Cancer Research

Activation-Induced Cytidine Deaminase Contributes to Pancreatic Tumorigenesis by Inducing Tumor-Related Gene Mutations

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Abstract

Pancreatic ductal adenocarcinoma (PDAC) develops via an accumulation of various gene mutations. The mechanism underlying the mutations in PDAC development, however, is not fully understood. Recent insight into the close association between the mutation pattern of various cancers and specific mutagens led us to investigate the possible involvement of activation-induced cytidine deaminase (AID), a DNA editing enzyme, in pancreatic tumorigenesis. Our immunohistochemical findings revealed AID protein expression in human acinar ductal metaplasia, pancreatic intraepithelial neoplasia, and PDAC. Both the amount and intensity of the AID protein expression increased with the progression from precancerous to cancerous lesions in human PDAC tissues. To further assess the significance of ectopic epithelial AID expression in pancreatic tumorigenesis, we analyzed the phenotype of *AID* transgenic (*AID* Tg) mice. Consistent with our hypothesis that AID is involved in the mechanism of the mutations underlying pancreatic tumorigenesis, we found precancerous lesions developing in the pancreas of *AID* Tg mice. Using deep sequencing, we also detected *Kras* and *c-Myc* mutations in our analysis of the whole pancreas of *AID* Tg mice. In addition, Sanger sequencing confirmed the presence of *Kras*, *c-Myc*, and *Smad4* mutations, with the typical mutational footprint of AID in precancerous lesions in *AID* Tg mice separated by laser capture microdissection. Taken together, our findings suggest that AID contributes to the development of pancreatic precancerous lesions by inducing tumor-related gene mutations. Our new mouse model without intentional manipulation of specific tumor-related genes provides a powerful system for analyzing the mutations involved in PDAC. *Cancer Res; 75(16); 3292–301. ©2015 AACR.*

Introduction

Pancreatic ductal adenocarcinoma (PDAC) is a highly lethal carcinoma with a 5-year relative survival rate of <6% (1). Improving the prognosis of PDAC will require a more complete understanding of the molecular mechanisms that underlie pancreatic carcinogenesis.

Various genetic abnormalities are detected in human PDAC, including mutations of *KRAS*, *INK4A/CDKN2A*, *TP53*, *SMAD4*, and *BRCA2* (2–7). PDAC is considered to develop through a precancerous lesion, defined as pancreatic intraepithelial neoplasia (PanIN), ranging from low-grade PanIN (PanIN-1A/1B) to high-grade PanIN (PanIN-2/3; ref. 8). Molecular analysis of PanINs reveals that PanINs harbor many of the same genetic alterations found in PDAC. Especially, *KRAS* mutations are detected even in low-grade PanIN (9), suggesting that *KRAS* mutations may account for the initiation of PDAC. This concept is further supported by findings from a genetically engineered mouse model in which pancreas-specific expression of mutant *Kras* ($Kras^{G12D}$) in mice recapitulates the human PanIN-to-PDAC sequence (10). In the PDAC mouse model, PanIN coincides with or is preceded by acinar–ductal metaplasia (ADM), which is characterized by the replacement of acinar cells with cells coexpressing a ductal marker (e.g., cytokeratin 19) and an acinar cell marker (e.g., amylase). Moreover, inhibition of ADM formation reduces $Kras^{G12D}$-induced PanINs (11–13). On the basis of the observations that PanINs are frequently associated with ADM and lobular atrophy in human PDAC (14, 15), pancreatic carcinogenesis might depend on the ADM-to-PanIN-to-PDAC sequence via the accumulation of various genetic mutations. The mechanism underlying the induction of mutations during PDAC development, however, is not fully understood.

Several human enzymes capable of inducing nucleotide alterations were recently identified (16). Among them, the importance of the AID/APOBEC family, a cytidine deaminase family that acts as a DNA and RNA editor, in tumorigenesis through induction of genetic instability is highlighted. One APOBEC family protein, activation-induced cytidine deaminase

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(AID), is considered to act on single-strand DNA during the transcriptional stage, where it deaminates deoxycytidine (dC) to uracil (U), resulting in C:G>T:A transitions. Under physiologic conditions, AID is expressed only in activated B lymphocytes, and is essential for somatic hypermutation and class-switch recombination in immunoglobulin genes (17). Our group previously reported that AID can be aberrantly expressed in epithelial cells, however, where it induces somatic mutations and genetic aberrations in tumor-related genes (18–23). Constitutive and ubiquitous AID expression in transgenic mice induces the development of epithelial tumors, including lung adenoma and carcinoma, as well as lymphomas (24). We recently revealed that AID is involved in tumor development in various digestive organs, including liver, stomach, colon, bile duct, and Barrett esophagus, by the induction of genetic mutations (18–23).

A recent genome-wide analysis revealed a distinct mutation pattern of various types of cancer, implying the involvement of specific extrinsic or intrinsic mutagens in each type of cancer (25, 26). A recent report showed that C:G>T:A transitions are most frequently observed in human PDAC (27). Interestingly, AID is one of the mutagens that preferentially induce C:G>T:A transitions (17). Accordingly, we hypothesized that aberrant AID expression in the human pancreas has an important role in pancreatic carcinogenesis through the induction of multiple mutations in tumor-related genes. In the current study, we demonstrated AID expression in human ADM, PanIN, and PDAC, but not in normal pancreatic ducts. Furthermore, we found that *AID* transgenic (*AID* Tg) mice develop precancerous lesions in the pancreas, accompanied by the accumulation of various mutations in tumor-related genes, including *Kras*, *c-Myc*, and *Smad4*, suggesting important roles of AID in pancreatic tumorigenesis. To our knowledge, this is the first genetically engineered mouse model that spontaneously develops precancerous lesions in the pancreas without intentional manipulation of specific tumor-related genes.

