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Trisomy of the *Dscr1* gene suppresses early progression of pancreatic intraepithelial neoplasia driven by oncogenic *Kras*

Jang Choon Lee, Jimin Shin, Kwan-Hyuck Baek *

Department of Molecular and Cellular Biology, Samsung Biomedical Research Institute, Sungkyunkwan University School of Medicine, Suwon, Gyeonggi 440-746, Republic of Korea

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ABSTRACT

Individuals with Down syndrome exhibit remarkably reduced incidence of most solid tumors including pancreatic cancer. Multiple mechanisms arising from the genetic complexity underlying Down syndrome has been suggested to contribute to such a broad cancer protection. In this study, utilizing a genetically engineered mouse model of pancreatic cancer, we demonstrate that trisomy of the Down syndrome critical region-1 (*Dscr1*), an endogenous calcineurin inhibitor localized on chromosome 21, suppresses the progression of pancreatic intraepithelial neoplasia-1A (PanIN-1A) to PanIN-1B lesions without affecting the initiation of PanIN lesions mediated by oncogenic $Kras^{G12D}$. In addition, we show that *Dscr1* trisomy attenuates nuclear localization of nuclear factor of activated T-cells (NFAT) accompanied by upregulation of the $p15^{Ink4b}$ tumor suppressor and reduction of cell proliferation in early PanIN lesions. Our data suggest that attenuation of calcineurin–NFAT signaling in neoplastic pancreatic ductal epithelium by a single extra copy of *Dscr1* is sufficient to inhibit the progression of early PanIN lesions driven by oncogenic *Kras*, and thus may be a potential mechanism underlying reduced incidence of pancreatic cancer in Down syndrome individuals.

1. Introduction

Down syndrome is the most common genetic disorder in human arising from the presence of an extra copy of all or part of chromosome 21. Although most effects caused by trisomy 21 are deleterious, including delays in mental and physical development and increased risk of acute megakaryoblastic leukemia (AMKL) in children with Down syndrome, the incidence of most solid tumors, including pancreatic cancer, has been shown to be remarkably reduced in Down syndrome adults [1–6]. Such observations suggest that increased dosage of one or more genes on chromosome 21 is responsible for such a broad cancer protection, and elucidating the underlying mechanisms could potentially reveal new targets for cancer prevention and treatment.

Recent studies have demonstrated that multiple mechanisms derived from the genetic complexity of Down syndrome contribute to reduced cancer incidence in individuals with Down syndrome. The increased dosage of *Ets2* gene has been shown to suppress the incidence of intestinal tumors driven by Apc^{Min}, the multiple intestinal neoplasia mutation in the adenomatous polyposis coli (*Apc*) gene, in Ts65Dn mice, a Down syndrome mouse model [7]. In addition, tumor xenografts studies in Ts65Dn and Tc1 Down syndrome model mice have suggested that cancer protection observed in Down syndrome population is, in part, attributable to angiogenesis suppression by trisomy 21 [8,9]. Furthermore, a more recent study has shown that trisomy for 104 genes orthologous to those located on human chromosome 21 increases survival of NPcis mice spontaneously developing various tumors such as lymphomas, sarcomas and carcinomas, and this cancer protection is attributed to a shift of the tumor spectrum away from rapidly lethal sarcoma and toward adrenal tumors but not either increased *Ets2* dosage or reduced tumor angiogenesis [10]. Taken together, these observations suggest that, depending on types of tumors, distinct mechanisms may contribute to cancer protection in the Down syndrome population.

Sustained calcineurin–NFAT signaling promotes pathological cell growth and proliferation, leading to several disorders such as atherosclerosis and hyperinsulinaemia [11,12]. In addition, constitutively active NFAT expression enhances cell transformation and metastatic potential of cancer cells [13,14]. Furthermore, NFAT upregulation has been observed in several human malignancies,

Abbreviations: Dscr1, Down syndrome critical region-1; NFAT, nuclear factor of activated T-cells; PanIN, pancreatic intraepithelial neoplasia; PDA, pancreatic ductal adenocarcinoma; *Dyrk1a*, dual-specificity tyrosine-(Y)-phosphorylation regulated kinase 1A; CK-19, cytokeratin-19; CDK, cyclin-dependent kinase; TUNEL, terminal deoxyribonucleotidyl transferase-mediated dUTP nick end labeling.

* Corresponding author. Address: Department of Molecular and Cellular Biology, Samsung Biomedical Research Institute, Sungkyunkwan University School of Medicine, 300 Cheoncheon-dong, Jangan-gu, Suwon, Gyeonggi 440-746, Republic of Korea. Fax: +82 31 299 6169.

E-mail address: khbaek@skku.edu (K.-H. Baek).

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most notably in human pancreatic carcinomas [15]. These observations suggest that calcineurin–NFAT pathway plays a crucial role in development and progression of certain types of human neoplasia including pancreatic cancer. Notably, two genes, Down syndrome critical region-1 (*Dscr1*) and dual-specificity tyrosine-(Y)-phosphorylation regulated kinase 1A (*Dyrk1a*), on chromosome 21 have been shown to function as negative regulators for calcineurin–NFAT pathway and their expressions are elevated in Down syndrome tissues [16–21]. Hence, reduced pancreatic cancer incidence in Down syndrome individuals may be due, in part, to attenuated calcineurin–NFAT pathway by increased expression of those negative regulators afforded by the extra copy of chromosome 21. However, it has never been examined whether the modest excess of either DSCR1 or DYRK1A afforded by a single extra copy impedes calcineurin–NFAT signaling evoked by oncogenic activation in pancreatic ductal epithelial cells, and thus suppresses development of pancreatic cancer. In addition, the role calcineurin–NFAT signaling pathway in development and progression of pancreatic cancer still remains to be further verified in spontaneous animal tumor models closely recapitulating the characteristics of human pancreatic tumorigenesis

