a result, the survival rate of patients with pancreatic cancer has not substantially improved over the last few decades. Therefore, it is very important to develop effective chemotherapeutic and chemopreventive agents and to elucidate causative factors and mechanisms underlying pancreatic carcinogenesis. Epidemiological studies have suggested that several agents may have a chemopreventive potential against pancreatic cancer [253]. Here, we focus on the inflammatory factors in pancreatic carcinomas and the chemotherapeutic and chemopreventive agents having anti-inflammatory activity.

Agents that inhibit COX-2

COX-2 selective inhibitors

As described, COX-2 expression is elevated both in CP and PDACs. Selective COX-2 inhibitors prevent the growth of cancer cells [254]. In addition, preclinical and clinical data suggest that selective COX-2 inhibitors have the potential to prevent pancreatic cancer prolongation [255, 256]. It is thus likely that increased expression of COX-2 is an important contributor to pancreatic tumor formation, and compounds that inhibit the activity and/or expression level of this enzyme are potentially of great interest as candidate chemopreventive agents against pancreatic carcinogenesis. On the other hand, although major efforts have been made to develop selective inhibitors of COX-2 as chemopreventive agents against pancreatic cancer, efforts to identify agents that can selectively suppress the expression of COX-2 at the gene level appear to be equally important. It is also likely that the combination of suppression of COX-2 gene expression and selective inhibition of its enzyme activity may provide a most effective approach to pancreatic cancer prevention. Therefore, developing a simple screening system, which could detect the suppression of COX-2 gene expression, might be useful in searching for novel chemopreventive agents.

Nimesulide (4-nitro-2phenoxymethanesulfonanilide), a preferential COX-2 inhibitor of non-steroidal anti-inflammatory drugs (NSAIDs) [257], is used clinically as an anti-inflammatory drug in several European countries. This compound is a potent anti-inflammatory agent with fewer ulcerogenic effects than other NSAIDs, and severe side effects have not been reported [257].

Nimesulide has been demonstrated to suppress the development of precancerous lesions (atypical hyperplasia) and PDACs in BOP-treated hamsters. Proliferating cell nuclear antigen labeling indices of pancreatic ducts were also significantly reduced by nimesulide [258]. Therefore, the mechanism underlying the chemopreventive effect of nimesulide was suggested to be the inhibition of cell growth.

Funahashi et al. also evaluated the efficacy of nimesulide in preventing the progression of mPanINs using a *LSL-KRAS^{G12D};PDX-1-Cre* mouse model. Treatment with nimesulide inhibited COX-2, significantly decreased PGE_2 levels in the pancreas, and led to reduced mPanINs, particularly later-stage lesions (mPanIN2 and mPanIN3) [259]. These data clearly suggest that COX-2 and COX-2-derived prostanoids are critical mediators in pancreatic carcinogenesis. Inhibition of COX-2 may represent an intriguing strategy to prevent pancreatic cancer in high-risk patients.

NSAIDs

NSAIDs are some of the most commonly used pharmaceuticals worldwide. They are used for the prevention and treatment of inflammatory diseases. Aspirin is the most frequently used NSAID and has been reported to reduce cancer risk in several organs such as the colon [260]. However, in the pancreas, epidemiological data on aspirin use are controversial. A cohort study of post-menopausal women has shown that current use of aspirin is associated with reduced risk of pancreatic cancer (the multivariate-adjusted RR=0.57) [261], whereas another cohort study of nurses demonstrated that more than 20 years of regular aspirin use is associated with increased risk (RR=1.58) [262]. It is difficult to understand these epidemiological data because regular users of aspirin possibly include CP patients.

In GEM models, aspirin treatment has been shown to delay the progression of PanINs in *LsL-KrasG12D;Pdx1-Cre* mice and to partially inhibit the development of invasive carcinomas in *LSL-KrasG12D;LsL-Trp53R172H;Pdx1-Cre* mice [263]. Meanwhile, the NF-κB pathway has been implicated in pancreatic cancer biology [264]. Aspirin is a surrogate pharmacological inhibitor of the NF-κB pathway, and aspirin treatment inhibited tumor formation in mice [265, 266].

Laboratory studies indicate that aspirin may inhibit pancreatic carcinogenesis, but epidemiologic data to support this finding are limited. To identify aspirin as an effective chemotherapeutic and chemopreventive agent against pancreatic cancer, further detailed studies of its role might be warranted.

Takahashi et al. investigated the effects of prostanoid synthesis inhibitors, such as indomethacin, phenylbutazone, and aspirin, on the development of BOP-initiated hamster pancreatic tumors [267]. The incidence of pancreatic carcinoma was significantly lower in hamsters receiving phenylbutazone than in the controls and the numbers of carcinomas per hamster were significantly reduced by indomethacin and phenylbutazone treatment compared with the control group value. Aspirin also showed a tendency to decrease pancreatic tumor incidence, although not significantly. Thus, inhibition of prostanoid synthesis might help reduce the development of pancreatic cancer.

Other (COX-2 specific and non-specific) NSAIDs, including etodolac, sulindac, ibuprofen, celecoxib, and NS-398,

have also shown efficacy in cellular and animal models [268–272], but they have not been fully evaluated for the prevention and/or treatment of pancreatic cancer.

For instance, some case report studies have also shown that NSAIDs such as aspirin, sulindac, indomethacin, ketoprofen diclofenac, and naproxen could induce acute pancreatitis [273–278]. To resolve the discrepancy, further investigation is needed to better elucidate how COX-2 inhibition might affect pancreatitis.

The effect of dual COX-1/2 inhibitor sulindac on mPanIN and PDAC development was studied using caerulein-treated K-*Ras*$^{+/G12V}$;*Elas-tTA/tetO-Cre* mice. Sulindac treatment for 3 months after caerulein exposure for 3 months significantly reduced the numbers of high-grade PanIN lesions and PDACs [240]. These results suggest that inflammation is a key contributor to the effect of pancreatitis not only in promoting mPanIN formation but also in inducing progression to PDAC.

