2019) and authentication of genome editing was described previously (39). Cells were passaged no more than twice before experiments were performed.

DNA isolation

On standard histologic sections, tumor tissue was macrodissected guided by hematoxylin and eosin (H&E)-stained section. Sections (5 × 10 μm) from FFPE tumor samples were used for DNA extraction. For the UW OncoPlex sequencing assay, DNA was extracted from FFPE material using the Qiagen FFPE DNA Extraction Kit according to the manufacturer's directions. DNA concentrations were quantified with the Qubit Fluorometer, using a Quant-iT dsDNA High Sensitivity Assay Kit (Invitrogen). For the Myriad HRD Plus sequencing assay, sections from FFPE tissue were first incubated in proteinase K followed by DNA extraction performed using the Promega Maxwell 16 LEV FFPE Plus Kit (AS1290, Promega) according to the manufacturer's instructions.

Targeted next-generation sequencing

Targeted next-generation sequencing was performed in a subset of cases using the UW OncoPlex assay as described previously (27), and for all cases we performed the HRD Plus Assay (Myriad Genetics). The HRD Plus assay has been described previously in detail for *BRCA1/2* sequencing (41); here, it was modified to also include the entire coding region of *ATM* and a panel of 106 additional genes (Supplementary Table S1). Briefly, the HRD Plus assay uses a custom hybridization capture method employing Integrated DNA Technologies' xGen Hybridization Capture Technology (Integrated DNA Technologies). Sequencing was performed on an Illumina HiSeq2500 using a 200 cycle HiSeq Rapid SBS kit v2 and a HiSeq Rapid PE cluster kit v2.

Sequence analysis

Average coverage for *ATM* after alignment to the target regions and removal of nonclonal reads was 687 (range, 217–1,262). Novel variants identified by tumor sequencing using the HRD Plus assay were classified using a process that is consistent with the published standards and guidelines for clinical testing from the American College of Medical Genetics and Genomics (42). Variants were classified into one of five categories: deleterious, suspected deleterious, variant of uncertain significance, favor polymorphism, and polymorphism. Both deleterious and suspected deleterious variants were considered pathogenic. Variant classifications are stored in a classification database and can be retrieved each time they are observed during routine testing.

Statistical analysis

Clinicopathologic characteristics of tumors with and without ATM loss were compared using the χ^2 test and the Wilcoxon rank-sum test for categorical and continuous variables, respectively. Metastasis-free survival (MFS) was estimated with Kaplan–Meier curves, both univariate and adjusted for Gleason score and TMA set (43). A proportional hazards frailty model was used to evaluate the prognostic effect of ATM loss adjusted for known prognostic factors, while accounting for clustering within TMA sets (44). All analyses were performed with SAS v9.4 (SAS Institute).

Results

Genetic validation of ATM IHC assay

ATM IHC was initially validated using isogenic CWR22Rv1 cell lines with and without biallelic *ATM* inactivation derived via CRISPR/Cas9 genome editing (39). The clone with *ATM* knockout was previously confirmed to harbor a biallelic 33-nucleotide insertion in *ATM* (c.3383_3384ins33; p.Q1128_E1129insTASANSF*), resulting in a premature stop codon (39). These cells lacked detectable ATM protein by immunoblotting (**Fig. 1A**) and by IHC performed on a FFPE cell block with the same antibody clone (**Fig. 1B**). Next, we performed ATM IHC on a TMA containing 77 primary Gleason pattern 5 tumors procured from consecutive radical prostatectomies at our institution (27). Overall, 84% (65/77) were evaluable for ATM IHC, defined by adequate nuclear staining in internal control benign glands and stromal cells. Of these, 12% (8/65) had ATM protein loss. Of the 65 evaluable cases, 52 had previously generated UW OncoPlex sequencing data (27), including seven cases with ATM protein loss (13%; **Fig. 1C**). Of the seven cases with ATM protein loss and sequencing data available for comparison, four cases had a deleterious *ATM* mutation and two had shallow *ATM* genomic deletions (LOH implying monoallelic loss; **Table 1**; **Fig. 1C**). The final case had no detectable *ATM* alterations, although tumor content was noted to be low, precluding accurate copy-number calls. Of the four deleteriously mutated *ATM* cases, three had definitive LOH suggesting biallelic inactivation; the remaining case was uncertain due to low tumor content, however, biallelic loss was favored (**Table 1**). Of the remaining 45 cases without ATM protein loss, one had a pathogenic *ATM* missense mutation (p.R3008C) without LOH that was evident only on manual review of the sequencing data as the variant allele fraction was below the threshold of the variant calling algorithm. Of note, this mutation has been reported in clonal hematopoiesis of indeterminate potential and chronic lymphocytic leukemia and could represent a hematolymphoid clone given the low variant allele fraction (45). In summary, the negative predictive value of the ATM IHC assay for lack of underlying pathogenic *ATM* mutation was 98% (44/45) and the assay was 80% (4/5) sensitive for pathogenic *ATM* mutations and 100% (3/3) sensitive for biallelic *ATM* alteration.

To verify these sequencing results on a different platform, we reisolated DNA and resequenced all seven samples with ATM protein loss, this time using the Myriad HRD Plus assay. All four pathogenic mutations were confirmed (of which two had definitive LOH; **Table 1**). The additional two samples with isolated LOH by the OncoPlex assay also showed isolated LOH by the Myriad assay and the single sample that was previously low tumor content and without detectable alterations on the OncoPlex assay showed *ATM* LOH on Myriad resequencing (**Table 1**). Taking the results from both DNA-sequencing assays together, all seven cases with ATM protein loss had evidence of at least monoallelic *ATM* inactivation, however, only three of these cases had evidence of biallelic inactivation of *ATM* by one or both sequencing assays. These data suggest that targeted next-generation sequencing assays may be insensitive for biallelic deleterious *ATM* genomic (or epigenomic) alterations leading to protein loss and may be complemented by IHC staining assays.

