Low Incidence of High-Grade Pancreatic Intraepithelial Neoplasia Lesions in a Crmp4 Gene–Deficient Mouse Model of Pancreatic Cancer

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A R T I C L E   I N F O

Article history:
Received 26 September 2019
Received in revised form 30 January 2020
Accepted 30 January 2020
Available online xxxx

A B S T R A C T

Pancreatic intraepithelial neoplasia (PanIN), the most common premalignant lesion of the pancreas, is a histologically well-defined precursor to invasive pancreatic ductal adenocarcinoma (PDAC). However, the molecular mechanisms underlying the progression of PanINs have not been fully elucidated. Previously, we demonstrated that the expression of collapsin response mediator protein 4 (CRMP4) in PDAC was associated with poor prognosis. The expression of CRMP4 was also augmented in a pancreatitis mouse model. However, the role of CRMP4 in the progression of PanIN lesions remains unclear. In the present study, we examined the relationship between CRMP4 expression and progression of PanIN lesions using genetically engineered mouse models. PanIN lesions were induced by peritoneal injection of the cholecystokinin analog caerulein in LSL-KrasG12D; Pdx1-Cre (KC-Crmp4 wild-type, WT) mice and LSL-KrasG12D; Pdx1-Cre; Crmp4+/− (KC-Crmp4 knockout, KO) mice. We analyzed pancreatic tissue sections from these mice and evaluated PanIN grade by hematoxylin and eosin staining. CRMP4 expression was examined and the cellular components assessed by immunohistochemistry using antibodies against CRMP4, CD3, and α-smooth muscle actin (SMA). The incidence of high-grade PanIN in KC-Crmp4 WT mice was higher than that in KC-Crmp4 KO animals. CRMP4 expression was not only in epithelial cells but also in α-SMA-positive cells in stromal areas of PanIN lesions. The CRMP4 expression in stromal areas correlated with PanIN grade in WT mice. These results suggested that the expression of CRMP4 in stromal cells may underlie the incidence or progression of PanIN.

Introduction

Pancreatic ductal adenocarcinoma (PDAC) constitutes a leading cause of cancer death [1,2], primarily owing to the lack of effective early detection methods and poor efficacy of existing therapies. Moreover, even among the 10% to 20% of patients who received a diagnosis of surgically resectable PDAC, most ultimately die of recurrent and metastatic disease [3]. These low survival rates are attributed in part to the fact that PDAC metastases have often progressed to the point where surgical removal cannot provide a cure. In order to improve the cancer mortality, detection and treatment in the early phase are necessary. Toward this end, analyses of PDAC pathological specimens and of genetically engineered PDAC mouse models have suggested that PDAC develops from pancreatic intraepithelial neoplasia (PanIN) [4,5].

PanIN represents the most common pancreatic precursor lesion. An activating K-ras point mutation is almost uniformly present in early stage PanIN, whereas subsequent inactivating mutations in p16, p53, and Smad4 occur in advanced lesions [6–9]. The development of genetically engineered mouse models with PDAC, such as Pdx1-Cre; LSL-KrasG12D [10], Pdx1-Cre; LSL-KrasG12D; Ink4a/Arflox/lox [11], and Pdx1-Cre; LSL-KrasG12D; LSL-p53R172H [7], has facilitated our understanding of the molecular mechanisms of pancreatic neoplasia [10,12].

Recently, collapsin response mediator proteins (CRMPs), also known as the dihydropyrimidinidase-like protein (DPYSL) family, have been shown to be involved in malignant tumors [13–20]. Altered expression of different CRMPs has been observed in various malignant tumors including lung, breast, colorectal, prostate, liver, gastric, pancreatic, and neuroendocrine lung cancer [21]. CRMPs were originally identified as the intracellular signaling mediators of a repulsive axon guidance molecule, semaphorin-3A (Sema3A) [22]. The CRMP family consists of five members, CRMP1-5 [23,24], which are highly expressed in the developing and adult nervous system. CRMPs are involved in axon guidance, axonal elongation, cell migration, synapse maturation, and the generation of neural polarity.
In these developmental processes, CRMPs play critical role in regulating cytoskeletal rearrangement, which is largely mediated by their phosphorylation-dependent interaction with F-actin and microtubules [27,28]. CRMPs can be phosphorylated by various kinases including Cdk5, GSK3β, Rho-kinase, and Fyn [29–34]. In addition, CRMPs have also been implicated in a variety of cellular and molecular events such as inflammation and cell growth in peripheral tissues or organs as well as in the central nervous system [35–37].

