ORIGINAL ARTICLE

Somatic loss of *ATM* is a late event in pancreatic tumorigenesis

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Abstract

Understanding the timing and spectrum of genetic alterations that contribute to the development of pancreatic cancer is essential for effective interventions and treatments. The aim of this study was to characterize somatic *ATM* alterations in noninvasive pancreatic precursor lesions and invasive pancreatic adenocarcinomas from patients with and without pathogenic germline *ATM* variants. DNA was isolated and sequenced from the invasive pancreatic ductal adenocarcinomas and precursor lesions of patients with a pathogenic germline *ATM* variant. Tumor and precursor lesions from these patients as well as colloid carcinoma from patients without a germline *ATM* variant were immunolabeled to assess ATM expression. Among patients with a pathogenic germline *ATM* variant, somatic *ATM* alterations, either mutations and/or loss of protein expression, were identified in 75.0% of invasive pancreatic adenocarcinomas but only 7.1% of pancreatic precursor lesions. Loss of ATM expression was also detected in 31.0% of colloid carcinomas from patients unselected for germline *ATM* status, significantly higher than in pancreatic precursor lesions [pancreatic intraepithelial neoplasms ($p = 0.0013$); intraductal papillary mucinous neoplasms, $p = 0.0040$] and pancreatic ductal adenocarcinoma ($p = 0.0076$) unselected for germline ATM status. These data are consistent with the second hit to *ATM* being a late event in pancreatic tumorigenesis.
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Introduction

Pancreatic ductal adenocarcinoma (PDAC) is a lethal disease with a 5-year survival rate of just 11% [1]. The incidence of PDAC in the United States has increased, with more than 55,000 people diagnosed with the disease in 2021. Patients with PDAC often harbor germline pathogenic variants in pancreatic cancer susceptibility genes [2–4], and clinical care guidelines now recommend offering patients with PDAC and their

first-degree relatives germline genetic testing [5,6]. Furthermore, individuals with a strong family history of PDAC or individuals with a known pathogenic germline variant in a pancreatic cancer susceptibility gene are recommended to consider annual surveillance once they reach age criteria [7,8].

ATM codes for a member of the phosphoinositide 3-kinase-related protein kinase (*PIKK*) family and the protein is a crucial component of DNA damage response that ultimately plays a role in cell death, cell survival, cell cycle arrest, apoptosis, and DNA repair [9,10]. Approximately 2–3% of patients with PDAC and 1.6% of patients with surgically resected intraductal papillary mucinous neoplasm (IPMN) [11], a pancreatic cancer precursor lesion, carry a pathogenic germline *ATM* variant [2,12–17]. Importantly, individuals with a pathogenic germline *ATM* variant have a significantly increased risk of developing PDAC. We recently estimated age-specific risk of pancreatic cancer in 130 pancreatic cancer kindred with a pathogenic germline *ATM* variant using a modified segregated analysis and found that the cumulative risk of pancreatic cancer was 1.1% (95% CI: 0.8–1.3%) by age 50, 6.3% (95% CI: 3.9–8.7%) by age 70, and 9.5% (95% CI: 5.0–14.0%) by age 80 [18]. These cumulative risk estimates are consistent with a previously published odds ratio of pancreatic cancer in pathogenic germline *ATM* variant carriers of 5.71 (95% CI: 4.38–7.30) compared to publicly available gnomAD control data [4].

PDAC develops from noninvasive precursor lesions that include pancreatic intraepithelial neoplasia lesions (PanINs) and IPMNs. Studying these precursor lesions is important to further our understanding of pancreatic tumorigenesis. In particular, understanding the timing and spectrum of genetic alterations that contribute to the development of PDAC is essential for effective interventions and treatments. For example, cancers from patients with germline and/or somatic mutations that lead to defects in homology-directed repair or mismatch repair may be more susceptible to poly(ADP-ribose) polymerase (PARP) 1 inhibitors and immunotherapy, respectively [19–21]. Similarly, the loss of ATM-mediated DNA repair in neoplastic cells may increase their susceptibility to ionizing radiation and ATR inhibitors. *In vitro* studies of multiple pancreatic cancer cell lines demonstrated significant radiosensitivity after shRNA knockdown of ATM expression, and we have reported significant pathologic response to radiation therapy in a patient with pancreatic cancer and a pathogenic germline *ATM* variant [22]. Additionally, studies of pancreatic and prostate cancer cell lines indicated ATM loss increased sensitivity to ATR inhibitors [23–25]. Furthermore, preclinical *in vitro* and *in vivo* studies have shown that inhibitors of PARP, ATR, and DNA-dependent protein kinase (DNA-PK) act synergistically to target pancreatic cancer cells with ATM loss and may improve outcomes when used as maintenance therapy after folinic acid, fluorouracil, irinotecan, and oxaliplatin (FOLFIRINOX) induction therapy [26,27].

A recent study of cancers arising in patients with pathogenic germline *ATM* variants found that 17 of 19 (89.5%) evaluable cancers had either somatic loss of heterozygosity (LOH) encompassing the variant or additional somatic mutations in *ATM* [28]. The important role of ATM in pancreatic tumorigenesis is further supported by an *in vivo* study indicating that loss of ATM expression accelerates pancreatic cancer formation [29]. However, the timing of *ATM* inactivation is still poorly understood.