Agents that inhibit iNOS

As mentioned, increased expression of iNOS has been frequently detected in pancreatic cancers and severe AP patients [164, 165]. We have demonstrated that an iNOS inhibitor, ONO-1714, can effectively suppress the development of atypical hyperplasia and carcinomas, especially invasive adenocarcinomas, in hamster pancreas after treatment with BOP [167]. The results indicated that iNOS plays important roles in the development of pre-neoplastic lesions at an early stage of pancreatic carcinogenesis and also in carcinoma invasion and expansion in later stages. ONO-1714 also attenuated rat diaphragmatic dysfunction associated with cerulein-induced AP through the reduction of iNOS activity and lipid peroxidation [166]. These results could serve as basis of clinical research to assess whether the use of iNOS-selective inhibitors is a promising approach to the management of patients with pancreatitis and pancreatic cancer.

Agents that inhibit oxidative stress

There is increased oxidative stress in experimental animals as well as in patients with CP, and suppression of oxidative stress by antioxidative agents has been demonstrated to reduce the severity of pancreatitis in animals and humans. Moreover, suppressive effects of antioxidative agents on pancreatic cancer development have also been demonstrated in animals.

Treatment with the flavonoid quercetin markedly reduced the severity of cerulein-induced pancreatitis, malondialdehyde, and the serum levels of TNF-α, IL-1β, and IL-6 in mice [279]. Quercetin ameliorates the severity of cerulein-induced AP by acting as an anti-inflammatory and antioxidant agent. Treatment of mice with green tea polyphenol attenuates the degree of cerulein-induced mouse pancreatitis by reducing the activation of NF-κB, the production of pro-inflammatory cytokines, and the formation of lipid peroxidation [280]. Patients with topical pancreatitis received oral curcumin, which reduced erythrocyte malondialdehyde levels and increased the glutathione levels compared with a placebo [281]. When methionine was supplemented along with selenium, β-carotene, vitamin C, and vitamin E, CP symptoms were improved [282, 283].

Furthermore, treatment with the combined antioxidant supplement decreased the serum levels of the free radical marker 9-*cis*,11-trans linoleic acid, which was initially significantly higher in CP patients [282, 283]. A randomized clinical trial for combined antioxidant supplementation has been reported. One hundred twenty-seven CP patients were randomly assigned to receive either an antioxidant supplement, which contained selenium, ascorbic acid, β-carotene, α-tocopherol, and methionine, or a placebo. After 6 months, reduction in the levels of thiobarbituric acid-reactive substances and superoxide dismutase, which are markers of oxidative stress, was observed in the antioxidant group compared with the placebo group. Pain was also diminished in patients receiving the supplement. Significantly fewer painful days per month compared with the placebo group were reported by questionnaire [284]. Combined antioxidant supplementation might appear to be more promising for CP treatment than single antioxidant supplementation.

Protochatechuic acid, green tea extracts, and butylated hydroxyanisole are antioxidative agents which have demonstrated inhibitory effects on pancreatic cancer development during the post-initiation stage of the BOP-initiated hamster model [285–287]. Sarcophytol A, which is known to be an anti-tumor promoter, and methionine, which is an essential amino acid and associated with antioxidation, have also been shown to suppress pancreatic carcinogenesis in the BOP-treated hamster model [288, 289]. Woutersen et al. [290] reported that antioxidant products, such as β-carotene, selenium, and vitamin C, inhibit pancreatic carcinogenesis in azaserine-treated rat model. Therefore, it is considered that treatment with antioxidants may have practical application in chemoprevention of pancreatitis and pancreatic cancer.

Anti-hyperlipidemic/anti-type II diabetic agents

A high-calorie diet and low physical activity are associated with an increased risk of pancreatic cancer. Moreover, they are also closely associated with hyperlipidemia [291]. High serum TG levels are known to cause pancreatitis. It is also known that hypertriglyceridemia often precedes hyperglycemia in type II diabetes. Interestingly, Syrian golden hamsters are in a hyperlipidemic state even under normal diet conditions [211]. Thus, hyperlipidemia in hamsters may also be an enhancing factor for PDAC development.

Peroxisome proliferator-activated receptor γ (PPARγ) is a member of nuclear receptor superfamily of ligand-activated nuclear transcription factors, which prominently express in adipose tissue and in the immune system [292, 293]. Moreover, PPARγ is involved in the regulation of lipid and glucose homeostasis and also controls inflammation [294].

Thiazolidinediones (TZDs) are ligands for PPARγ, and one of the TZD derivatives, pioglitazone, has been clinically accepted as an anti-diabetic drug. Our previous study showed that dietary intake of pioglitazone improves hyperlipidemia and suppresses the incidence and multiplicity of pancreatic tumors in BOP-treated hamsters; the PDAC incidences in the BOP + 800 ppm pioglitazone group and the BOP-alone group were 38 vs 80 % ($P<0.01$) and the multiplicities were 0.55±0.15 vs 1.37±0.22 ($P<0.01$), respectively [211]. It is well established that administration of TZDs improves hyperlipidemia and hyperglycemia in animal models [295, 296]. Thus, anti-hyperlipidemic drugs may deserve more consideration as candidate chemopreventive agents against pancreatic cancer.

Metformin (1,1-dimethylbiguanide hydrochloride) is a biguanide class of oral hypoglycemic agents and it is the world's most widely used anti-diabetic drug for the treatment of type II diabetes mellitus. In a study using a hamster model, hamsters fed a high-fat diet and metformin had lower numbers of pancreatic carcinoma, proliferative lesions, and pre-neoplastic lesions than hamsters fed a high-fat diet alone [297]. In addition, administration of metformin significantly decreased the growth of pancreatic carcinoma cells xenografted into the flank of nude mice [298]. A large case–control clinical trial regarding the use of metformin for pancreatic cancer risk was conducted from 2004 to 2008 involving 973 pancreatic cancer patients (including 259 diabetes mellitus patients) and 863 controls (including 109 diabetes mellitus patients) [299]. Diabetes mellitus patients who were administrated metformin had a significantly lower risk of pancreatic cancer compared to those who did not receive metformin (OR, 0.38; 95 % confidence interval (CI), 0.22–0.69, $P=0.001$). These recent studies clearly suggest that the administration of metformin is positively associated with a decreased risk of pancreatic cancer in diabetes mellitus patients. Based on these positive associations between hyperinsulinemia, diabetes, and pancreatic cancer, therapeutic targets aimed at treating diabetes should decrease the risk of pancreatic malignancy. Metformin has been found to inhibit the production of inflammatory cytokines such as TNF-α and IL-6 as well as VEGF, probably via inactivation of NF-κB and HIF-1α [300–302]. Additionally, antioxidant and tumor growth inhibition activities have been shown for the potential function of metformin [303, 304]. These data imply that metformin may appear to exert a protective role