Patients and Methods

Patients

The study group comprised 20 patients who underwent potentially curative resection for primary PDAC at Kyoto University Hospital (Kyoto, Japan) from 2006 to 2011 (Table 1). Written informed consent for the use of their resected tissues was obtained from all patients in accordance with the Declaration of Helsinki, and the Kyoto University Graduate School and Faculty of Medicine Ethics Committee approved the study.

Table 1. Clinicopathologic features of 20 patients with PDAC

Feature	Value
Age at surgery	
Median (range)	69 (45–84)
Sex	
Male	11
Female	9
Tumor size, cm	
Median (range)	2.9 (1.3–4.5)
Tumor differentiation	
Well differentiated	2
Moderately differentiated	16
Poorly differentiated	1
Unknown differentiated	1
TNM staging	
IA	1
IB	2
IIA	2
IIB	15
III	0
IV	0

Mice

The generation of *AID* Tg mice with constitutive and ubiquitous AID expression was described previously (24). Wild-type (WT) C57BL/6J mice were purchased from Japan SLC, Inc. For immunohistochemistry, tissue samples were removed from the mice, flushed with 1×PBS, fixed overnight in 4% (w/v) formaldehyde, and embedded in paraffin. Tissue samples were also frozen immediately in liquid nitrogen for nucleotide extraction.

Histology and immunohistochemistry

Paraffin-embedded human and mouse pancreatic tissues were sectioned and stained with hematoxylin and eosin. Paraffin-embedded mouse pancreatic tissues were also stained with Alcian blue and nuclear fast red. Immunohistochemistry was performed according to the manufacturer's recommendations, typically using a citric acid unmasking protocol followed by standard detection with 3,3-diaminobenzidine using a LSAB+ kit (Dako) or VECTASTAIN ABC Kit (Vector Laboratories). All sections were counterstained with hematoxylin. A polyclonal antibody against human AID was generated using purified recombinant AID protein as an immunogen (28). In addition, we used the following primary antibodies: cytokeratin 19 [Developmental Studies Hybridoma Bank, TROMAIII, developed by Dr. Rolf Kemler (Max-Planck Institute of Immunobiology, Freiburg, Germany)], amylase (Santa Cruz Biotechnology), phospho-Erk1/2 (Cell Signaling Technology), and NFκB p65 (Cell Signaling Technology). Visual assessment of AID staining based on the intensity of immunoreactivity was classified as no staining (−; no appreciable staining), weak positive staining (+; any percentage of neoplastic cells with weak intensity, or <50% of neoplastic cells labeled with strong intensity), and strong positive staining (++; >50% of neoplastic cells labeled with strong intensity). For ADM and PanIN quantification, hematoxylin and eosin staining using one representative slide per mouse was quantified. Ten randomly selected microscopic fields were observed in each slide, and the number of lesions was calculated manually.

Deep sequencing on selected genes

Genome DNA was extracted from frozen pancreatic tissues of mice using a DNA Mini Kit (Qiagen) according to the manufacturer's protocol. We analyzed 1 *AID* Tg mouse and 1 *WT* mouse at 13 months of age. The mutational status of *Kras*, *c-Myc*, and *Trp53* DNA was investigated by deep sequencing analyses. Target regions were designed within the range from 388 to 409 bp. The primer sequences are described in Supplementary Table S1. Each region was amplified with high-fidelity PCR using Phusion High-Fidelity DNA Polymerase (FINNZYMES), purified by gel extraction methods. A 500 ng aliquot of each sample was dA-tailed and ligated with adaptors containing tag sequences, followed by emulsion PCR and sequencing using the GS Junior System (Roche) according

to the manufacturer's protocol. Deep sequencing data were analyzed with NextGENe software, v2.3.4 (SoftGenetics). We identified low-abundance somatic mutations using a strict variant filtering process. We excluded nucleotide changes that were common between *WT* and *AID* Tg mice. Candidate low-abundance mutations were validated by repeated deep sequencing using independent amplicons derived from the same samples. According to our previous result (29), we picked the mutations that presented at a frequency greater than 0.1%.

Laser capture microdissection and DNA purification

Sections for laser capture microdissection (7–10 μm) were cut from formalin-fixed, paraffin-embedded tissue blocks and placed on slides. The slides were stained with hematoxylin and eosin, and then coated with laser mount. Next, normal pancreatic parenchyma, ADM lesions, and PanIN lesions were separately microdissected in that order using a laser capture microdissection technique from PALM Technologies (Carl Zeiss Microimaging) or Leica AS LMD (Leica). Dissected tissues were catapulted into caps by defocused laser pulses. Representative photographs before and after microdissection of PanIN are shown in Supplementary Fig. S1. Next, the xylene was removed from the dissected samples and the DNA was extracted using the QIAamp DNA Micro Kit (Qiagen) according to the manufacturer's protocol.