Similar to other CRMP family proteins, CRMP4 is highly expressed in the central nervous system. CRMP4 expression is also observed in malignant tumors originated from various organs including the intestine, liver, pancreas, and prostate [14,38–45]. However, the roles of CRMP4 in tumorigenesis or tumor progression remain unknown. Overexpression of CRMP4 suppresses the invasion ability and inhibits tumor metastasis of prostate cancer cells [14]. Consistent with this, lower expression of CRMP4 mRNA in hepatocellular carcinoma tissues is associated with shorter recurrence-free survival and subsequent adverse prognosis [42]. In contrast, high expression levels of CRMP4 mRNA in gastric cancers are significantly associated with shortened recurrence-free survival [39]. Previously, we reported that CRMP4 expression is associated with poor prognosis through the promotion of liver metastasis of pancreatic cancer [43]. In addition, CRMP4 knockdown using siRNA reduces the cellular invasion of Capan-1 cells, a human pancreas adenocarcinoma cell line. CRMP4 expression was also found to be enhanced in the pancreatic parenchyma and the infiltrated lymphocytes in pancreatic tissue of a pancreatitis mouse model [46]. In turn, a meta-analysis of chronic pancreatitis has shown a relative risk of 13.3 for developing malignancy [47], and chronic pancreatitis is considered to have a strong relationship with carcinogenesis and pancreatic cancer [48]. Together, these findings suggest that CRMP4 is involved in the pathogenesis of pancreatic cancer, although direct causality has not been demonstrated. To further clarify the role of CRMP4 in pancreatic carcinogenesis, in this study, we examined the role of CRMP4 in the progression of PanIN in a genetically engineered mouse model of pancreatic cancer.

Materials and Methods

Ethics Statement

All animal procedures were performed according to the Guide for the Care and Use of Laboratory Animals (Japanese Association for the Laboratory Animal Science) and the Guide for Yokohama City University. Specific approval for the mouse experiments was obtained from the Institutional Animal Care and Use Committee of Yokohama City University School of Medicine with the protocols F-A-15-042 and F-A-16-019 “Biological function of CRMP4 in metastasis and invasion with pancreatic cancer model mice.” All surgical procedures were performed under isoflurane (Pfizer, New York, NY) and pentobarbital sodium (Kyoritsu seiyaku, Tokyo, Japan) anesthesia, and all efforts were made to minimize the number of animals used and their suffering.

Reagents

Antibodies purchased were as follows: polyclonal rabbit anti-CRMP4 (AB5454, Merck Millipore, Darmstadt, Hessen, Germany), monoclonal rabbit anti-CD3 (ab5690, Abcam, Cambridge, Cambridgeshire, UK), and monoclonal mouse anti-α-smooth muscle actin (SMA) (14-9760, eBioscience, San Diego, CA). Alexa Fluor 488 Goat Anti-rabbit-IgG and Alexa Fluor 594 Goat Anti-mouse-IgG antibodies were purchased from Life Technologies (Carlsbad, CA).

Purification of Recombinant CRMP4 Peptide

GST-fused CRMP4 fragment was expressed in Escherichia coli BL21 strain [35], and CRMP4 was affinity-purified using glutathione-resin following digestion with PreScission protease (GE Healthcare, Little Chalfont, Buckinghamshire, UK) [35].

Animals

Wild-type (WT) C57BL/6JInsSlc male mice were purchased from Japan SLC (Hamamatsu, Shizuoka, Japan). All mice were maintained at the animal care facility of Yokohama City University under a 12-hour light/12-hour dark cycle at 23°C ± 1°C, with free access to water and food. All mice were fed commercially available MF feed (Oriental Yeast Kogyo Co., Tokyo, Japan).

We used a genetic strategy to selectively knock-in the K-ras mutation in the pancreas. Using the Cre/loxP system, we crossed LSL-KRAS<sup>G12D</sup> mice and Pdx1-Cre mice to generate LSL-KRAS<sup>G12D</sup>; Pdx1-Cre (KC-CrmP4 WT) mice, which exhibit conditional K-ras mutation in the pancreas [10,49]. Crmp4<sup>−/−</sup> mice were established as previously described (Acc. No. CD0637K) and maintained on a 129/SV X C57BL/6J hybrid background [50–52]. The Pdx1-Cre transgenic mouse strain, LSL-KRAS<sup>G12D</sup> knock-in mouse strain, and Crmp4<sup>−/−</sup> mouse strain were intercrossed. LSL-KRAS<sup>G12D</sup> knock-in and Crmp4<sup>−/−</sup> mice were interbred to generate Pdx1-Cre; LSL-KRAS<sup>G12D</sup>; Crmp4<sup>−/−</sup> (KC-CrmP4 KO) mice (Figure 1, A and B).

Genotyping of Animals

Genotyping of the CRMP4 allele was performed by polymerase chain reaction (PCR) using the following primers: Crmp4<sup>+</sup> fourth intron Rv, 5′-CAC TGG CCT GGC TGA AGA TCA A-3′; Crmp4<sup>−</sup> WT Fw, 5′-GTC AAG CTG CTA AAG GAG CCT-3′; and CDB-Neo Fw, 5′-GCC GAG GAT CTC GTC GTG ACC-3′. The PCR was performed with 30 cycles of 95°C for 30 seconds, 62°C for 30 seconds, and 72°C for 1 minute to obtain a 790-bp mutant allele and a 427-bp WT allele.

Genotyping of the LSL-Kras<sup>G12D</sup> allele was performed by PCR using the following primers: Kras Y116-common, 5′-TCC GAA TTT AGT GAC TAC AGA TG-3′; Crmp4<sup>−</sup> WT Fw, 5′-GTC AAG CTG CTA AAG GAG GAT CTC GTC GTG ACC-3′. The PCR was performed with 35 cycles of 95°C for 30 seconds, 60°C for 30 seconds, and 72°C for 30 seconds. The expected product size of Y117/Y116 is 327 bp for LSL, and that of Y116/Y118 is 450 bp for WT. Genotyping of the Pdx1-Cre allele was performed by PCR using the following primers: Pdx1-Cre Rv, 5′-GGT GTA CCG TCA GTA AAT TTG-3′ and Pdx1-Cre Fw, 5′-CTG GAC TAC ATG CTC GTC GTG ACC-3′. The PCR was performed with 35 cycles of 95°C for 30 seconds, 60°C for 30 seconds, and 72°C for 30 seconds to obtain a 650-bp product size.

Acute Pancreatitis-Induced PanIN Lesions in Conditional K-ras Mutant Mice

In order to investigate the role of CRMP4 in PanIN development, acute pancreatitis was induced in KC mice [53] by treatment with the cholecytokinin analog caerulein (C9026, Sigma-Aldrich, St. Louis, MO) [54]. Working aliquots (1 ml) at 100 μg/ml were stored at −20°C until use, at which time it was dissolved in phosphate buffered saline (PBS), pH 7.4, at a concentration of 10 μg/ml. Acute pancreatitis was induced by seven hourly intraperitoneal injections of caerulein (50 μg/kg) at the age of 7-10 weeks, which were repeated 48 hours later [53]. PanIN lesions developed with 3-4 months. The pancreas was removed at day 73-216 from the last injection. Subsequent to euthanasia with an excessive dose of isoflurane and pentobarbital, mice were perfusion-fixed with 4% (wt/vol) paraformaldehyde in PBS. To obtain macroscopic and microscopic findings of both KC-CrmP4

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WT mice and KC-Crm4 KO mice, the pancreas was removed together with the spleen and duodenum. Collected pancreases were fixed in 4% paraformaldehyde in PBS for 12 hours at 4°C.

Figure 1. Experimental design. (A) Targeting endogenous KrasG12D expression to the mouse pancreas. Pdx1-Cre allele crossed to LSL-KrasG12D allele. (B) Genetic makeup of the KC-Crm4 KO mouse. LSL-KrasG12D; Pdx1-Cre (KC-Crm4 WT) mice were crossed with Crmp4−/− mice. (C) Treatment protocol for caerulein in LSL-KRASG12D; Pdx1-Cre (KC-Crm4 WT) (n = 19) and LSL-KRASG12D; Pdx1-Cre, Crmp4−/− (KC-Crm4 KO) mice (n = 11).

