

Dietary Administration of *Aloe Vera* Gel Extract Inhibits Intestinal Polyp Formation in Min Mice Fed a High-Fat Diet

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Abstract

Objectives: *Aloe vera* gel extract (AVGE) was recently produced using a new procedure with supercritical carbon dioxide fluid. AVGE was proven safe in acute toxicity and subchronic toxicological tests, and was shown to contain five phytosterols (*Aloe*-sterols). AVGE may inhibit the accumulation of visceral fat due to the functions of *Aloe*-sterols, which may, in turn, contribute to preventing metabolic syndrome. A meta-analysis previously revealed that obesity was positively associated with colon cancer. Therefore, we examined the effects of the dietary administration of AVGE on intestinal polyp formation in *Apc*-deficient Min mice fed a high-fat diet (HFD).

Methods: Male Min mice were divided into normal diet (ND), HFD, and AVGE groups. The ND group was fed an AIN-93G diet, the HFD group was given modified high-fat AIN-93G diets, and the AVGE group was fed HFD 0.0125% containing AVGE for 65 days.

Results: Between days 56 and 65, body weights were significantly lower in the HFD group than in the ND group, and were slightly lower in the AVGE group. The total number of polyps (≥ 0.5 mm in diameter) in the small intestine and total (small and large) intestine was significantly lower in the AVGE group than in the HFD group. When intestinal polyps were categorized by their size into 0.5-1.4, 1.5-2.4, or ≥ 2.5 mm, the number of large polyps (≥ 2.5 mm) in the small intestine was significantly lower in the AVGE group than in the HFD group. Plasma HMW adiponectin levels were significantly higher in the AVGE group than in the HFD group.

Conclusion: The dietary administration of AVGE reduced the total number of polyps and number of large polyps. By combining our results with other findings, the inhibition of cell proliferation by HMW adiponectin was suggested as one of the mechanisms underlying the suppression of intestinal polyp formation.

Keywords: APC-deficient Min mice; *Aloe vera* gel extract (AVGE); High molecular weight (HMW) adiponectin

Introduction

Aloe barbadensis Miller (*Aloe vera*) is a traditional medicinal plant. *Aloe vera* gel (inner leaf) has been used to treat burns and other wounds. Tanaka et al. [1] recently produced *Aloe vera* gel extract (AVGE) using a new procedure with supercritical carbon dioxide fluid, and demonstrated that it was safe using in the acute toxicity test and 90-d subchronic toxicological test in rats. AVGE has been shown to ameliorate serum alanine aminotransferase (ALT) and γ -glutamyl transpeptidase (GPT) levels in obese men. Saito et al [2] reported that AVGE prevented ethanol-induced fatty liver in mice by inhibiting the mRNA expression of lipogenic genes in the liver. AVGE contains five minor phytosterols, called *Aloe*-sterols: lophenol, 24-methyl-lophenol, 24-ethyl-lophenol, cycloartanol, and 24-methylene-cycloartanol. Among these phytosterols, lophenol and cycloartanol were previously identified to be anti-diabetic compounds that reduced visceral fat weight in Zucker diabetic fatty rats [3]. Furthermore, Nomaguchi et al. [4] found that these compounds acted as ligands of peroxisome proliferator-activated receptors (PPAR)- α and - γ , which regulate the metabolism of glucose and lipids in a diet-induced obesity mouse model. AVGE may inhibit the accumulation of visceral fat, which may, in turn, contribute to preventing metabolic syndrome.

The *Apc*-deficient Min (multiple intestinal neoplasia) mouse is a model for human familial adenomatous polyposis (FAP) in which many tumors are characteristically detected in the intestinal tract within three months [5]. Serum triglyceride levels are markedly higher (10-fold) in this mouse than in the wild-type mouse. Moreover, mRNA levels of lipoprotein lipase (LPL), which catalyzes the hydrolysis of triglycerides

into fatty acids and monoacylglycerol, were markedly lower in the liver and small intestine [6]. Niho et al. [7] also reported that the LPL selective inducer suppressed both hyperlipidemia and intestinal polyp formation in Min mice. Larsson and Wolk [8] showed that obesity positively correlated with colon cancer in a meta-analysis. Baltgalvis et al. [9] demonstrated that a high-fat diet (HFD), which induced obesity, increased the total number of polyps in Min mice. Adipose tissue produces and secretes various proinflammatory and anti-inflammatory factors, including adiponectin [10]. Adiponectin produces diverse biological effects including anti-inflammatory properties [11]. Fenton et al [12] reported that adiponectin blocked cell proliferation in Min mouse colon epithelial cells. Plasma adiponectin levels were shown to be low in obese humans [13]. Some dietary factors, such as soy protein, fish oil, and linoleic acid have previously been found to increase plasma adiponectin levels in rodent models [14-16].