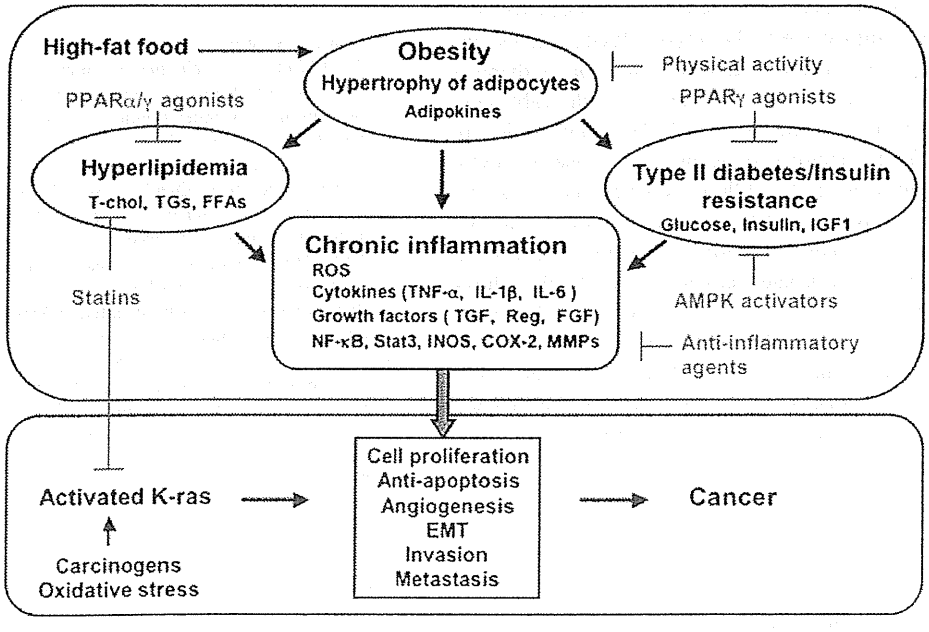

Fig. 2 Cancer promotion by chronic inflammation and its prevention by anti-inflammatory agents. K-*ras* mutations plus inflammatory status including CP, hyperlipidemia, type II diabetes/insulin resistance, and obesity effectively cause PDAC development. Chemopreventive agents targeting these inflammatory factors may prevent PDAC development. *TG* triglyceride, *FFA* free fatty acid, *ROS* reactive oxidative species, *TNF* tumor necrosis factor, *IL* interleukin, *TGF* transforming growth factor, *FGF* fibroblast growth factor, *NF-κB* nuclear factor-κB, *Stat3* signal transducer and activator of transcription 3, *iNOS* inducible nitric oxide synthase, *COX-2* cyclooxygenase-2, *MMP* matrix metalloproteinase, *EMT* epithelial to mesenchymal transition

against the development and progression of pancreatic cancer through improvement of inflammation.

Statins, inhibitors of 3-hydroxy-3-methylglutaryl coenzyme-A reductase, are widely used for the treatment of lipid disorders, especially hypercholesterolemia [305]. Furthermore, several preclinical studies revealed their pleiotropic actions, such as apoptosis induction, anti-angiogenesis, tumor growth suppression, metastasis suppression, anti-inflammation, and K-ras prenylation inhibition properties [306–308]. Thus, the use of statins as pancreatic cancer chemopreventive agents is expected.

Treatment with pravastatin started after the induction of CP attenuates progression of pancreatic inflammation in a rat experimental model. Pravastatin also downregulated the expression level of pro-inflammatory cytokines such as TNF-α and markedly increased the production of anti-inflammatory cytokines such as IL-10 [309]. In experimental data, statins were associated with a decreased risk of pancreatic cancer [310, 311]. Furthermore, one case–control study demonstrated 67 % risk reduction in incidence of pancreatic cancer among statin users (adjusted OR, 0.33; 95 % CI, 0.26–0.41; $P<0.01$) [312]. However, other observational studies suggested that there is no association between statin use and pancreatic cancer risk [313]. Thus, the use of statins as a chemopreventive agent remains a matter of debate.

Still some studies have declared the availability of statins for adjuvant chemotherapy. A dramatic synergism between lovastatin and troglitazone (a TZD-type PPARγ agonist and used for improving insulin sensitivity in type II diabetes mellitus patients) in anti-cancer at clinically achievable concentrations has been indicated [314]. The combination of fluvastatin with the anti-cancer agent gemcitabine (the cytosine arabinoside analog 2′,2′-difluorodeoxycytidine) is an effective cytotoxic, proapoptotic treatment in vitro and in vivo against MIAPaCa-2 cells harboring a mutated K-*ras* by a mechanism of action mediated, at least in part, by the inhibition of prenylation of K-ras and rhoA proteins [315]. These results support the probability of statins for the adjuvant chemotherapy treatment of pancreatic cancer. Further investigations may be needed to ensure the combination chemotherapy of anti-diabetic drugs or anti-cancer agents and statins for pancreatic cancer.

Conclusions

Epidemiological studies and animal model studies have shown an increase of pancreatic cancer risk by inflammatory status, such as CP, hyperlipidemia, type II diabetes, and obesity (Fig. 2). Acinar/ductal cell damages caused by exposure to alcohol, carcinogens derived from tobacco, lipids, high amounts of glucose, pancreatic enzyme/bile reflux by ductal obstruction, and following cellular proliferation stimulated by further exposure to pro-inflammatory cytokines, chemokines, adipokines, and growth factors are considered to favor the development of pro-tumorigenic environments. In addition, K-*ras* activating mutations, which can be induced by tobacco carcinogens or ROS, are essential for pancreatic cancer development. Indeed animal model studies have demonstrated that chemical or genetic induction of K-*ras* mutations plus inflammation effectively causes PDAC development.