In this study we characterized the timing and spectrum of somatic *ATM* alterations in invasive adenocarcinomas and precursor lesions from patients with PDAC and a pathogenic germline *ATM* variant using a targeted gene panel and immunohistochemistry (IHC). We also explored the role of ATM loss in pancreatic tumorigenesis in precursor lesions and invasive carcinoma, stratified by histologic subtype, in patients unselected for germline *ATM* status using immunohistochemical labeling (IHC).

Materials and methods

Ethics approval

The study was reviewed by and approval obtained from the Johns Hopkins Institutional Review Board.

Patient selection

Twenty-four patients (P1–P24) with surgically resected pancreatic cancer and pathogenic germline *ATM* variants previously identified in research studies were collected from the Johns Hopkins Hospital, Memorial Sloan Kettering Cancer Center, University of Pittsburgh Medical Center, and Radboud University Medical Center Nijmegen. All patients included in the study had formalin-fixed paraffin-embedded (FFPE) tissue available that included invasive carcinoma, PanINs, IPMNs, suitable for DNA sequencing (15 of 24 patients), and/or immunostaining (24 of 24 patients). The histomorphology and clinicopathologic features of 16 of these patients were reported previously [22].

Pathologic review

Hematoxylin and eosin (H&E)-stained slides from FFPE resection specimens and tissue microarrays (TMAs) were reviewed. Invasive carcinomas and precursor lesions, including pancreatic intraepithelial neoplasia (PanIN) and intraductal papillary mucinous neoplasm (IPMN), were identified for laser capture microdissection and IHC. Invasive pancreatic carcinomas were classified by histologic subtype according to World Health Organization criteria [30]. Neoplastic precursor lesions were graded for dysplasia based on consensus recommendations [31].

Laser capture microdissection of invasive carcinoma and precursor lesions

Ten to 20 serial tissue sections from FFPE tissue blocks were cut onto membrane slides (Carl Zeiss Microscopy, LLC, White Plains, NY, USA, Catalog No.: 415190-9041-000) at 10-μm thickness. Deparaffinization and staining were performed as described previously [32]. Sixteen invasive carcinoma samples were microdissected from 14 patients. Morphologically and regionally distinct precursor lesions were microdissected, including one IPMN region and 13 PanIN regions from eight patients (Table 1). Microdissection was performed using a Leica LMD7000 laser-capture microscope (Leica Microsystems, Wetzlar, Hesse, Germany).

DNA extraction

DNA from laser-capture microdissected tissue (invasive carcinoma or precursor lesions) or four to six 4-μm sections of FFPE blocks containing duodenum (non-neoplastic tissue) was extracted using the QIAamp DNA FFPE Tissue Kit (Qiagen, Germantown, MD, USA, Catalog No.: 56404) and deparaffinization solution (Qiagen, Catalog No.: 19093) as previously described [11]. Extracted DNA was quantified with the Qubit 3.0 Fluorometer (Thermo Fisher Scientific, Waltham, MA, USA) using the Qubit 1× dsDNA BR Assay Kit (Thermo Fisher Scientific, Catalog No.: Q32853). Sufficient microdissected neoplastic DNA for sequencing was available from 15 of the 24 patients.

Targeted gene sequencing

DNA sequence libraries for each sample were prepared using the Nextera Flex for Enrichment Kit protocol (Illumina, San Diego, CA, USA, Catalog No.: 20025524). Sequence libraries were PCR amplified before amplification with a custom Illumina AmpliSeq panel (Illumina) according to the manufacturer's specifications (supplementary material, Table S1). Fragment size and yields of amplified libraries were determined using an Agilent Bioanalyzer 2100 (Agilent, Santa Clara, CA, USA, Catalog No.: G2939BA) and a Qubit 3.0 Fluorometer (Thermo Fisher Scientific) using the Qubit 1× dsDNA HS Assay Kit (Thermo Fisher Scientific, Catalog No.: Q33230). Amplified libraries were sequenced using an Illumina MiSeq genome analyzer and the MiSeq Reagent Kit version 2 (500 cycles) (Illumina, Catalog No.: MS-102-2003) configured to produce 2 × 150 bp paired-end reads.

Analysis of genetic data

Sequence reads were aligned to the human genome hg19 using Burrows-Wheeler Aligner (BWA) (SourceForge, San Diego, CA, USA) [33]. Somatic mutation and germline variant calling was performed using the Genome Analysis Toolkit (GATK) Mutect2 and haplotype caller pipelines (Broad Institute, Cambridge, MA, USA) [34,35]. Mutations were filtered with GATK FilterMutectCalls (Broad Institute). Default settings were used. Somatic mutations and germline variants were annotated with ANNOVAR (GitHub, San Francisco, CA, USA) [36]. Somatic mutations were visually inspected in IGV (Broad Institute) [37].