Adiponectin binds to two adiponectin receptors, AdipoR1

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and AdipoR2. AdipoR1 is ubiquitously expressed while AdipoR2 is abundantly expressed in the liver. AdipoR1 activates the AMP-activated protein kinase pathway, whereas AdipoR2 activates the PPAR- α pathway, which has been shown decrease triglyceride content in the liver [13].

We previously demonstrated that the number of polyps (≥ 2.5 mm in diameter) in Min mice fed a high-fat diet was reduced by the oral administration of AVGE and AVGE ameliorated reductions in plasma high molecular weight (HMW) adiponectin levels [17].

In the present study, we examined the effects of the dietary administration of AVGE on intestinal polyp formation in Min mice fed a high-fat diet and measured plasma lipid and adiponectin levels. We also analyzed the mRNA levels of AdipoR2 and PPAR- α gene in the liver.

Materials and Methods

Materials and animals

AVGE was dissolved in 0.5% carboxymethylcellulose (CMC) solution and added to modified high-fat AIN-93G diets (HFD; Oriental Yeast Co. Ltd., Tokyo, Japan) at 0.0125%. We already demonstrated that the number of polyps (≥ 2.5 mm in diameter) in Min mice fed a high-fat diet was reduced by the oral administration of AVGE (12.5 mg/kg). Based on these findings [17], we calculated the concentration of AVGE in the diet to ensure that the same amount of AVGE was consumed on a daily basis. A HFD was also contained 0.5% CMC solution without AVGE. These were provided by Morinaga Milk Industry Co. Ltd. (Zama, Japan). Male C57BL/6J-APC^{Min/+} mice (Min mice) were originally purchased from Jackson Laboratories (Bar Harbor, ME, USA) and were bred with female C57BL/6J-APC^{+/+} mice purchased from Charles River Japan, Inc. (Tokyo, Japan). The *Apc*-deficient Min mouse is a model for human familial adenomatous polyposis. Min mouse adenomas were previously reported to develop more uniformly in the small intestine [18]. The presence of the mutant *APC* allele was detected in DNA from the tail using an allele-specific PCR assay as described by Jacoby et al. [18]. These mice were maintained under the management of laboratory animals in Nanakuri Laboratory of Animal Models for Human Diseases, Fujita Health University. They were kept in groups of one or two in plastic cages on woodchip bedding and were fed the normal diet AIN-93G (ND; Oriental Yeast Co., Ltd., Tokyo, Japan) in an animal facility controlled at a temperature of $23 \pm 5^\circ\text{C}$, $60 \pm 5\%$ humidity, and with a 12-h light/dark cycle. The care and use for the animals was according to the 'Guidelines for the Management of Laboratory Animals in Fujita Health University' and the experimental protocols were approved by the Institutional Animal Care and Use Committee of Fujita Health University.

Experimental Designs

Experiments were performed as follows. Five or six-week-old male Min mice were divided into ND (n=10), HFD (HFD + 0.5% CMC, n=21), and AVGE (HFD + AVGE, n=20) groups. The ND, HFD, and AVGE groups were given these diets *ad libitum* for 65 days. We decided the final experimental day when significant weight loss was observed in the HFD group. Mice were observed and food intake was measured daily. At the end of the experiment, all mice were anesthetized with Nembutal, exsanguinated via the heart into heparin-coated syringes, and carefully autopsied. After sacrifice, the small intestine, large intestine, and liver were removed from each mouse. The intestines were used for polyp assessments and the liver was kept at -80°C until

the analysis of mRNA expression levels. However, as one mouse in the AVGE group, which was moribund and autopsied on the 49th day, had small intestinal polyps, the mice alive on that day were counted as the effective number.

Intestinal polyp count and size comparison

The number and size of polyps were determined according to the procedure described in Ushida et al. [19]. Briefly, the entire intestine was flushed with saline and cut longitudinally. It was then spread on filter paper with the lumen side up and fixed in 10% neutral buffered formalin. