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*Original Article*

# Sesamol suppresses cyclooxygenase-2 transcriptional activity in colon cancer cells and modifies intestinal polyp development in *Apc*$^{Min/+}$ mice

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**Excessive prostaglandin production by cyclooxygenase-2 in stromal and epithelial cells is a causative factor of colorectal carcinogenesis. Thus, compounds which inhibit cyclooxygenase-2 transcriptional activity in colon epithelial cells could be candidates for anti-carcinogenic agents. A cyclooxygenase-2 transcriptional activity in the human colon cancer cell line DLD-1 has been measured using a β-galactosidase reporter gene system. Using this system, we demonstrated that the decrease in basal cyclooxygenase-2 transcriptional activities at 100 μM sesamol, one of the lignans in sesame seeds, was 50%. Other compounds in sesame seeds such as sesamin, sesamolin, ferulic acid, and syringic acid did not exhibit significant suppression of cyclooxygenase-2 transcriptional activity at up to 100 μM. In a following experiment, 6-week-old male *Min* mice, *Apc*-deficient mice, were divided into a non-treated and 500 ppm sesamol groups. At the age of 15 weeks, it was found that treatment with sesamol decreased the number of polyps in the middle part of small intestine to 66.1% of the untreated value. Moreover, sesamol suppressed cyclooxygenase-2 and cytosolic prostaglandin $E_2$ synthase mRNA in the polyp parts. The present findings may demonstrate the novel anti-carcinogenetic property of sesamol, and imply that agents that can suppress cyclooxygenase-2 expression may be useful cancer chemopreventive agents.**

*Key Words*: **cyclooxygenase-2, reporter gene assay, sesame, sesamol, *Min* mice**

The sesame plant (*Sesamun indicum*, Linn.) is well known for its edible seeds and oil.[1] Sesame seeds are characterized by the presence of fatty acids (linoleic acid, linolenic acid, oleic acid, palmitic acid and stearic acid), oil-soluble lignans (episesamin, sesamin, sesaminol, sesamol and sesamolin) and other phenol compounds (γ-tocophenol, ferulic acid and syringic acid). The nonfat portion of sesame seed is only 1–2% by wet weight. Recently, multiple biological functions of sesame seeds, such as inhibition of inflammation and carcinogenesis, have been elucidated. In experimental studies, sesamol was shown to inhibit development of spontaneous development of preneoplastic hepatocytic foci in rats.[2] Sesamin reduced the incidence of chemically induced rat mammary gland cancers.[3] Moreover, sesame oil has been reported to inhibit growth of human colon cancer cells *in vitro*.[4] These effects of sesame seed and its constituents were partly associated with its hydroxyl radical scavenging activity, inhibitory activity of lipid peroxidation and anti-mutageneic activity.[5–7] However, the desirable biological functions have not entirely been elucidated yet.

Recent accumulating evidence has indicated that prostaglandins (PGs) are implicated in colon carcinogenesis.[8] Expression levels of cyclooxygenase-2 (COX-2) are increased in colon carcinoma tissues compared to that of normal colonic mucosa. Therefore, inhibitors against COX-2 have been studied extensively for their ability to suppress colon carcinogenesis. It has been also reported that COX-2 gene knockout causes significant reduction in number and size of intestinal polyps in a mouse model for human familial adenomatous polyposis, *Apc*-deficient *Min* mice.[9] Thus, it is likely that agents that can suppress COX-2 expression at the transcriptional level may be equally advantageous.

As reported in previous papers,[10–12] we have constructed a β-galactosidase reporter gene system to test the effects of compounds on COX-2 transcriptional activity in a human colon cancer cell line, DLD-1 cells. In the present study, effects of five sesame seeds constituents on the transcriptional activity of COX-2 were investigated and one constituent, sesamol, was found to suppress basal COX-2 transcriptional activity. In a further experiment, we investigated the suppressive effect of sesamol on intestinal polyp development and on COX-2 expression levels in *Min* mice.

## Materials and Methods

**Chemicals.** Sesamin were obtained from Cayman Chemical, (Ann Arbor, MI), ferulic acid, sesamol and syringic acid were from Sigma-Aldrich Co. (St. Louis, MO). Sesamolin was from Nagara, Ltd. (Gifu, Japan).

**Cell culture.** DLD-1 cells, a human colon adenocarcinoma cell line, were obtained from the Japanese Collection of Research Bioresources Cell Bank (Osaka, Japan). Construction of DLD-1/COX-2-B2-βGal-BSD cells has been reported in our previous papers.[13] The cells were maintained in DMEM medium supplemented with 5% heat-inactivated fetal bovine serum (FBS; Hyclone Laboratories Inc., Logan, UT) and antibiotics (100 μg/ml streptomycin and 100 U/ml penicillin) at 37°C in 5% $CO_2$.

**Measurements of cell viability.** Cell viability in each culture was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Cells at a density of $2.0 \times 10^4$ cells per well were seeded in 96-well tissue culture plates

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and treated with sesame constituents for 48 h. After treatment, the cells were further incubated in a medium containing 0.5 mg/mL of MTT for 1 h. The MTT formazan produced by living cells was dissolved in dimethyl sulfoxide and absorbance at 595 nm was measured on a microplate Reader (Bio-Rad Laboratories, CA).

**Reporter gene assay for COX-2 promoter-dependent transcriptional activity.** DLD-1/COX-2-B2-βGal-BSD cells were seeded at a density of $2.0 \times 10^4$ cells per 96-well tissue culture plate and precultured for 24 h. After treatment with the test reagents, the total β-galactosidase activities of the cells in each well were determined by colorimetric assay using *o*-nitrophenyl-β-d-galactopyranoside (ONPG) as described previously.[10,11] The background β-galactosidase activity of DLD-1 cells was determined in non-treated DLD-1/B2-βGal-BSD cells, and the value was set as 0. Basal β-galactosidase activity of non-treated DLD-1/COX-2-B2-βGal-BSD cells was set as 100%. The percent β-galactosidase activity of each treatment was calculated from triplicate wells. The viable cell number was assessed by the MTT assay. All assays, including MTT assay, were carried out in triplicate and each experiment repeated at least three times.

**Quantitative real-time polymerase chain reaction (PCR) analysis.** Total RNA was isolated using TRIzol Reagent (Invitrogen, NY), treated with DNase (Invitrogen, Grand Island, NY) and 1 μg aliquots in a final volume of 20 μL were used for synthesis of cDNA using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Real-time PCR was carried out using Fast Start Universal SYBR Green Mix (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions. Primers for COX-1 (5'-TGA TGC TCT TCT CCA CGA and 5'-GCA GGA AAT AGC CAC TCA AG), COX-2 (5'-GTG CCA ATT GCT GTA CAA GC and 5'-TAC AGC TCA GTT GAA CGC CT), cPGES (5'-AGT CAT GGC CTA GGT TAA C and 5'-TGT GAA TCA TCA TCT GCT CC), EP1 (5'-ACC CTG CAT CCT GAG CAG CAC TGG CCC TCT and 5'-CGA TGG CCA ACA CCA CCA ACA CCA GCA GGG), EP2 (5'-AGG ACT TCG ATG GCA GAG GAG AC and 5'-CAG CCC CTT ACA CTT CTC CAA TG), EP3 (5'-TGA CCT TTG CCT GCA ACC TG and 5'-AGA CAA TGA GAT GGC CTG CC), EP4 (5'-TCC CGC TCG TGG TGC GAG TGT TC and 5'-GAG GTG GTG TCT GCT TGG GTC AG), mPGES-2 (5'-AAG ACA TGT CCC TTC TGC and 5'-CCA AGA TGG GCA CTT TCC) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (5'-TGT CAG CAA TGC ATC CTG CA and 5'-TTA CTC CTT GGA GGC CAT GT) were employed. For evaluation of human mRNA levels, indicated primers as shown below were used. Human EP1 (5'-TCT ACC TCC CTG CAG CGG CCA CTG and 5'-GAA GTG GCT GAG GCC GCT GTG CCG GGA), human EP2 (5'-ATG GGC AAT GCC TCC AAT GAC TCC CAG and 5'-CTC CAG GGA ACA ATT TCA AAA T), human EP4 (5'-CCT CCT GAG AAA GAC ACT GCT and 5'-AAG ACA CTC TCT GAG TCC T), and human GAPDH (5'-CCA CCC ATG GCA AAT TCC and 5'-TGG GAT TTC CAT TGA TGA CAA). To assess the specificity of each primer set, amplicons generated from the PCR reaction were analyzed for melting curves.

**Western blot analysis.** EP1–4 protein levels were analyzed by western blot. DLD-1/COX-2-B2-βGal-BSD cells were seeded at a density of $2 \times 10^5$/well in 24-well plates, and incubated with 50 and 100 μM sesamol for 24 and 48 h. After treatment, cells were lysed in 100 μl lysis buffer [0.0625 M Tris–HCl (pH 6.8), 20% 2-mercaptoethanol, 10% glycerol, 5% sodium dodecyl sulfate]. Samples were separated in 10% polyacrylamide gel electrophoresis–sodium dodecyl sulfate gels and transferred onto polyvinylidene difluoride membranes (Millipore, MA). Abs against the EP1, EP2, EP4 (Cayman Chemical Co. Ann Arbor, MI) and EP3 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) was used at a 1:2,000 dilution. Peroxidase-conjugated secondary Abs for anti-rabbit IgG were obtained from GE Healthcare (Buckingham shire, UK). Blots were developed with ECL western blotting detection reagents (GE Healthcare).

**Animals.** Female C57BL/6-*Apc*$^{Min/+}$ mice (*Min* mice) were purchased from The Jackson Laboratory (ME). Mice were housed per plastic cage with sterilized softwood chips as bedding in a barrier-sustained animal room at 24 ± 2°C and 55% humidity on a 12 h light/dark cycle. Sesamol was well mixed at a concentration of 500 ppm in AIN-76A powdered basal diet (CLEA Japan, Tokyo, Japan).