In humans, a number of epidemiological studies have suggested reduced pancreatic cancer risk with the use of anti-inflammatory agents. However, this is difficult to prove in randomized control studies because of the relatively low incidence of pancreatic cancer in humans and the absence of early biomarkers to predict pancreatic cancer. Thus, in vivo carcinogenesis studies using animal models are important to support the epidemiological findings and provide direct evidence. Some anti-inflammatory agents that target COX-2, iNOS, oxidative stresses, insulin resistance, and hyperlipidemia have indeed been shown to exert suppressive effects on pancreatic carcinogenesis in animal models, indicating that factors related to inflammation are candidate targets for pancreatic cancer prevention.

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Mouse Model for ROS1-Rearranged Lung Cancer

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Abstract

Genetic rearrangement of the *ROS1* receptor tyrosine kinase was recently identified as a distinct molecular signature for human non-small cell lung cancer (NSCLC). However, direct evidence of lung carcinogenesis induced by *ROS1* fusion genes remains to be verified. The present study shows that *EZR-ROS1* plays an essential role in the oncogenesis of NSCLC harboring the fusion gene. *EZR-ROS1* was identified in four female patients of lung adenocarcinoma. Three of them were never smokers. Interstitial deletion of 6q22–q25 resulted in gene fusion. Expression of the fusion kinase in NIH3T3 cells induced anchorage-independent growth *in vitro*, and subcutaneous tumors in nude mice. This transforming ability was attributable to its kinase activity. The ALK/MET/ROS1 kinase inhibitor, crizotinib, suppressed fusion-induced anchorage-independent growth of NIH3T3 cells. Most importantly, established transgenic mouse lines specifically expressing EZR-ROS1 in lung alveolar epithelial cells developed multiple adenocarcinoma nodules in both lungs at an early age. These data suggest that the *EZR-ROS1* is a pivotal oncogene in human NSCLC, and that this animal model could be valuable for exploring therapeutic agents against *ROS1*-rearranged lung cancer.

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Introduction

Lung cancer is the leading cause of cancer death around the world [1]. Lung adenocarcinoma (LADC), the most common form of non-small-cell lung cancer (NSCLC), comprises several different genomic subsets defined by unique oncogenic alterations, and a considerable proportion of LADC cases harbor driver alterations in the *EGFR*, *KRAS* and *ALK* genes at the mutually exclusive manner with rare exceptions [2–5]. Understanding the molecular basis of cancer allows us to develop therapeutic agents that target genetic druggable aberrations identified in cancer genomes. Tyrosine kinase inhibitors (TKIs) that target the EGFR and ALK proteins are particularly effective in the treatment of LADC carrying *EGFR* mutations and *ALK* fusions, respectively [2–6]. However, the development of an effective TKI requires experimental validation of the genetic aberrations as actionable and druggable. Transgenic mouse models harboring *EGFR* mutations or *EML4-ALK* gene fusions have successfully demonstrated the oncogenic potential of the alterations and the efficacy of TKI therapy [7,8]. Genetic rearrangement of the *ROS1* was recently identified as a distinct molecular signature for human LADC [9–16]. In the present study, we established a mouse model of *ROS1* fusion, and showed that *EZR-ROS1* as an essential driver oncogene in lung carcinogenesis.

Results

Identification of EZR-ROS1 Fusion Gene in LADC of Never-smokers

Whole transcriptome high-throughput sequencing of tumor specimens is one of the most effective methods for identifying fusion oncogenes [17]. Analysis of five LADC cases of never-smokers without *EGFR/KRAS/ALK* alterations using transcriptome sequencing identified 56 reads overriding the in-frame *EZR-ROS1* gene fusion point connecting *EZR* exon 10 to *ROS1* exon 34 in one tumor. RT-PCR analysis of matched non-cancerous tissues confirmed tumor-specific expression of the fusion transcript (Figure 1A). In addition, transcriptome sequencing clearly demonstrated a specific increase in the expression of the fused 3′ portion of *ROS1* (exons 34 to 43) after the breakpoint, suggesting that the *EZR-ROS1* fusion transcript causes aberrant overexpression of *ROS1* tyrosine kinase domain along with the 5′ portion of *EZR* (Figure 1B). SNP array comparative genomic hybridization (array CGH) data showed that this fusion gene was generated by a large interstitial deletion spanning ~41.5 Mb on chromosome 6q22-q25 (Figure 1C). Genomic PCR and sequencing analysis also revealed the deletion of 41.5 Mb causing somatic fusions of the