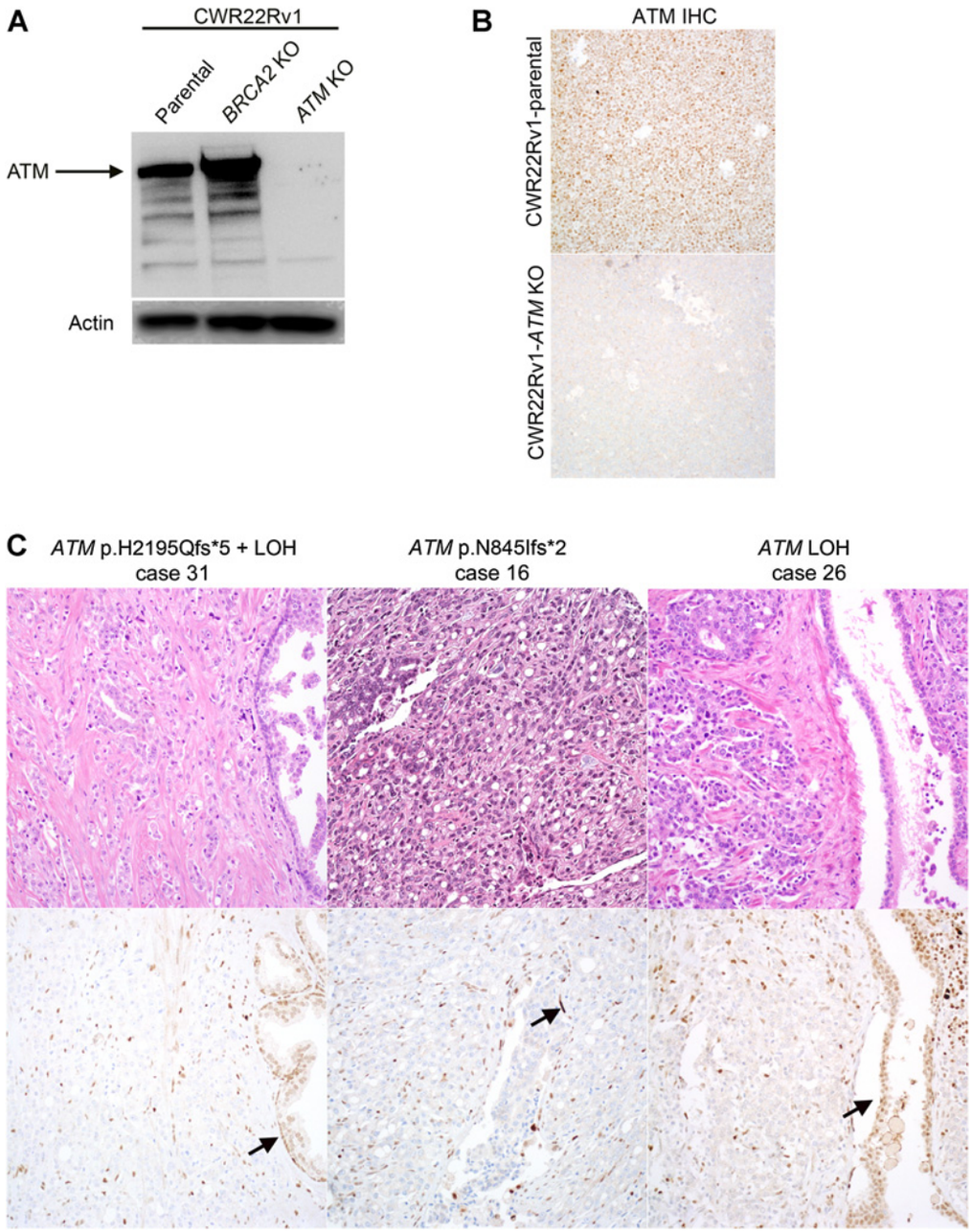

Figure 1.

Genetic validation of ATM IHC assay. **A,** Immunoblotting for ATM in cell lysates of isogenic CWR22Rv1 cell lines with and without biallelic *ATM* or *BRCA2* inactivation via CRISPR/Cas9 genome editing (40). **B,** ATM IHC in FFPE cell pellets of CWR22Rv1 cell lines with and without biallelic *ATM* inactivation. **C,** H&E staining (top row) and ATM IHC (bottom row) in representative primary Gleason pattern 5 prostate tumors with ATM protein loss and available *ATM* sequencing results (ref. 27; all images reduced from 200×). Benign glands (arrows) or stromal/endothelial nuclei provide an internal positive control in each case. KO, knockout.

ATM protein expression and somatic *ATM* status in primary prostate tumors with germline pathogenic *ATM* mutations

Next, we assessed the prevalence of ATM protein loss in primary prostate cancer cases with known *ATM* germline mutations. We identified 20 cases with pathogenic germline *ATM* mutations and available primary tumor tissue at our institution (6, 12). Tumor tissue for all cases was resequenced on the Myriad HRD Plus platform and mutations were confirmed in 19 of 20 cases (**Table 2**). These cases were generally high-grade, high-stage tumors, with more than half harboring Gleason grade group 5 cancer (**Table 2**). Of the 19 cases with confirmed germline *ATM* alterations, 74% (14/19) had ATM protein loss upon immunostaining of standard histologic sections of the dominant tumor nodule. Of the cases with ATM protein loss, 79% (11/14) had homogeneous loss in the entire sampled dominant tumor

Table 1. Next-generation sequencing results for tumors with ATM protein loss in primary Gleason pattern 5 cohort.