**Protocol for Animal experiments.** Ten female *Min* mice at 5 weeks of age were given 500 ppm sesamol, for 8 weeks. The animals in each cage were all in the same treatment group. Food and water were available *ad libitum*. The animals were observed daily for clinical signs and mortality. Body weights and food consumption were measured weekly. The intestinal tract was removed and separated into the small intestine, cecum and colon. The small intestine was divided into the proximal segment (4 cm in length) and then the proximal (middle) and distal halves of the remainder. Polyps in the proximal segments were counted and all

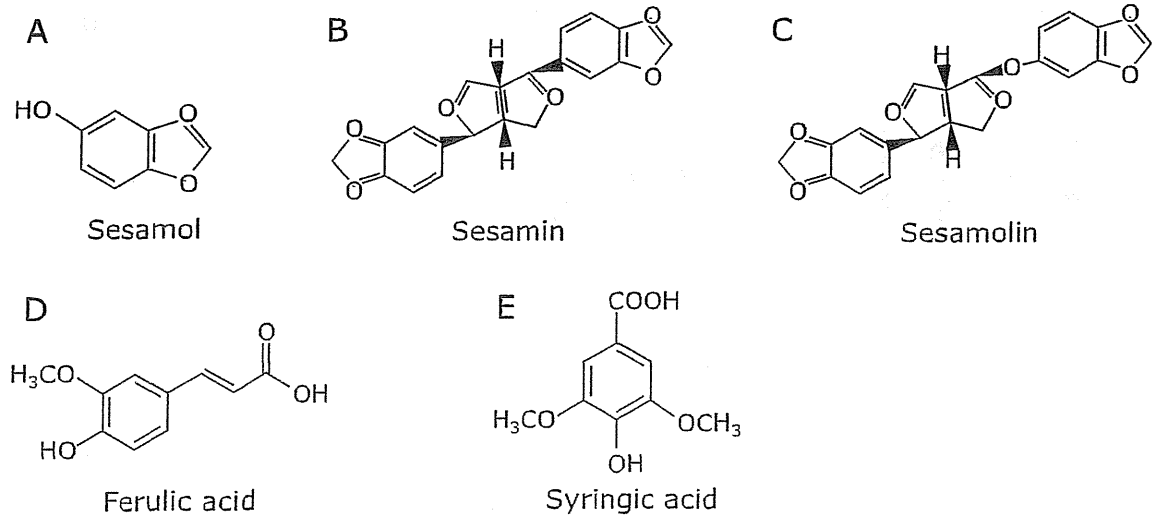


**Fig. 1.** Chemical structures of the five constituents in the sesame seed. (A), sesamol; (B), sesamin; (C), sesamolin; (D), ferulic acid; (E), syringic acid.



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polyps in the proximal segment were picked up under a stereoscopic microscope and the remaining intestinal mucosa (non-polyp part) was removed by scraping, and then both stored at −80°C for further analysis. Other segments were opened longitudinally and fixed flat between sheets of filter paper in 10% buffered formalin. The numbers and sizes of polyps and their distributions in the intestine were assessed with a stereoscopic microscope. The experiments were performed according to the "Guidelines for Animal Experiments in the National Cancer Center" and were approved by the Institutional Ethics Review Committee for Animal Experimentation in the National Cancer Center.

**Statistical analysis.** All the results are expressed as mean ± SD. values, with statistical analysis using the Student's *t* test, except for the COX-2 promoter activity investigation and mRNA examination in the human cell line. The Bonferroni *z* test was used for statistical analyses of the COX-2 promoter activity and of human mRNA levels. Differences were considered to be statistically significant at $p<0.05$.

## Results

**Suppression of COX-2 promoter activity in human colon cancer cells by sesamol.** Five compounds, shown in Fig. 1, were tested at various concentrations up to 100 μM with regard to their effects on COX-2 promoter activity. Remarkable suppression of cell proliferation rates by five compounds was not observed at the concentrations up to 100 μM in MTT assay. Among five constituents in the sesame seed, only sesamol significantly suppressed COX-2 prompter activity in a dose-dependent manner. Decrease in COX-2 promoter activities by sesamol at 100 μM was 50% (Fig. 2A). The other four compounds, ferulic acid, sesamin, sesamolin, syringic acid exhibited weak or no suppression of COX-2 promoter activity (Fig. 2B–E).

**Suppression of intestinal polyp formation in *Min* mice by sesamol.** Administration of 500 ppm sesamol to *Min* mice for 8 weeks did not affect body weights, food intake or clinical signs throughout the experimental period. Average daily food intake did not significantly differ among the groups, being 3.9 and 3.5 g per mouse per day for the 0 and 500 ppm group of *Min* mice, respectively. In addition, there were no changes observed in any organ weights that might have been attributable to toxicity.

Table 1 summarizes data for the number and distribution of intestinal polyps in the basal diet and sesamol-treated groups. Almost all polyps developed in the small intestine, with only a few in the colon. The total number of polyps tended to be decreased by administration of 500 ppm sesamol to 75% of the untreated control value. Reduction of polyps was observed in the middle part, and was by 66% ($p<0.05$ vs 0 ppm). In the other parts of small intestine and colon, treatment with sesamol lowered the number of polyps without significant difference.

Fig. 3 shows the size distribution of intestinal polyps in the basal diet and sesamol-treated groups. The maximal number of polyps was observed in the size range between 0.5 and 2.0 mm in diameter. Administration of sesamol significantly reduced the numbers of polyps sized <0.5 mm in diameter.

**Decrease of inflammation-related factors mRNA levels in intestinal polyp parts by sesamol.** Inflammation-related factors mRNA expressions in intestinal polyp parts and non-polyp parts were investigated (Fig. 4). Real-time PCR revealed that treatment with 500 ppm sesamol for 8 weeks significantly suppressed COX-2, and cPGES, mRNA levels in the intestinal polyp parts to 48% ($p<0.01$) and 54% ($p<0.05$) of sample value, respec-

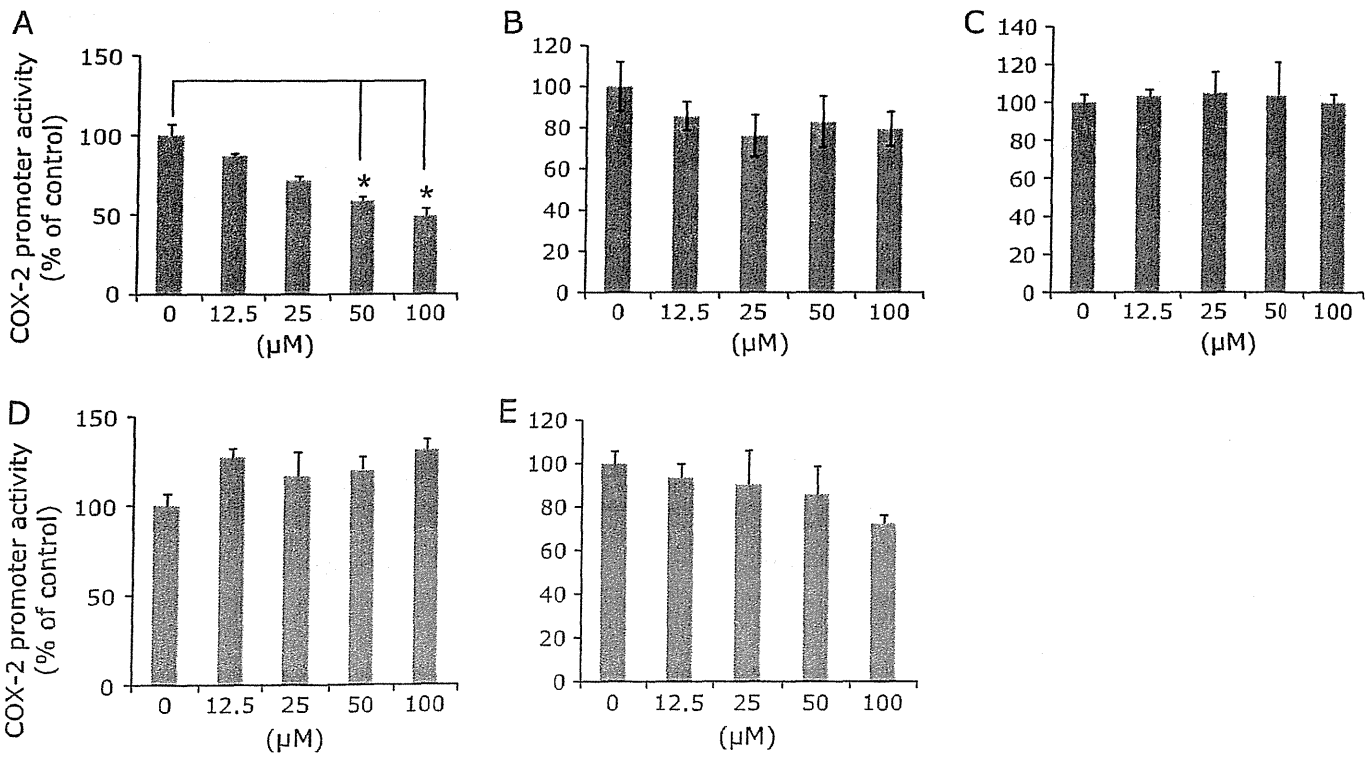


**Fig. 2.** Effects of treatment with sesame constituents on reporter gene activity in DLD-1/COX-2-B2-β-gal-BSD cells. DLD-1/COX-2-B2-β-gal-BSD cells were seeded in 96-well multiwell plates at a density of $2 \times 10^5$ cell/well and cultured in medium containing sesame constituents at concentrations up to 100 μM. After 48 h, the COX-2 promoter activity was evaluated by β-galactosidase activity and was normalized for viable cell numbers assessed by MTT assay. The columns indicate the values of the mean percentages of triplicate wells of promoter activity of DLD-1/COX-2-B2-β-gal-BSD cells. The data are representative of more than three independent experiments. Bars indicate the SD. *$p<0.05$. (A), sesamol; (B), sesamin; (C), sesamolin; (D), ferulic acid; (E), syringic acid. COX; cyclooxygenase, cPGES; cytosolic PGES, mPGES; microsomal PGES, PGES; prostaglandin E synthase.

**Table 1.** Number of intestinal polyps/mouse in *Min* mice

| Sesamol (ppm) | No. of mice | Small intestine | | | Colon | Total |
|---|---|---|---|---|---|---|
| | | Proximal | Middle | Distal | | |
| 0 | 9 | 4.9 ± 4.6 | 17.1 ± 5.6 | 22.1 ± 11.7 | 0.7 ± 1.3 | 44.8 ± 15 |
| 500 | 8 | 2.4 ± 1.5 | 11.3 ± 5.6* | 19.9 ± 4.4 | 0.1 ± 0.4 | 33.6 ± 9.2# |

Data are mean ± SD. *Significantly different from the control untreated group at $p<0.01$. #$p = 0.087$.