Subcloning and sequencing of the tumor-related genes

Genomic DNA from the laser capture microdissection samples was prepared using the DNA Micro Kit (Qiagen) according to the manufacturer's protocol. The primers used for amplifying *Kras*, *c-Myc*, and *Smad4* are described in Supplementary Table S2. After amplifying each gene using high-fidelity Phusion Taq polymerase (FINNZYMES), the PCR products were subcloned by insertion into the *Eco*RI–*Xho*I sites of the pcDNA3 vector (Invitrogen) and further subjected to capillary sequence analyses.

Results

Expression of AID protein in human PDAC and precancerous lesions

To clarify the specific expression and precise localization of AID protein in human pancreatic tissues, immunohistochemistry was performed using various paraffin-embedded specimens from patients with PDAC. We found ADM in 9 of 20 (45%), PanIN-1A/1B in 9 of 20 (45%), and PanIN-2/3 in 6 of 20 (30%) PDAC specimens. We examined the AID immunoreactivity in each lesion. AID immunoreactivity was detected in all the PDAC tissues [20/20 (100%)], with strong and weak expression detected in 17 of 20 (85%) and 3 of 20 (15%) cases, respectively. Weak AID expression was detected in 7 of 9 (78%) ADM lesions, 5 of 9 (56%) low-grade PanIN, and in 6 of 6 (100%) high-grade PanIN lesions, whereas no strong AID expression was detected in ADM or PanIN lesions (Fig. 1A; Table 2). AID protein expression tended to increase with progression from precancerous lesions to invasive cancer. Notably, we occasionally detected AID protein expression in acinar cells adjacent to human PDAC tissues. In normal pancreatic ducts, we observed no AID protein expression (Fig. 1B). We confirmed that no immunostaining was obtained when nonimmunized serum or PBS was used instead of the antibodies against AID (Fig. 1B). AID protein expression was also detected in ADM and PanIN lesions in human chronic pancreatitis as well as PDAC tissues (Fig. 1C). Immunohistochemistry of serial sections confirmed that these AID protein expressions were accompanied by p65 nuclear translocation, suggesting NFκB activation (Fig. 1C).

Development of ADM and PanIN in *AID* Tg mouse pancreas

Previous studies reported that *AID* Tg mice with systemic and ubiquitous AID expression develop four types of tumors, including lymphoma, hepatocellular carcinoma, gastric cancer, and lung cancer (30). In the current study, we focused on the pancreatic phenotypes, and examined the pancreatic pathology in detail in *AID* Tg mice at 3, 6, 11, and 13 months of age. *AID* Tg mice developed tubular structures with both acinar and ductal differentiation, which is consistent with human ADM. ADM showed coexpression of acinar (amylase) and ductal (CK19) markers, consistent with a previous report (Fig. 2A) (31). *AID* Tg mice also developed ductal lesions with histologic and molecular characteristics of human PanIN, including a high acidic mucin content indicated by Alcian blue staining and CK19 expression (Fig. 2A). These ductal lesions were low-grade PanINs that weakly expressed amylase and CK19, consistent with a previous study (32). Another previous report revealed that MAPK signaling activation is required for the initiation and maintenance of pancreatic precancerous lesions in the PDAC mouse model (33). Therefore, we next examined phospho-Erk1/2 (p-Erk1/2) expression in pancreatic precancerous lesions that developed in *AID* Tg mice, and confirmed the activation of MAPK signaling in both ADM and PanIN in *AID* Tg mice (Fig. 2A).

We found ADM in 2 of 5 (40%), 4 of 4 (100%), and 7 of 7 (100%) *AID* Tg mice at 6, 11, and 13 months of age, respectively. We also found PanIN lesions in 1 of 4 (25%) and 4 of 7 (57%) *AID* Tg mice at 11 and 13 months of age, respectively, whereas no PanIN was detected at 3 and 6 months of age (Table 3). At 13 months of age, remarkable atrophic changes in pancreas, decreased acinar cells, and ductal lesions were observed in some *AID* Tg mice (e.g., *AID* Tg 13M-2). Thus, the frequencies of ADM and PanIN increased with age, and ADM development appeared to precede the development of PanIN (Fig. 2B and C). On the other hand, the *WT* mice did not develop ADM or PanIN, even at 13 months of age (Fig. 2C; Table 3).

Mutational profiles of *AID* Tg mice pancreas analyzed by deep sequencing

To investigate the mutational profiles of tumor-related genes in the *AID* Tg mouse whole pancreas, we extracted DNA from the pancreas of 1 *AID* Tg and 1 *WT* mouse at 13 months of age, and subjected them to deep sequencing on the selected amplicons of the tumor-related genes. We selected three representative tumor-related genes, including *Kras* and *Trp53* that are frequently mutated in human PDAC, and *c-Myc*, which was recently reported to play a significant role in the progression and maintenance of PDAC (34). An overall mean coverage depth of 10,975 was achieved for each nucleotide site. An overall mean coverage depth of 4,950, 15,300, and 12,595 was achieved for each nucleotide site in *Kras*, *Trp53*, and *c-Myc*,