ID	Alt ID (Ref 25)	ATM IHC	OncoPlex *ATM* mutation	OncoPlex LOH	OncoPlex germline suspected?	Myriad *ATM* mutation	Myriad LOH	Myriad germline suspected
ATM26	61466	Loss	LOH	Yes	No	LOH	Yes	No
ATM16	71497	Loss	p.N845Ifs*2	Yes	Yes	p.N845Ifs*2	Yes	No
ATM19	72889	Loss	p.K2589*	Uncertain, biallelic favored*	Yes	p.K2589*	Uncertain	Uncertain
ATM30	71513	Loss	∼28 bp indel that takes out the exon 11/intron 11 splice boundary	Yes	No	c.1774_1802+4delins6	Yes	No
ATM31	71514	Loss	p.H2195Qfs*5	Yes	No	p.H2195QInfs*5	No	No
ATM27	N/A	Loss	LOH	Yes	No	LOH	Yes	No
ATM42	71515	Heterogeneous loss	None	Uncertain*	No	LOH	Yes	No
N/A	69210	Intact	p.R3008C	No	No	N/A	N/A	N/A

Abbreviation: N/A, not assessed.
*Low tumor content.

nodule, suggesting that the *ATM* mutation was likely an early clonal event (**Fig. 2**). Among the cases with ATM protein loss, which were evaluable for copy-number alteration or a second pathogenic *ATM* alteration, 70% (7/10) had genomic evidence of potential biallelic inactivation. The term "potential" biallelic inactivation is used because in cases with two *ATM* alterations, the phase (*cis* vs. *trans*) of the mutations was not conclusively determined (**Table 2**). Of interest, two of the three cases with protein loss, but lacking evidence of LOH, had a second *ATM* mutation detected that was classified as a variant of unknown significance (VUS; p.W3055C; p.A1272N), suggesting the potential for biallelic *ATM* inactivation in these cases as well. In contrast, among five cases with germline *ATM* mutations and intact ATM protein, four were evaluable for copy-number alteration and none had an evidence of LOH or a second somatic *ATM* mutation, suggesting the development of a sporadic prostate cancer in individuals with an incidental *ATM* germline lesion.

Prevalence of ATM loss among large set of surgically treated patients

Next, we assessed the prevalence of ATM protein loss in several previously described sets of TMAs prepared from genetically unselected radical prostatectomy specimens. There were a total of 1,076 patients with tumor tissue sampled in these TMAs, which included the primary Gleason pattern 5 TMA set described above. Overall, 82% (885/1,076) of these samples were interpretable for ATM staining and the subset of 831 with complete clinicopathologic data and clinical follow-up information are presented in **Table 3**. ATM loss was present in 3% (25/831) of these evaluable tumors overall. Patients with ATM loss underwent prostatectomy more recently ($P = 0.028$), had higher Gleason grade ($P < 0.0001$), pathologic stage ($P = 0.024$), and CAPRA-S score ($P = 0.002$). ATM loss was identified more commonly in Grade group 5 (17/181; 9%) compared with all other grades (8/650; 1%; $P < 0.0001$). Of the cases with ATM loss, seven were from the primary Gleason pattern 5 TMA and one case overlapped with the germline cases described above, and were already sequenced (**Tables 1** and **2**). The remaining 17 cases were processed for DNA sequencing and restaining for ATM using standard histologic sections to assess for ATM protein expression heterogeneity (**Table 4**; **Fig. 2**). Of these, 80% (4/5) with homogeneous ATM protein loss and 50% (6/12) with heterogeneous ATM protein loss had detectable pathogenic *ATM* alterations. One additional case with heterogeneous protein loss had a mutation potentially affecting a canonical splice site in ATM (c.6808-1G>C), suggesting pathogenicity (**Fig. 2**). Of the mutated cases, only one was inferred to have a germline pathogenic *ATM* mutation based on variant allele frequency in the tumor sequencing, while the remainder were inferred somatic alterations or indeterminate between germline and somatic based on tumor sequencing. Among the mutated cases with homogeneous protein loss and evaluable copy-number alteration data, two of two had potential biallelic inactivation of *ATM*. Among the mutated cases with heterogeneous protein loss and evaluable copy-number alteration data, three of five had potential biallelic inactivation, with one additional case harboring an additional VUS in *ATM*.

Clinical outcomes of primary prostate tumors with ATM protein loss

Because many of the TMA sets that we stained for ATM had clinical follow-up information available after radical prostatectomy, we compared biochemical recurrence-free survival (BCR-FS), MFS, and prostate cancer–specific survival (PCSS) in men with and without ATM protein loss from the TMA sets. As described above, the prevalence of ATM loss was strongly correlated with Gleason grade group. In addition, the different TMA sets varied considerably both in the proportion of cases with ATM loss and with respect to survival probabilities based on their varied design and differing study populations. Thus, while ATM loss was significantly associated with an increased risk of metastasis in univariate analysis [HR, 2.32; 95% confidence interval (CI), 1.23–4.39; $P = 0.009$], it was not significantly associated with increased risk of metastasis after adjusting for Gleason grade and including TMA set as a random variable in a proportional hazards frailty model (adjusted HR, 1.06; 95% CI, 0.55–2.05; $P = 0.857$). Similar results were seen with a standard (unadjusted) and adjusted Kaplan–Meier analysis (Supplementary Fig. S1). Furthermore, ATM loss was not significantly associated with BCR-FS, MFS, or PCSS in random-effects Cox models adjusted for age, race (White vs. non-White), radical prostatectomy year, and Gleason grade group (Supplementary Table S2). Concordant results were observed if CAPRA-S replaced Gleason grade group (data not shown).

Table 2. Genomic and clinicopathologic characteristics of prostate tumors with pathogenic germline *ATM* mutations.

