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ORIGINAL ARTICLE

PDZK1 induces resistance to apoptosis in esophageal adenocarcinoma cells

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Abstract

Background Esophageal cancer is a lethal malignancy with a poor prognosis. The incidence of esophageal adenocarcinoma, which develops from Barrett's esophagus (BE), has recently been increasing. In a previous study, we found that PDZK1 expression is higher in long segment BE compared to that in short-segment BE. However, the function of PDZK1 in the mucosa of BE is unclear.

Aims Clarify the role of PDZK1 in BE mucosa using PDZK1 overexpressed cells.

Methods Human adenocarcinoma-derived OE33 cells were used as a parental cell line and transfected to generate PDZK1 overexpressed OE33 cells (PC cells) or transfected with empty vector as control cells (NC cells). Cell growth of NC and PC cells in 10% fetal bovine serum was evaluated by cell counting. The effect of PDZK1 on proteasome inhibitor (PSI)-induced apoptosis was qualified by fluorescence microscopy and quantified by flow cytometry. Expression of apoptosis-related proteins was evaluated by western blotting.

Results There were no significant differences in cell growth between NC and PC cells. PSI significantly increased apoptosis in NC cells, but not in PC cells. In response to PSI, increased levels of cleaved-caspase3 and decreased pro-caspase3 levels were found in NC cells, but not in PC cells. In NC cells, PSI significantly decreased Bcl-2 expression without affecting Bax levels. In contrast, high expression of both Bcl-2 and Bax was observed in PC cells.

Conclusion Overexpression of PDZK1 protein induces an apoptosis-resistant phenotype in BE cells, which may be a potential therapeutic target.

Keywords PDZK1 · Barrett's esophagus · Esophageal adenocarcinoma · Apoptosis

Introduction

Esophageal cancer (EC) is a lethal malignancy with a poor prognosis. The incidence of EC has been reported to be increasing in every country, especially in Asian countries [1]. The two main histological subtypes of EC are squamous cell carcinoma (SCC) and esophageal adenocarcinoma (EAC). For a long time, SCC was histologically the most common type of EC in the United States; however, the incidence of SCC has been reported to be decreasing and EAC is now the predominant type of EC [2]. The incidence of EC is reported to be associated with socioeconomic development. Specifically, the incidence of SCC negatively correlates with the human development index (HDI) and the incidence of EAC positively correlates [3], suggesting the incidence of EAC will increase in developed countries. Akita Prefecture is reported to be the region in Japan with the highest rate of EC-associated mortality and SCC remains the vast majority of ECs [4]. The incidence of EC in Japan as a whole includes increases in both SCC and EAC, but the increased rate of EAC is higher than that of SCC [4]. In addition, the prognosis of EAC is poor with the 5-year survival of patients with EAC being 20% [5]. Therefore, investigating the epidemiology, characteristics, and therapy of EAC is important for future public health in Japan.

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Known risk factors of EC include being male, consumption of red and processed meat, and a high-fat diet. Moreover, alcohol consumption and tobacco smoking are strong risk factors of SCC, while gastrointestinal reflux disease and obesity are risk factors of EAC. Histological investigation of EC background mucosa has indicated that SCC grows from atypical squamous cells and EAC from the mucosa of Barrett's esophagus (BE). In addition, the risk of EAC with long-segment BE (LSBE) has been reported to be higher than that with short-segment BE (SSBE) [6]. In our recent study, we determined the expression of several oncogene-related proteins are upregulated in the mucosa of BE, including PDZK1 [7]. PDZK1 is a PDZ protein that has four PDZ domains of PSD95, D1g, and ZO-1, and plays a role in cancer growth, metastasis, and resistance to chemotherapeutic drugs [8]. The expression and function of PDZK1 have been recognized in various organs and tissues, including kidney with the highest levels of all human organs [9], gastrointestinal tract, breast cancer [10], lung, and liver [11]. However, the function of PDZK1 in BE mucosa is unclear. Therefore, in the current study, we clarified the role of PDZK1 in the mucosa of BE using a PDZK1 overexpressing cell line.