Immunohistochemistry

IHC for the ATM protein was performed as previously described using an anti-ATM primary antibody (Abcam, Cambridge, UK, Catalog No.: 32420, 1:100 dilution) and 4-μm tissue sections from archived FFPE tissue blocks or tissue microarrays (Oncology Tissue Services, The Johns Hopkins University, Baltimore, MD, USA) [38]. Tissue sections from patients with surgically resected PDAC and a pathogenic germline *ATM* variant included 25 invasive carcinoma samples from 22 patients, four IPMN samples from four patients, and 24 PanIN samples from 12 patients (Table 1). Tissue microarrays (TMAs) were generated from patients unselected for germline AT status and grouped by lesion type. TMAs contained samples from 105 patients with IPMN (80 low-grade IPMN and 47 high-grade IPMN samples; 94 nonintestinal-type and 15 intestinal-type samples), 51 patients with PanIN (53 samples), and 29 patients with colloid carcinoma (30 samples). Nuclear ATM labeling was scored by board-certified pathologists (DH and LDW). ATM was determined to be lost when >90% of neoplastic cells lacked positive labeling, as described previously [39].

Statistical analysis

Prism6 (GraphPad, Boston, MA, USA) was used for all statistical analyses. To assess differences in ATM loss between different pancreatic lesions, a Fisher's exact test was used. A p value < 0.05 was considered significant. As the TMAs included multiple samples for some patients, the most severe ATM characterization for a given lesion type, for example loss of ATM, was used for each patient when conducting statistical analyses.

Results

Characteristics of patients with germline *ATM* alterations

For patients with germline *ATM* alterations, clinicopathologic and *ATM* variant data are detailed in Table 1. In the patient cohort, 62.5% were female (15 of 24 patients), predominantly white (12 of 14 patients with race data; 85.7%) and had a median age range of 60–69 years. The most common cancer histological type among patients was infiltrating ductal adenocarcinoma (20 of 24 patients; 83.3%). Colloid carcinoma was diagnosed in three patients (12.5%) and adenosquamous carcinoma in one patient (4.2%). Of the 15 patients with information on receipt of neoadjuvant therapy, five received neoadjuvant chemotherapy (33.3%) including gemcitabine and nab-paclitaxel (three patients), gemcitabine and paclitaxel (one patient), and FOLFIRINOX (one patient). *ATM* germline variants were classified as pathogenic or likely

Table 1. Clinicopathologic information for patients with a pathogenic germline ATM variant.

ID	Pathogenic germline ATM variant			Age (years)	Sex	Race	Histologic subtype	Neoadjuvant therapy[§]	Pathologic stage	Pathologic response
	Genomic variant*	Transcript variant[†]	Protein variant[‡]							
P1	chr11:108173655-108173656_AG>A	c.5396delG	p.Ser1799MetfsTer8	50–59	Male	White	Ductal adenocarcinoma	No	pT1c pN1	N/A
P2	chr11:108224608-108,224,608_G>A	c.8786+1G>A	Splicing	50–59	Male	White	Ductal adenocarcinoma	No	pT1a pN0	N/A
P3	chr11:108188128-108188129_TT>T	c.6228delT	p.Leu2077PhefsTer5	60–69	Female	No data	Ductal adenocarcinoma	No	pT1c pN0	N/A
P4	chr11:108115702-108,115,702_C>T	c.850C>T	p.Gln284Ter	70–79	Male	White	Ductal adenocarcinoma	No	pT2 pN1	N/A
P5	chr11:108098354-108098354_G>A	c.3G>A	p.Met1Ile	60–69	Male	White	Ductal adenocarcinoma	No	pT2 pN2	N/A
P6	chr11:108121755-108121757_AGA>A	c.1564_1565delGA	p.Glu522IlefsTer43	60–69	Male	White	Ductal adenocarcinoma	No	pT2 pN0	N/A
P7	chr11:108206605-108,206,605_C>T	c.8185C>T	p.Gln2729Ter	70–79	Female	No data	Ductal adenocarcinoma	No	pT2 pN1	N/A
P8	chr11:108214064-108214074_AIT TCAGTGCC>A	c.8395_8404delTTTCAGTGCC	p.Phe2799LysfsTer4	<50	Male	White	Colloid carcinoma	No data	pT1a pN0	N/A
P9	chr11:108202604-108202604_A>C	c.7630-2A>C	Splicing	50–59	Male	White	Colloid carcinoma	No	pT3 pN2	N/A
P10	chr11:108181032-108181032_C>T	c.5908C>T	p.Gln1970Ter	70–79	Female	Other	Adenosquamous carcinoma	No	pT1c pN0	N/A
P11	chr11:108202604-0820026477_A>T	c.7630-2A>T	Splicing	60–69	Female	White	Ductal adenocarcinoma	Gemcitabine + nab-paclitaxel	ypT2 ypN2	Poor
P12	chr11:108100051-108100051_G>A	c.331+1G>A	Splicing	60–69	Female	White	Ductal adenocarcinoma	Gemcitabine + nab-paclitaxel	ypT2 ypN2	Poor
P13	chr11:108121561-108121561_C>T	c.1369C>T	p.Arg457Ter	70–79	Female	White	Ductal adenocarcinoma	Gemcitabine + nab-paclitaxel	ypT2 ypN2	Moderate
P14	chr11:108121755-108121757_AGA>A	c.1564_1565delGA	p.Glu522IlefsTer43	<50	Male	White	Ductal adenocarcinoma	FOLFIRINOX	ypT2 ypN2	Poor
P15	chr11:108198361-108198385_TTCTT ATACAGAACAATCCCAGCCT>T	c.6976-10_6989delTTCTTATAC AGAACAATCCCAGCCT	Splicing	70–79	Female	White	Ductal adenocarcinoma	No	pT2 ypN0	N/A
P16	chr11:108235935-108235935_C>T	c.8977C>T	p.Arg2993Ter	70–79	Female	Other	Ductal adenocarcinoma	Gemcitabine + paclitaxel	ypT1c ypN2	Poor
P17	chr11:108128198-108128198_T>G	c.2251-10T>G	Splicing	70–79	Male	No data	Ductal adenocarcinoma	No data	pT1N0	N/A
P18	chr11:108121755-108121757_AGA>A	c.1564_1565delGA	p.Glu522Ilefs*43	70–79	Female	No data	Colloid carcinoma	No data	pT1	N/A
P19	chr11:108196885-108196886_A>AA	c.6908dupA	p.Glu2304Glyfs*69	60–69	Female	No data	Ductal adenocarcinoma	No data	No data	N/A
P20	chr11:108122615-108122616_AA>A	c.1660delA	p.Thr554Argfs*2	60–69	Female	No data	Ductal adenocarcinoma	No data	pT3N0	N/A
P21	chr11:108205825-108205825_C>T	c.8140C>T	p.Gln2714Ter	50–59	Female	No data	Ductal adenocarcinoma	No data	No data	N/A
P22	chr11:108216545-108216545_C>T	c.8494C>T	p.Arg2832Cys	60–69	Female	No data	Ductal adenocarcinoma	No data	No data	N/A
P23	chr11:108123641-108123641_I>G	c.1898+2T>G	Splicing	60–69	Female	No data	Ductal adenocarcinoma	No data	pT3N0	N/A
P24	chr11:108153571-108153576_TITATT>T	c.3712_371delTTTATT	p.Leu1238Lysfs*6	70–79	Female	No data	Ductal adenocarcinoma	No data	No data	N/A