ID	ATM IHC	*ATM* mutation from germline sequencing	Germline sequencing platform	*ATM* mutation from tumor sequencing	Myriad LOH	Two pathogenic alterations in *ATM*?	Age	Race	Gleason (primary)	Gleason (secondary)	Gleason (sum)	Pathologic stage
ATM6	Loss	p.L2005fs*	Research	p.Q2007Rfs*11 + p.W3055C (VUS)	No	No	74	W	4	5	9	T3a N1 MX
ATM11	Heterogeneous loss	p.K750K	Research	p.K750K + p.A1272N (VUS)	No	No	63	W	4	4	8	T2 N0 MX
ATM13	Loss	S1905I fs*25	Clinical	p.S1905Ifs*25	No	No	44	W	4	3	7	T3b N0 MX
ATM7	Loss	p.P292L	Research	p.P292L + p.C430*	No	Yes	66	W	4	5	9	T3a N0 MX
ATM3	Loss	p.D2795fs*	Research	p.F2799Kfs*4	Yes	Yes	64	W	5	4	9	T2 N0 MX
ATM5	Heterogeneous loss	c.8988–1G>A	Research	c.8988–1G>A	Yes	Yes	53	W	4	5	9	T3a N0 MX
ATM10	Loss	p.K750K	Research	p.K750K	Yes	Yes	64	W	4	4	8	T2 N0 MX
ATM12	Loss	Duplication exon 48	Clinical	Large genomic rearrangement + p.I2356Vfs*2	Uncertain	Yes	55	W	4	5	9	T3b N0 MX
ATM15	Loss	p.K2756*	Research	p.K2756* + del exons 33-62	No	Yes	61	W	5	4	9	T3b N1 MX
ATM20	Loss	p.L1107*	Research	p.L1107*	Yes	Yes	60	W	4	3	7	T2 N0 MX
ATM18	Loss	p.K2756*	Research	p.K2756*	Uncertain	Uncertain	54	W	4	3	7	T3a N0 MX
ATM8	Loss	p.G1458fs*	Research	p.G1458Qfs*15	Uncertain	Uncertain	75	W	4	5	9	T3a N0 MX
ATM1	Heterogeneous loss	p.T452fs*	Research	p.T452Nfs*21	Uncertain	Uncertain	52	W	3	4	7	T2 N0 MX
ATM14	Loss	c.8786+1G>A	Research	c.8786+1G>A	Uncertain	Uncertain	50	W	4	5	9	T3a N0 MX
ATM4	Intact	p.R2598*	Research	p.R2598*	No	No	69	W	4	5	9	T3a N0 MX
ATM9	Intact	p.2546_2548del	Research	p.R2547_S2549del	No	No	49	W	4	5	9	T3a N0 MX
ATM17	Intact	p.S274fs*	Research	p.L275*	No	No	47	W	4	5	9	T2 N0 MX
ATM21	Intact	c.7629 +2 T>C	Clinical	c.7629+2T>C	Uncertain	Uncertain	60	W	3	4	7	T3a N0 MX
ATM22	Intact	p.S160Afs*23	Clinical	p.S160Afs*23	No	No	65	W	4	5	9	T3b N1 MX
ATM2	Intact	p.R447*	Research	No variants found	Uncertain	Uncertain	36	W	3	3	6	T2 N0 MX

Abbreviation: W, white.

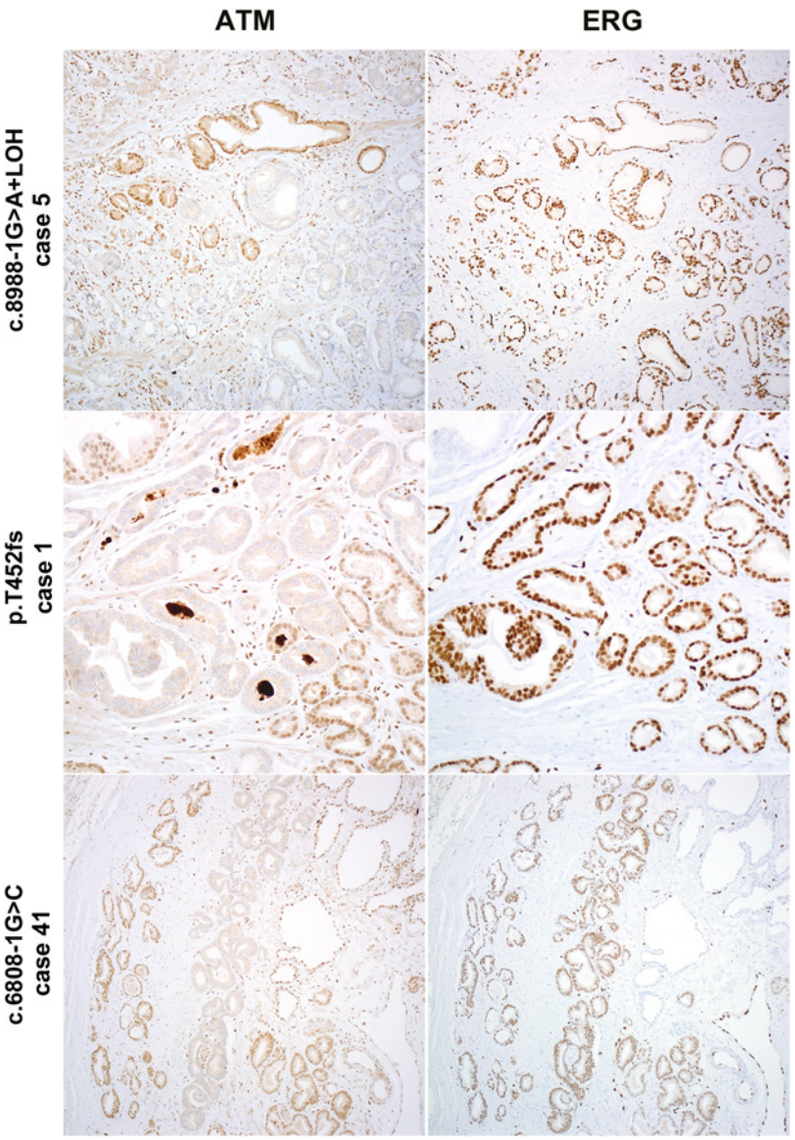

Figure 2.

Primary prostate tumors with heterogeneous ATM protein loss. Heterogeneous ATM immunostaining (left column) in representative tumors with germline or inferred somatic *ATM* mutation. ERG immunoreactivity (right column), indicating underlying *ERG* gene rearrangement, is homogeneous in all cases, suggesting the *ERG* rearrangement preceded subclonal *ATM* inactivation (all images reduced from 200×).