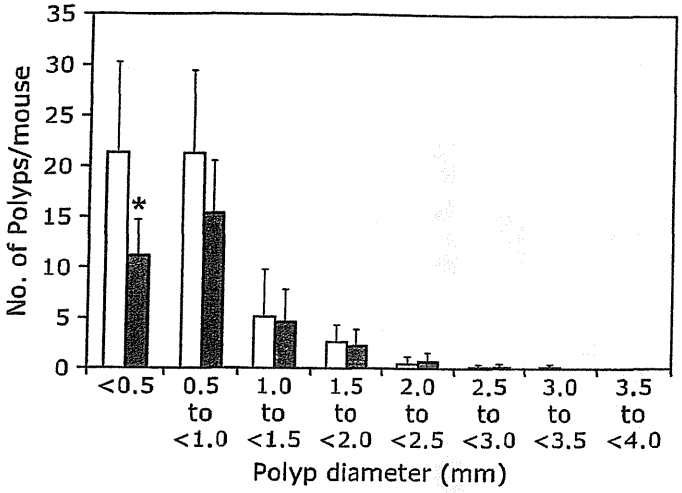


**Fig. 3.** Effects of sesamol on the size distribution of intestinal polyps in *Min* mice. *Min* mice were fed a basal diet (open box) or a containing 500 ppm (black filled box) sesamol for 8 weeks. The number of polyps per mouse in each size class is given as a mean ± SD. *$p<0.05$.

tively. mPGES2, EP1 and EP2 mRNA levels tended to be reduced in intestinal polyp parts by sesamol. COX-1 and EP4 mRNA expression levels in non-polyp parts and polyp parts of small intestine were not suppressed by sesamol treatment. Only EP3 did not increase its expression levels in the intestinal polyp parts compared to those of mucosa parts, and rather decreased its expression by sesamol treatment in the intestinal polyp parts.

**Decrease of prostaglandin $E_2$ receptor expression levels in human colon cancer cells by sesamol.** To clarify the effects of sesamol on EP1 and EP2 mRNA levels in human cells, we treated DLD-1/COX-2-B2-β-gal-BSD cells with 50 and 100 μM sesamol for 48 h. Sesamol significantly suppressed EP1 and EP2 mRNA levels in a dose-dependent manner (Fig. 5). However, mRNA levels for EP4 were increased and EP3 was not clearly detected (data not shown). We further confirmed the EP1–4 protein expression levels in the cells with or without sesamol treatment. As shown in Fig. 5D, sesamol down-regulated EP1 and EP2 protein levels in dose- and time-dependent manner. As in the case of EP4, sesamol treatment for 24 h slightly increased its protein levels, whereas 48 h treatment decreased EP4 protein levels. EP3 expression levels seem to be very low in these cells, as shown in Fig. 5D.

## Discussion

In the present study, sesamol was found to suppress basal transcriptional activity of the COX-2 gene in human colon cancer DLD-1 cells. Previously, we have reported that mono-benzonic compounds such as resorcinol and resacetophenone suppress COX-2 transcriptional activity,[10,11] but their inhibitory activities are almost 5-times less than that of sesamol. Thus, sesamol may have a notable potential to suppress COX-2 expression for a natural compound.

The underlying mechanism of suppression of COX-2 transcriptional activity by sesamol is not clear. Protein-tyrosine kinases (PTKs), including the epidermal growth factor receptor, are well known to be involved in the induction of COX-2 expression.[14,15] Signals from activated PTKs are transduced to the downstream transcription factor NF-κB, mainly by the Ras and mitogen-activated protein kinase pathways. It is also known that activation of NF-κB has been reported to play an important role in the regulation of COX-2 expression. However, our preliminary experiment that aimed to evaluate the effects of sesamol on NF-κB transcriptional activity failed to show its reduction at concentrations up to 100 μM in human colon cancer cells (data not shown). Further studies are needed to elucidate the molecular mechanisms responsible for the inhibition of COX-2 transcriptional activity by sesamol.

We next aimed to show the suppressive potential of sesamol on intestinal polyp development in *Min* mice. Administration of 500 ppm sesamol tended to reduce the total number of intestinal polyps development compared to that of the untreated group. Further analysis revealed that treatment with sesamol decreased the number of polyps in the middle part of the small intestine. It has been reported that indomethacin, a COX inhibitor, and nimesulide, a COX-2 selective inhibitor, mainly reduce the number of polyps in the middle to distal part of the small intestine.[16,17] Thus, sesamol with a COX-2 suppressive function has a similar inhibitory potential for polyp development. For instance, LPL inducers such as NO-1886 or PPAR ligands effectively reduce the number of polyps in the proximal part of the small intestine.[18,19]

In the polyp parts of *Min* mice, it was confirmed that sesamol could suppress expression levels of COX-2 mRNA. In addition, cPGES mRNA was reduced by sesamol treatment, and this is the first report that suggests suppressive effects of sesamol on cPGES as far as we know. Moreover, a tendency to suppression was observed in the expression levels of $PGE_2$ receptor subtypes EP1 and EP2 in the polyp parts of *Min* mice. Using $PGE_2$ receptor subtype-knockout mice, the roles of these receptors in colon carcinogenesis have been investigated.[20–23] These observations suggest that EP1, EP2 and EP4 are promotive receptors in colorectal carcinogenesis, and EP3 plays suppressive roles. EP1 signals transmitted by increased intracellular $Ca^{2+}$ concentrations activate protein kinase C (PKC). However, the actual signal transduction mechanisms are not known in detail.[24,25] Stimulation of EP2 and EP4 receptors in both cases involves coupling with stimulatory G protein, leading to activation of adenylate cyclase. As a result, increased cAMP levels activate cAMP-dependent protein kinase (PKA) and increase a transcriptional factor that binds to cAMP-responsive element, that plays a role in cell growth and cell survival. Thus, it may be worthwhile to develop functional inhibitors or specific suppressors for EP1, EP2 and EP4. However, it is regrettable that there are a few inhibitors for $PGE_2$ receptor subtypes. To add to the novel potential of sesamol, we confirmed the effect of sesamol on human EP1 and EP2 mRNA levels. We found suppression of EP1 and EP2 mRNA levels by sesamol treatment. Down-regulation of EP1 and EP2 protein was also confirmed. These data imply a double suppressive potential exists in sesamol regarding cell growth function of $PGE_2$. Suppression of COX-2 may reduce production of growth lipid mediator $PGE_2$, and down regulation of $PGE_2$ receptors such as


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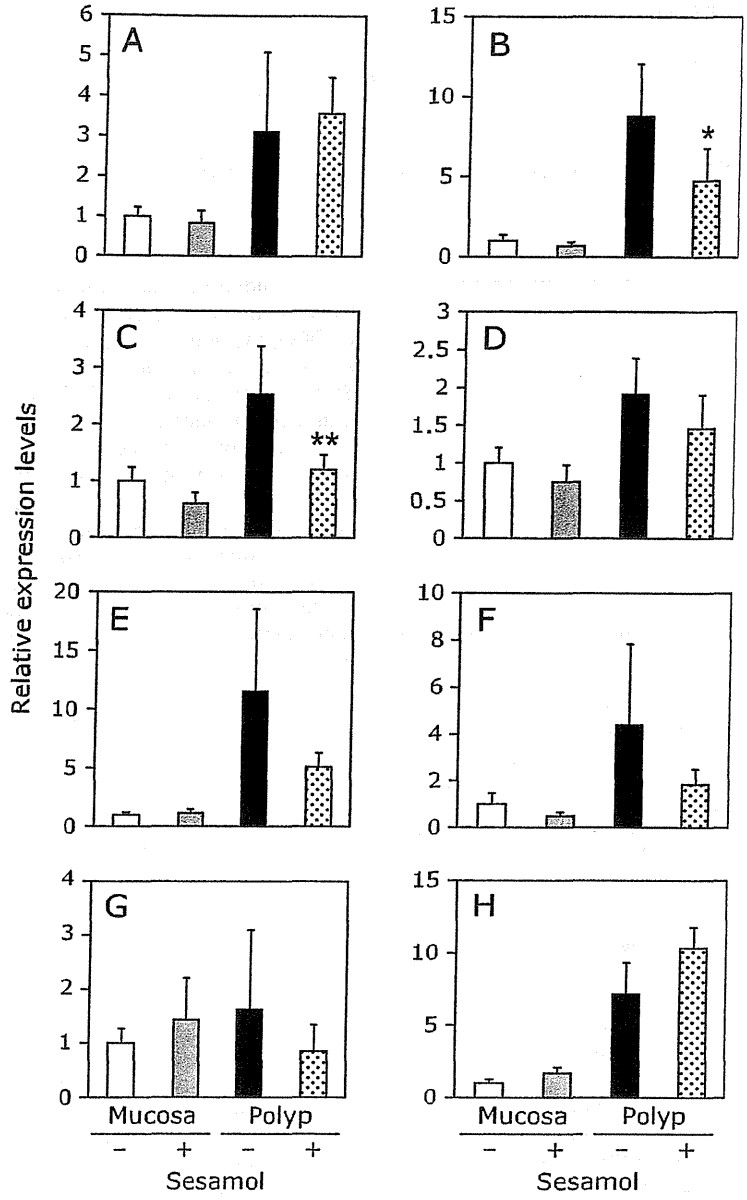


**Fig. 4.** Changes of inflammation-related factors in intestinal non-polyp mucosa parts and/or polyp parts of Min mice. Quantitative real-time PCR analysis were performed to determine COX-1 (A), COX-2 (B), cPGES (C), mPGES2 (D), EP1 (E), EP2 (F), EP3 (G), EP4 (H) mRNA expression levels in the polyps or non-polyp mucosa parts of *Min* mice, given diets containing sesamol at doses of 500 ppm for 8 weeks. Data are normalized with GAPDH expression level. Data are mean ± SD, $n = 6$. $^{**}p<0.01$, $^{*}p<0.05$ vs 0 ppm.

EP1 and EP2 may additionally suppress tumor growth through a transmembrane G protein-coupled receptor.

In summary, sesamol suppressed the transcriptional activity of COX-2 gene in DLD-1 cells. Moreover, our *in vivo* data imply that agents that can suppress COX-2 expression at the gene level may be useful cancer chemopreventive agents. Further information of the mechanisms by which sesamol suppresses COX-2 expression may clarify the anti-inflammatory and anti-carcinogenetic properties of sesamol.