For immunohistological evaluation of CRMP4, two investigators, I. K. and K. Y., independently assessed the stained sections. These investigators were blinded to the sources of sections. The intensity of cytoplasmic immunoreactivity was scored as follows: 0, no staining; 1, mild; 2, moderate; and 3, strong. In statistical analyses, scores 0 and 1 were defined as CRMP4 negative, whereas 2 and 3 were defined as CRMP4 positive. For measurement of colocalization between CRMP4, and α-SMA, we quantitated the number of CRMP4-, α-SMA-, and CD3-positive cells using serial sections, respectively (n = 3).

**Immunohistochemistry**

Paraffin-embedded pancreas sections (3 μm thick) were deparaffinized in xylene and rehydrated sequentially in ethanol. Slides were washed in deionized water, stained with hematoxylin (131-09665, Wako, Osaka, Japan) and eosin (051-06515, Wako) in 60% ethanol, then dehydrated sequentially in ethanol, cleared with xylenes, and mounted with Entellan new (Merck KGaA, Darmstadt, Hessen, Germany). All slides were analyzed by a pathologist (L.K.) for findings of PanIN development. The pathologist was blinded to the tissue genotype. Each slide was classified as normal acinar/ductal cells; acinar-toductal metaplasia; PanIN-1A, -1B, -2, or -3; or PDAC based on the classification consensus [55].

**Immunofluorescence Staining**

The sections were incubated with primary antibody in 2% normal goat serum at 4°C overnight. The Vector ABC-HRP system kit was employed for signal amplification. The proteins were visualized using 3,3′-diaminobenzidine (ImmPACT DAB; K-6100, Vector Laboratories) staining. Finally, slides were counterstained with hematoxylin, dehydrated sequentially in ethanol, cleared with xylenes, and mounted with Entellan new (Merck KGaA, Darmstadt, Hessen, Germany). All slides were analyzed by a pathologist (L.K.) for findings of PanIN development. The pathologist was blinded to the tissue genotype. Each slide was classified as normal acinar/ductal cells; acinar-toductal metaplasia; PanIN-1A, -1B, -2, or -3; or PDAC based on the classification consensus [55]. Brieﬂy, PanIN-1 constitutes flat (1A) or papillary (1B) lesions of the columnar epithelium with basally oriented, round nuclei. PanIN-2 represents papillary lesions with nuclear hyperchromasia, crowding, and pseudostratiﬁcation. PanIN-3 indicates papillary, micropapillary, or cribiform lesions with nuclear pleomorphism, frequent loss of nuclear polarity, and mitoses [56,57]. The tissue samples were evaluated at high optical power (objective, 40) for all PanIN lesions in a randomly selected microscopic field. PanINs grade were presented as 0, no staining; 1, mild; 2, moderate; and 3, strong. In statistical analyses, scores 0 and 1 were deﬁned as CRMP4 negative, whereas 2 and 3 were deﬁned as CRMP4 positive. For measurement of colocalization between CRMP4, and α-SMA or CD3, we quantitated the number of CRMP4−, α-SMA−, and CD3−positive cells using serial sections, respectively (n = 3).

**Immunofluorescence Staining**

The sections were incubated with primary antibody in 2% NGST at 4°C overnight, followed by incubation with Alexa Fluor-conjugated secondary antibodies. Primary antibodies used were polyclonal anti-CRMP4 (1:1000) and monoclonal anti-α-SMA (1:500). The samples were counterstained with...
DAPI (340-07971, Wako, Osaka, Japan), and examined and photographed with confocal microscope (Fluo View FV1000, Olympus, Tokyo, Japan).