Timing of ATM loss in primary prostate cancer progression

The higher frequency of ATM loss heterogeneity among cases in the TMA study (71% or 12/17) compared with those in the germline study (21% or 3/14) was interesting and suggested that ATM inactivation may occur later in tumors with inferred somatic mutations (which comprised the majority in the TMA study) compared with those with germline mutations. However, given that multifocal and multiclonal tumors are common in primary prostate cancer, it is also conceivable that the tumors with heterogeneous ATM expression represent collisions between independent clones. To resolve this, and to begin to elucidate the timing of ATM loss in primary prostate cancer, we assessed ERG status in cases with heterogeneous ATM loss. *ERG* gene rearrangements are present in approximately half of all prostate cancer cases in European ancestry populations (46) and are likely among the earliest genomic alterations during prostatic tumorigenesis, occurring in some cases of prostatic intraepithelial neoplasia (PIN; refs. 47–49) and before *PTEN* genomic deletion (50, 51). Accordingly, ERG expression (a highly validated surrogate marker of underlying *ERG* gene rearrangement; ref. 38) is almost always ubiquitously present in all tumor cells from a given tumor nodule if *ERG* is rearranged, unless the tumor represents a collision between two independent clones (51). Among cases with heterogeneous ATM loss (including three germline cases and 12 from the TMA study described above), ERG was expressed in 53% (8/15) of cases and was uniformly expressed in both ATM-positive and ATM-negative tumor cells in all cases, including two germline cases and six TMA cases (**Fig. 2**). These data strongly suggest that in cases with inferred somatic *ATM* genomic alteration (the majority in the TMA study), *ATM* inactivation commonly occurs subclonally and subsequent to *ERG* gene rearrangement, and that tumors with heterogeneous ATM expression do not commonly represent collisions of independent clones.

Because ATM loss was most commonly homogeneous and apparently clonal in primary prostate tumors with pathogenic germline *ATM* mutations, this raised the question of how early ATM inactivation may occur in patients with germline mutations. To begin to assess this, we screened 16 ATM protein-negative cases with known or

Table 3. Clinicopathologic features of cases with and without ATM loss on TMA screening.

Variable	ATM loss ($n = 25$)[a]	ATM intact ($n = 806$)[a]	*P*
Age (years), median (IQR)	63 (56–66)	59 (55–64)	0.073
Race, *n* (%)			
White	23 (92%)	599 (74%)	0.143
Black	2 (8%)	193 (24%)	
Other	0	14 (2%)	
RP year, median (IQR)	2004 (2001–2008)	2001 (1998–2005)	0.028
PSA (ng/mL), median (IQR)	7.7 (5.9–12.8)	7.3 (5.0–11.5)	0.271
RP grade group, *n* (%)			
1 (Gleason score 6)	0	117 (15%)	<0.0001
2 (Gleason score 3+4)	3 (12%)	209 (26%)	
3 (Gleason score 4+3)	2 (8%)	214 (27%)	
4 (Gleason score 8)	3 (12%)	102 (13%)	
5 (Gleason score 9–10)	17 (68%)	164 (20%)	
Pathologic stage, *n* (%)			
T2 N0	5 (20%)	281 (35%)	0.024
T3a N0	9 (36%)	310 (39%)	
T3b N0	9 (36%)	106 (13%)	
Tx N1	2 (8%)	101 (13%)	
CAPRA-S	6.0 (4.0–9.0)	4.0 (3.0–6.0)	0.002

Abbreviations: IQR, interquartile range; RP, prostatectomy.
[a]Sample sizes vary due to missing values.

inferred germline *ATM* alterations from the above cohorts for high-grade PIN, the presumptive precursor for invasive carcinoma in the prostate. We exclusively selected PIN lesions present on slides without invasive tumor by H&E, because recent studies have suggested that retrograde intraductal spread of invasive carcinoma can masquerade as PIN (52). We identified 11 cases that met these criteria, of which nine (82%) were evaluable for ATM IHC in the PIN lesions. Only one of nine (11%) showed ATM loss in the PIN (**Fig. 3**). Taken together, with our finding that ATM loss in germline cases is most commonly homogeneous and likely clonal, these findings suggest that ATM loss commonly occurs at or just after initial tumor invasion, rather than in precursor lesions in the prostate.

Discussion

Pathogenic mutations in the homologous recombination DNA repair pathway, including the *BRCA2, BRCA1*, and *ATM* genes, are common in advanced prostate cancer, occurring in nearly 20% of mCRPC cases (4). Nearly half of these alterations have proven to be inherited at the germline level, comprising close to 10% of men with mCRPC (3, 4). Historically, the *BRCA2, BRCA1*, and *ATM* genes have been grouped together in most analyses, given that all encode proteins that are key components of homology-mediated DNA repair. The first large-scale sequencing studies in prostate cancer showed that there is a twofold enrichment of mutations in these genes in metastatic compared with primary cancers (4, 53), suggesting that these alterations are associated with aggressive disease. Indeed, a number of earlier studies had already confirmed this hypothesis for germline *BRCA2* mutations (54–57). More recent studies have found that germline alterations in *BRCA2* and *ATM* (considered together) are significantly more common in lethal compared with indolent primary prostate cancer (6), are associated with grade reclassification in active surveillance cohorts (58), and with high-grade disease in surgical cohorts (7). The correlation of germline *BRCA2* and *ATM* mutations with adverse pathologic features, including more advanced Gleason scores and higher PSA levels (6), likely drives a large part of this association with adverse outcomes. Considering all homologous repair gene mutations in aggregate, our group has previously shown that aggressive histologic subsets of primary prostate cancer, such as ductal carcinomas (59), primary Gleason pattern 5 disease (27), and intraductal cancer (60) have mutation rates approaching or exceeding those in metastatic disease.

While nearly all prior studies have considered *BRCA2* and *ATM* mutations together because both genes encode proteins functioning in the homologous DNA repair pathway, emerging evidence from clinical trials suggests that the roles of these two genes may in fact be quite distinctive. Prospective phase II and phase III trials of PARP inhibitors (including olaparib, rucaparib, niraparib, and talazoparib) in patients with CRPC have shown that the response rates are much more limited in patients with the *ATM* mutation compared with patients with the *BRCA2* mutation (9–13, 61). ATM is a sensor of double-strand DNA damage, and is a kinase with a diverse range of substrates; these clinical data suggest that some of its key functions in prostate cancer may be independent of its role in homology-mediated DNA repair. In fact, ATM has also been shown to modulate growth factor signaling and to play a role in other stress response pathways (2). Accordingly, our study is the first to examine the clinicopathologic features and outcomes of *ATM*-deficient primary prostate tumors separately from those with *BRCA2* deficiency. This work was largely made possible by the development of a robust clinical grade ATM IHC assay, which we genetically validated and subsequently used to screen hundreds of prostate tumors for ATM loss. Importantly, we confirm that ATM loss is highly enriched in Grade group 5 prostate cancers and is associated with poor outcomes in univariate analyses of surgically treated patients. However, this association with aggressive disease appears to be largely driven by a correlation with high-grade group, and we show here that ATM loss is not independently prognostic of outcomes in multivariate models, although these data require confirmation in validation cohorts.

Table 4. Genomic and clinicopathologic features of tumors with ATM protein loss on TMA screening.

ID	ATM IHC	*ATM* mutation from tumor sequencing	Germline or somatic (inferred)	LOH	Two pathogenic alterations in *ATM*?	Age	Race	Gleason (primary)	Gleason (secondary)	Gleason (sum)	Pathologic stage
ATM25	Loss	p.L1722Rfs*25 + c.2124+1G>A	Somatic	No	Yes	71	W	3	4	7	T3b N0 MX
ATM23	Loss	p.Q1361*	Somatic	Uncertain	Uncertain	57	W	4	5	9	T3b N0 MX
ATM28	Loss	p.R447* + del exons 1-32	Germline and somatic	No	Yes	65	W	4	5	9	T3b N1 MX
ATM24	Loss	p.S743*	Somatic	Uncertain	Uncertain	59	W	4	5	9	T3b N0 MX
ATM29	Loss	None	None		No	66	B	4	5	9	T3a N0 MX
ATM38	Heterogeneous loss	c.185+1G>A	Somatic	Yes	Yes	56	W	4	4	8	T3b N0 MX
ATM41	Heterogeneous loss	c.6808-1G>C	Uncertain	Uncertain	Uncertain	63	W	3	4	7	T2 N0 MX
ATM40	Heterogeneous loss	Complex large rearrangement + p.V2766K (VUS)	Somatic	No	No	55	W	4	3	7	T3a N0 MX
ATM37	Heterogeneous loss	p.Q2220Rfs*15	Somatic	No	No	59	W	4	4	8	T2 N0 MX
ATM33	Heterogeneous loss	p.Q513*	Uncertain	Uncertain	Uncertain	58	W	3	4	7	T2 N0 MX
ATM39	Heterogeneous loss	p.Q893*	Somatic	Yes	Yes	67	W	4	4	8	T3b N0 MX
ATM32	Heterogeneous loss	Large genomic rearrangement	Somatic	Yes	Yes	67	W	4	5	9	T3a N0 MX
ATM34	Heterogeneous loss	None	None		No	72	W	3	4	7	T2 N0 MX
ATM35	Heterogeneous loss	None	None		No	70	W	4	5	9	T3a N0 MX
ATM36	Heterogeneous loss	None	None		No	51	W	4	3	7	T2 N0 MX
ATM43	Heterogeneous loss	None	None		No	52	W	4	5	9	T3b N0 MX
ATM44	Heterogeneous loss	None	None		No	49	W	4	5	9	T3b N1 MX

Abbreviation: W, white.

Although ATM loss appears not to be prognostic in primary prostate cancer, it will still be critically important to identify ATM-deficient prostate tumors for trials of targeted therapies, including ATR inhibitors as well as other rational therapies (e.g., radium-223). Similar to ATM, ATR is a PI3K-like kinase, however, unlike ATM, which responds largely to double-strand DNA breaks, ATR responds to single-strand breaks and other types of DNA damage. Because of its atypical nature, ATR inhibitors have historically lagged in development behind other serine–threonine kinase inhibitors, such as AKT inhibitors (18). However, several potent and relatively specific ATR inhibitors have been characterized and have shown synthetic lethal activity in ATM-deficient tumors *in vitro* and in early clinical trials (18, 19). Given these promising initial results, larger biomarker-selected trials are currently ongoing (e.g., NCT04095273) and will require screening of large populations of patients with prostate cancer for inclusion. Combined with previous germline cohorts (7), this study demonstrates that screening of grade group 5 tumors will likely be most fruitful for trial recruitment, with 9% of such patients (and as many as 13% of primary Gleason pattern 5 tumors) harboring ATM loss.

Equally important to defining which populations to screen for these trials is determining which assay will be utilized for the screening protocol. Our data strongly suggest that if germline *ATM* sequencing is used for enrollment, at least a quarter of enrolled patients with prostate cancer may be unlikely to respond because they lack ATM protein loss and biallelic inactivation and most likely have sporadic cancers. We found that the presence of ATM protein loss strongly enriched for underlying potential biallelic inactivation of *ATM*, with 70% of cases with ATM protein loss (and none of the cases without protein loss) harboring a likely somatic "second hit" in the *ATM* gene by DNA sequencing. It will require careful clinical trials to resolve whether *ATM* may function in a haploinsufficient manner as seen with other tumor suppressor genes (62) and to discern whether patients with monoallelic inactivation may also respond to targeted therapy similarly to those with biallelic inactivation. In this setting, ATM IHC will likely prove very useful to determine whether *ATM* deficiency is likely to be monoallelic or biallelic, because next-generation sequencing may miss complex rearrangements of the gene or epigenomic gene silencing and germline sequencing will not detect somatic or epigenomic *ATM* inactivation. If *ATM* is not a haploinsufficient tumor suppressor gene, then screening with germline DNA assays alone will be inadequate for

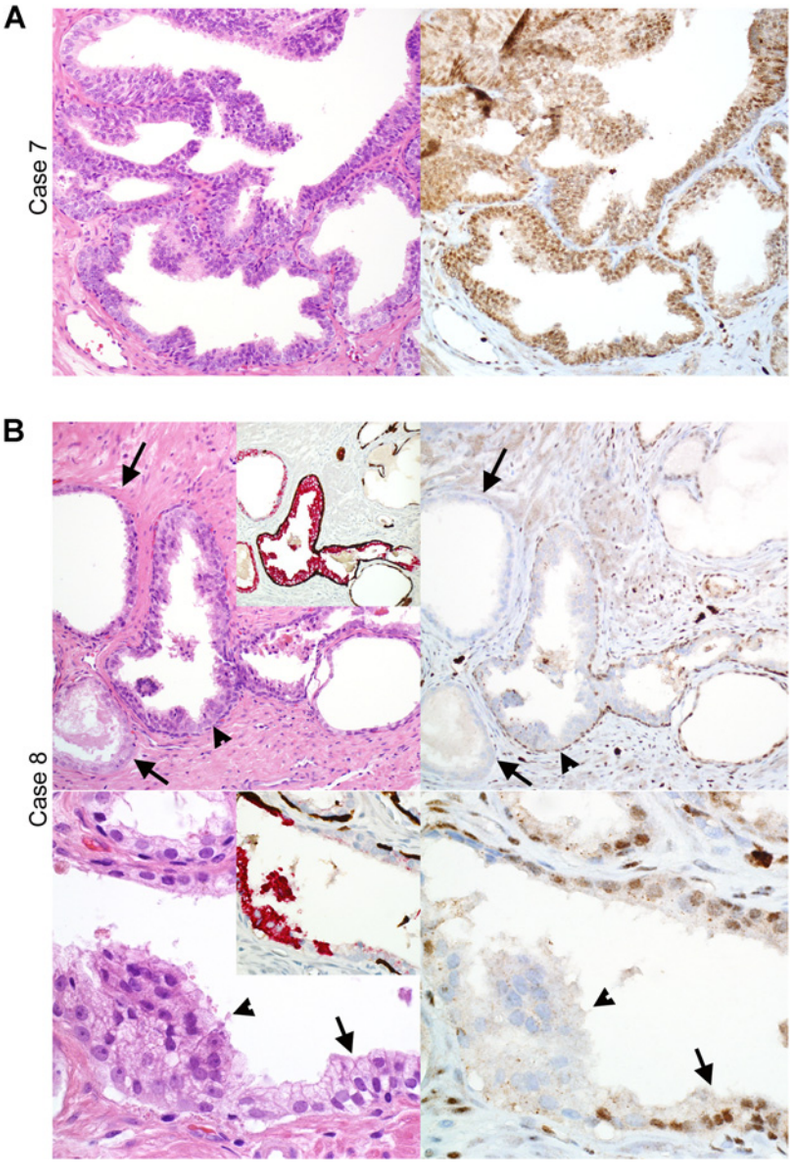

Figure 3.
ATM expression in high-grade PIN. **A,** Focus of PIN from prostatectomy showing intact ATM. This prostatectomy specimen harbored invasive tumor in additional tissue blocks with homogeneous ATM loss and two pathogenic *ATM* mutations [*ATM* p. P292L (germline) and p.C430*; all images reduced from 200×). **B,** Focus of PIN showing ATM loss in luminal cells, with expression of ATM in surrounding basal cells (arrowheads, top). Inset shows multiplex immunostaining for PIN4 (p63 and high molecular weight cytokeratin in brown highlight basal cells, while racemase in red highlights luminal PIN cells). There are two nearby atypical glands with a patchy basal cell layer on PIN4 IHC also showing loss of ATM (arrows). This prostatectomy specimen harbored invasive tumor in additional tissue blocks with homogeneous ATM loss and a pathogenic *ATM* germline mutation (*ATM* p.G1458Qfs*15; all images reduced from 200×). Higher magnification view of adjacent area to that depicted in top panels shows ATM loss in focal area of PIN with enlarged nucleoli (arrowheads, bottom). Inset shows multiplex immunostaining for PIN4 (p63 and high molecular weight cytokeratin in brown highlight basal cells, while racemase in red highlights luminal PIN cells). ATM is intact in surrounding, morphologically benign-appearing cells (arrows; all images reduced from 630×).

enrollment in biomarker-selected clinical trials and the addition of somatic sequencing with ATM IHC assays will also be important. While there have been limited preclinical studies examining *ATM* haploinsufficiency in prostate cancer, previously published studies in breast cancer mouse models seem to suggest that *Atm* can function in a haploinsufficient manner in some genetic contexts (63, 64). In contrast, other preclinical studies found no increased mutagenic response to radiation in mice with hemizygous loss of *Atm* in germline and somatic tissue (65, 66), implying potential context-dependent effects. Additional work in prostate cancer preclinical models will be important to evaluate for potential evidence of *ATM* haploinsufficiency.

Our study is among the first to shed light on the underlying heterogeneity in ATM-deficient tumors and the likely timing of ATM loss in the setting of germline and somatic pathogenic mutations. Among tumors with germline *ATM* mutations, only 21% had heterogeneous ATM loss in the dominant nodule of the primary tumor, suggesting subclonal inactivation. Thus, when ATM loss occurs in patients with germline *ATM* mutations, it is most commonly a relatively early driver event, similar to *ERG* gene rearrangement. Analogous to *ERG* rearrangement, which is only infrequently seen in PIN (49), we demonstrate that ATM is only rarely lost in the presumptive prostate cancer precursor lesion (PIN) in cases with germline and apparent clonal ATM inactivation. This suggests that similar to *ERG* rearrangement, ATM loss likely occurs just after the point of tumor invasion. In contrast to these germline cases, in the TMA study where most *ATM* alterations were inferred to be somatic (based on variant allele frequency), nearly 70% of cases had heterogeneous or likely subclonal ATM loss. In cases with heterogeneous ATM loss, we demonstrate that ATM inactivation most likely occurred after *ERG* gene rearrangement given that ERG was expressed in all tumor cells.