## Acknowledgments

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## Abbreviations

| | |
|---|---|
| cPGES | cytosolic PGES |
| COX | cyclooxygenase |
| FBS | fetal bovine serum |
| mPGES | microsomal PGES |
| MTT | 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide |
| NF-κB | nuclear factor-κB |
| ONPG | *o*-nitrophenyl-β-D-galactopyranoside |
| PGs | prostaglandins |
| PGES | prostaglandin E synthase |
| PTKs | protein-tyrosine kinases |

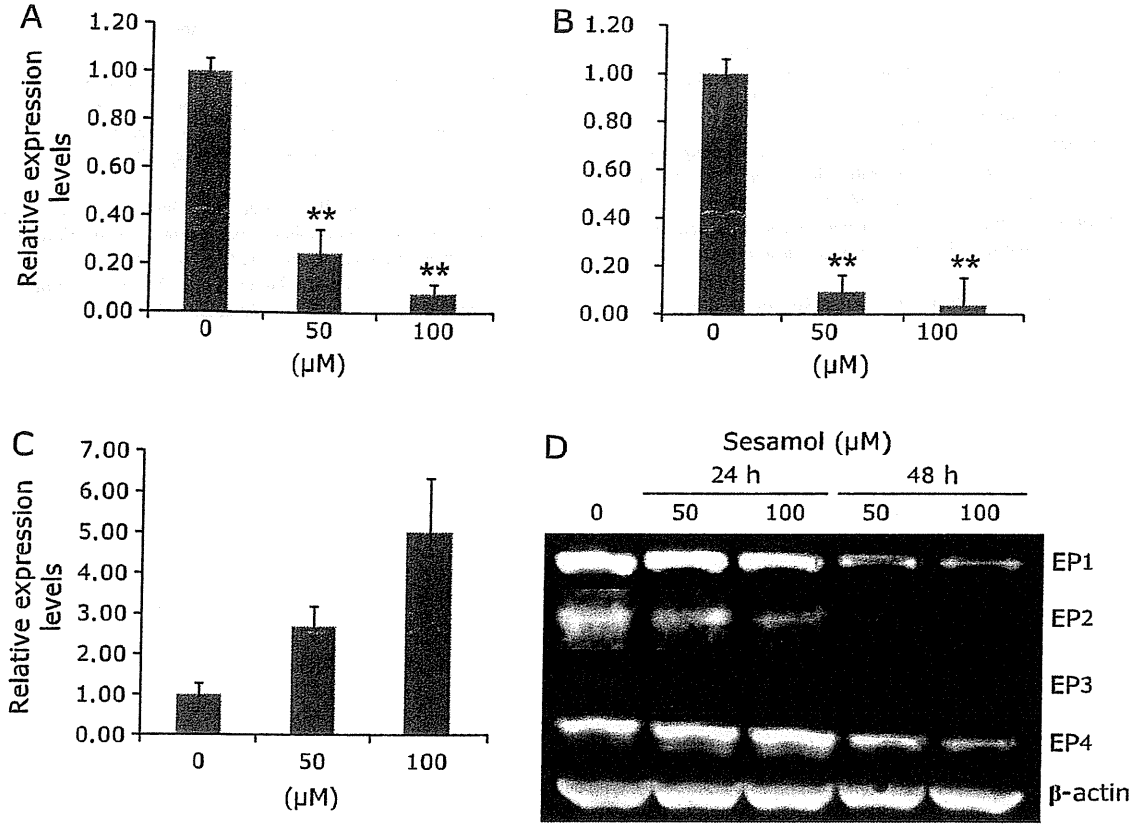


**Fig. 5.** Prostaglandin $E_2$ receptor expression in human colorectal cancer cells with or without sesamol treatment. DLD-1/COX-2-B2-β-gal-BSD cells were seeded in 6-well multiwell plates at a density of $2 \times 10^6$ cell/well and cultured in medium containing 50 and 100 μM sesamol for 48 h. After 48 h treatment, quantitative real-time PCR analysis was performed to determine EP1(A), EP2(B), EP4(C) mRNA levels. Data are normalized with GAPDH, Data are mean ± SD, $n = 3$.**$p<0.001$ vs 0 ppm. (D) EP1–4 protein was detected by western blot analysis, using DLD-1/COX-2-B2-β-gal-BSD cells (24-well plates at a density of $2 \times 10^5$ cell/well) with treatment of 50 and 100 μM sesamol for 24 and 48 h.

## Conflict of Interest

No potential conflicts of interest were disclosed.

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# Bi-directional Regulation Between Adiponectin and Plasminogen Activator-inhibitor-1 in 3T3-L1 Cells

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**Abstract.** *Background: Adiponectin (APN) and plasminogen activator inhibitor-1 (Pai-1) are adipocytokines, and low levels of serum APN and high levels of PAI-1 are observed in obese patients. Moreover, both APN and Pai-1 are known to be involved in colorectal carcinogenesis. Recently, we demonstrated that serum Pai-1 levels are elevated in APN-deficient mice. We hypothesized that Pai-1 expression levels could be depressed by APN. Thus, we aimed to clarify the bi-directional regulatory mechanisms between APN and Pai-1. Materials and Methods: We investigated the expression levels of APN and Pai-1 during 3T3-L1 pre-adipocyte differentiation, and examined the role of AMP-activated protein kinase (AMPK) and peroxisome proliferator-activated receptor (PPAR)-γ on APN and Pai-1 expression at early and late differentiation stages. Results: In the early phase of differentiation, Pai-1 expression increased and APN slightly decreased. Reduction of Pai-1 or activation of PPARγ resulted in elevation of APN, and supplementation of APN with activation of AMPK resulted in reduction of Pai-1. In the late phase of differentiation, APN increased its expression and Pai-1 decreased. Supplementation of Pai-1 resulted in a slight reduction of APN. Conclusion: It is suggested that APN and Pai-1 expressions are inversely-regulated. Understanding of the regulatory system between APN and Pai-1 may lead to finding novel methods for colorectal cancer prevention.*

Adiponectin (APN; 30 kDa protein) is one of the adipocytokines discovered in adipose tissue (1), and abundant amounts of APN are detected in plasma (3-30 μg/ml). A decrease of APN levels is associated with insulin-resistant type-2 diabetes, coronary artery disease, and the development of cancer, including colorectal cancer (2-6). There are two APN receptors, AdipoR1 and AdipoR2 (7). The physiological function of APN is evoked by binding to these receptors. It is known that AdipoR1 activates AMP-activated protein kinase (AMPK) and AdipoR2 activates peroxisome proliferator-activated receptor-α (PPARα) (8, 9).

We have been studying the involvement of APN in colorectal cancer risk. *Adenomatous Polyposis Coli* (*Apc*)-deficient Min mice ($Apc^{Min/+}$), a model of familial adenomatous polyposis (FAP), with APN deficiency were used to investigate the effects of *APN* knockout on intestinal polyp development. APN-deficient *Min* mice show a 2- or 3-fold increase in the total number of intestinal polyps developed compared with APN wild-type *Min* mice, regardless of gender (10). APN-deficient C57BL/6J mice treated with azoxymethane (AOM) demonstrated increased incidence and multiplicity of colorectal tumors, including adenomas and adenocarcinomas. *Min* mice exhibited an increase in serum plasminogen activator inhibitor-1 (Pai-1) levels with decreasing expression levels of *APN*. In addition, the tendency for elevation of serum Pai-1 levels was observed with APN-deficiency in C57BL/6J mice at the age of 55 weeks (10).

Pai-1 is one of the adipocytokines whose levels increase with obesity. Pai-1 directly inhibits tissue plasminogen activator (tPA) and urokinase-type plasminogen activator (uPA). tPA and uPA activate plasminogen to produce plasmin through serine protease activity, and physiologically breakdown blood clots. Pai-1 is also reported to possess/exhibit multifunctional factors. Although the molecular mechanisms are not fully-established, Pai-1 was found to modulate cell proliferation and stimulate angiogenesis (11, 12). PAI-1 is known to be induced by triglyceride (TG), very low-density lipoprotein (TG-rich lipoprotein), transforming growth factor-β (TGFβ), various

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*Key Words:* Adiponectin, Pai-1, AMPK, PPAR, pre-adipocyte.

growth factors, tumor suppressor p53, nuclear factor kappa B (NFκB) and Wnt signaling (13-17), all of which are plausibly involved in carcinogenesis.

Adipocytokines can affect each other. Among them, APN is known to act as a major regulator of other adipocytokines. For instance, APN stimulates AMPK in the hypothalamus to promote food intake under starvation conditions and inhibit leptin activation (18). In peripheral tissues, especially in skeletal muscle, APN activates AMPK, insulin receptor substrate-1 and fatty acid transport protein-1, to stimulate fatty acid combustion and glucose intake. It is interesting that these types of activation can be inhibited by tumor necrosis factor α (TNFα), another adipocytokine. Thus, it is assumed that APN deficiency affects the action elicited by other adipocytokines or the production of other adipocytokines, such as Pai-1. Therefore, we hypothesized that Pai-1 expression levels might also be depressed by APN.

In the present study, we aimed to clarify the bi-directional regulatory mechanisms between APN and Pai-1. We investigated the expression levels of APN and Pai-1 during 3T3-L1 pre-adipocyte differentiation, and examined the role of AMPK and PPARγ on APN and Pai-1 expression at the early and late differentiation stage. We demonstrated that APN can suppress Pai-1 expression through activation of AMPK, and Pai-1 can suppress APN expression through inhibition of a transcription factor, PPARγ.

## Materials and Methods

*Cell culture and induction of adipocyte maturation in the 3T3-L1 cell line.* 3T3-L1 cells (JCRB Cell Bank, Osaka, Japan) were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum (Hyclone Laboratories Inc., Logan, UT, USA) (basal medium). Induction of differentiation into adipocyte phenotypes was performed by treating confluent cells with 0.5 mM 3-isobutyl-1-methylxanthine (IBMX; Sigma-Aldrich Co., St. Louis, MO, USA), 1 μM dexamethasone (Sigma-Aldrich) and 1.6 μM insulin (Life Technologies, Co., Carlsbad, CA, USA) in basal medium for two days (Figure 1A). After the treatment, the medium was replaced by basal medium, and the cells were incubated for three days (indicated as day 5 in Figure 1A) and 16 days (indicated as day 18 in Figure 1A).

Mouse recombinant adiponectin (R&D Systems Inc., Minneapolis, MN, USA), metformin (Wako Pure Chemical Industnes, Osaka, Japan), troglitazone (Sigma-Aldrich) and PNU74654 (Wnt-I; Sigma-Aldrich) were applied to adipocyte cells on day 2 and the cells were incubated until day 5. Mouse recombinant Pai-1 (Merck, Darmstadt, Germany) was applied to adipocyte cells on day 15 and the cells were incubated until day 18.

*Mouse fat tissue samples.* Five abdominal fat tissue samples from 15-week-old male APN-deficient mice and APN wild-type mice were obtained from our previous experiment reported elsewhere (10).

*Western blot analysis.* Protein expression was analyzed by western blot. Cells ($2\times10^5$) were seeded in 24-well plates. After treatment, cells were lysed in 100 μl lysis buffer [0.0625 M Tris-HCl (pH 6.8), 20% 2-mercaptoethanol, 10% glycerol, 5% sodium dodecyl sulfate] and Halt Phosphatase Inhibitor Cocktail (Thermo Fisher Scientific Inc., Waltham, MA, USA) added. Equal amounts of protein were separated in 5-20% gradient polyacrylamide gel electrophoresis-sodium dodecyl sulfate gels and transferred onto polyvinylidene difluoride membranes (Merck-Millipore, Billpore, MA, USA). Antibodies against p-AMPK and AMPK (Cell Signaling Technology, Danvers, MA, USA) were used at a 1:1,000 and 1:2,000 dilution, respectively. Blots were developed with enhanced chemiluminescence western blotting detection reagents (GE Healthcare, Buckingham Shire, UK).

*Quantification of mRNA expression by quantitative real-time Polymerase Chain Reaction (qRT-PCR).* Total RNA was isolated from cultured adipocyte and tissue samples using TRIzol Reagent (Invitrogen, Grand Island, NY, USA). One-microgram aliquots in a final volume of 20 μl were used for synthesis of cDNA using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Real-time PCR was carried out using a CFX96™ (Bio-Rad Laboratories, Inc., Hercules, CA, USA) with FastStart Universal SYBR Green MIX (×2) (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions. Primers for mouse *APN* (5' AGGATGCTA CTGTTGCAAGCTCTC, 5' CAGTCAGTTGG TATCATGGTAGAG), glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*; 5' TTGTCTC CTGCGACTTCA, 5' CACCACCCTGT TGCTGTA), Pai-1 (5' ACAGCCTTTGTCATCTCAGCC, 5' AGGG TTGCACTAAACAT GTCAG) were employed. The data were normalized by *GAPDH*. To assess the specificity of each primer set, amplicons generated from the PCR reaction were analyzed for melting curves.

*Statistical analysis.* Statistical analysis was performed using Student's *t*-test. Differences were considered to be statistically significant at $p<0.05$.

## Results

*Difference in APN and Pai-1 expression pattern during 3T3-L1 pre-adipocyte differentiation.* 3T3-L1 is a suitable cell line to examine differences in molecular change during pre-adipocyte differentiation. Thus, expression levels of APN and Pai-1 were examined in 3T3-L1 cells at day 5 after the initiation of differentiation (early phase) and at day 18 after the initiation of differentiation (late phase). In the early phase, Pai-1 mRNA levels were significantly higher compared to those of undifferentiated 3T3-L1 cells (Figure 1B). In the late phase, *Pai-1* mRNA levels were significantly lower than those of undifferentiated 3T3-L1 cells. Comparing the expression levels of Pai-1 in early and late phases, an obvious reduction was observed in differentiated 3T3-L1 cells, while a slight induction of Pai-1 was observed in undifferentiated 3T3-L1 cells (Figure 1B). Comparing day 5 and day18, induction of APN was observed in both undifferentiated and differentiated 3T3-L1 cells in the late phase (Figure 1C). *APN* mRNA levels in differentiated cells tended to be lower compared to those of undifferentiated 3T3-L1 cells in the early phase, and higher when cells were in the late phase (Figure 1C and D).

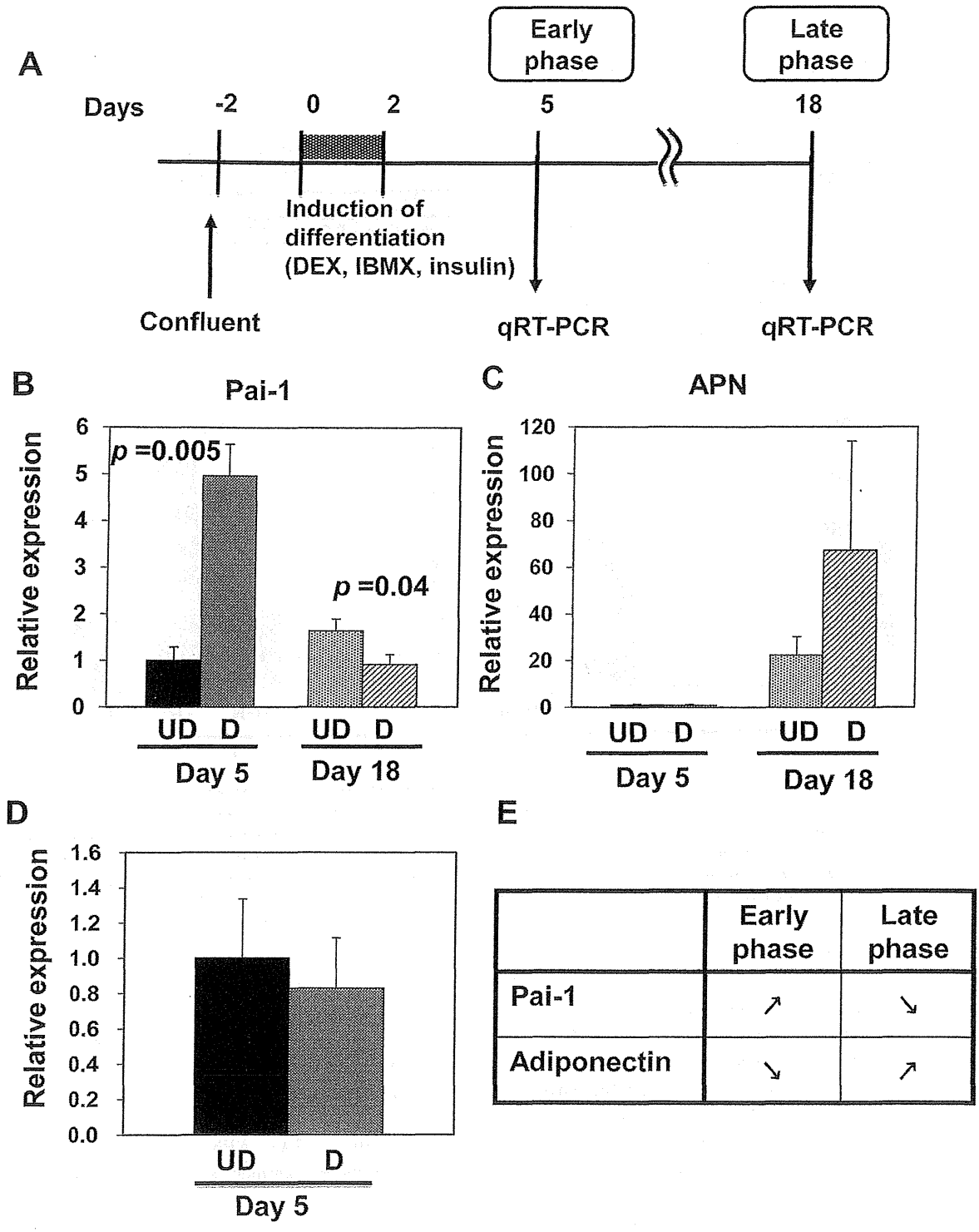


Figure 1. *Adiponecti n(APN) and plasminogen activator inhibitor-1(Pai-1) expression levels in 3T3-L1 pre-adipocytes. A: Illustration of differentiation protocol for 3T3-L1 pre-adipocytes is shown. Day 5 after the initiation of differentiation is defined as the 'early phase' and day 18 is defined as the 'late phase'. Quantitative real time-polymerase chain reaction (qRT-PCR) for Pai-1 (B) and APN (C) was performed at day 5 and day 18. The data are normalized by glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Relative Pai-1 and APN mRNA expression levels are plotted as the ratio of the untreated and undifferentiated control culture values. Data are means±SD (n=3). Similar results were obtained from more than three separate experiments. D: The data focused on low relative expression levels of Figure 1C. E: Summary of APN and Pai-1 expression patterns during 3T3-L1 pre-adipocyte differentiation. DEX, Dexamethasone; D, differentiated; IBMX, 3-isobutyl-1-methylxanthine; UD, undifferentiated.*

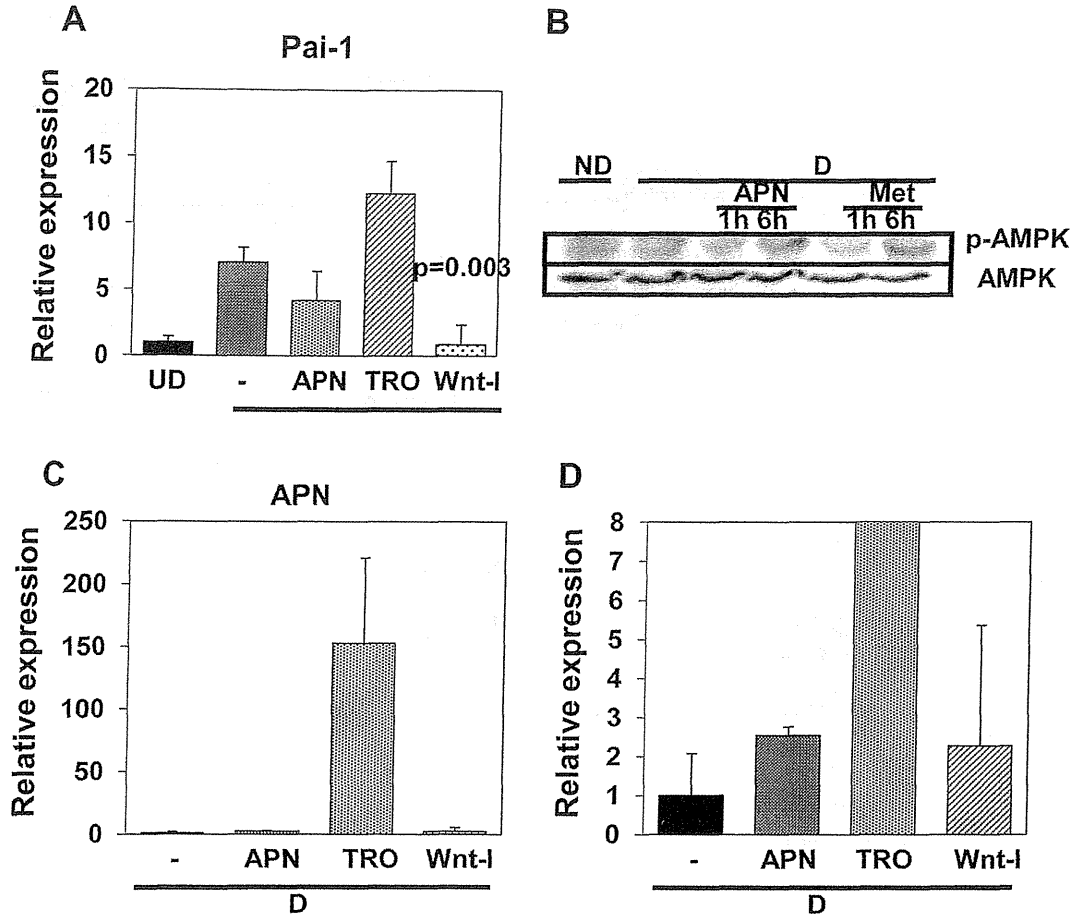


Figure 2. *Effects of adiponectin (APN), peroxisome proliferator-activated receptor (PPAR)γ ligand and Wnt inhibitor on 3T3-L1 cells at the early stage of differentiation. At day 2 after the initiation of differentiation, 3T3-L1 cells were treated with mouse recombinant protein APN (10 μg/ml), troglitazone (TRO), PPARγ ligand (10 μM) and Wnt inhibitor PNU74654 (20 μM) for three days. Quantitative real time-polymerase chain reaction (qRT-PCR) for Pai-1 (A) and APN (C and D) was performed. Relative Pai-1 and APN mRNA expression levels are plotted as the ratio of the untreated and undifferentiated control culture value. Data are means±SD (n=3). Similar results were obtained from three separate experiments. B: 3T3-L1 cells were treated with APN (10 μg/ml) and metformin (MET) an AMPK activator (5 mM) for 1 or 6 h, and AMPK and phosphorylated AMPK were examined by western blot. D: The data focused on low relative expression levels of Figure 1C. D, differentiated; UD, undifferentiated.*

*Correlation between adiponectin and Pai-1 in the early phase*. To clarify the relation between APN and Pai-1, 3T3-L1 cells were treated with APN at a dose of 10 μg/ml on day 2, after the initiation of differentiation. At the early-phase time point (day 5), high *Pai-1* mRNA expression levels were observed and it was found that APN could slightly reduce Pai-1 expression levels compared to those differentiated cells not treated with APN (Figure 2A). Phosphorylation of AMPK was confirmed by western blotting after six hours treatment with 10 μg/ml APN and 5 mM metformin, used as a positive control, at day 2 after the initiation of differentiation (Figure 2B). In addition, we tried to induce an increase in APN expression by treatment with troglitazone, a PPARγ ligand. As expected, treatment with 10 μM troglitazone markedly induced APN as shown in Figure 2C. However, treatment with troglitazone did not suppress but rather increased Pai-1 expression. On the other hand, we tried to reduce the high levels of Pai-1 by inhibiting Wnt/β-catenin signaling. PNU74654, a Wnt inhibitor, at a dose of 20 μM successfully suppressed Pai-1 expression levels (Figure 2A). APN expression levels under this treatment were examined, and almost a 2-fold elevation was observed (Figure 2C and D).