**Immunoblot Analysis**

After euthanization, the brains were immediately removed from Crmp4+/- and Crmp4+/− mice and homogenized in immunoprecipitation buffer: 20 mM Tris-HCl (pH 8.0), 150 mM NaCl, 5 mM EDTA, 1 mM NaF, 0.5 mM Na3VO4, 1% Triton X-100, and 2% Protease Inhibitor Cocktail (P8340; Sigma-Aldrich). The lysates were centrifuged at 20,400 g for 5 minutes at 4°C, and supernatants were normalized for total protein concentration. Equal amounts of total protein were separated by 8% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to a nylon membrane using Ez Fastblot (#2332590, ATTO, Tokyo, Japan). After being blocked with TTBS buffer (25 mM Tris HCl pH 7.4, 137 mM NaCl, 2.7 mM KCl, and 0.2% Tween-20) supplemented with 5% skim milk, the membrane was incubated with primary antibody: anti-CRMP4 antibody (1:2000) diluted in 10% bovine serum albumin at 37°C for 30 minutes. Following 2% skim milk wash, the membrane was incubated with secondary antibody in signal enhancer HIKARI Solution B (#02267-41, Nacalai Tesque, Kyoto, Japan) at 37°C for 30 minutes. After reaction with Lumina Forte (AWBLUF0500, Millipore Merck KGa), an ImageQuant 400 (GE Healthcare) was used to detect the signals.

**Statistical Analyses**

Data are presented as the means ± standard deviation and were analyzed with the built-in Student’s t test using SPSS computer software package version 26.0 for Windows (SPSS Inc., Chicago, IL). P values of < .05 were considered significant. The relationship between the progression of PanIN grade and the expression of CRMP4 was analyzed using Fisher’s exact test in R software for Windows version 3.3.2 (R Foundation for Statistical Computing, Vienna, Austria). In all cases, a group size was chosen that produced statistically unambiguous results.

**Results**

**Low-Grade PanIN Progression in KC-Crmp4 KO Compared to KC-Crmp4 WT Mice**

Gross pathological findings included swelled pancreas with hard nodular lesions in KC-Crmp4 WT and KC-Crmp4 KO mice. No obvious difference was observed between WT and KO mice (Figure 2, A and B). Histological examination revealed pathological lesions in the pancreatic tissues of WT and KO mice that were similar to those in human PanIN (Figure 2, C and D). Notably, PanIN-2 lesions were mainly observed in KC-Crmp4 WT mice (Figure 2C, Table 1), which showed a papillary structure with pseudostratification and enlarged nuclei. Conversely, PanIN-1 lesions were mainly observed in KC-Crmp4 KO mice (Figure 2D, Table 1), which consisted of tall columnar cells with basally located nuclei and abundant supranuclear mucin.

Table 1 shows the grade of PanIN in both genotypes. High-grade PanIN lesions (PanIN-2 and -3) were observed more frequently in KC-Crmp4 WT (Table 1). The acinar-to-ductal metaplasia was similarly observed in both groups. The observation period was 139.2 ± 41.2 days for KC-Crmp4 WT and 126.0 ± 23.1 days for KC-Crmp4 KO mice (Student’s t test, P = .340).

**Increased Expression of CRMP4 in PanIN Lesions**

We first validated the sensitivity and specificity of the anti-CRMP4 antibody. Western blot analysis using brain lysate revealed that the band detected in Crmp4+/− samples was absent in Crmp4−/− (Figure 3A). In immunohistochemistry using pancreatic tissues, CRMP4 expression was detected in the ductal and acinar cells in Crmp4+/− but not in Crmp4 KO

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**Figure 2.** High-grade PanIN lesions in KC-Crmp4 WT mice. (A, C) Representative findings of PanIN lesions developed in KC-Crmp4 WT mice. (A) Gross appearance of PanIN-2 lesions. Scale bar, 1 cm. (C) Prominent papillary architecture and mild-to-moderate cytologic atypia including hyperchromasia, increased nuclear size, crowding, pseudostratification, and some loss of polarity are observed. The arrows indicate the representative feature of PanIN-2. Scale bar, 20 μm. (B, D) Representative PanIN lesions in KC-Crmp4 KO mice. (B) Gross appearance of PanIN-1 lesions. (D) Flat epithelial lesions consisting of tall columnar cells with basally located nuclei and abundant supranuclear mucin are observed. The arrowheads indicate representative feature of PanIN-1. Scale bar, 20 μm. KC-Crmp4 KO: LSL-KrasG12D; Pdx1-Cre; Crmp4−/−.
tissue (Figure 3, E-H). In WT mice, weak expression of CRMP4 was detected in some of the nuclei of the ductal and acinar cells (Figure 3F). CRMP4 expression was also detected in the cytoplasm of cells in PanIN lesions (Figure 3, G and H) and in the stroma surrounding PanIN lesions (Figure 3, G and H, black arrowheads).