Whether the presence of subclonal loss implies that patients are less likely to respond to targeted therapy is an unanswered question that will hopefully be resolved in future trials of ATR inhibitors and other

molecularly targeted approaches. Studies to date have suggested that DNA repair gene mutations are most commonly present in the primary tumor and all metastases (26, 67), implying that they are relatively early drivers in most cases. If this is the case, then even patients with heterogeneous ATM loss in the primary tumor may have metastases with homogeneous ATM loss that will respond to therapy. On the other hand, it is conceivable that some cases may have late subclonal loss in the primary tumor, potentially even after metastatic spread has occurred, in which case response to targeted therapy may not be assured and testing of the metastatic deposit will be critical. In an attempt to shed light on these possibilities, we examined the medical records of all cases in this study with heterogeneous ATM protein loss in the primary tumor to identify cases with available metastatic tissue samples. Unfortunately, no cases had available tissue from metachronous metastases, although one case had tissue sampled from a synchronous pelvic lymph node metastasis (ATM44). Interestingly, in this case, there was clear heterogeneous ATM loss in the tumor, with intact ATM in a synchronous pelvic lymph node metastasis (Supplementary Fig. S2). Notably, this case did not have a detectable *ATM* mutation in the primary tumor, likely due to the subclonal nature of ATM loss identified by IHC. Consistent with this, cases that had heterogeneous ATM loss in our study were less likely to have any detectable underlying *ATM* mutation (50%), compared with those with homogeneous ATM protein loss (80%), highlighting the challenges of utilizing DNA-sequencing assays in potentially genetically heterogeneous tumors. This is particularly true in prostate cancer, where copy-number alterations and rearrangements are common causes of somatic genomic inactivation and may be missed by sequencing in a mixed population of tumor cells (68). Thus, while IHC may be useful to screen primary tumors for subclonal loss, it is equally important to follow-up in heterogeneous cases with sequencing or IHC evaluation of metastatic deposits to further evaluate trial eligibility.

Our study has some limitations that warrant discussion. First, all sequencing was done using a panel-based approach, where there may be limited sensitivity for shallow copy-number alterations and complex genomic rearrangements which can lead to ATM deficiency. This could be a possible explanation for cases with ATM IHC loss, but lacking an apparent genomic alteration. Although less likely, epigenomic silencing of the *ATM* locus remains another possibility. Second, although we screened a very large cohort of primary prostatectomies for ATM loss, this study was done using selected TMAs, which may not be representative of underlying population or tumor heterogeneity. The analyses of clinical outcomes are based on the combined data from nine TMA sets, which differ with respect to design, clinical characteristics, dates of surgery, postprostatectomy treatment, and time of TMA construction. Although the data are retrospective, patients were selected for each TMA on the basis of clinical characteristics rather than outcome, reducing differences in study design. Furthermore, incorporation of TMA set as a random effect in the proportional hazards frailty models addresses the clustering within TMA. Despite the large sample size, only 25 patients exhibited ATM loss, which may have affected stability of multivariate models. Importantly, the lack of a validation cohort in the prognostic analyses is a significant limitation and additional studies in independent cohorts are required to establish the validity of our findings.

In conclusion, we present the largest study to date using a genetically validated ATM protein IHC assay to interrogate more than 1,000 primary prostate cancers for ATM deficiency. We show that ATM loss is strongly associated with the highest Gleason grades, and that the negative prognostic impact of ATM loss is primarily driven by Gleason grade. Because not all *ATM*-mutated prostate cancers demonstrate ATM protein loss, and not all cases with protein loss have underlying genomic *ATM* alterations, we propose a combined interrogation approach utilizing both ATM IHC and next-generation sequencing assays for upcoming clinical trials. These trials will make possible a head-to-head comparison of the assays to select the most predictive biomarker. We anticipate that some combination of these assays will lead to optimal detection of ATM inactivation in prostate cancer for consideration of genomically targeted strategies in this subset of patients.

Disclosure of Potential Conflicts of Interest

D.C. Salles reports non-financial support from Myriad Genetics during the conduct of the study. C.C. Pritchard reports personal fees from AstraZeneca outside the submitted work. A.M. De Marzo reports grants from NIH, NCI and The Department of Defense during the conduct of the study, Janssen Research and Development (sponsored research project unrelated to this manuscript) and Myriad Genetics (sponsored research project unrelated to this manuscript), and personal fees from Cepheid Inc (consulting fees, for project unrelated to this manuscript) outside the submitted work. J.S. Lanchbury reports personal fees from Myriad Genetics, Inc. (employee and stockholder) during the conduct of the study. K.M. Timms reports personal fees from Myriad Genetics, Inc. (employee and stockholder) during the conduct of the study and outside the submitted work. E.S. Antonarakis reports grants and personal fees from Janssen, and Sanofi, Dendreon, Merck, Bristol Myers Squibb, and AstraZeneca outside the submitted work, personal fees from Pfizer and Clovis, Eli Lilly, and Amgen outside the submitted work, grants from Johnson & Johnson, Genentech, Novartis, and Constellation outside the submitted work, and has a patent for AR-V7 liquid biopsy technology issued, licensed, and with royalties paid from Qiagen. T.L. Lotan reports non-financial support from Myriad Genetics (provided sequencing assays used) during the conduct of the study and grants from Roche/Ventana outside the submitted work. No potential conflicts of interest were disclosed by the other authors.

Authors' Contributions

H. Kaur: Conceptualization, data curation, investigation, writing-original draft, writing-review and editing. **D.C. Salles:** Data curation, investigation. **S. Murali:** Data curation, investigation. **J.L. Hicks:** Methodology. **M. Nguyen:** Methodology. **C.C. Pritchard:** Conceptualization, data curation, investigation. **A.M. De Marzo:** Conceptualization, data curation, investigation. **J.S. Lanchbury:** Data curation, funding acquisition, investigation, methodology. **B.J. Trock:** Data curation, visualization, writing-review and editing. **W.B. Isaacs:** Conceptualization, data curation, investigation, writing-original draft, writing-review and editing. **K.M. Timms:** Conceptualization, data curation, formal analysis, investigation, writing-original draft, writing-review and editing. **E.S. Antonarakis:** Conceptualization, resources, data curation, formal analysis, funding acquisition, investigation, writing-original draft, project administration, writing-review and editing. **T.L. Lotan:** Conceptualization, data curation, formal analysis, supervision, validation, investigation, writing-original draft, project administration, writing-review and editing.

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