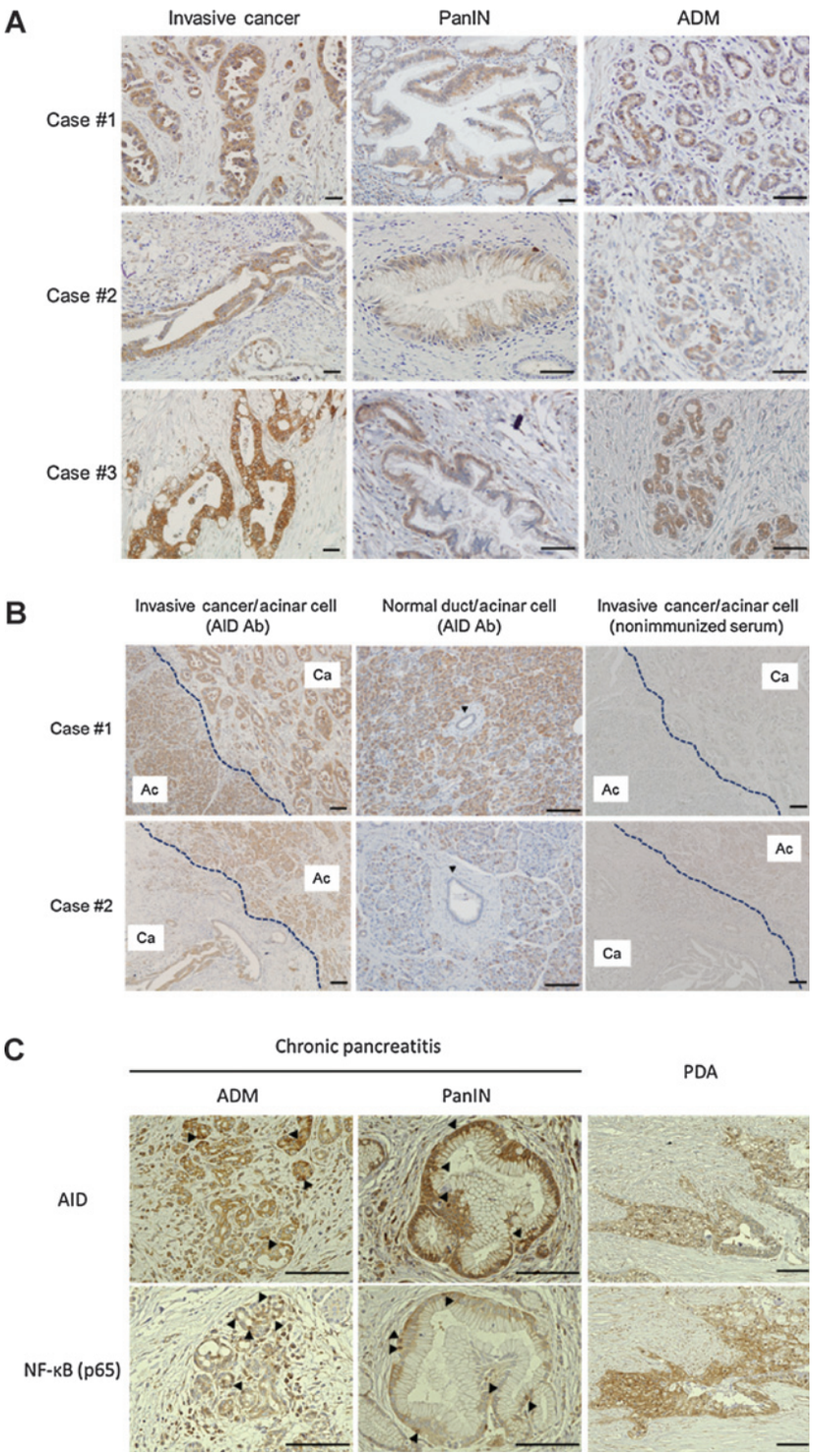

Figure 1.
Representative images of immunohistochemistry for AID in ADM, PanIN, and PDAC (invasive cancer). A, the majority of the invasive cancers strongly expressed AID protein. On the other hand, human ADM and PanIN lesions only weakly expressed AID protein (scale bars, 50 μm). B, several PDAC specimens showed AID protein expression in acinar cells adjacent to invasive cancer (Ac, acinar cell; Ca, invasive cancer; left). On the other hand, normal ducts showed no AID protein expression (middle, arrowhead). Right, negative control with nonimmunized serum (scale bars, 100 μm). C, AID protein expression was detected in ADM and PanIN lesions in human chronic pancreatitis as well as PDAC tissues (top, arrowheads). Immunohistochemistry of serial sections confirmed that these AID protein expressions were accompanied by p65 nuclear translocation, suggesting NFκB activation (bottom, arrowheads; scale bars, 100 μm).

respectively. Deep sequencing of the selected genes identified that *Kras* and *c-Myc* were mutated in *AID* Tg, but not in *WT* mouse pancreas. We found three and four point mutations in *Kras* and *c-Myc*, respectively (Table 4). The positions of the *Kras* mutations were codon 10 (c.30A>G, p.G10G), codon 20 (c.60G>A, pT20T), and c.37+20C>T. On the other hand, deep sequencing revealed that *Trp53* was not mutated in *AID* Tg or *WT* mouse pancreas (Table 4).

Recent reports led us to speculate on the molecular process underlying the mutation induction by analyzing the

Table 2. Semiquantitation of AID immunoreactivity in human ADM, PanIN, and PDAC

Accompanied lesion, *n*		AID immunoreactivity, *n*			Frequency of +[a]	Frequency of ++[a]
		−	+	++		
ADM	9	2	7	0	7/9	0/9
PanIN-1A/1B	9	4	5	0	5/9	0/9
PanIN-2/3	6	0	6	0	6/6	0/6
Invasive cancer	20	0	3	17	3/20	17/20

NOTE: Accompanied lesion indicates the number of cases with each lesion in PDAC specimens ($n = 20$). Number of cases with different AID immunoreactivity is shown. −, negative; +, weak positive; ++, strong positive.

[a]Number of cases with + or ++ AID immunoreactivity/number of cases analyzed.

pattern of nucleotide alterations (35–38). Among the cytidine deaminase family proteins, AID shows a strong preference for deaminating C residues flanked by a 5′-purine (G or A; refs. 38–40). In contrast, APOBEC3 family enzymes and APOBEC1 favor C residues flanked by 5′-T (41, 42). Therefore, we investigated the pattern of nucleotide alterations in the *AID* Tg mouse pancreas. Of a total of seven point mutations, three mutations (43%) were C:G>T:A transitions. In addition, all three C:G>T:A transitions were in the context of GpCpX, showing the typical mutational footprint of AID (Table 5).

To further elucidate the global mutational profile of *AID* Tg mouse pancreas, we performed whole-exome sequencing for DNA from pancreatic tissues of *AID* Tg and *WT* mice (Supplementary Materials and Methods). We targeted approximately 20,000 genes, sequenced 4.93 and 4.39 Gbp, and achieved 99.19- and 88.51-fold coverage in *AID* Tg and *WT* mouse, respectively (Supplementary Table S3). According to the variant filtering process (Supplementary Fig. S2A), we identified 117 somatic mutations in 42 genes with 37% of C:G>T:A transitions in *AID* Tg pancreatic tissue, indicating broad spectrum of genotoxic effect of AID on entire genome (Supplementary Fig. S2B; Supplementary Table S4).

Mutations of tumor-related genes in precancerous lesions of *AID* Tg mouse pancreas

Deep sequencing revealed *Kras* and *c-Myc* mutations in the *AID* Tg mouse pancreas, and thus we regarded these two genes as candidate target genes for AID. To clarify the distribution of mutations in the pancreas of the *AID* Tg mouse, we performed Sanger sequencing for each lesion. We analyzed 1 *AID* Tg mouse that developed pancreatic precancerous lesions (ADM and PanIN) and 1 *WT* mouse at 13 months of age. Mutations of *Kras*, *c-Myc*, and *Smad4* were assessed in the normal pancreatic parenchyma of the *WT* mouse, normal pancreatic parenchyma without precancerous lesions of the *AID* Tg mouse, and precancerous lesions (ADM and PanIN) of the *AID* Tg mouse that were separately isolated by laser capture microdissection, followed by subcloning and capillary sequencing. We collected 30 ADMs and 5 PanINs from two or three adjacent slides by laser capture microdissection.

The total number of amplified clones and DNA sequence reads, and the frequency of nucleotide alterations detected in each site or lesion from the *WT* and *AID* Tg mice are shown in Table 6. *Kras* and *c-Myc* mutations were found in ADM and PanIN of the *AID* Tg mouse, whereas *Smad4* mutations were found in normal pancreatic parenchyma as well as ADM and PanIN of the *AID* Tg pancreas. On the other hand, none of these mutations was detected in the normal pancreatic parenchyma of the *WT* mouse. These findings suggest that mutations of tumor-related genes detected by deep sequencing in the whole pancreas of the *AID* Tg mouse originated mainly from precancerous lesions, including ADM and PanIN.

The mutations and predicted amino acid changes in *Kras*, *c-Myc*, and *Smad4* detected in precancerous lesions of the *AID* Tg mouse pancreas are shown in Table 6. Of the 9 point mutations detected in *Kras*, *c-Myc*, and *Smad4*, two (22%) were C:G>T:A transitions in the context of GpCpX or ApCpX, a typical mutation pattern induced by AID (Table 6). These results indicate that the deamination induced by AID is involved in the mutational signature of pancreatic tumorigenesis in mice.

Discussion

Although it is well established that an accumulation of genetic abnormalities plays a crucial role in the development of PDAC, the mechanisms underlying the induction of tumor-related gene mutations has remained unexplained. A role for the AID/APOBEC family members in carcinogenesis was recently proposed (35–37). Given that more than half of the mutations in PDAC are C:G>T:A substitutions (27), a typical AID/APOBEC mutation pattern, we explored the possible contribution of AID to the mutagenesis underlying the development of PDAC. In the current study, we found substantial AID expression, not only in human PDAC, but also in precancerous lesions. Furthermore, we detected the accumulation of multiple mutations in tumor-related genes (*Kras*, *c-Myc*, and *Smad4*) in association with the development of pancreatic precancerous lesions in *AID* Tg mice.

Furthermore, we confirmed that the AID protein was expressed in human pancreatic precancerous lesions (ADM and PanIN) as well as in invasive cancer, whereas no AID expression was detected in normal pancreatic ducts. This AID expression in human pancreatic cancer was also supported by RNAseq datasets derived from The Cancer Genome Atlas (Supplementary Fig. S3; ref. 43). These findings suggest that AID is involved in the initiation of pancreatic cancer development. Our immunohistochemistry also suggests that these AID expressions in human precancerous and PDAC lesions could be induced by some inflammatory stimulus through NFκB activation as previously shown in other organs (19–23). Inflammation-induced AID expression in pancreatic epithelial cells was further demonstrated by our preliminary data using mouse pancreatitis model (data not shown). Interestingly, we also found AID expression in some acinar cells located close to invasive cancers in several PDAC samples. Based on findings in recent mouse models that ADM/PanIN arise from acinar cells

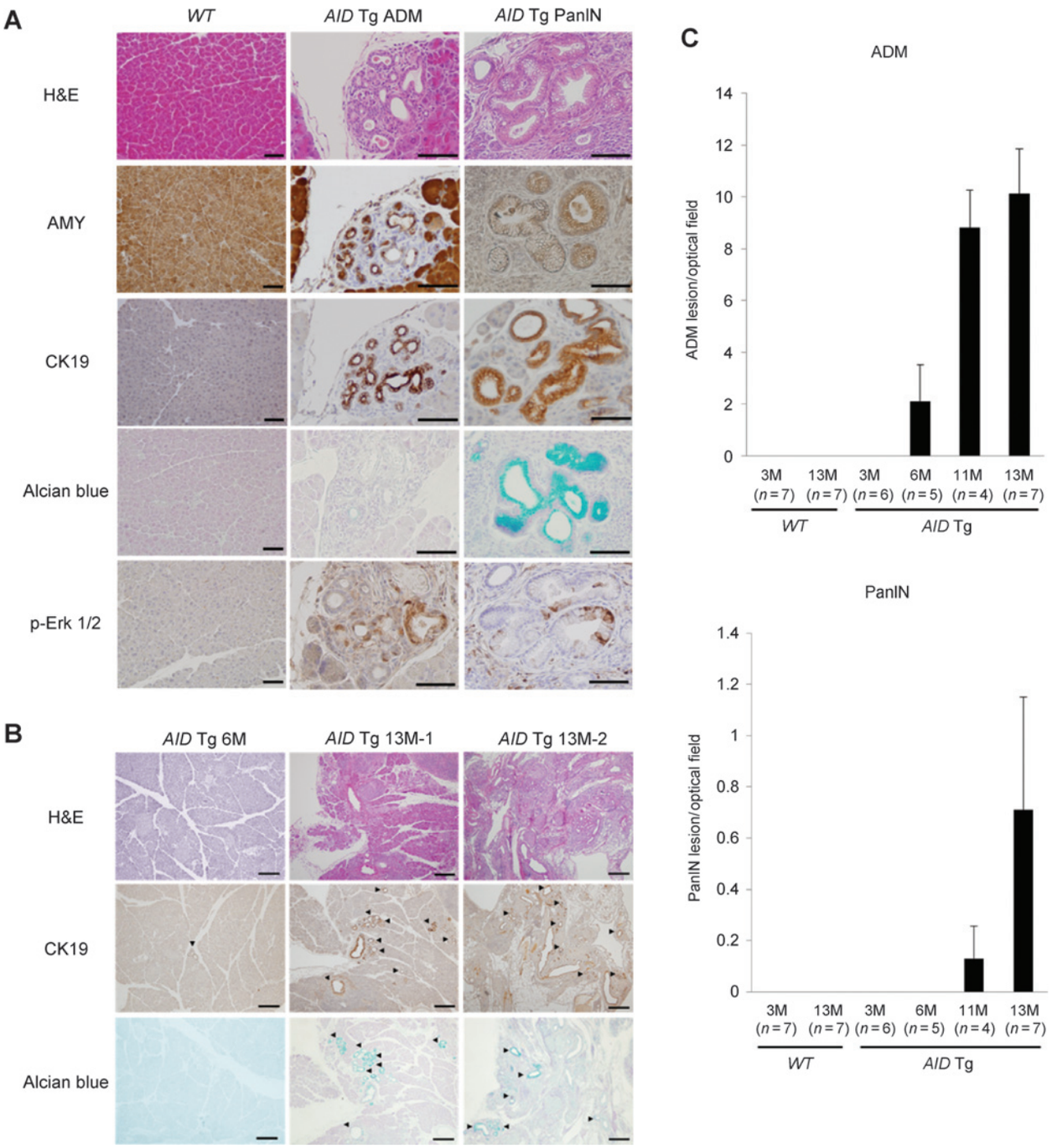

Figure 2.
ADM and PanIN lesions developed in *AID* Tg mice. A, immunohistochemical analysis of ADM (middle panels) and PanIN (right) lesions that developed in *AID* Tg mice and acinar cells in *WT* mice (left) at 13 months of age. ADM expressed amylase (acinar marker) and CK19 (ductal marker). PanIN lesions expressed CK19 and weakly expressed amylase. Alcian blue staining showed acidic mucin content in PanIN lesions, but not in ADM. ADM and PanIN lesions also expressed p-Erk1/2 (AMY, amylase; CK19, cytokeratin 19; p-Erk1/2, phospho-Erk1/2; scale bars, 100 μm). B, *AID* Tg mice developed a few ADM lesions (CK19, positive; Alcian blue staining, negative) at 6 months of age (arrowhead, left). On the other hand, *AID* Tg mice developed many PanIN lesions (CK19, positive; Alcian blue staining, positive) at 13 months of age (arrowhead in the middle and on the right; M, months of age; scale bars, 200 μm). C, *AID* Tg mice developed ADM and PanIN lesions by 6 and 11 months of age, respectively. The frequencies of these lesions increased with age. On the other hand, *WT* mice developed no precancerous lesions (*n*, number of mice). Data, mean SEM of the number of ADM or PanIN lesions per optical field.

Table 3. Frequency of ADM and PanIN in *AID* Tg mice at each age

	WT		*AID* Tg			
Type of lesion	**3 mo**	**13 mo**	**3 mo**	**6 mo**	**11 mo**	**13 mo**
ADM	0/7 (0)	0/7 (0)	0/6 (0)	2/5 (40)	4/4 (100)	7/7 (100)
PanIN	0/7 (0)	0/7 (0)	0/6 (0)	0/5 (0)	1/4 (25)	4/7 (57)

NOTE: Number of mice that developed ADM or PanIN lesion/total mice is shown. Values in parentheses indicate percentage.
Abbreviation: mo, months of age.

(32, 44), AID expression in acinar cells might have a role in the development of pancreatic precancerous lesions. In addition, the AID expression level and frequency increased with the progression from low-grade PanIN (PanIN-1A/1B) to high-grade PanIN (PanIN-2/3) and subsequent invasive cancer, suggesting the possible contribution of AID to both the initiation and progression of human pancreatic cancer.

We previously demonstrated the development of tumors in the liver, stomach, and lung of *AID* Tg mice (30), revealing important roles of AID in the development of various epithelial tumors. In the current study, to further assess the potential role of AID in pancreatic tumorigenesis, we examined the pancreatic phenotype of *AID* Tg mice, and found that precancerous lesions developed in a considerable number of animals. Indeed, *AID* Tg mice developed ADMs and PanINs at 6 and 11 months of age, respectively. The development of PanINs coincided with, or was preceded by, ADMs, and the number of these lesions increased with age. These findings were also observed in the conventional PanIN/PDAC mouse model induced by mutant *Kras* (10), and mimic the pathologic findings of human pancreatic precancerous lesions that PanINs are frequently associated with ADM (14, 15). Thus, the *AID* Tg mouse pancreas appears to recapitulate the ADM-to-PanIN sequence, suggesting the contribution of AID to the initiation of PDAC. Although we observed the development of ADM and PanIN, we detected no PDAC development in the *AID* Tg mice. This could be due to an insufficient observation period. Almost all *AID* Tg mice develop lethal lymphoma and die at 12 to 13 months of age (24), and thus we could not observe the phenotypes of *AID* Tg mice beyond 13 months of age. Further studies to analyze the generation of pancreas-specific *AID* transgenic mice are required.

The *KRAS* gene mutation is thought to play an essential role in the initiation of PDAC. Indeed, *KRAS* mutations are frequently detected, even in low-grade human PanIN (9). This concept was further confirmed by a mouse model in which pancreas-specific induction of mutant *Kras* ($Kras^{G12D}$) led to the development of PanIN and even PDAC (10). A recent study utilizing a more sensitive method, however, demonstrated various gene mutations in addition to *KRAS* in human low-grade PanIN, suggesting that genes other than *KRAS* are involved in the initiation of PDAC (9). In this regard, our *AID* Tg mouse model appears to be a unique and powerful tool for analyzing the genes involved in PDAC initiation because it spontaneously develops ADM and PanIN without the intentional manipulation of specific tumor-related genes. Among the representative tumor-related genes analyzed by deep sequencing in the current study, *Kras* and *c-Myc* were frequently mutated in the *AID* Tg mouse pancreas but not in the *WT* mouse. This observation suggests that AID induces mutations in tumor-related genes closely related to human pancreatic carcinogenesis. On the other hand, *Trp53* mutation, which is known to be induced by AID in gastric cancer, was not found in the *AID* Tg mouse pancreas. This organ specificity of AID-targeted genes may be dependent on the organ-specific gene transcriptions, as previously reported (30).

We found several *Kras* gene mutations in the *AID* Tg mouse pancreas, including 5′-UTR (c.-4G>A), codon 6 (c.11C>A, p.L6I), codon 8 (c.24G>T, p.V8V), codon 10 (c.30A>G, p.G10G), and c.37+20C>T. Nevertheless, we found no mutations consistent with the hot spots of the *Kras* mutation in human PDAC (codons 12, 13, and 61). Among the *Kras* mutations, mutations in codon 10 are reported in human and mouse colorectal cancer (45–47) and activate the Raf–MEK–ERK pathway (48). Given the moderate level of phospho-Erk1/2 expression in ADM and PanIN of *AID* Tg mice, suggesting activation of the canonical MAPK cascade, *Kras* mutations might be drivers for the formation of precancerous lesions. As for *c-Myc* gene, all the mutations found in the *AID* Tg mouse pancreas were nonsynonymous. Moreover, all these mutant c-Myc preoteins were predicted by Sorting Tolerant From Intolerant (SIFT) 5.2.2 algorithm (49) to have altered function suggesting possible involvement in the formation of precancerous lesions. Further experiments are required, however, to determine whether these mutations found in *AID* Tg mouse pancreas are pathogenic.