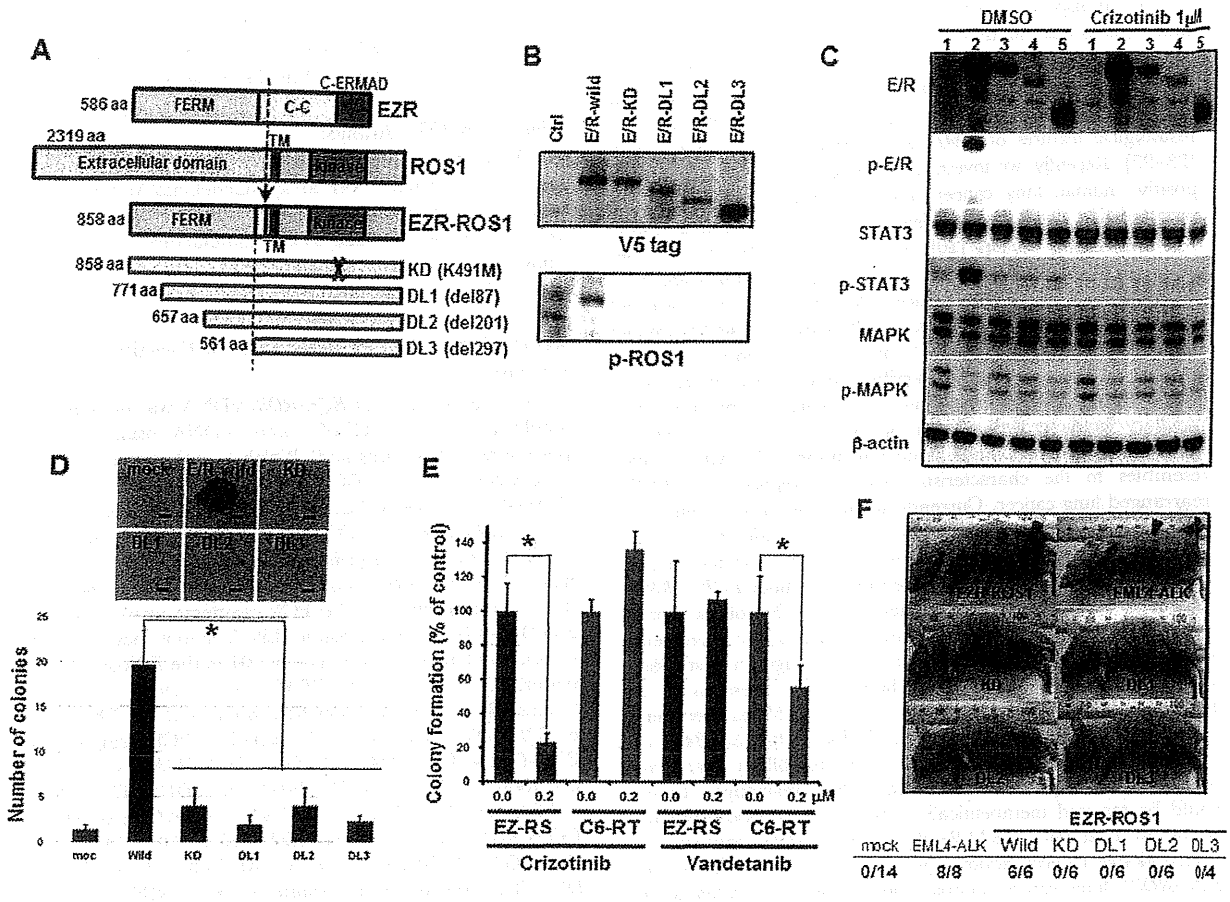

Figure 2. Oncogenic activity of the *EZR-ROS1* fusion gene. (A) Schematic representation of EZR, ROS1, EZR-ROS1, and deletions/mutations of EZR-ROS1 genes. The domain organization is shown. C-C: coiled-coil domain; TM: transmembrane; C-ERMAD: C-terminal ERM associated domain. (B) ROS1 phosphorylation in wild-type and mutant EZR-ROS1 (E/R)-expressing NIH3T3 clones. Cell lysates from each clone were immunoblotted with anti-V5-tag (top) and anti-phosphorylated ROS1 (Tyr-2274, bottom) antibodies. (C) Suppression of ROS 1 kinase activity of EZR-ROS1 by crizotinib inhibits STAT3 activation. NIH3T3 cells transfected with 1: empty vector, 2: wild-type EZR-ROS1, 3: KD 4: DL1, 5: DL3 were serum starved and treated for 2 hr with DMSO or 1 μM of crizotinib, and immunoblotted with the relevant antibodies. β-actin was used as a loading control. E/R: EZR-ROS1, p-E/R: phosphorylated EZR-ROS1 detected with an anti-phosphotyrosine-2274 antibody of ROS1. (D) Soft agar colony formation of wild-type and mutant EZR-ROS1 expressing NIH3T3 clones. A representative picture of colony formation for each clone is plotted at the top (scale bar, 100 μm). The number of colonies obtained for each clone is plotted at the bottom. *P<0.05. (E) Crizotinib-induced suppression of anchorage-independent growth of NIH3T3 cells expressing EZR-ROS1. Bar graph showing the percentage of NIH3T3 colonies induced by *EZR-ROS1* or *CCDC6-RET* after treatment with 200 nM of crizotinib or vandetanib with respect to those formed by DMSO-treated cells. EZ-ROS: EZR-ROS1, C6-RET: CCDC6-RET. *P<0.05. (F) Representative pictures of mice subcutaneously transplanted with NIH3T3 cells expressing wild-type, kinase domain-mutated, or amino-terminal-deleted EZR-ROS1. An EML4-ALK-expressing NIH3T3 clone was used as a positive control. The number of tumors per injection in each transfectant is shown below the photographs.
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and DL mutants-expressing clones produced tumors (Figure 2F), confirming that *in vivo* tumorigenic activity of *EZR-ROS1* requires ROS1 kinase activity.

Development of LADC in EZR-ROS1 Transgenic Mice

To further evaluate the role of *EZR-ROS1* in lung carcinogenesis, we generated transgenic mice expressing the fusion gene under the control of a type 2 alveolar epithelium-specific surfactant C gene promoter [20] (Figure 3A). We obtained four independent lines (TgA, B, C and D) with different copy number of the transgene (Figure S3) and detected lung adenocarcinoma nodules in all lines examined except TgD. Analysis of fusion protein expression level among them revealed no expression in TgD (Figure S4). The birth rate of transgene-positive progenies was low in TgC (Transgene-positive F1 progeny number : total F1 number; 1:3), and we failed to keep up a TgC line, then we mainly analyzed one line (TgA), which harbors approximately four copies of the transgene. RT-PCR and immunoblot analysis verified lung-specific *EZR-ROS1* mRNA and protein expression, and indicated phosphorylation of the EZR-ROS1 fusion protein (Figure 3B). Although endogenous *Ezrin* was ubiquitously expressed in many tissues, endogenous *Ros1*-transcript was detected only in stomach, kidney and lung. Protein expression levels of endogenous ROS1 were very weak compared with the levels of the fusion gene in the transgenic mice (Figure S4). Even at the four-week-old, multiple lesions over 1 mm in diameter were detected in the transgenic mice, and tumors occupied over 40% of sectioned surface of lung (Figure 3C and Figure S5). Computed tomography examination

The transgenic mice showed an emergence of multiple adenocarcinoma nodules at an early point, and the fast progression of the tumors. These features are broadly similar to the *EML4-ALK* mouse model [8]. Several groups reported that mucinous cribriform pattern and signet ring cell are characteristic histological features of *EML4-ALK* positive human lung cancer [23–25]. Recently, we investigated histopathology of *ROS1*-fusion positive human lung cancers [16]. Although other researchers reported that signet ring cell feature was not common in *ROS1*-rearranged lung cancers [10], we found that 53% of the cases harbored mucinous cribriform or signet ring cell features similar to the *ALK*-rearranged lung cancers but that the rest showed papillary/lepidic growth pattern. *EZR-ROS1*-positive tumors seemed less well differentiated, and showed more frequently histological features of mucinous cribriform or signet ring cell. Our mouse model of *EZR-ROS1* lung cancer generally demonstrated papillary/lepidic growth pattern, but in some cases, we observed accumulation of cytoplasmic mucin in tumor cells, which quite resembles to the characteristic histology reported in *ROS1*-rearranged lung cancer. Currently we have no answer why only part of mice harbored tumors with mucin accumulation.