*Genomic variant shown based on GRCh37 version of human genome.
[†]Transcript variant shown based on NM_000051.3.
[‡]Protein variant shown based on NP_000042.3.
[§]FOLFIRINOX – folinic acid, fluorouracil, irinotecan, and oxaliplatin.

pathogenic based on American College of Medical Genetics guidelines. Pathogenic or likely pathogenic variants included six nonsense variants (25.0%), eight frameshift deletions (33.3%), one frameshift insertion (4.2%), seven splicing variants (29.2%), and two missense variants (8.3%).

Immunohistochemical analysis of invasive carcinoma and precursor lesions from patients with germline *ATM* alterations

To determine the prevalence of loss of ATM expression, we immunolabeled histological sections containing invasive carcinoma, PanIN, and/or IPMN from 24 patients. In total, 25 invasive carcinoma samples from 22 patients, 24 PanIN samples (all low-grade) from 12 patients, four IPMN samples (three low-grade and one high-grade) each from a different patient were characterized (Table 1). Loss of ATM expression was observed in invasive carcinoma samples from 17 of 22 patients (77.3%) (Table 2). Loss of ATM expression was also observed in one of four IPMNs (25.0%). Of note, the IPMN sample with loss of ATM expression was the only precursor lesion analyzed with high-grade dysplasia (Figure 1). We found that ATM expression was intact for PanINs, all low-grade, that were assessed (Table 2). Loss of ATM expression was statistically more prevalent in invasive carcinoma compared to PanIN (Fisher's exact test; $p < 0.001$) (Table 3).

Targeted sequencing of invasive carcinoma and precursor lesions

Invasive carcinomas, PanINs, and/or IPMN from 15 patients were sequenced using the custom amplicon panel covering the coding regions of *ATM*, *KRAS*, *GNAS*, *TP53*, *CDKN2A*, and *SMAD4* (supplementary material, Table S1). In total, 15 invasive carcinoma samples from 12 patients, 12 PanIN samples from seven patients, and one IPMN sample were sequenced (Table 1).

Across all samples, target regions were sequenced to a mean depth of $204\times$ (range: $60–451\times$), with 83.1% (range: 42.5–92.2%) of target bases covered at a minimum of $50\times$. Coverage was similar for invasive carcinoma ($217\times$ mean coverage; 84.2% at $50\times$), precursor lesions ($194\times$ mean coverage; 85.5% at $50\times$), and normal tissue samples ($200\times$ mean coverage; 80.0% at $50\times$) (supplementary material, Table S2).

Somatic mutations identified in sequenced samples are presented in Figure 2 and supplementary material, Table S3. Somatic mutations in *ATM* were identified in six of 15 sequenced invasive carcinoma samples (40.0%) representing five of 12 patients (41.7%) and one of 12 sequenced PanIN samples (8.3%) had a somatic mutation in *ATM*. No *ATM* somatic mutations were identified in the single sequenced IPMN sample. *ATM* somatic mutations identified included truncating mutations (nonsense, frameshift indel, and splicing;

Table 2. Samples from patients with pathogenic germline *ATM* variant available for immunolabeling and targeted sequencing.