Table 4. Mutations in tumor-related genes in the whole pancreas of *AID* Tg and *WT* mice determined by deep sequencing

Gene	**Mouse**	**Mutated nucleotide positions**	**Total number of mutated nucleotides**	**Total bp sequenced**	**Mean coverage**
Kras	*WT*	0	0	2077600	5300
	AID Tg	3	27	1803200	4600
Trp53	*WT*	0	0	3880000	10000
	AID Tg	0	0	7992800	20600
c-Myc	*WT*	0	0	5165670	12630
	AID Tg	4	51	5137040	12560

NOTE: Deep sequencing revealed *Kras* and c-*Myc* mutations in the *AID* Tg mouse.

Table 5. The mutational signature in *AID* Tg mouse pancreas

Gene	**Mutation signature (*n*)**	**GpCpX context of C:G>T:A transitions**
Kras	C:G>T:A (2)	2/2[a]
	T:A>C:G (1)	NA
c-Myc	T:A>C:G (2)	NA
	C:G>T:A (1)	1/1[a]
	A:T>C:G (1)	NA

NOTE: The mutational signature in *AID* Tg mouse pancreas included C:G>T:A transitions in the context of GpCpX.
Abbreviations: NA, not applicable.
[a]GpCpX/C:G>T:A transitions.

Table 6. Mutations in tumor-related genes in pancreatic lesions of the *AID* Tg mouse

Gene	Pancreatic lesion		Nucleotide change	Amino acid change	Mutated/total clones	Mutation frequencies (/10^4)	Sequence context of C:G>T:A
Kras	*WT*	Normal[a]	(−)	(−)	0/76	0	(−)
	AID Tg	Normal[a]	(−)	(−)	0/93	0	(−)
		ADM	c.−4G>A	(−)[b]	1/96	0.90	ApCpX
			c.11C>A	p.L6 I	1/96	0.90	(−)
		PanIN	c.24G>T	p.V8V	2/94	1.83	(−)
c-Myc	*WT*	Normal[a]	(−)	(−)	0/30	0	(−)
	AID Tg	Normal[a]	(−)	(−)	0/29	0	(−)
		ADM	c.1092C>T	p.R157C	1/42	0.79	GpCpX
		PanIN	(−)	(−)	0/48	0	(−)
Smad4	*WT*	Normal[a]	(−)	(−)	0/48	0	(−)
	AID Tg	Normal[a]	c.1479G>C	p.V347L	1/47	1.51	(−)
			c.1488G>T	p.D350Y	1/47	1.51	(−)
		ADM	c.1457G>T	p.K339N	1/48	1.48	(−)
			c.1492G>T	p.G351V	1/48	1.48	(−)
		PanIN	c.1545G>T	p.V369F	1/46	1.54	(−)

NOTE: Mutation frequencies were calculated per the total bases analyzed $\times 10^{-4}$.
[a]Normal pancreatic parenchyma without precancerous lesions.
[b]Amino acids not expressed because of UTR.

Recent studies demonstrated that the mutation signature that accumulates in tumor tissues provides a key to identifying the cause of mutations during tumorigenesis (25, 37). In this regard, the mutation signature of AID is characterized by C:G>T:A alterations that occur in the GpCpX or ApCpX sequences (38–40, 50–52). In our deep sequence analysis of the *AID* Tg mouse, C:G>T:A transitions in the context of GpCpX were detected in 3 of 7 (43%) mutations, supporting the notion that mutations in tumor-related genes found in *AID* Tg mouse pancreas are induced by AID. More importantly, microdissection and subsequent Sanger sequence analysis detected *Kras* and *c-Myc* mutations with the characteristic AID signature exclusively in precancerous lesions of *AID* Tg mice. These results further support the notion that AID is involved in the induction of mutations during the development of pancreatic precancerous lesions in *AID* Tg mice. In contrast to our *AID* Tg mice, the characteristic AID signature is not evident in human pancreatic cancer in the previous global analyses (25). One explanation for this discrepancy may be the involvement of DNA error-prone polymerases or mismatch repair proteins that can produce any type of mutations following AID-induced cytidine deamination (53). Indeed, in chronic lymphocytic leukemia and malignant B-cell lymphomas, T>G transversions at ApTpN and TpTpN trinucleotides are shown to be induced by an error-prone polymerase η following AID-induced cytidine deamination (54). Considering the involvement of various DNA error-prone polymerases or mismatch repair proteins in human pancreatic cancer (55), it is likely that these factors affect the mutational signature in human pancreatic cancer.

In summary, we detected aberrant AID expression in PDAC as well as in human precancerous lesions. We also demonstrated the induction of tumor-related gene mutations and the development of precancerous lesions in the *AID* Tg mouse pancreas. These findings suggest that AID is involved in cancer initiation through the induction of tumor-related gene mutations in the pancreas. Further studies are required to clarify the mechanism of AID-induced gene mutations in the pancreas and the actual target genes that lead to the development of PDAC.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): Y. Sawai, Y. Kodama, A. Kurita, T. Masui
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): Y. Sawai, Y. Kodama, T. Shimizu, Y. Eso, A. Kurita, H. Marusawa
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