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ORIGINAL RESEARCH

Increased Bcl-xL Expression in Pancreatic Neoplasia Promotes Carcinogenesis by Inhibiting Senescence and Apoptosis

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SUMMARY

Bcl-xL expression increased with the progression of pancreatic neoplasia from pancreatic intraepithelial neoplasia (PanIN)-1 to pancreatic ductal adenocarcinoma. Bcl-xL overexpression in low-grade PanINs inhibits oncogene-induced senescence, whereas it inhibits apoptosis in high-grade PanINs, accelerating carcinogenesis. Bcl-xL deficiency increased senescence in PanINs.

BACKGROUND & AIMS: Bcl-xL, an anti-apoptotic Bcl-2 family protein, is overexpressed in 90% of pancreatic ductal adenocarcinoma (PDAC) cases. However, Bcl-xL expression in pancreatic intraepithelial neoplasias (PanINs) and its significance in PDAC carcinogenesis remain unclear. The aim of this study was to elucidate the significance of Bcl-xL expression in PanINs.

METHODS: We investigated the expression levels of Bcl-xL in pancreas-specific KrasG12D (P-KrasG12D) mice and human PanINs and PDAC. We examined the impact of Bcl-xL expression on Kras-mutated pancreatic neoplasia using Bcl-xL–overexpressing P-KrasG12D mice and Bcl-xL–knockout P-KrasG12D mice.

RESULTS: In P-KrasG12D mice, the number of PanINs increased and their grades progressed with age. In total, 55.6% of these mice developed PDAC at 12–14 months. According to the immunohistochemistry of mouse pancreas and human resected specimens, Bcl-xL expression was increased significantly in PanIN-1 compared with that in normal pancreatic ducts, and augmented further with the progression of pancreatic neoplasia in PanIN-2/3 and PDAC. Oncogene-induced senescence was observed frequently in PanIN-1, but rarely was detected in PanIN-2/3 and PDAC. Bcl-xL overexpression significantly accelerated the progression to high-grade PanINs and PDAC and reduced the survival of P-KrasG12D mice. Bcl-xL overexpression in P-KrasG12D mice suppressed oncogene-induced senescence in PanIN-1 and inhibited apoptosis in PanIN-3. Bcl-xL deficiency in P-KrasG12D mice induced cellular senescence in PanIN-2/3.

CONCLUSIONS: Bcl-xL expression increases with the progression from PanIN-1 to PDAC, whereas oncogene-induced senescence decreases. Bcl-xL overexpression increases PDAC incidence rates by inhibiting oncogene-induced senescence and apoptosis in PanINs. Conversely, Bcl-xL deficiency induced senescence in PanINs. Anti–Bcl-xL treatments may have the potency to suppress the progression from PanINs to PDAC. *(Cell Mol Gastroenterol Hepatol 2017;4:185–200; http://dx.doi.org/10.1016/j.jcmgh.2017.02.001)*

Keywords: Kras; PanINs; Bcl-2 Family Protein.

Pancreatic ductal adenocarcinoma (PDAC) is one of the most highly aggressive forms of human cancer, with <5% of patients surviving after 5 years, and the early detection of PDAC remains difficult.[1,2] The number of patients with PDAC has increased steadily, and PDAC has increased to the fourth leading cause of cancer-related death in the United States.[3] Pancreatic intraepithelial neoplasias (PanINs) are the most frequent and well-characterized precursor lesions of PDAC.[4,5] PanINs are classified into PanIN-1, 2, and 3, depending on the degree of cytologic and architectural atypia.[6] Kras mutations, the most frequent genetic alteration of PDAC, are identified in more than 90% of low-grade PanIN-1/2, as well as high-grade PanIN-3 and PDAC in human beings.[7,8] A pancreas-specific Kras mutant mouse model, which develops PanINs and PDAC over time,[9] is suitable for studying both PanINs and PDAC.

Bcl-xL is one of the anti-apoptotic Bcl-2 family proteins.[10] Bcl-xL inhibits Bak or Bax activation, which is an essential event for apoptosis execution.[11,12] Previous reports have clarified that increased Bcl-xL expression is observed in 90% of PDAC cases based on immunohistochemical analysis using tumor tissues of PDAC patients.[13,14] However, the expression levels in PanINs remain unclear. The impact of Bcl-xL overexpression on PDAC carcinogenesis also remains uncharacterized.

In the present study, Bcl-xL expression showed a graded increase with the progression of pancreatic neoplasia from

Abbreviations used in this paper: **IHC, immunohistochemistry; KO, knockout; P-KrasG12D, Pdx1-Cre LSL-KrasG12D; PanIN, pancreatic intraepithelial neoplasia; PDAC, pancreatic ductal adenocarcinoma; SA–β-gal, senescence-associated β-galactosidase; siRNA, small interfering RNA; Tg, transgenic; TUNEL, terminal deoxynucleotidyl transferase–mediated deoxyuridine triphosphate nick-end labeling.**

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PanIN-1 to PDAC. Bcl-xL overexpression accelerated PDAC carcinogenesis with the decrease of senescence in PanIN-1 and apoptosis in PanIN-3, reducing survival in Kras mutant mice. Conversely, Bcl-xL deficiency induced senescence in PanINs. This study showed the function of Bcl-xL and its effect on the process of pancreatic carcinogenesis.