Preabsorption of anti-CRMP4 antibody with antigen peptide reduced the immune signal (data not shown). These findings supported the specificity of the anti-CRMP4 antibody used.

**CRMP4-Positive Cells in Stromal Areas Coincide with αSMA-Positive Cells**

To examine the immunohistochemical features of the CRMP4-positive cells in the stromal areas, pancreatic tissues were stained with an antibody against αSMA, the most common marker of fibroblasts/myofibroblasts, and against CD3, a general marker of T cells. Serial sections of PanIN-2 lesions were stained using hematoxylin and eosin along with antibodies against CRMP4, CD3, and αSMA. CRMP4-positive spindle-shaped cells were observed in the stromal areas (Figure 4). The staining pattern of CRMP4 was similar to that of αSMA but not of CD3 (Figure 4, B and D, black arrowheads). Quantitative colocalization analysis was performed. The percentage of colocalization between CRMP4 and αSMA or CRMP4 and CD3 was 39.0% ± 1.95% or 13.7% ± 0.98%, respectively (n = 3, P = .003, Figure 5, A and B). Immunofluorescence staining of PanIN-2 lesion (Figure 6, C and D, yellow arrowhead) illustrated strong expression of CRMP4. Moreover, some stromal cells surrounding the PanIN lesions coexpressed CRMP4- and αSMA-positive cells (Figure 6, F, G and H, white arrowhead).

**Immunohistochemical Analysis of CRMP4 in PanIN Lesions and Stromal Areas**

To examine the relationship between expression of CRMP4 and the PanIN grade, we evaluated CRMP4 expression in the epithelial cells and stromal cells in PanIN lesions in WT mice. The expression levels of CRMP4 in the epithelial cells of PanIN lesions showed no correlation with PanIN development (Table 2, P = .588). In stromal cells, however, high-grade PanINs were observed more frequently in the CRMP4-positive group than in the CRMP4-negative group (Table 2, P = .023). These results indicated that the expression levels of CRMP4 in the stromal cells of PanIN lesions correlated with the progression of high-grade PanIN in the mouse model of pancreatic cancer. PanIN-1 was found in almost every tissue examined, while PanIN-2 or -3 was rarely seen.

**Discussion**

In this study, we demonstrated that PanIN progression was suppressed in KC-Crmp4 KO mice compared with KC-Crmp4 WT mice. CRMP4 expression was increased in both epithelial cells and the stromal areas of PanIN lesions, especially in αSMA-positive cells. These findings suggested that CRMP4 may participate in the development of PanIN. In addition, CRMP4 expression was observed in the epithelium of PanIN lesions as well as in normal acinar and ductal cells. This expression profile of CRMP4 in the pancreatic tissue of mice coincides with that in human surgical specimens of PDAC [43]. Whether CRMP4 is also involved in PanIN in human pancreatic cancers thus represents an important issue to be addressed in future studies.

Our study revealed that CRMP4 is expressed in both epithelial cells and in stromal areas, especially in αSMA-positive cells (Figures 4 and 6). Previous studies have shown that the formation of PanIN accompanies the accumulation of a desmoplastic stroma and abundant immune infiltrates [48,58]. It has been suggested that the desmoplastic reaction by inflammation participates in the development of PanIN [59,60]. αSMA-positive cells in stromal areas of PanIN lesions are considered to constitute an active type of pancreatic stellate cells [61]. It is generally accepted that the quiescent type of pancreatic stellate cells, which contain vitamin A, become changed to active type by tissue injury or inflammation [62]. The active type of pancreatic stellate cells accelerates the production of extracellular matrix such as fibrillar collagen [63].

| Table 1 | PanIN Grade in KC-Crmp4 WT or KC-Crmp4 KO Mice |
|---------|-----------------------------------------------|
| Grade of PanIN | KC-Crmp4 WT | KC-Crmp4 KO |
| Normal or 1 | (n = 19) | (n = 11) |
| 2 or 3 | (26.3%) | (63.6%) |
| 3 | (73.7%) | (36.4%) |

Histological analysis of PanIN progression in KC-Crmp4 KO compared to KC-Crmp4 WT mice (χ² test; P = .044).