ID	No. immunostained samples			No. sequenced samples		
	PanIN*	IPMN†	Invasive carcinoma	PanIN*	IPMN†	Invasive carcinoma
P1	2	–	1	1	–	–
P2	5	1	–	3	1	1
P3	2	1	–	–	–	1
P4	1	–	1	–	–	1
P5	1	–	2	–	–	2
P6	3	–	1	1	–	1
P7	1	–	1	–	–	1
P8	–	1	1	–	–	–
P9	1	–	2	1	–	2
P10	–	–	2	–	–	2
P11	–	–	1	–	–	1
P12	2	–	1	1	–	–
P13	1	–	1	1	–	–
P14	–	–	1	–	–	1
P15	4	–	1	4	–	1
P16	–	–	1	–	–	1
P17	1	–	1	–	–	–
P18	–	1[H]	1	–	–	–
P19	–	–	1	–	–	–
P20	–	–	1	–	–	–
P21	–	–	1	–	–	–
P22	–	–	1	–	–	–
P23	–	–	1	–	–	–
P24	–	–	1	–	–	–
Total	24	4	25	12	1	15

IPMN, intraductal papillary mucinous neoplasm; PanIN, Pancreatic intraepithelial neoplasm.
*PanINs were all low-grade.
†IPMNs were all low-grade except where indicated by superscripted H.
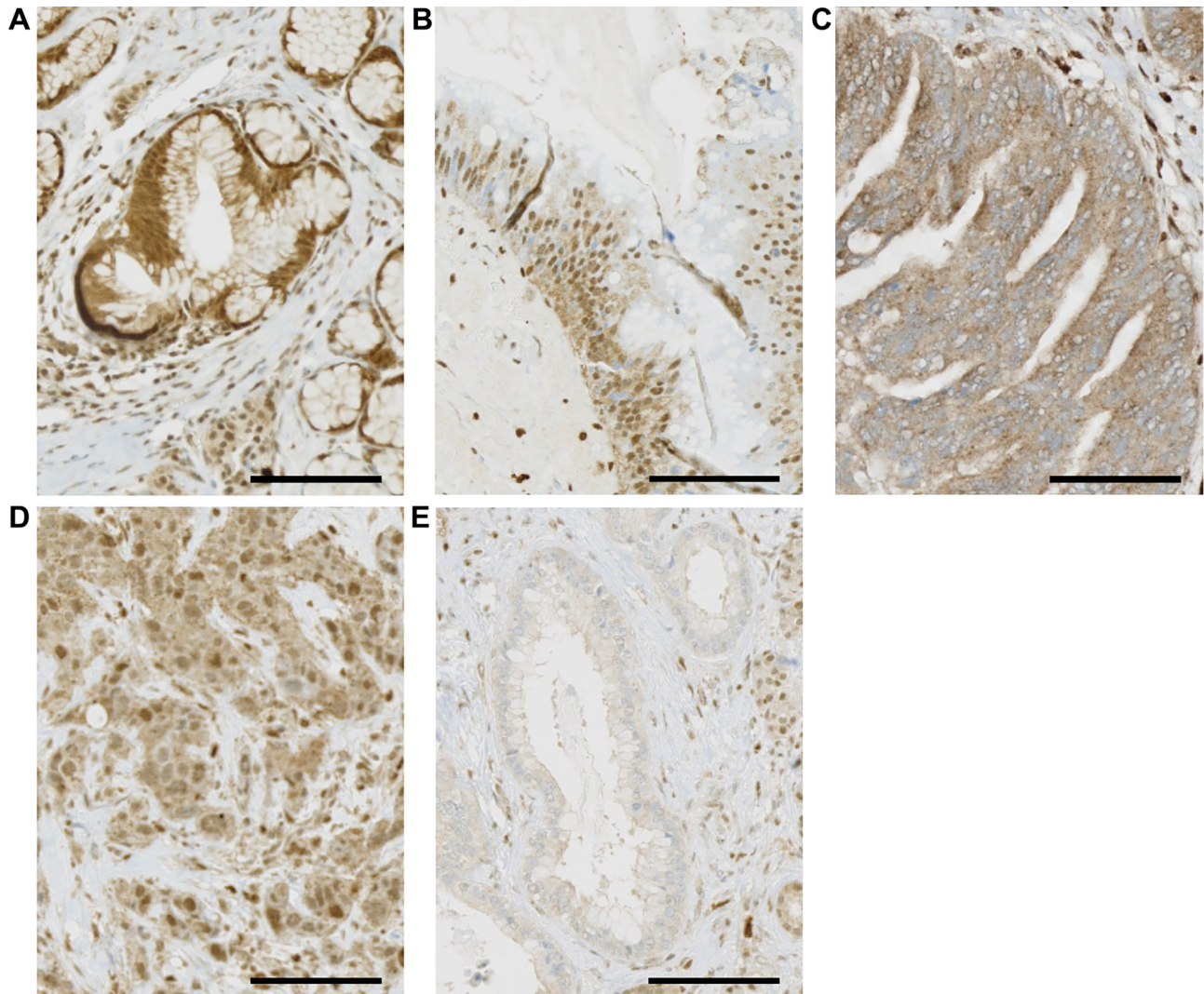
Figure 1. ATM IHC images of PDAC and pancreatic cancer precursor lesions. Black scale bar, 100 μm. (A) PanIN showing retention of nuclear ATM staining. (B) Low-grade IPMN showing retention of nuclear ATM staining. (C) High-grade IPMN showing loss of nuclear ATM staining. (D) PDAC showing retention of nuclear ATM staining. (E) PDAC showing loss of nuclear ATM staining.