Materials and methods

Reagents

All chemicals were prepared immediately before use. Proteasome inhibitor (PSI; *N*-carbobenzoxy-L-isoleucyl-L-g-t-butyl-L-blutamyl-L-alanyl-L-leucilual) was purchased from Calbiochem (San Diego, CA, USA) and Hoechst 33432 (HO342) was purchased from Dojindo Molecular Technologies, Inc. (Kumamoto, Japan). The Annexin-V Propidium Iodide Apoptosis Detection Kit was obtained from Sigma Chemical Co. (St. Louis, MO, USA). Dimethyl sulfoxide (DMSO) and fetal bovine serum (FBS) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Trypsin was purchased from Gibco (Thermo Fisher Scientific, Waltham, MA, USA).

Rabbit anti-caspase3 monoclonal antibody, which detects full-length caspase-3 as well as the large subunit of caspase-3 resulting from cleavage during apoptosis, rabbit anti-Bax polyclonal antibody, and mouse anti-Bcl-2 monoclonal antibody were each purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). Mouse monoclonal anti-β-actin was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Secondary antibodies included peroxidase-conjugated goat anti-rabbit IgG and peroxidase-conjugated gout anti-mouse IgG and were purchased from Jackson ImmunoReserch Inc. (West Grove, PA, USA).

Expression vector construction and cell cloning

Expression vector construction and viral production were performed as previously described [12]. Briefly, Human Stomach Total RNA (TaKaRa, Ohtsu, Japan) was reverse-transcribed and the resulting complementary DNA (cDNA) was amplified using Prime STAR HS DNA polymerase (TaKaRa) with the following primers: PDZK1-f, 5′-GTT GGGATCCATGACCTCCACCTTCAACCCCCGAG-3′ and PDZK1-r, 5′-TGGTCTCGAGCTATTACTTGTTTT CATCACATC-3′. Sequences of the full-length PDZK1 cDNA were confirmed by sequencing after cloning into a pCR-Blunt II-TOPO cloning vector (Invitrogen, Carlsbad, CA, USA). PDZK1 cDNA in the TOPO cloning vector was excised and transferred to a pBABE-puro retroviral vector (Cell Biolabs, San Diego, CA, USA). To generate the viral particles, the pBABE constructs were co-transfected with a pVSV-G vector (Clontech, Mountain View, CA, USA) into gpIRES-293 cells using the FuGENE6 transfection reagent (Roche Diagnostics, Basel, Switzerland). After 48 h of transfection, the culture medium was collected and the viral particles were concentrated by centrifugation at 15,000×*g* for 3 h at 4 °C. The viral pellet was then resuspended in Dulbecco's Modified Eagle's Medium (DMEM; Sigma-Aldrich) and was added to OE33 cells (DS Pharma Biomedical, Osaka, Japan), which is an adenocarcinoma cell line established from EAC on BE [13]. Infected OE33 cells were then grown in DMEM containing puromycin (1 μγ/mL; Thermo Fisher Scientific) to clone the overexpressing PDZK1 cell line (PC cells). Empty pBabe-puro vector was used to transfect and clone the control cell line (NC cells). The NC cells and PC cells were confirmed for PDZK1 expression (Fig. 1) and then used in the current study.

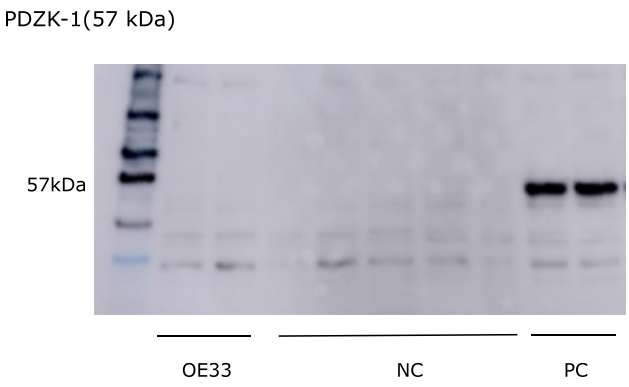

Fig. 1 PDZK1 expression. The expression of PDZK1 (57 kDa) in parental OE33 cells, OE33 cells transfected with empty vector (NC), and PDZK1 overexpressing OE33 cells (PC) cells was assessed by western blotting

Cell growth analysis by cell count

NC or PC cells were seeded into 10-cm culture dishes (Falcon, Corning, NY, USA) containing DMEM supplemented with 10% FBS (1×10^5 cells/dish). At days 1, 4, and 7 post seeding, the cells were trypsinized and collected. The number of cells was determined using an Eve™ Automated Cell Counter (NanoEntek: Seoul, Korea).