*Correlation between adiponectin and Pai-1 in the late phase*. To clarify the relation between APN and Pai-1 in the late phase, Pai-1 at a dose of 1 μg/ml was added to the medium at 15 days after the initiation of differentiation. At the late-phase time point (day 18), APN expression was slightly reduced by Pai-1 treatment compared to those of untreated

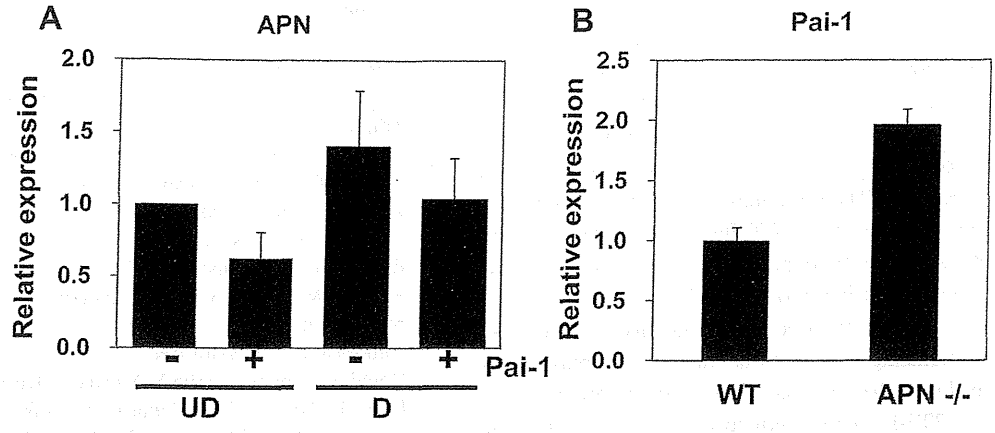


Figure 3. *Effects of plasminogen activator inhibitor-1 (Pai-1) on adiponectin (APN) mRNA levels and expression of Pai-1 in adiponectin-deficient mice. A: 3T3-L1 pre-adipocytes were treated with 1 μg/ml mouse recombinant Pai-1 at day 15 after the initiation of differentiation, and cells were collected for Quantitative real time-polymerase chain reaction (qRT-PCR) analysis at day 18. Relative APN mRNA expression levels are plotted as the ratio of the unstimulated-control culture value. Data are means±SD (n=3). Similar results were obtained from more than two separate experiments. B: qRT-PCR was performed on abdominal fat tissue from 12-week-old male adiponectin homozygous knockout mice (C57BL/6J background; n=5) and its control wild-type (WT) mice (n=5) as described in the Materials and Methods. Relative Pai-1 mRNA expression levels are plotted as the ratio of the value for the wild-type control fat tissue. Data are means±SD. APN(-/-), Homozygous adiponectin knockout mice; D, differentiated; UD, undifferentiated.*

differentiated and undifferentiated cells (Figure 3A). Furthermore, we confirmed an almost doubling of Pai-1 levels in the abdominal adipose tissue in *APN* homozygous knock-out mice compared to those of *APN* wild-type mice (Figure 3B).

## Discussion

The present study demonstrated seesaw patterns of APN and Pai-1 expression in the different stages of pre-adipocyte differentiation. Moreover, bi-directional regulation observed between APN and Pai-1 may be through activation of AMPK and PPARγ (Figure 4).

In the early phase of 3T3-L1 cell differentiation, Pai-1 expression increased and APN slightly decreased. Besides, the late phase of differentiation showed low Pai-1 and high APN (Figure 1E). PPARγ, sterol regulatory element-binding protein-1c (SREBP-1c) and CCAAT/enhancer-binding proteins (C/EBP) are known to be involved in the early changes during pre-adipocyte differentiation (19). In the late phase of pre-adipocyte differentiation, the canonical Wnt signaling pathway reduces adipogenesis (19). These signalings might affect expression patterns observed for APN and Pai-1.

Indeed, a PPARγ ligand remarkably induced APN expression in this study, especially in the early phase (Figure 4A). Another PPARγ ligand, pioglitazone was also found to induce APN expression (20). Of note, Pai-1 is reported to suppress PPARγ expression (21). In this study, APN induction did not effectively lower Pai-1, but addition of 10 μg/ml APN to the culture medium did reduce Pai-1 expression. Concentrations of APN

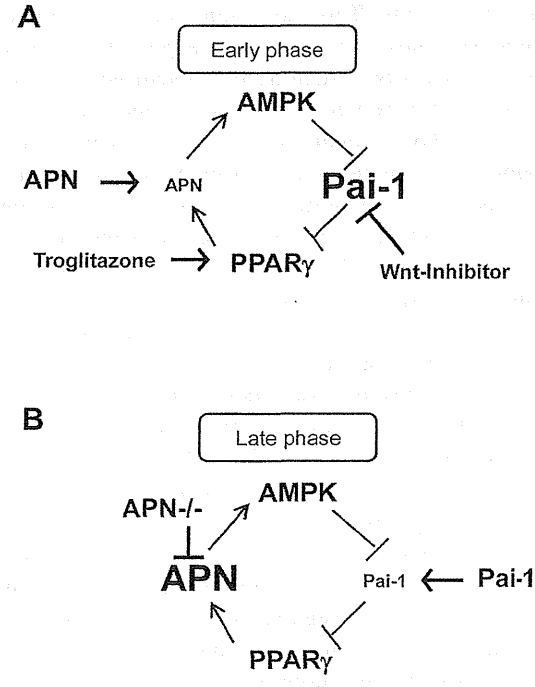


Figure 4. *Proposed mechanism for the seesaw regulation between adiponectin (APN) and plasminogen activator inhibitor-1 (Pai-1). A: Low APN and high Pai-1 expression levels in the early differentiation phase of 3T3-L1 cells may be regulated by AMP-activated protein kinase (AMPK) and peroxisome proliferator-activated receptor (PPAR)γ. Wnt inhibitor was used for suppression of Pai-1. Troglitazone was used for activation of PPARγ. APN was used for activation of AMPK. B: High APN and low Pai-1 expression levels in the late differentiation phase of 3T3-L1 cells may also be regulated by AMPK and PPARγ. Pai-1 was used for suppression of PPARγ. Abdominal fat tissue from APN-deficient mice (APN-/-) was used to determine the effects of APN deficiency on Pai-1 expression levels (→induction; —| suppression/inhibition).*

detected in plasma range from 3-30 μg/ml. Thus, a biologically appropriate dose might be used in this study. It has been reported that the activation of AMPK leads to the inhibition of adipogenesis (22). AMPK activation by APN resulted in suppression of Pai-1 expression. Similar findings were obtained in our recent experiment (10), in which APN-deficiency evoked hepatic Pai-1 induction. These findings suggest that in addition to the Pai-1-suppressive function of TNFα, APN-induced AMPK activation acts as a more direct physiological suppressor of Pai-1. Moreover, Wnt signal inhibitors lowered Pai-1 expression in the early phase. Pai-1 is reported to be a downstream target of Wnt/β-catenin signaling (17), and this might be the reason why APN is induced by a Wnt inhibitor. Summarizing effects in the early phase in 3T3-L1 cells, reduction of Pai-1 resulted in elevation of APN, and supplementation of APN resulted in reduction of Pai-1 (Figure 4A).

In the later phase of pre-adipocyte differentiation, low expression of Pai-1 and high expression of APN were observed. Addition of 1 μg/ml Pai-1 in the culture medium slightly reduced APN expression. Generally, the concentration of PAI-1 detected in human plasma is lower than 50 ng/ml. This dose may not be a biologically-appropriate dose, but may partly explain Pai-1 functions on specific occasions, such as in a localized area in the late phase of differentiation. We also examined the effect of APN-knockout conditions on Pai-1 expression using abdominal fat tissue samples from APN homozygous knock-out mice. Pai-1 expression was observed at a high level compared to that of fat tissue from APN wild-type mice. In the late phase of 3T3-L1 cells, supplementation of Pai-1 resulted in a slight reduction of APN (Figure 4B).

Both APN and Pai-1 are known to be involved in colorectal carcinogenesis. An absence of APN results in an increase of intestinal polyp development in *Min* mice (10), while a PAI-1 inhibitor was reported to reduce intestinal polyp development in *Min* mice (23). It is assumed that both induction of APN and inhibition/suppression of Pai-1 may be a useful approach in colorectal cancer prevention. Here, we have demonstrated a seesaw pattern of regulation between APN and Pai-1. Further studies are required to identify more direct regulatory mechanisms between APN and Pai-1, and the molecular targets identified might be utilized as novel chemopreventive targets.

## Conflicts of Interest

None.

## Acknowledgements

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ORIGINAL ARTICLE

# The preventive effects of low-dose enteric-coated aspirin tablets on the development of colorectal tumours in Asian patients: a randomised trial

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## ABSTRACT

**Objective** To evaluate the influence of low-dose, enteric-coated aspirin tablets (100 mg/day for 2 years) on colorectal tumour recurrence in Asian patients with single/multiple colorectal tumours excised by endoscopy.

**Design** A double-blinded, randomised, placebo-controlled multicentre clinical trial was conducted.

**Participants** 311 subjects with single/multiple colorectal adenomas and adenocarcinomas excised by endoscopy were enrolled in the study (152 patients in the aspirin group and 159 patients in the placebo group). Enrolment began at the hospitals (n=19) in 2007 and was completed in 2009.