Table 3. ATM expression status in invasive carcinoma, IPMN, and PanIN from patients with a pathogenic germline *ATM* variant as determined by immunolabeling.

Lesion morphology	Lost	Retained	Total	Percent lost	*P* value
PanIN	0	24	24	0	<0.0001
IPMN	1	3	4	25.0	0.1048
Invasive carcinoma	18	7	25	72.0	

Two-tailed *p* values (PanIN versus invasive carcinoma and IPMN versus invasive carcinoma) calculated using a Fisher's exact test.
IPMN, intraductal papillary mucinous neoplasm; PanIN, Pancreatic intraepithelial neoplasm.

three of seven mutations; 42.9%) and missense mutations (four of seven mutations; 57.1%).

Hotspot *KRAS* mutations were frequently identified in invasive carcinomas (13 of 15 samples; 86.7%), PanINs (11 of 12 samples; 91.7%), and the IPMN (one of one sample; 100%). While most sequenced samples had only one *KRAS* somatic mutation, the single sequenced IPMN sample (P2) had both p.G12D and p.Q61H somatic mutations (supplementary material, Table S2). The spectrum of *KRAS* somatic mutations identified included p.G12D (eight of 26 *KRAS* mutations; 30.8%), p.G12R

(seven of 26; 26.9%), p.G12V (six of 26; 23.1%), p.G13D (one of 26; 3.8%), and p.Q61H (four of 26; 15.4%). Somatic mutations in *GNAS*, *TP53*, and *SMAD4* were infrequent in sequenced invasive carcinoma and precursor samples (Figure 2; supplementary material, Table S2). No somatic mutations in *CDKN2A* were identified; however, we did not assess copy number alterations or chromosomal alterations in sequenced samples.

We did not observe the co-occurrence of *TP53* somatic mutations with either *ATM* somatic mutation
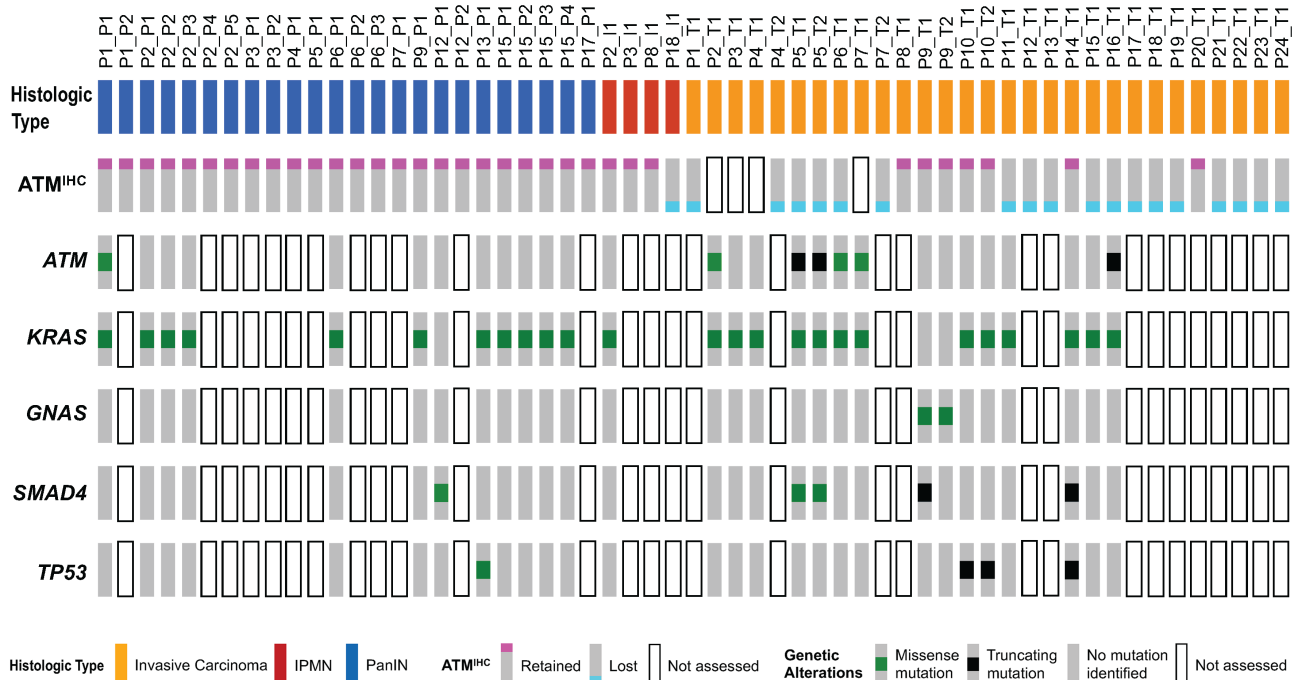
Figure 2. OncoPrint diagram showing immunolabeling and somatic mutation status for invasive carcinoma, PanIN, and IPMN samples in patients with a pathogenic germline *ATM* variant. ATM expression status (ATM^IHC) determined by immunolabeling. Lost ATM expression – blue square. Retained ATM expression – red square. Not assessed – white rectangle. Genetic alterations in *ATM*, *KRAS*, *GNAS*, *TP53*, and *SMAD4* determined by targeted next-generation sequencing. Missense somatic mutations – green square. Truncating somatic mutations – black square. No mutation identified – gray rectangle. Not assessed – white rectangle. Samples grouped by histologic type.