**Figure 3.** CRMP4 expression in normal pancreatic tissue and in PanIN lesions. (A) Western blot analysis for CRMP4 proteins in brain lysates from Crmp4+/− and Crmp4 KO mice. The band of 66 kDa corresponds to CRMP4, which is missing in brain lysates from Crmp4 KO mice. Hematoxylin and eosin stain (B-D) and immunostaining with anti-CRMP4 antibody (E-H) in pancreatic tissue. (B, E, F) Representative normal tissue in KC-Crmp4 KO mice. The band of 66 kDa corresponds to CRMP4, which is missing in brain lysates from CRMP4 antibody in KC-Crmp4 WT (F) and Crmp4 KO mice (E). Immunohistochemistry with anti-CRMP4 antibody in KC-Crmp4 WT (F) and Crmp4 KO mice (E). (C, G) Representative PanIN-1 in KC-Crmp4 WT mice. (D, H) Representative PanIN-2 in KC-Crmp4 WT mice. Expression levels of CRMP4 are relatively high in PanIN lesions (F-H). Arrows indicate PanIN. Arrowheads indicate stromal areas. Scale bar, 20 μm. Insets show higher magnified view of representative areas (B, E, F). Scale bar, 80 μm. The magnification is 1600 times.
as collagen, fibronectin, and laminin, thereby leading to pancreas fibrosis [59,63,64]. Therefore, the interaction between pancreatic stellate cells and pancreatic cancer cells or epithelial cells of PanIN may be involved in the proliferation of PDAC or PanIN through the desmoplastic reaction.

Repeated acute pancreatic injury and inflammation serve as contributing factors to the development of pancreatic cancer. In particular, intracellular activation of both pancreatic enzymes and the transcription factor NF-κB comprises an important mechanism that induces acute pancreatitis [65]. Recurrent pancreatic injury owing to genetic susceptibility, along with environmental factors such as smoking, alcohol intake, and conditions such as obesity, leads to increases in oxidative stress, impaired autophagy, and constitutive activation of inflammatory pathways. These processes can stimulate pancreatic stellate cells, thereby increasing fibrosis and encouraging chronic disease development [66]. Fibrosis has a pivotal role in inflammation and carcinogenesis. PDAC is unique among solid tumors because of the extremely dense desmoplastic reaction that surrounds the cancer cell glands of this tumor. The desmoplasia, containing myofibroblastic pancreatic stellate cells, extracellular matrix proteins, and immune cells, modulates the growth of the cancer by providing a scaffold for the cancer cells to grow, along with growth factors, angiogenesis factors, and immune modulators [67]. Considering that activated pancreatic stellate cells underlie the desmoplasia, CRMP4 expression in stellate cells surrounding PanIN lesions may have a critical role in the development of PanIN.