**Results** The subjects treated with aspirin displayed reduced colorectal tumourigenesis and primary endpoints with an adjusted OR of 0.60 (95% CI 0.36 to 0.98) compared with the subjects in the placebo group. Subgroup analysis revealed that subjects who were non-smokers, defined as those who had smoked in the past or who had never smoked, had a marked reduction in the number of recurrent tumours in the aspirin-treated group. The adjusted OR for aspirin treatment in non-smokers was 0.37 (CI 0.21 to 0.68, $p<0.05$). Interestingly, the use of aspirin in smokers resulted in an increased risk, with an OR of 3.44. In addition, no severe adverse effects were observed in either group.

**Conclusions** Low-dose, enteric-coated aspirin tablets reduced colorectal tumour recurrence in an Asian population. The results are consistent with those obtained from other randomised controlled trials in Western countries.

**The clinical trial registry website and the clinical trial number** http://www.umin.ac.jp (number UMIN000000697).

## Significance of this study

### What is already known on this subject?

- A considerable amount of evidence regarding the utility of aspirin as a cancer chemopreventive agent has been generated in Western populations.
- The evidence regarding aspirin as a cancer chemopreventive agent in Asian populations is limited. Moreover, no cancer chemopreventive drugs have been approved in Japan.
- The advantages of aspirin as a cancer chemopreventive agent are well recognised, as it has been in clinical use for a long period of time. In addition, aspirin's adverse effects and cost-effectiveness are well known.

### What are the new findings?

- We are the first to report the efficacy of low-dose, enteric-coated aspirin tablets in the suppression of colorectal tumour recurrence in Asian patients; these findings are consistent with the observations of other aspirin adenoma trials in Western populations.
- We report the safety of low-dose, enteric-coated aspirin tablets administered to patients as a cancer chemopreventive agent for 2 years.

### How might it impact on clinical practice in the foreseeable future?

- The evidence that aspirin is effective in the reduction of colorectal tumour recurrence in Asian patients may impact cancer preventive strategies in Japan and other Asian countries, including Korea and China.

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## INTRODUCTION

Among chemopreventive interventions, aspirin (acetylsalicylic acid) has been examined in numerous trials that support its suppressive effect on colorectal cancer (CRC) development. Aspirin is a synthetic medicine based on the structure of salicylates, which are commonly found in fruits and vegetables. Aspirin's antineoplastic effects have been mechanistically explained by its cyclooxygenase (COX) inhibitory activity. The use of aspirin as a cancer chemopreventive agent is advantageous because it has a long history of clinical use

and its adverse effects are well known. Moreover, the cost-effectiveness of aspirin administration to prevent other diseases, such as cardiovascular disease, has also been demonstrated.[1]

An early prospective cohort study of 662 424 adults (the Cancer Prevention Study II cohort) demonstrated that the CRC death rate decreased with frequent aspirin use. The decreased relative risk (RR) of CRC among frequent aspirin users (≥16 times/month for at least 1 year with doses greater than 160 mg) was 0.60 (95% CI 0.4 to 0.89) in men and 0.58 (95% CI 0.37 to 0.9) in women.[2] An updated analysis of this cohort (the Cancer Prevention Study II Nutrition cohort) demonstrated that long-term daily aspirin use (≥325 mg/day for ≥5 years) is associated with reduced incidence of CRC compared with non-users (RR=0.68, 95% CI 0.52 to 0.90 among men and women collectively).[3 4] The factors that may affect the impact of aspirin include the population, dose of aspirin and duration of intervention.[4] In the general population, trials of 75–325 mg/day aspirin for 3 years reduced the risk of recurrent of colorectal adenoma by 17%.[5] Moreover, the use of aspirin for 5 years or longer reduced the incidence and mortality of CRC by 30%–40% after 20-year follow-up.[6]

A considerable amount of evidence on the utility of aspirin has been generated in Western populations; however, the evidence for aspirin as a cancer chemopreventive agent in Asian populations is limited. Thus, it is important to present evidence that aspirin is also effective as a cancer chemopreventive agent in Asian populations.

We recently reported a double-blinded, randomised, placebo-controlled clinical trial of a high-risk CRC group, familial adenomatous polyposis, to evaluate the effect of low-dose, enteric-coated aspirin tablets. Secondary endpoint data from the trial revealed that subjects with a mean baseline polyp diameter of <2 mm administered aspirin displayed a significant reduction in mean polyp size.[7]

We investigated the effects of low-dose, enteric-coated aspirin tablets administered for 2 years in a double-blinded, randomised, placebo-controlled clinical trial in patients with a single/multiple colorectal adenomas and/or adenocarcinomas with invasions confined to the mucosa and excision by endoscopy. This population was considered to be a high-risk colorectal tumour group. Low-dose, enteric-coated aspirin tablets (100 mg/day) were chosen for the study because low-dose aspirin may circumvent the risk of upper GI toxicity.[8] In addition, the enteric coating may decrease gastric mucosal damage, as demonstrated in the MAJIC study targeting high-risk cardiovascular Japanese patients[9] as well as other short-term endoscopic studies.[10]

Here, we report the efficacy and safety of low-dose, enteric-coated aspirin tablets in the suppression of colorectal tumour recurrence in Asian patients with colorectal adenomas and/or adenocarcinomas with confined mucosal invasions that were excised by endoscopy.

## METHODS

### Trial methodology

In this double-blinded (both subjects and investigators), randomised, placebo-controlled trial using low-dose, enteric-coated aspirin tablets, the subjects received either 100 mg/day aspirin or placebo for 2 years. Each case was randomised by investigators using a computer-aided system from the Medical Research Support website. Using a minimisation algorithm, the primary examination selection was balanced with respect to three stratification variables: institution, age (≤60 and >60 years) and sex (male or female). The website was only available to the trial investigators. Subject enrollment and intervention assignment began at each hospital in January 2007, and the trial ended in July 2009. To further evaluate the effects of aspirin, follow-up for more than 2 years after the randomised trial was also planned. An Ethics Monitoring Committee was established for this multicentre trial (n=19) that was primarily based at Osaka Central Hospital. A system to ensure continuous follow-up of adverse events was also established. All hospitals participating in this trial obtained approval from their own ethics committees. This trial is registered and details are available at http://www.umin.ac.jp (number UMIN000000697), where the full trial protocol can be accessed.

### Trial population

The trial population (n=389) consisted of patients with single/multiple colorectal adenomas and/or adenocarcinomas with invasions confined to the mucosa. The colorectal tumours of all subjects participating in the trial were excised by endoscopy before the trial start. An endoscopic examination was performed twice before the start of the trial; the examinations occurred at an average of 488.4 ±472 (mean±SD) days apart to confirm that all colorectal tumours were excised. All of the subjects were Asian men or women 40–70-years-old living in Japan. The following are exclusion criteria for the trial: (1) patients with familial adenomatous polyposis, Lynch syndrome or colorectal resection; (2) patients currently taking an antithrombotic or anticoagulant, including aspirin; (3) individuals with a history of stroke or gastric/duodenal ulcers (with the exception of patients with confirmed scars resulting from the successful eradication of *Helicobacter pylori*); (4) patients with IBD, haemorrhagic diverticulitis or haemorrhagic tendency; (5) patients with a platelet count of ≤100 000/mm$^3$ or abnormal prothrombin time; (6) patients with a known aspirin allergy; (7) patients currently taking an anticancer drug; (8) pregnant patients or those who planned to become pregnant during the trial period; and (9) patients taking non-steroidal anti-inflammatory drugs (NSAIDs) for pain relief more than thrice weekly. We calculated that 266 randomised patients would achieve an 80% power (with a 5% type I error) to detect a 40% difference in the recurrence rate of adenoma given a 40% risk of recurrence in the placebo group.[11] However, data were unavailable to calculate an appropriate number of individuals to recruit from the Asian population; therefore, we set our recruitment goal in the initial aspirin protocol to 700 randomised patients.

Consent interviews were performed individually, and written informed consent was obtained from all patients.

### Investigational drug

Low-dose, enteric-coated aspirin tablets (100 mg per tablet) and the placebo tablets were kindly provided by Bayer Pharma AG (Leverkusen, Germany) and imported into Japan. The trial was financed by research funding from the Ministry of Health, Labor and Welfare, not by Bayer Pharma AG. We signed an agreement to certify that no conflicts of interest with Bayer Pharma AG existed. The investigational drugs were placed in blister packages (calendar sheets of 31 tablets), and both sides of the package were aluminium-laminated.

### Trial questionnaire

At the time of trial enrolment, the height, body weight, medical history, smoking history, alcohol ingestion and use of NSAIDs were investigated for each patient using a questionnaire. In addition, data regarding everyday meals were collected using a self-administered food-frequency questionnaire developed by the Department of Health Promotion and Preventive Medicine,

Nagoya-City University Graduate School of Medical Science, Aichi, Japan.[12] Non-smokers were defined as people who had smoked in the past or never smoked. Occasional drinkers were defined as people who drank less than twice a week.

To ensure the accurate characterisation of adverse effects and evaluation of tolerability, the subjects were asked to keep a treatment diary that documented their conditions during treatment, such as drug compliance and medical conditions, and a blister sheet was sent to the data centre every month.

### Trial endpoints

Colonoscopy was performed at least three times, twice before the start of the trial and once at the end of the trial. All the patients were given an oral lavage solution for colonic cleansing at the time of the colonoscopy for clear imaging, and a medical colonoscopy specialist carefully examined the patients from the rectum to the cecum. The final endoscopy examination was performed 2 years after the start of the trial. Recurrent tumours were further diagnosed by histology after tumour excision. The primary endpoint was the incidence of adenoma or adenocarcinoma recurrence. The data were analysed using logistic regression and ORs, and general factors, such as sex, age and the tumour number before the trial, were used to adjust occasional deviation during the randomised allocation. Each tumour was removed and examined histologically by a pathologist. Tumours were classified as adenomas or adenocarcinomas according to the 'Japanese Classification of Colorectal Carcinoma' criteria. The secondary endpoints included recurring tumour number, size and histology as well as the effects of lifestyle, such as smoking and alcohol drinking, and the frequency of adverse effects.

### Statistical analysis

The baseline characteristics of the two arms were compared using the $\chi^2$ test or the t test. The adverse effect rates of both arms were compared using the $\chi^2$ test. If needed, Fisher's exact probability was applied due to sparse data in a table. To adjust for potential confounding effects at baseline, logistic regression was performed.