or loss of ATM expression (Figure 2). In one patient (P5), with two sequenced invasive carcinoma samples, somatic mutations in *ATM* and *SMAD4* were identified in both samples. Notably the *ATM* somatic mutation (NM_000051:c.7629+1G>T) was the same in each sample, while the two *SMAD4* somatic mutations were different (NP_005350.1:p.H132N and NP_005350.1:p.R361T) (supplementary material, Table S3). These data suggest a common origin for both sequenced carcinomas, where the somatic mutation in *ATM* occurred before the somatic mutations in *SMAD4*.

ATM loss in patients unselected for germline *ATM* status

We next determined the prevalence of ATM loss in precursor lesions and invasive carcinoma in patients with PDAC unselected for *ATM* status. As we previously published data on the prevalence of ATM loss in invasive carcinoma from 555 patients with PDAC using the same antibody and scoring system [39], we determined the ATM expression status of PanIN and IPMN from 51 and 105 patients unselected for pathogenic germline *ATM* variants, respectively. The prevalence of ATM loss was similar in PanIN (two of 51 patients; 3.9%) and IPMN (nine of 105 patients; 8.6%) (Fisher's exact test; $p = 0.5058$), in low-grade IPMN (five of 80 patients; 6.3%) and high-grade IPMN (four of 47 patients; 8.5%) (Fisher's exact test; $p = 0.7251$), and intestinal-type IPMN (zero of 15 patients; 0%) and nonintestinal type

IPMN (nine of 94; 9.6%) (Fisher's exact test; $p = 0.3563$).

We next compared the prevalence of ATM loss in pancreatic precursor lesions to our previously published data on ATM loss in 555 patients with PDAC unselected for *ATM* germline status. We did not identify any statistically significant differences in ATM loss between PanIN (two of 51 patients; 3.9%) and PDAC (67 of 555 patients; 12.1%) between the two groups (Fisher's exact test; $p = 0.1040$) or IPMN (nine of 105 patients; 8.6%) and PDAC (67 of 555 patients; 12.1%) (Fisher's exact test; $p = 0.4038$).

We also determined the prevalence of ATM loss by IHC in 29 patients with colloid carcinoma unselected for *ATM* germline status. The prevalence of ATM loss in patients with colloid carcinoma (nine of 29 patients; 31.0%) was significantly higher compared to usual PDAC (67 of 555 patients; 12.1%) (Fisher's exact test; $p = 0.0076$), IPMN (nine of 105 patients; 8.6%) (Fisher's exact test; $p = 0.0040$), or PanIN (two of 51 patients; 3.9%) (Fisher's exact test; $p = 0.0013$). These data clearly indicate that loss of ATM is an important aspect of the pathogenesis of colloid carcinomas.

Interestingly, six patients had multiple samples assessed that were discordant for ATM expression status (supplementary material, Table S4). In general, discordant expression occurred within samples of the same histological type and grade (one patient with PanIN, one patient with low-grade IPMN, two patients with high-grade IPMN, and one patient with colloid carcinoma) and likely represented clonal heterogeneity. However,

one patient who had samples of low-grade and high-grade IPMN assessed had ATM loss restricted to the high-grade sample (supplementary material, Figure S1).

Discussion

ATM is a pancreatic cancer susceptibility gene frequently identified by germline genetic testing in patients with PDAC and their relatives [12,14]. In carriers of a pathogenic germline *ATM* variant, the risk of developing PDAC is high, at 9.5% by age 80 years [18]. However, despite these clear findings supporting the importance of *ATM* in the development of PDAC, little is known about the role of ATM in pancreatic tumorigenesis. For example, as *ATM* is a tumor suppressor gene, the remaining wildtype copy of *ATM* in individuals with a pathogenic germline *ATM* variant is predicted to be mutated or lost during tumorigenesis [40]. Understanding the prevalence, timing, and type of somatic alterations that result in loss of *ATM* during the development of PDAC is essential to improve clinical surveillance, early detection initiatives, chemoprevention, and targeted therapies.

The prevalence of somatic loss of *ATM* in invasive carcinoma from patients with PDAC and pathogenic germline *ATM* variants, by somatic mutation and/or loss of ATM expression, was 75% (18 of 24 patients; Figure 2 and Table 2). The 25% of patients without an identifiable somatic alteration in *ATM* in their invasive carcinoma suggests that either the wildtype *ATM* allele is not somatically altered or that somatic loss occurred by a mechanism not detected by the approaches used to assess somatic *ATM* status in this study. Regardless, these data clearly indicate that somatic loss of *ATM* in invasive carcinoma is common in patients with PDAC and a pathogenic germline *ATM* variant.