In this study, utilizing the well-characterized *Pdx-1-Cre;LSL-Kras^{G12D}* mouse model of preinvasive human pancreatic ductal adenocarcinoma (PDA) [22], we examined the inhibitory role of *Dscr1* in pancreatic tumorigenesis and evaluated the calcineurin–NFAT signaling pathway in neoplastic pancreatic ductal epithelium. In addition, we specifically addressed the effect of a single extra copy of *Dscr1* on progression of pancreatic intraepithelial neoplasia (PanIN) lesions driven by oncogenic *Kras^{G12D}* using *Dscr1* transgenic mice, a mouse model harboring a third copy of a *Dscr1* transgene targeted into the *Hprt* locus and controlled by the *Dscr1* native promoter [8]. We generated the *Pdx-1-Cre;LSL-Kras^{G12D}* mice trisomic for *Dscr1* and found that trisomy of the *Dscr1* inhibits the progression of early PanIN lesions through attenuating nuclear localization of nuclear factor of activated T-cells (NFAT) accompanied by compromised cell proliferation in neoplastic ductal epithelium.

2. Materials and methods

2.1. Mice

Pdx-1-Cre mice on a C57BL/6 background were obtained from the Jackson Laboratory (Bar Harbor, ME, USA). *LSL-Kras^{G12D}* mice on a 129SvEv background were provided by Dr. Tyler Jacks (MIT Cancer Center) and backcrossed onto a C57BL/6 background for more than 10 generations. *Dscr1* transgenic mice bearing a single extra copy of a *Dscr1* transgene inserted into the *Hprt* locus on a C57BL/6 background were generated as previously described [8]. *LSL-Kras^{G12D}* mice were crossbred with *Pdx-1-Cre* mice and then further crossed to *Dscr1* transgenic mice to generate *Pdx-1-Cre;LSL-Kras^{G12D};Dscr1* transgenic mice. Genotyping of mice was performed on genomic DNA extracted from tails using PCR as previously described [8,22,23]. *Pdx-1-Cre;LSL-Kras^{G12D}* mice littermates on a *Dscr1* wild-type background were used as controls in our studies. All animal studies were performed in accordance with guidelines of the Institutional Animal Care and Use Committee at the Sungkyunkwan University School of Medicine.

2.2. Histology

Mice were euthanized by CO_2 asphyxiation, and pancreatic tissues were removed, fixed with 10% neutral buffered formalin and embedded in paraffin following standard procedures. 4-μm serial sections were prepared and every fifth slide was stained with hematoxylin and eosin (H&E; Sigma, St. Louis, MO, USA) followed by examination under a bright-field microscope (Carl Zeiss, Jena, Germany). The stage of PanIN lesions was determined without knowledge of genetic background on three sections of each pancreas as previously described [18].

2.3. Immunohistochemistry

Paraffin-embedded sections were deparaffinized in xylene, rehydrated by sequential incubations in graded ethanol from 100% to 95% followed by distilled water and antigen retrieved in a pressure cooker containing a sodium citrate buffer (10 mM, pH 6.0). Sections were then incubated in dual endogenous enzyme block reagent (Dako, Glostrup, Denmark) to quench endogenous peroxidase activity, treated with an avidin/biotin blocking solution (Vector Laboratories, Burlingame, CA, USA) and further incubated in serum-free protein blocking solution (Dako). The sections were then incubated with rabbit antibodies to cyclin-dependent kinase 4 (CDK4; Santa Cruz Biotechnology, Santa Cruz, CA, USA), p15^{INK4b} and Ki-67 (Abcam, Cambridge, UK) overnight at 4 °C followed by visualization with Vectastain Elite ABC Universal kit and the DAB Peroxidase Substrate kit (Vector Laboratories) according to the manufacturer's instructions. To stain pancreatic tissues with mouse monoclonal antibodies against cytokeratin-19 (CK-19), NFATc2 (Abcam) and c-Myc (Santa Cruz Biotechnology), the sections processed as described above were further treated with mouse-on-mouse (M.O.M.) blocking reagent (Vector Laboratories) prior to incubation with the antibodies. After incubation with the primary antibodies overnight at 4 °C, sections were incubated with M.O.M. biotinylated anti-mouse IgG followed by visualization as described above. The sections were then counterstained with hematoxylin to detect cell nuclei, mounted and examined under a bright-field microscope.

2.4. TUNEL staining

To assess apoptosis in PanIN lesions, terminal deoxyribonucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) staining was performed on pancreatic tissue sections using the Dead-End labeling kit (Promega, Madison, WI, USA) following the manufacturer's instructions. In brief, sections were deparaffinized, rehydrated and antigen retrieved as described above. Sections were then treated with avidin/biotin blocking solution, blocked in M.O.M. blocking reagent and incubated with anti-CK-19 antibody followed by serial incubation with M.O.M. biotinylated anti-mouse IgG and Texas Red Avidin DCS (Vector Laboratories). The sections were then incubated in equilibration buffer for 15 min followed by further incubation with fluoresceinated rdNTPs and terminal deoxynucleotidyl transferase for 60 min at 37 °C. Sections were treated with 2× SSC buffer to stop reaction, counterstained with 1 μg/ml Hoechst dye (Sigma) to visualize cell nuclei, mounted and examined under a fluorescence microscope (Carl Zeiss).

2.5. Statistics

Student's two-tailed unpaired *t* test was used to determine the difference between two groups and *p* value less than 0.05 was considered as statistically significant.

3. Results and discussion

3.1. A single extra transgenic copy of Dscr1 inhibits early PanIN progression

The largest epidemiological study to date involving 17,897 individuals with Down syndrome has revealed that the incidence of