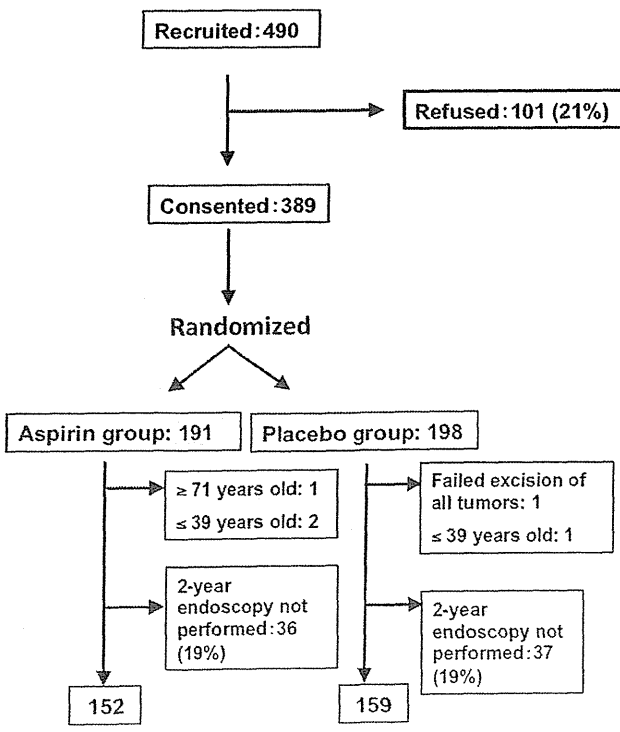


**Figure 1** Flowchart of subject recruitment.

We also examined the effect modification (interaction) of several factors, such as (1) sex, (2) age, (3) smoking and (4) alcohol drinking, on the main effect of aspirin by adding an interaction term to the logistic regression. In this analysis, we determined the ORs of the subgroups of the above factors and the difference in the ORs of the subgroups.

All statistical analyses were based on the intention-to-treat and performed using PC-SAS (V.9.3; SAS Inc., Cary, North Carolina, USA), with $p<0.05$ considered statistically significant.

## RESULTS

### Characteristics of the trial subjects

A total of 490 patients were screened, and 389 patients provided informed consent. Subject enrolment began in January 2007, and the trial ended in July 2009. Subject recruitment ended according to the planned time schedule. After randomisation, the aspirin and placebo group consisted of 191 and 198 subjects, respectively. At the end of the trial, 152 subjects from the aspirin group and 159 subjects from the placebo group underwent a 2-year follow-up endoscopy examination (figure 1). The characteristics of the subjects in the aspirin and placebo groups after randomisation are displayed in table 1. No significant differences between the two groups were observed with regard to the following characteristics: age, sex, smoking status, alcohol drinking status, height, weight, body mass index, tumour number upon entry into the trial, past history of CRC with invasion confined to the mucosa, treatment period, compliance (ie, whether patients correctly take medicine and follow the doctors' instructions (data not shown)), surgical history (data not shown) and family history of CRC (data not shown). The serum concentrations of alanine transaminase, aspartate amino transferase, γ-glutamyl transpeptidase and triglycerides were almost identical between the groups (data not shown).

### Colorectal tumour recurrence as the primary endpoint

In total, 96 patients did not experience colorectal tumour recurrence in the aspirin group (total 152), and 86 patients in the placebo group (total 159) did not recur. In crude analyses, the subjects in the aspirin group tended to demonstrate a reduced number of colorectal tumours, which was the primary endpoint, compared with subjects in the placebo group. The OR was 0.69 (95% CI

**Table 1** Characteristics of the two groups

| | Aspirin | | Placebo | |
|---|---|---|---|---|
| Number | 152 | | 159 | |
| Age | 60.0 | ±7.3* | 60.5 | ±6.6 |
| Sex | | | | |
| Male | 121 | (79.6%) | 125 | (78.6%) |
| Smoking | | | | |
| Current smokers | 45 | (29.6%) | 34 | (21.4%) |
| Alcohol | | | | |
| Drinker† | 83 | (54.6%) | 92 | (57.9%) |
| Height | 164.7 | ±6.8 | 165.5 | ±7.3 |
| Weight | 64.3 | ±9.7 | 65.6 | ±10.1 |
| BMI‡ | 23.6 | ±2.7 | 23.9 | ±2.8 |
| Number of tumours upon trial entry | 5.3 | ±5.7 | 5.1 | ±7.0 |
| Past CRC history | 40 | (26.3%) | 39 | (24.5%) |
| Treatment period | 751 | ±67 days | 764 | ±90 days |

*SD.
†Alcohol drinker: drinks more than three times a week.
‡Body mass index (BMI)=Weight (kg)/height (m) squared.
CRC, colorectal cancer.

**Table 2** The effects of aspirin on colorectal tumour development in smokers

| Subanalysis | No. of subjects with (+) or without (–) colorectal tumour | | | Adjusted OR (95% CI) |
|---|---|---|---|---|
| | – | + | Total | |
| Current smoker | | | | |
| Placebo group | 26 | 19 | 45 | 1 |
| Aspirin group | 14 | 20 | 34 | 3.45 (1.12 to 10.64), p=0.03 |
| Non-smoker* | | | | |
| Placebo group | 60 | 54 | 114 | 1 |
| Aspirin group | 82 | 36 | 118 | 0.37 (0.21 to 0.68), p=0.01 |

Adjusted OR, OR is adjusted by sex, age and the number of tumours prior to the trial.
*Non-smoker: never smoked and former smokers.

0.44 to 1.08); despite a marginal difference, the value was not statistically significant. To adjust for potential confounders, such as sex, age and the number of recurrent tumours, we performed logistic regression and obtained a significant OR value of 0.60 (95% CI 0.36 to 0.98). The OR for the number of recurrent tumours <4 in the aspirin group was 0.34 (0.09 to 1.26), and the OR for a tumour >3 mm in longitudinal diameter was 0.86 (0.63 to 1.16), but the value was not statistically significant.

### The effects of smoking and drinking on colorectal tumour recurrence

Using a logistic regression with smoking as the interaction term and aspirin as the effect, we observed that smoking displays strong effect modification on the main effect of aspirin (p for interaction=0.004). Namely, the OR for non-smokers was 0.37 (95% CI 0.21 to 0.68), and this value was significantly different from the OR for smokers (OR 3.44, 95% CI 1.12 to 10.64) after adjustment for age, sex and the number of tumours (table 2). In contrast, no significant effect modification for sex (p=0.68), age (p=0.53) or alcohol consumption (p=0.32) was observed. With regard to sex, the OR was 0.48 (95% CI 0.15 to 1.55) and 0.63 (95% CI 0.36 to 1.08) among men and women, respectively. For age, the OR was 0.68 (95% CI 0.36 to 1.28) and 0.49 (95% CI 0.22 to 1.08) for subjects aged <60 and ≥60 years, respectively. For alcohol consumption, the OR was 0.72 (95% CI 0.37 to 1.40) and 0.44 (95% CI 0.21 to 0.95; p<0.05) for drinkers and occasional drinkers, respectively.

In addition, no severe adverse effects, such as cardiovascular events, were reported in either group. GI bleeding was not observed. Of note, colorectal adenocarcinomas were observed in four subjects: two cases from the aspirin group (one adenocarcinoma with invasion confined to the mucosa, and one adenocarcinoma with muscularis propria invasion) and two in the placebo group (two adenocarcinomas with invasion confined to the mucosa). The remaining tumours were tubular adenomas; villous adenomas were not identified. In addition, three high-grade dysplasias were detected; one case was observed in the aspirin group, and two cases were noted in the placebo group. The adenocarcinomas were 10–20 mm in diameter. The lesions were localised to the transverse colon (n=2), the descending colon (n=1) and the sigmoid colon (n=1).

## DISCUSSION

In the present trial, we enrolled subjects with single/multiple colorectal adenomas and/or adenocarcinomas with invasions confined to the mucosa that were excised by endoscopy. Patients treated with low-dose, enteric-coated aspirin tablets for 2 years were shown to have a low risk of incidental colorectal tumour development, and this appeared to be reduced after adjustment for sex, age and the number of baseline tumours. Moreover, smoking significantly modified the preventive effect of aspirin.

In a meta-analysis of subjects with a history of colorectal adenoma or cancer in four randomised adenoma prevention trials (nearly 3000 patients), aspirin reduced the occurrence of advanced lesions (ie, tubulovillous adenomas, villous adenomas, adenomas ≥1 cm in diameter, adenomas with high-grade dysplasia or invasive cancer) by 28% (adenoma 17%; RR=0.83; 95% CI 0.72 to 0.96).[3] Our trial also demonstrated reduced adenoma occurrence (OR=0.69), and similar effects were obtained compared with the meta-analysis by Cole *et al.*[5] However, the ORs we used have a predictable effect on the comparison of the two sets of analyses. Regarding the limitations of our trial, the number of subjects enrolled is rather small, but the tumour recurrence results are consistent with previous studies. Thus, our data demonstrate that aspirin is also useful as a CRC chemopreventive agent in an Asian population. Of note, the first Asian adjuvant study (ASCOLT, NCT00565708) is ongoing, wherein patients with Dukes C or high risk Dukes B CRC are treated with aspirin (200 mg/day for 3 years).

Although the daily aspirin doses administered for vascular disease prevention are as effective as high-dose (1200 mg/day) aspirin,[11] analyses comparing moderate (300–325 mg/day) and lower (81–160 mg/day) aspirin doses trials (AFPPS[11] and APACC[13]) revealed that the reduced risk of all adenoma recurrence was only observed with lower doses.[5] Our trial using low-dose, enteric-coated aspirin tablets (100 mg/day) was designed in light of these trials, thereby confirming that low-dose, enteric-coated aspirin tablets effectively reduce recurrence. In addition, low-dose regimens may have an advantage in that the lower doses potentially reduce adverse effects. Aspirin has been reported to induce GI bleeding at a rate of 1–2 GI bleeds per 1000 person-years.[14] In our trial, no severe adverse effects due to aspirin treatment were observed.

Aspirin's antineoplastic effects are explained by COX-dependent and -independent mechanisms. In humans, aspirin inhibits COX-1 and COX-2 at high doses[15] and appears to effectively inhibit prostaglandin synthesis in the colon.[16] COX-independent mechanisms underlying aspirin's antineoplastic effects are attributed to the modulation of nuclear factor κ B: the induction of spermidine/spermine N1-acetyltransferase, caspase-8 and -9; and the activation of 5′ adenosine monophosphate-activated protein kinase, Erk and β-catenin.[17–23]

Despite copious information regarding aspirin's functions, the mechanism by which smoking negates aspirin's CRC chemopreventive effects remains unclear. A strong association between antiplatelet therapy resistance (aspirin resistance) and smoking has been reported. Specifically, a statistically significant interaction exists based on the multivariate analysis (risk ratio 11.47, CI 6.69 to 18.63, p<0.0001),[24] which is likely due to smoking-induced platelet hyperactivity and chronic inflammation.[25] In addition, smoking-induced decreased basal GI blood flow may also be involved.[26] Thus, it is suggested that smoking negated aspirin's chemopreventive effects in CRC. However, the evidence is limited. It is important to review and generate additional aspirin trial data to examine the association between NSAIDs use and smoking history and to determine whether the benefits of aspirin are limited to non-smokers.

In conclusion, although the size of this trial is small, the results are consistent with the observations of other aspirin adenoma trials; thus, aspirin may be useful for chemoprevention in Asian