Microscopic analysis of apoptotic cells

PSI is known to induce cell apoptosis in various cells by inhibiting the ubiquitin–proteasome pathway [14] and was used as an inducer of apoptosis in the current study. Morphological assessment of apoptosis was performed using HO342 dye under fluorescence microscopy. Briefly, NC or PC cells were grown on 35-mm cell culture dishes (Falcon) until confluent. After treatment with 10 μM PSI or 0.5% DMSO for 8 h, the cells were incubated with HO342 dye for 15 min at 37 °C. The stained cells were examined using an IX83 inverted fluorescence microscope (Olympus, Tokyo, Japan). Photographic images (MPEG format) were taken from three random microscopic fields. Both live cells and apoptotic cells have cell-membrane function and take up the blue HO342 dye. Apoptosis was characterized morphologically based on condensed chromatin (apoptotic body) [15].

Flow cytometric quantification of dead cells

Quantitative analysis of cell death was performed using flowcytometry. NC or PC cells were cultured in 35-mm cell culture dishes (Falcon) until confluent. After treatment with 10 μM PSI or 0.5% DMSO for 6 h, cells were collected using a cell scraper (Iwaki, AGC techno glass, Shizuoka, Japan), pelleted by centrifugation, and resuspended in phosphate-buffered saline (PBS). Cells (1.0×10^6) were incubated with 100-μl PI and annexin-V solution for 15 min at 37 °C, measured using a FACSCalibur flow cytometer (Becton Dickinson Bioscience, Franklin Lakes, NJ, USA), and analyzed using CELLQuest Pro ver 6 software (Becton Dickinson Bioscience). Under normal physiological conditions, cells maintain a strictly asymmetric distribution of phospholipids in the two leaflets of the cellular membrane with phosphatidylserine (PS) facing the cytosolic side. However, upon induction of apoptosis, this membrane asymmetry is rapidly lost without a concomitant loss of membrane integrity [16]. Cell surface exposure of PS, ultimately resulting in the loss of membrane integrity, can be detected using fluorescein isothiocyanate (FITC)-labeled annexin V, which has a high affinity for PS residues [17]. Annexin-V positive cells include both early apoptotic (PI negative) cells and late apoptotic (PI positive) cells, and PI positive cells include late apoptotic (annexin-V positive) cells and necrotic (annexin-V negative) cells.

Western blotting

NC or PC cells were cultured in 10-cm cell culture dishes (Falcon) until confluent. After treatment with 10 μM PSI or 0.5% DMSO for 8 h, the cells were lysed on ice with RIPA buffer (Nakalai Tesque, Kyoto, Japan). Cellular debris was removed by centrifugation at 13,000×*g* at 4 °C for 15 min and the supernatant was collected. Supernatant protein concentrations were determined using a Thermo Science NanoDrop One spectrophotometer (Thermo Fisher). The supernatant samples were then electrophoresed and the separated proteins transferred to a nitrocellulose or polyvinylidene difluoride (PVDF) membrane (GE Healthcare Amersham, Little Chalfront, UK) using a Trans-Blot SD Semi-Dry Electrophoretic Transfer Cell (Bio-Rad, Hercules, CA, USA). After blocking with skim-milk for 60 min at room temperature and washing twice, the membranes were incubated with the primary antibodies specific for caspase3 (1:200), bcl-2 (1:1000), bax (1:1000), or β-actin (1:1000) overnight at 4 °C. The membranes were washed twice and then incubated with the appropriate secondary antibody for 1 h at room temperature. The immunostained blots were visualized using a Chem-Lumi One Super kit (Nakalai Tesque). The intensity of each band was measured relative to β-actin using ImageJ software.

Statistical analysis

Statistical analyses were performed using SPSS version 22.0 software (IBM Inc., Armonk NY, USA). The experiments were repeated at least three times and the results are presented as mean ± standard error (SE). Comparison between groups was conducted using Student's *t*-test or multiple comparisons by Bonferroni's method. A P-value < 0.05 was considered statistically significant.

Results

Transfection of OE33 cells with PDZK1 and the effect on cell growth

Based on western blot analyses, parental OE33 cells and OE33 cells transfected with empty vector (NC cells) did not express PDZK1 protein (Fig. 1). However, PDZK1 expression was clearly upregulated in OE33 cells that transfected the PDZK1 expression vector (PC cells). Analysis of growth kinetics over seven days demonstrated there were no significant differences in cell growth between NC and PC cells,

suggesting PDZK1 expression did not influence BE cell growth (Fig. 2).

PDZK1 suppressed PSI-induced apoptosis

In a fluorescence microscopy qualification analysis of apoptosis using HO342 dye, PSI clearly increased the level of apoptosis in NC cells (Fig. 3a, b). However, PSI-induced apoptosis was clearly suppressed in PC cells, suggesting that overexpression of PDZK1 conferred apoptosis resistance to OE33 cells. Quantification of apoptosis by flow cytometry revealed that PSI significantly induced apoptosis in NC cells, but not in PC cells, confirming the antiapoptotic effect of PDZK1 on BE cells (Fig. 4a, b).

Molecular mechanism of anti-apoptotic effect of PDZK1

In response to PSI stimulation, the levels of cleaved-caspase3 were significantly upregulated in NC cells, but not in PC cells. Increased cleaved-caspase3 in PSI-stimulated NC cells correlated with reduced levels of pro-caspase3. In contrast, PSI stimulation failed to affect the levels of both pro-caspase3 and cleaved-caspase3 in PC cells (Fig. 5), suggesting the anti-apoptotic mechanism of PDZK1 on BE cells. In NC cells, PSI significantly decreased the expression of Bcl-2 without affecting Bax levels. In contrast, high expression of both Bcl-2 and Bax was found in PC cells, regardless of PSI stimulation (Fig. 5).

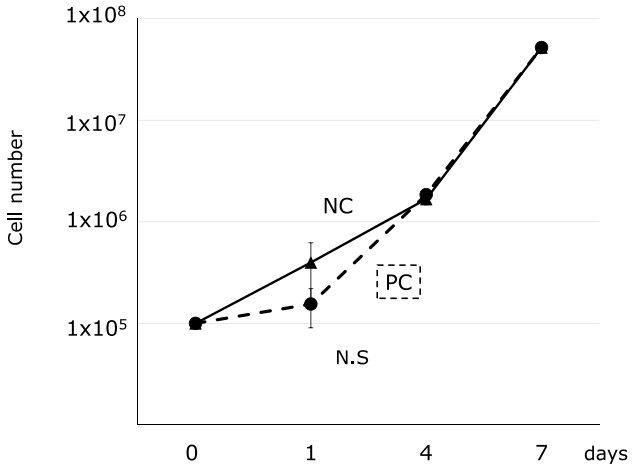

Fig. 2 Effect of PDZK1 overexpression on cell growth. NC or PC cells were seeded into 10-cm culture dishes containing DMEM/10% FBS (1×10^5 cells/dish) and the number of cells in each dish were counted using an automated cell counter on days 1, 4, and 7 post seeding. There were no significant differences in cell growth between NC and PC cells. Data are presented as mean ± SEM of three independent experiments, each performed with triplicate samples

Discussion

In the current study, we determined for the first time the anti-apoptotic phenotype-inducing effect of PDZK1 in BE cells. PDZK1 is a scaffolding protein and therefore directly binds to a variety of receptors and transport proteins [18], resulting in a variety of functions in organs and cells. For example, PDZK1 interacts with C-terminal residues of uric acid transporters in renal tubular cells and modulates its metabolism [19], mediates cholesterol metabolism in hepatocyte [20], and is involved in high-density lipoprotein (HDL)-signaling in vascular cells via binding to the HDL-receptor [21]. In a previous human study, we found that PDZK1 gene expression is higher in the mucosa of LSBE compared to that of SSBE [7], suggesting a possible function of PDZK1 on the elongation of BE. For cells to proliferate and develop into cancer, they must achieve several phenotypes, such as rapid growth, resistance against harmful stimuli, and/or the ability to metastasize to remote organs. Therefore, in this study we investigated the effect of PDZK1 on cell growth and apoptosis using BE cells.

We first investigated the effect of PDZK1 on cell growth and found that PDZK1 had no influence on cell growth of BE cells. The relationship between PDZK1 and cell growth has been previously reported in many studies. Some reports indicate that PDZK1 overexpression decreases cell growth. For instance, in renal cell carcinoma, PDZK1 expression exhibits tumor suppressive effects such as the inhibition of cell proliferation, migration, and invasion [22]. In addition, low-level PDZK1 expression has been reported in clear cell carcinoma of kidney tissues [10] and in gastric cancer cells [23], which correlates with increased growth of cancer cells and poor prognosis. In contrast, a cell-growth promoting effect of PDZK1 has also been reported. PDZK1 expression is upregulated in breast cancer cells and promotes their growth, while it is not expressed in normal breast tissue [24]. The fact that PDZK1 had no effect on cell growth in our study indicate that several other functional proteins, such as transporters or receptors, may vary among the cell types reported.

Next, we investigated the effect of PDZK1 on apoptotic cell death, which is one of the important phenotypes regarding cell survival. Consistent with what is observed in various cells, treatment of the NC cells with PSI significantly induced apoptosis; however, the overexpression of PDZK1 significantly suppressed the PSI-induced apoptosis in PC cells. This is similar to that in breast cancer cells where the anti-apoptotic function of PDZK1 has also been reported [24], [25]. In addition, increased PDZK1 expression in multiple myeloma cells induces an apoptosis-resistant phenotype against various chemotherapeutic drugs

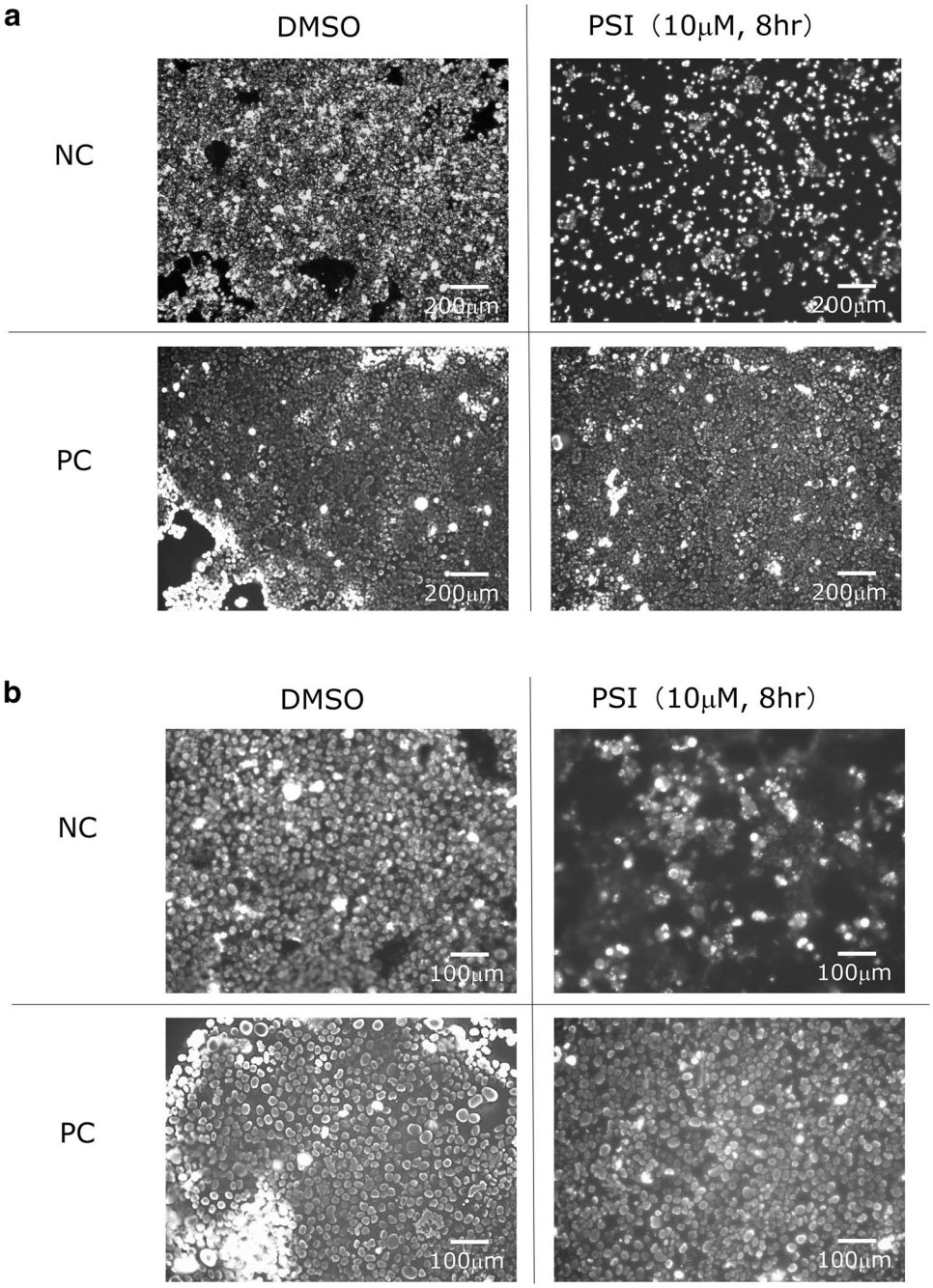

Fig. 3 Morphological assessment of PSI-induced apoptosis. Confluent NC or PC cells were treated with 10 μM PSI or DMSO for 8 h. After treatment, HO342 dye was added for 15 min and cell morphology then assessed using an inverted fluorescence microscope. **a** × 10 and **b** × 20 objective lens. Photographic images were obtained from three random microscopic fields. Representative images are shown. PSI clearly increased the level of apoptosis in NC cells but not in PC cells

[26]. The anti-apoptotic phenotype induced by PDZK1 in the current study may enable BE cells to survive against harmful stimuli, such as excessively refluxed gastric acid, immune reactions, or oxidative stress, thereby resulting in the elongation of BE.

In this study, we also investigated the molecular mechanism by which PDZK1 induced the anti-apoptotic phenotype in BE cells and found a direct effect of PDZK1 on the caspase-3-involved apoptotic signaling pathway. In the upstream portion of the apoptotic signaling pathway, activation of mitochondria localized Bax and Bcl-2 proteins drives the pro-apoptotic and anti-apoptotic effects, respectively. The conformation change of Bax protein induces the release of cytochrome C from mitochondria into the cytosol, activates caspases, and enhances the apoptosis signal. In BE cells with high PDZK1 expression (PC cells), elevated Bcl-2 expression was observed, which was not suppressed by PSI. Since higher Bcl-2 levels have been reported in human BE and EAC compared to cells in normal esophageal mucosa [27], the overexpression of PDZK1 in BE cells may be involved in the increase of Bcl-2 levels, thereby raising the malignant potential in the BE cells. Elongation of

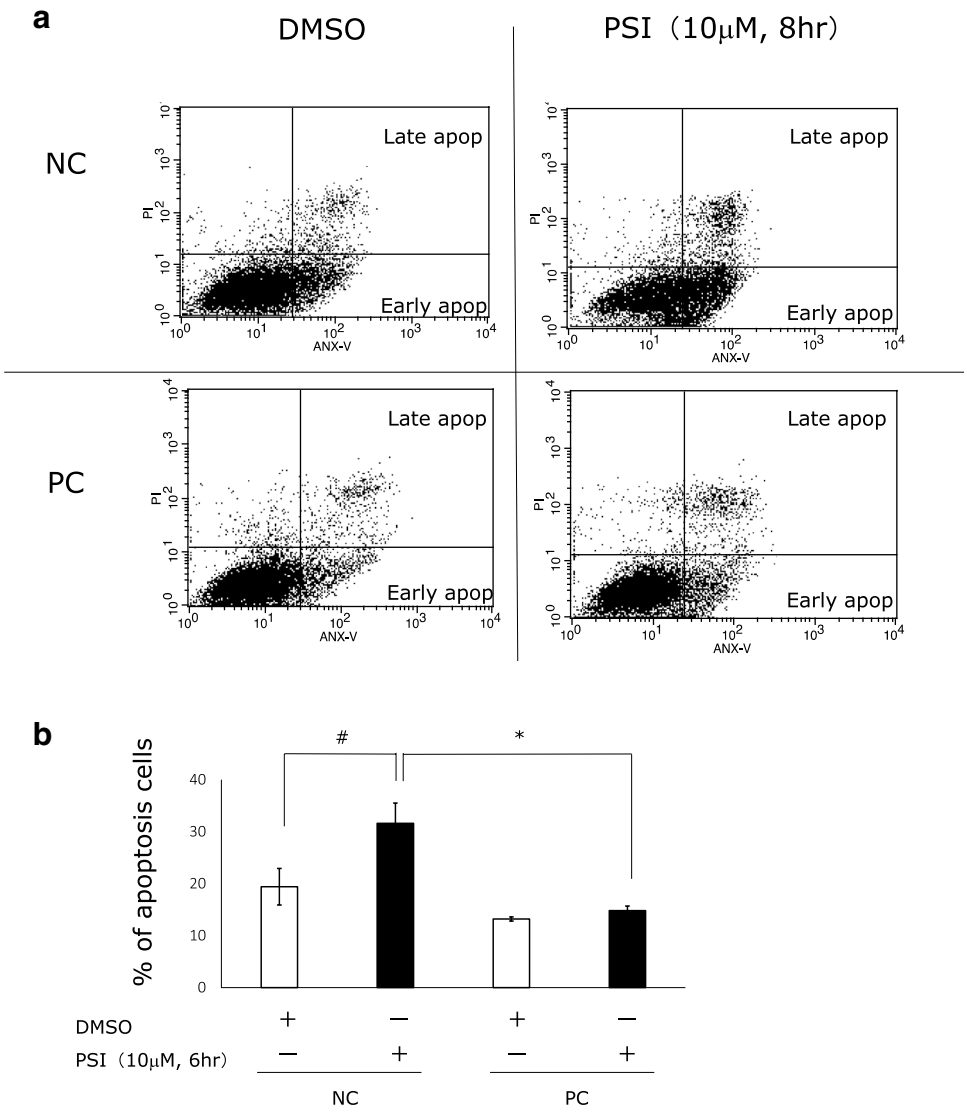

Fig. 4 Quantification of PSI-induced apoptosis. Confluent NC or PC cells were stimulated with 10 μM PSI or 0.5% DMSO for 6 h. The cells were then collected, resuspended in PBS, and 1.0×10^6 cells of each sample incubated with 100 μl PI and Annexin-V solution for 15 min at 37 °C. The stained cells were evaluated using a FACSCalibur flow cytometer and analyzed using CELLQuest Pro ver 6 software. Data are presented as mean ± SEM of three independent experiments, each performed with triplicate samples. **a** Representative flow cytometry scatter plots. **b** Analysis of each group for the percentage (%) of apoptotic cells. #$p < 0.05$ vs. NC cells treated with DMSO, *$p < 0.001$ vs NC cells treated with PSI. PSI significantly induced apoptosis in NC cells, but not in PC cells

BE has been associated with an accumulation of oxidative DNA damage, which has been reported to cause telomerase activation and mutation of the *p53* gene, a checkpoint gene of proliferation and apoptosis [28]; this may result in the progression of BE to carcinogenesis. Fibrates are drugs used for the treatment of hyperlipidemia and have been reported to activate the nuclear hormone receptor peroxisome proliferator-activated receptor-alpha (PPARα) and reduce the expression of PDZK1 [29]. These drugs may be good candidates for the suppression of BE elongation and its malignant transformation. As there is currently no clinical data available regarding the effect of fibrates on the reduction of BE, it would be interesting to investigate this issue.

Although we report for the first time the novel function of PDZK1 in BE cells, this study had limitations. First, we used only one cell line. As the function of PDZK1 is dependent on the type of cells, as we described above, the effect of PDZK1 should be evaluated in additional BE-derived cell lines. As a non-neoplastic BE-derived cell line, BAR-T or Seg-1 has widely been used for the study of BE development, however, BAR-T behaves like a fibroblast and is doubted its BE origin [30] and Seg-1 exhibits typical lung epithelial characteristics [31]. Therefore, human EAC cell lines containing fewer genomic alterations, such as OE33 or OE19 [32], were selected in our study. Although we tried PDZK1 transfection in both OE33 and OE19, we were able to establish PDZK1 overexpressed OE33 but not OE19. It must be noted that in this study we used an EAC cell line with a forced expression vector, which may have affected the amount of PDZK1 expressed. Therefore, the effect of PDZK1 expression may be different in native BE cells. Second, although the suppressive effect of PDZK1 on PSI-induced apoptosis of BE cells was apparent, as well as increased levels of cleaved caspase-3, a final executer of apoptosis, many signaling molecules may be involved in apoptotic pathways. Therefore, the precise mechanism should be further examined in future

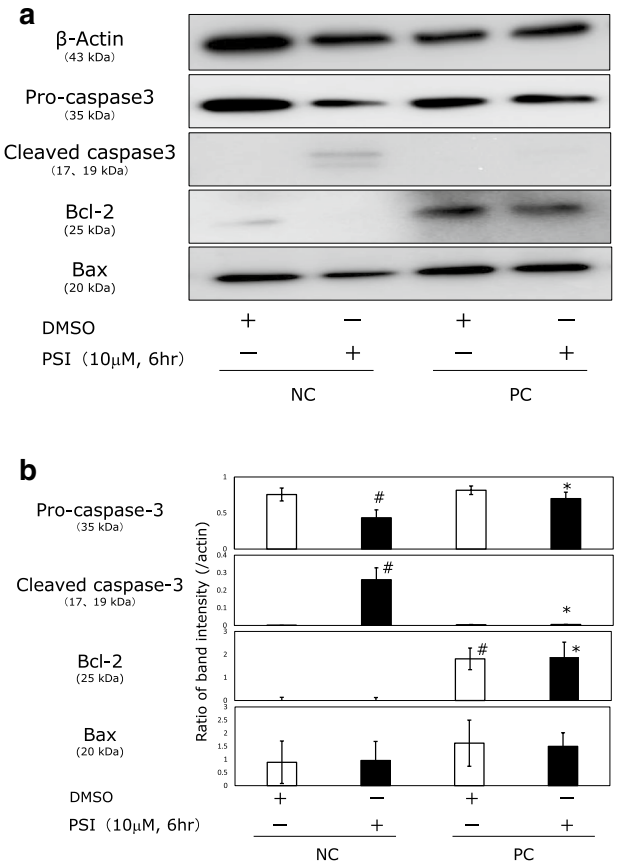

Fig. 5 Expression of apoptosis-related proteins. Confluent NC or PC cells were treated with 10 μM PSI or 0.5% DMSO for 6 h. The cells were then collected and resuspended in PBS. Protein levels of pro-caspase3, cleaved-caspase3, Bcl-2, Bax, and β-actin were assessed by western blotting. **a** Representative western blot images. **b** Quantification of western blot protein bands. The intensity of each band was measured relative to β-actin using ImageJ software. Data are presented as mean ± SEM of three independent experiments. #$p < 0.05$ vs. NC treated with DMSO, *$p < 0.05$ vs. NC treated with PSI

studies. Third, in the current study we employed PSI as an inducer of apoptosis. In the clinical setting, gastric acid, bile acid, oxidative stress, and cytokines correlate with the elongation of BE [33]. Therefore, these stimuli should also be examined in a future study. The limitation of our previous study should be also noted. In our previous study, we found that the gene expression of PDZK1 was significantly higher in LSBE compared to SSBE. However, the expression of PDZK1 gene in EAC was not significantly upregulated compared with its background BE mucosa. Therefore, we speculated that PDZK1 plays an important role in the elongation of BE, which might increase the risk of incidence of EAC. Since the expression of PDZK1 protein in SSBE, LSBE and EAC has not been reported, this issue should be further investigated.

Taken together, our current results demonstrated the overexpression of PDZK1 induced an apoptosis-resistant phenotype in BE cells, thereby possibly potentiating the elongation and neoplastic transformation of BE. Therefore, PDZK1 in BE cells is a potential target for increasing their susceptibility to therapeutic drugs.

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Author contributions OH, AS, and KG contributed substantially to the conception or design of the work and the acquisition, analysis and interpretation of the data. KG, YH, SF, MO, TM, and HM collected data in this study. OH drafted the manuscript and AS, EU, YF, and KN critically revised it for important intellectual content. All authors approved the final version to be published and agreed to be accountable for all aspects of the work, including to ensure that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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Compliance with ethical standards

Ethical statement None.

Conflict of interest The all authors declare that they have no conflict of interest.

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