Materials and Methods

Genetically Engineered Mouse Models and Mouse Strains

The *Pdx1-Cre* transgenic (Tg) strain on a C57BL/6/FVB background and the *LSL-KrasG12D* knock-in strain on a C57BL/6/129 background were obtained from the Mouse Models of Human Cancer Consortium (National Cancer Institute–Frederick, Bethesda, Maryland). The LSL-KrasG12D allele is expressed at endogenous levels after Cre-mediated excision of a transcriptional stopper element.[9,15] A heterozygous hemagglutinin-hBcl-xL transgenic strain expressing the human Bcl-xL gene under the regulation of the CAG promoter on a C57BL/6/129 background (Bcl-xL Tg mice) using a hemagglutinin-tagged human Bcl-xL expression plasmid (pcDNA3HAbcl-xL) was described previously.[16] Mice carrying a *bcl-x* gene with 2 loxP sequences in the promotor region and a second intron (*bcl-x*$^{flox/flox}$ mice) were described previously.[17] These strains were intercrossed to produce the experimental cohorts. Mice were genotyped by polymerase chain reaction analysis (primer sequences are provided in the Supplementary Materials and Methods section). All mice were maintained with free access to food and water in a specific pathogen-free facility. They were treated with humane care under approval from the Animal Care and Use Committee of Osaka University Medical School.

Histologic Evaluation

Formalin-fixed, paraffin-embedded mouse pancreas tissues were sectioned (4 μm) and stained with H&E. PanIN lesions and carcinoma were classified according to histopathologic criteria.[18] To quantify the progression of PanIN lesions, the number and their grades from each sample were obtained from 10 high-power ($\times$200) microscopic fields. The area of normal-appearing pancreatic tissue was measured using the Hybrid Cell Count analysis of a digital microscope image (BZ-X700; Keyence, Osaka, Japan). Terminal deoxynucleotidyl transferase–mediated deoxyuridine triphosphate nick-end labeling (TUNEL) staining, which is used to detect apoptotic cells,[19,20] was performed as previously described.[21]

Immunohistochemistry

The sections of mouse pancreas were processed further for immunohistochemistry (IHC). The sections were immersed in 1$\times$ Target Retrieval Solution (pH 6.0) (Dako, Glostrup, Denmark) for Bcl-xL and p21 staining. The samples were heated to 120°C in a decloaking chamber for antigen retrieval. The endogenous peroxidase activity was quenched by incubating with 3% hydrogen peroxide in methanol for 30 minutes. After sections were blocked in 5% goat serum for 1 hour, they were incubated with antibodies against Bcl-xL (1:200, #2764; Cell Signaling Technology, Danvers, MA) and p21 (1:400, sc-471; Santa Cruz Biotechnology, Santa Cruz, CA) overnight at 4°C. After rinsing with phosphate-buffered saline, the slides were incubated with secondary antibody (SignalStain Boost IHC detection reagent; Cell Signaling Technology) for 30 minutes. After washes with phosphate-buffered saline, the sections were incubated with 3,3'-diaminobenzidine for 30 seconds and counterstained with hematoxylin. In every staining set, negative controls were applied by omission of the primary antibody.

Evaluation of the Immunohistochemistry Results

Immunohistochemical staining for Bcl-xL was defined as a detectable immunoreaction in the cytoplasm. The number of positive cells was estimated semiquantitatively. The quantity of the immunostaining was evaluated as follows: 0 corresponded to no positive immunostaining, 1 corresponded to less than 20%, 2 corresponded to 20%–75%, and 3 corresponded to more than 75% positive cells.[22] The intensity of the immunostaining was evaluated as follows: 0 corresponded to no staining, 1 corresponded to weak staining, 2 corresponded to moderate staining, and 3 corresponded to intense staining.[23] A combined total score for the immunostaining was calculated by adding both the quantitative score and the intensity score. The total score for each sample was the average score obtained from 10 high-power (magnification, $\times$200) microscopic fields.

Senescence-Associated β-Galactosidase Assay

To assess senescence in vivo, mouse pancreatic cryosections were stained for senescence-associated β-galactosidase (SA–β-gal) activity according to a previously described procedure.[24] Briefly, the frozen pancreas sections were fixed in 0.2% glutaraldehyde with 2% formaldehyde for 1 minute and incubated overnight in SA–β-gal solution from the Senescence β-Galactosidase Staining Kit (Cell Signaling Technology) at 37°C. This staining kit also was used for the evaluation of senescence in vitro according to the manufacturer's protocol. A total of approximately 500 cells was counted in 4 randomly selected fields to determine the percentage of senescent cells.

Human Samples

Formalin-fixed, paraffin-embedded samples were obtained from 9 patients with PDAC accompanied by PanIN lesions and 7 patients with other types of pancreatic tumors (neuroendocrine tumor, 2; serous cystic neoplasm, 2; solid pseudopapillary neoplasm, 1; epithelial cyst, 1; and hamartoma, 1) who underwent pancreas resection without preoperative therapy between March 2009 and March 2012. The use of resected samples was approved by the Institutional Review Board for Clinical Research at Osaka University Hospital (14435). Written informed consent was obtained from each patient. These samples were sectioned (4 μm) and stained with H&E. The sections also were