A recent study suggested that somatic mutations in *TP53* are mutually exclusive with inactivation of the *ATM* gene [28]. In support of this observation, we did not identify any *TP53* somatic mutations in any precursor or invasive carcinoma samples that had either a somatic mutation in *ATM* or loss of ATM expression (Figure 2); however, this observation was not statistically significant. Analysis of additional samples is necessary to confirm mutual exclusivity of *TP53* somatic mutation and somatic *ATM* mutations or loss of ATM expression in patients with a pathogenic germline *ATM* variant.

We were also able to determine the prevalence of ATM loss in colloid carcinomas unselected for germline *ATM* status using IHC. We found that ATM loss was common in colloid carcinomas (nine of 29 samples; 31.0%) and that ATM loss was significantly more prevalent in this subtype of pancreatic cancer compared to PDAC unselected for subtype, PanIN, and IPMN. These data indicate that loss of ATM is an important event in the development of colloid carcinomas, which is also supported by our previous observations of an increased prevalence of colloid carcinomas in patients with pathogenic germline *ATM* variants [22].

Several lines of evidence from our study suggest that loss of ATM is a relatively late event in pancreatic tumorigenesis. Somatic mutations and/or loss of expression of ATM were almost completely restricted to invasive carcinoma in patients with PDAC and a pathogenic germline *ATM* variant, with only one of 28 immunolabeled precursor lesions and one of 13 sequenced precursor lesions demonstrating loss of ATM expression or a somatic mutation in *ATM*, respectively (Table 2; Figure 2). The precursor lesion with loss of ATM expression was a high-grade IPMN. All other immunolabeled precursor lesions were low-grade and had intact ATM expression. Of note, we could not establish whether the high-grade IPMN sampled was a precursor lesion or represented ductal cancerization. The precursor lesion with a somatic mutation in *ATM* was a PanIN, and the mutation resulted in a missense change of unknown functional consequence (p.R832C; Supplementary Table S3). While immunostaining of the PanIN demonstrated retention of ATM expression, invasive carcinoma from the same patient demonstrated loss of ATM expression, indicating a different mechanism for somatic loss of ATM in the patient's invasive carcinoma compared to their PanIN.

While we did not see a significant difference in the prevalence of ATM loss between precursor lesions and PDAC in patients unselected for germline *ATM* status, the total number of PanIN and IPMN samples included in our study was small, and a larger study incorporating more patients with precursor lesions is necessary to elucidate differences in prevalence. Notably, however, one patient with IPMN with both high-grade and low-grade dysplasia had ATM loss that was restricted to the high-grade component (supplementary material, Table S3 and Figure S1). The late biallelic inactivation of *ATM* in germline *ATM* alteration carriers is consistent with evidence that patients with pathogenic *ATM* variants do not have an increase in pancreatic precursor lesions [22].

Finally, we also found that ATM loss was statistically more prevalent in colloid carcinomas compared with PDAC unselected for histologic subtype, IPMN, and PanIN from patients unselected for germline *ATM* status. Furthermore, lack of ATM expression in the more common precursor to colloid carcinoma, intestinal-type IPMN, was not detected in this study. This might have been due to the small numbers available for study, or it might reflect the late occurrence of *ATM* alterations in the progression of IPMN to colloid carcinoma. Given the evidence that ATM loss is a later event in patients with pathogenic germline *ATM* variants, additional studies are warranted to assess the utility of ATM loss as a biomarker of progression.

In summary, we presented an analysis of ATM loss during pancreatic tumorigenesis in patients with a pathogenic germline *ATM* variant and those unselected for germline *ATM* status. We found that loss of ATM was a common event in patients with a pathogenic germline *ATM* variant who developed PDAC, as well as in patients with colloid carcinoma unselected for germline

ATM status. We also found that loss of ATM was likely a later event in pancreatic tumorigenesis. These observations could have implications for the use of ATM loss as a biomarker of neoplastic progression and patient care.

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Author contributions statement

NJR conceptualized the study. CLW, JH, DSK, REB, ADS, MGG, LAAB, RHH, APK, TL, LDW and NJR designed the experiments. CLW, JH, DSK, REB, ADS, AD, LZ, MGG, LAAB, RHH, APK, TL and NJR provided resources. RMP, ZJ, DH, VK, CG, LZ, RHH, APK, LDW and NJR curated the data. RMP, ZJ, DH, VK, CG, KF, NN, BH, MS, TL, LDW and NJR acquired, analyzed, and interpreted the data. RMP and NJR wrote the manuscript. RMP, ZJ, DH, VK, CG, KF, NN, BH, MS, CLW, JH, DSK, REB, ADS, AD, LZ, MGG, LAAB, RHH, APK, TL, LDW and NJR reviewed and edited the manuscript. NJR was responsible for supervision and administration of the study.

Data availability statement

Histological images and targeted gene panel data are available on request from the corresponding author in accordance with institutional policies.

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SUPPLEMENTARY MATERIAL ONLINE

Figure S1. Low-grade and high-grade IPMN samples from individual patient discordant for ATM expression

Table S1. Targeted capture regions

Table S2. Summary sequencing statistics for sequenced samples

Table S3. Somatic mutations identified in patient samples

Table S4. Discordant ATM expression in patients unselected for germline *ATM* status