Our previous study demonstrated that CRMP4 was augmented in CD3-positive cells in a pancreatitis mouse model [46]. In comparison, our present study showed that CRMP4-positive cells were coincident with αSMA-positive cells, whereas the expression of CRMP4 was barely detectable in CD3-positive cells. The discrepancy might be attributed to differences in the genetic background of the mouse models, the period of observation, and other experimental conditions. For example, WT and CRMP4 KO mice were examined in acute pancreatitis models [46], whereas WT and CRMP4 KO with K-ras mutant background mice were examined in our current study. It has been shown that K-ras-expressing pancreatic acinar cells initiate microinflammation and that an interaction exists between PanIN and αSMA-positive cells, which may contribute to the formation and progression of PanIN lesions [68]. Furthermore, in our previous study,
Figure 6. Double immunofluorescence labeling with CRMP4 and αSMA in PanIN-2 lesion in KC-Crmp4 WT mice. Representative image of PanIN-2 lesion stained with anti-CRMP4 antibody (A), anti-αSMA antibody (B), DAPI (C), and merged image (D). Scale bar, 50 μm. The epithelial cells were CRMP4 positive (yellow arrowhead). Some stromal cells surrounding the PanIN lesions coexpress CRMP4 and αSMA (white arrowhead). Magnified images of boxed areas in the epithelial cells of PanIN lesion and in stromal area in panel D were shown in panels E and F, respectively. Scale bar, 25 μm. The representative CRMP4 and αSMA double-positive cells (white arrowhead) in stromal areas surrounding the PanIN-1A (opened arrowhead) were shown in panels G and H. Scale bar, 25 μm. All sections represent PanIN lesions in a KC-Crmp4 WT mouse.
CRMP4/CD3 double-positive cells were observed mainly in the phase of acute pancreatitis [46], whereas in the present study, CRMP4-positive cells were observed in the recovery phase from acute pancreatitis. It is possible that the number of CD3-positive cells may decrease in recovery phase. CRMP4 may be involved in PanIN or pancreatic cancer pathogenesis through several mechanisms. First, CRMP4 may promote the inflammation pathway in the development of PanIN. It has been shown that K-ras mutation and cell injury mediated by inflammation play important roles in the development of PanIN lesions [53]. Previous studies have demonstrated that CRMP4 is involved in inflammation pathways [46,51]. We reported that both acute pancreatitis and chronic pancreatitis augment CRMP4 expression and its phosphorylation in infiltrated CD3 T-cells [46]. Therefore, CRMP4 may play a pivotal role in pancreatic inflammation. Notably, deletion of CRMP4 in a spinal cord injury mouse model promotes the recovery of locomotion via neuroprotection and limited scar formation because CRMP4 deletion suppresses the activation of microglia or macrophages and reactive astrocytes following injury [51]. Therefore, we speculated that CRMP4 may participate in the development of PanIN through the acceleration of inflammation.

Second, CRMP4 may help to develop PanIN lesions through reconstitution of the cytoskeleton, as this may induce morphologic changes comparable to those observed during PanIN development [69]. In a pancreatitis mouse model, it was found that RAS-related C3 botulinum substrate 1 (Rac1), which is an effector molecule of EGF and K-ras, was necessary for caerulein-induced acinar morphologic changes and filamentous actin redistribution [70,71]. CRMP4 is also known to regulate the actin and microtubule growth cone cytoskeleton in hippocampal neurons [27,28,72]. Moreover, the alternatively spliced short (CRMP4a) and long (CRMP4b) isoforms are known to be involved in many biological processes [73]. In particular, CRMP4a suppresses RhoA activity, leading to reduced cytoskeleton realignment and cell motility in prostate cancer [38]. However, further studies are needed to clarify the molecular mechanism of CRMP4 in PanIN development.

In conclusion, the incidence of high-grade PanIN was low in the CRMP4-KO mouse model of pancreatic cancer. The expression of CRMP4 in the stroma cells correlated with the progression of PanIN lesions. These findings suggest that CRMP4 inhibition may serve as a therapeutic strategy to prevent PanIN development and PDAC.

Acknowledgements

We thank Dr. Hideki Taniguchi for providing LSL-KRASG12D2 mice. We thank Ms. Harumi Sakurada and Ms. Takako Okada for their excellent technical assistance. We thank Dr. Haruko Nakamura, Yuko Kawamoto-Kondo, Akihiko Fakasahi-Jitsuji, Hiroko Makihara, Motokazu Koga, and Tatsumi Hamasato for technical advice. We would like to thank Editage (www.editage.com) for English language editing. This work was supported by Grants-in-Aid from the Japanese Ministry of Education, Culture, Science, Sports, and Technology for Fundamental Research (14246462070 to LE, and 17K10707 to Y.Y.) and Creation of Innovation Centers for Advanced Interdisciplinary Research Areas Program in the Project for Developing Innovation Systems from the MEXT (to Y.G.). This work was also supported by grants from the Takei Foundation, Eli Lilly and Company, and Sanofi in Japan.

Table 2

| Grade of PanIN | Epithelial Cells | Stromal Cells |
|----------------|-----------------|---------------|
| 1A or 1B       | Negative: 3     | Negative: 3   |
|                | Positive: 1     | Positive: 1   |
| 2 or 3         | Negative: 4     | Negative: 4   |
|                | Positive: 10    | Positive: 10  |

Relationship between PanIN grade and CRMP4 expression in epithelial cells (Fisher’s exact test; two-sided, P = .588) and stromal cells (Fisher’s exact test; two-sided, P = .023).

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Conflict of Interest

None.
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