The *EZR-ROS1* fusion gene was specifically detected in lung cancer specimens of female never-smokers without *EGFR, KRAS,* and *ALK* alterations. It was estimated that ~2% of patients in White and Asian lung cancer cohorts had *ROS1*-rearrangements, which occur at significantly higher rates in younger, non-smoking, female individuals [10,11,16]. Although each alteration is infrequent, *ROS1* fusions with many kinds of 5′ partner genes (*CCDC6, CD74, EZR, FIG, KDELR2, LRIG3, SDC4, SLC34A2 and TPM3*) have been reported in lung, brain, biliary tract, and ovarian cancers [9–16,26–28]. These *ROS1*-rearranged tumors could be targeted therapeutically with specific kinase inhibitors, including crizotinib [10,14,27,29]. Two LADC patients had a remarkable clinical response to crizotinib [10,14]. Thus, our *EZR-ROS1* lung cancer animal model could be valuable for evaluating the therapeutic potential of these compounds and novel drugs as well as biological features of *ROS1*-rearranged lung cancer *in vivo*.

Materials and Methods

Clinical Samples

Tissue specimens from lung cancer patients were provided by the National Cancer Center Biobank, Japan. High-molecular weight genomic DNA and RNA were extracted from fresh frozen tumor specimens and non-cancerous lung tissues. Written informed consent was obtained from each patient. The study protocol was approved by the Ethical Committee of National Cancer Center, Tokyo, Japan.

Analysis of Whole-transcriptome Sequence Data

Insert cDNA libraries (150–200 bp) were prepared from 2 μg of total RNA using the mRNAseq Sample Preparation Kit (Illumina). The libraries were subjected to paired-end sequencing of 50 bp on the HiSeq2000 (Illumina), according to the manufacturer's instructions. Paired-end reads were mapped to known RNA sequences in the RefSeq, Ensembl, and LincRNA databases using the Bowtie program as described previously [30].

RT-PCR, Genomic PCR and Sequencing

Total RNA was reverse-transcribed to cDNA using Superscript III (Life Technologies). cDNA or genomic DNA was subjected to PCR amplification using Ex-Taq (Takara Bio) and primers EZR-e10-CF1 (GAAAAGGAGAGAAACCGTGGAG) and ROS1-e34-CR1 (TCAGTGGGATTGTAACAACCAG). The PCR products were directly sequenced by Sanger sequencing using the BigDye terminator kit (Life Technologies).

SNP Array CGH Analysis

Chromosomal copy number for the tumors was determined using high-resolution SNP arrays (GeneChip Mapping 250K-Nsp array, Affymetrix). Genomic DNA was labeled and hybridized to the SNP arrays according to the manufacturer's instructions, and copy numbers were calculated from the hybridization signals using the CNAG program [31].

Vector Cloning, and Generation of Deletion and Point Mutants

The coding region of *EZR-ROS1* cDNA was obtained by PCR amplification from LCY66 tumor cDNA using Phusion Taq polymerase (New England Biolabs) and primers EZR-H1F1 (CACCATGCCGAAACCAATCAATGTCCGAGTT) and ROS1-H1R1 (ATCAGACCCATCTCCATATCCACTCTG). *EML4-ALK* cDNA and *CCDC6-RET* cDNA were amplified from an *EML4-ALK*-positive primary lung cancer sample (E13;A20) and from a *CCDC6-RET*-positive primary lung cancer sample (C1;R12), respectively. The PCR products were subcloned into a pcDNA3.1D-V5-His plasmid (Life Technologies). Replacement of lysine with methionine at codon 491 in the *EZR-ROS1* gene was performed using a PrimeSTAR site-directed mutagenesis kit (Takara Bio). N-terminal deletion mutants of the FERM domain of *EZR-ROS1* cDNA were constructed by PCR using the primers EZR-FERM-AF (CACCATGGTGGCTGAGGAGCTCATC-CAGGACATC) and ROS1-H1R1 for DL1, EZR-FERM-BF (CACCATGATCAACTATTTCGAGATAAAAAACAAG) and ROS1-H1R1 for DL2, and EZR-FERM-CF (CACCATGAC-CATCGAGGTGCAGCAGATGAAGGC) and ROS1-H1R1 for DL3. The plasmids were transfected into NIH3T3 cells using Lipofectamine 2000 reagent (Life Technologies), and stable clones were isolated by G418 selection (0.7 mg/ml). For the colony formation assay, cells were embedded and cultured in 0.4% soft agar in triplicate and the number of colonies was counted after 21 days. Quantification of anchorage–independent growth under the condition with or without crizotinib (S1068, Selleck) and vandetanib (S1046, Selleck) after 9 days was performed with CytoSelect-96 kit (Cell Biolabs). The compound solution was added to the top layer of soft agar every 3 days.

Immunoblot Analysis

Whole cell lysates were extracted with CelLytic M reagent (#C2978, Sigma), and subjected to SDS-PAGE followed by blotting onto a PVDF membrane. Detection of Western blots was performed with the WesternBreeze Chemiluminescent Immunodetection kit (Life Technologies) using primary antibodies against ROS1 (#9202, Cell Signaling Technology), phosphorylated-ROS1 (Tyr2274) (#3078, Cell Signaling Technology), STAT3 (#610189, BD), phosphorylated-STAT3 (Tyr705) (#9138, Cell Signaling Technology), p44/42 MAPK (#4695, Cell Signaling Technology), phosphorylated-p44/42 MAPK (Thr202/Tyr204) (#9106, Cell Signaling Technology), Ezrin (#4135, Cell Signaling Technology), p53 (#6243, Santa Cruz), and b-actin (#A5441, Sigma).

Suppression of ROS 1 Kinase Activity of EZR-ROS1 by Crizotinib

Transfected NIH3T3 cells (empty vector, wild-type EZR-ROS1, KD/DL mutants) were serum starved for 2 hr, then

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Suppression of intestinal polyp development in $Apc^{Min/+}$ mice through inhibition of P-glycoprotein using verapamil

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P-glycoprotein (P-gp; encoded by the *Mdr1a* gene) is known to be associated with colon tumorigenesis through transcriptional activation and/or epigenetic modification. We investigated whether inhibition of P-gp function might decrease intestinal tumorigenesis. We used verapamil as an inhibitor of P-gp function in $Apc^{Min/+}$ mice, which lack a functional *Apc* gene product. We determined the number of intestinal polyps and 1-year survival rates after the ingestion of 10, 25, and 50 mg/kg/day verapamil contained in dry pellets. The number of polyps in $Mdr1a^{+/+}Apc^{Min/+}$ mice fed with pellets containing verapamil was significantly lower than that in mice fed with verapamil-free pellets. The 1-year survival rate of verapamil-fed mice was also improved in a dose-dependent manner. These results were similar to data from P-gp knockout mice. These results indicated that it might be possible to use verapamil to inhibit polyp development during the early stage of colon carcinogenesis. Thus, we propose a novel chemopreventive agent for colorectal cancer that acts by inhibiting P-gp function. *European Journal of Cancer Prevention* 22:8-10 © 2012 Wolters Kluwer Health | Lippincott Williams & Wilkins.

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Keywords: chemoprevention, colorectal cancer, Mdr1a protein, verapamil

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Introduction

P-glycoprotein (P-gp) is encoded by the *Mdr1a* gene and is expressed in many tissues including the small and large intestine, hepatobiliary tract, and placenta. P-gp belongs to the ATP-binding cassette B subfamily and has a major role in the efflux of xenobiotics as a self-defense mechanism (Tanigawara, 2000). This efflux system is known to be a cause of multidrug resistance, which decreases the effects of drug treatments.

We previously demonstrated that P-gp increased the incidence of intestinal polyp development by the direct involvement of TCF/LEF transcription factor activation (Yamada *et al.*, 2003) and/or epigenetic modification (Mochida *et al.*, 2003). Therefore, we hypothesized that it may be possible to control intestinal polyp development by inhibiting P-gp function.

Verapamil has been widely used in the treatment of ischemic heart disease since the 1960s (Haas and Igarashi, 1968). This agent is characterized by intestinal absorption, and it is also a substrate of P-gp. Numerous studies have demonstrated that verapamil can inhibit the efficiency of the efflux function of P-gp through an antagonistic action (Cornwell *et al.*, 1987; Qian and Beck, 1990).

In this study, we examined whether inhibition of P-gp function using verapamil or P-gp deficiency would decrease the number of intestinal polyps that developed in mice lacking a functional *Apc* gene product ($Apc^{Min/+}$), which is a model of human familial adenomatous polyposis.

Methods

Mice

The *Mdr1a* knockout mice (FVB/N) were obtained from Taconic Farms (Germantown, New York, USA), whereas the $Apc^{Min/+}$ (C57B1/6J) mice were supplied by Jackson Laboratories (Bar Harbor, Maine, USA). The mice were maintained under specific pathogen-free conditions at the Center of Biomedical Research, Kyushu University (Fukuoka, Japan). Generation of $Mdr1a^{-/-}Apc^{Min/+}$ mice is described elsewhere (Mochida *et al.*, 2003).

We analyzed 14 mice, which were fed a verapamil-free diet; and 15 mice, which were fed with verapamil. Mice were killed at 5–12 months of age for the analysis of intestinal polyps. All animal experiments were carried out according to the Guidelines for Animal Experiments in the Faculty of Medical Sciences, Kyushu University.

Feed

The mouse feed was compounded with three different doses of verapamil (Vasolan; Eisai, Japan), that is 10, 25, and 50 mg/kg/day. In addition, a verapamil-free control group was also maintained. According to the data sheet provided by the company (Eisai), the maximum acceptable dose of verapamil for an adult dog is 25 mg/kg/day. On the basis of this, we calculated the appropriate dose for mice.

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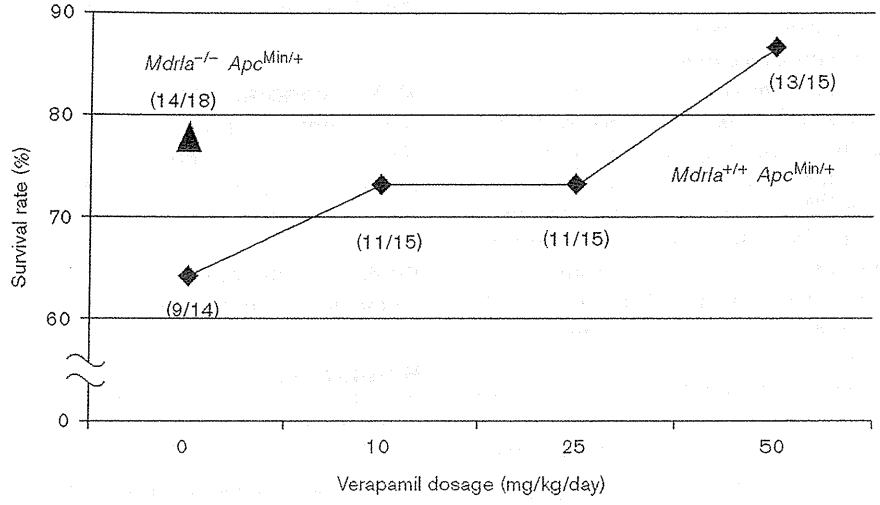
Table 1 The number of intestinal polyps in each mouse

Mouse strain	Dosage of verapamil (mg/kg/day)	Small intestine			Small intestine	Large intestine	Total
		Distal	Middle	Proximal			
Mdr1a$^{+/+}$*Apc*$^{Min/+}$	0	39.8±5.1	20.4±4.3	6.0±1.5	66.4±9.8	3.0±0.8	69.2±9.7
	10	16.2±2.5*	7.4±1.4*	3.3±0.8	26.8±3.9*	1.6±0.6	28.4±4.2*
	25	15.5±2.1*	8.5±1.5*	3.2±0.8	27.1±3.3*	2.2±0.5	29.3±3.3*
	50	14.2±2.0*	6.9±1.1*	3.2±0.8	24.3±3.3*	2.9±0.7	27.2±3.3*
Mdr1a$^{-/-}$*Apc*$^{Min/+}$	0	11.2±2.7*	4.4±1.1*	2.1±0.6	17.8±4.0*	1.6±0.6	19.4±4.4*

Values are mean±SEM.
*$P<0.001$ Dunnett's multiple comparison test.

Fig. 1



One-year survival rates of *Mdr1a*$^{+/+}$*Apc*$^{Min/+}$ (♦) and *Mdr1a*$^{-/-}$*Apc*$^{Min/+}$ mice (▲). The number of mice that survived for 1 year and the original number of mice are given in parentheses.

Genotyping

Genotyping was carried out for each mouse tail sample using a Genomic DNA Purification Kit (Qiagen, Germany), according to the manufacturer's protocol. Allele-specific PCR primers for the *Apc* and *Mdr1a* genes were produced according to previously published methods (Mochida *et al.*, 2003).

Counts of intestinal polyps and statistical analysis

Intestinal polyps were counted according to previously published methods (Mochida *et al.*, 2003). All statistical analyses were carried out using the GraphPad Prism5 program (GraphPad Software Inc., San Diego, California, USA). The correlations of the number of polyps in the mice that ate pellets with and without verapamil and P-gp knockout mice were tested using Dunnett's method. Survival rates were calculated by dividing the number of mice that survived for 1 year by the original number of mice.

Results

Inhibition of P-glycoprotein function using verapamil decreased the number of intestinal polyps

The number of intestinal polyps in *Mdr1a*$^{+/+}$*Apc*$^{Min/+}$ mice that survived for 1 year is shown in Table 1. The mean number of polyps in the small intestines of *Mdr1a*$^{+/+}$*Apc*$^{Min/+}$ mice that were fed pellets containing 10, 25, and 50 mg/kg/day verapamil was significantly lower, that is 26.8, 27.1, and 24.3, respectively, than that in mice fed with verapamil-free diet, which had a mean polyp count of 66.4. In the distal and middle parts of the small intestine, the mean number of polyps was decreased in a dose-dependent manner ($P < 0.001$).

We also crossed *Mdr1a*$^{-/-}$ mice with *Apc*$^{Min/+}$ mice to generate *Mdr1a*$^{-/-}$*Apc*$^{Min/+}$ mice and tested whether verapamil had similar effects on intestinal polyp development as in mice lacking P-gp. We compared the number of intestinal polyps in the *Mdr1a*$^{+/+}$*Apc*$^{Min/+}$ mice treated with verapamil with the P-gp knockout mice (*Mdr1a*$^{-/-}$*Apc*$^{Min/+}$). The number of polyps in *Mdr1a*$^{+/+}$*Apc*$^{Min/+}$ mice with 50 mg/kg/day verapamil was similar to that in *Mdr1a*$^{-/-}$*Apc*$^{Min/+}$ mice (Table 1).

Survival benefits of inhibiting P-glycoprotein function with verapamil

A major cause of lower survival rates in *Apc*$^{Min/+}$ mice is the development of intestinal polyps with bleeding. Thus, we

calculated the 1-year survival rates of *Mdr1a*$^{+/+}$*Apc*$^{Min/+}$ mice, which were treated with or without verapamil (Fig. 1). The survival rate of control mice fed with verapamil-free pellets was the lowest (9/14; 64.3%). Mice began to die from the age of 5 months. The survival rates of the verapamil-fed groups increased in a dose-dependent manner as follows: 11/15 (73.3%) with 10 mg/kg/day, 11/15 (73.3%) with 25 mg/kg/day, and 13/15 (86.7%) with 50 mg/kg/day verapamil. The survival rate of *Mdr1a*$^{+/+}$*Apc*$^{Min/+}$ mice fed with 50 mg/kg/day verapamil was higher than that of *Mdr1a*$^{-/-}$*Apc*$^{Min/+}$ mice (14/18; 77.8%).

Discussion

Chemoprevention is a strong strategy to overcome colorectal cancer, but it is still a challenging issue (Herszenyi *et al.*, 2008). This study showed that the number of intestinal polyps in *Mdr1a*$^{+/+}$*Apc*$^{Min/+}$ mice was significantly decreased by inhibiting P-gp function using verapamil (Table 1). The number of polyps was similar to that found in *Mdr1a*$^{-/-}$*Apc*$^{Min/+}$ mice; hence, we considered that the inhibition of P-gp function using verapamil produced the same effects as P-gp knockout. The maximum effect was observed in the distal and middle parts of the small intestine because the majority of verapamil absorption occurred in these parts. The greater effectiveness in the distal and middle parts of the small intestine may also be attributable to differences in contact duration with the verapamil-supplemented diet.

To check that there was no quantitative difference in P-gp expression between the control and verapamil-fed groups, we analyzed P-gp expression in the small intestine by immunohistochemical staining. We detected no compensatory feedback increase in P-gp expression, regardless of the verapamil intake (data not shown).

Furthermore, we found that the 1-year survival rates of *Mdr1a*$^{+/+}$*Apc*$^{Min/+}$ mice fed with pellets containing verapamil were improved in a verapamil dose-dependent manner (Fig. 1). Verapamil has been used as an inhibitor of P-gp function (Fujimoto *et al.*, 2009; Munić *et al.*, 2010) and it might be effective for prolonging the lifespan, because the survival rates of mice fed with 50 mg/kg/day verapamil tended to be higher than those of *Mdr1a*$^{-/-}$*Apc*$^{Min/+}$ mice. In addition to reducing small intestinal polyp formation, the effects of verapamil on lifespan elongation should be examined in the near future.

We demonstrated that the inhibition of P-gp function led to improved survival rates and lower intestinal tumorigenesis in mice. The inhibition of P-gp function had similar effects to a defective P-gp gene. Verapamil is a medicine that has already been widely used in humans. This will make it easier to use this candidate cancer chemopreventive agent in humans. In addition, there have been no reports of specific side effects after long-term treatment with verapamil in patients with hypertension or atherosclerosis. We believe that a cancer chemopreventive agent should first be applied in a high-risk cancer group, rather than the general population. In this study, we provided evidence that verapamil was effective in a mouse model of familial adenomatous polyposis. We, then, propose that inhibiting P-gp function using verapamil could be a potent method for preventing colorectal cancer.

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Conflicts of interest

There are no conflicts of interest.

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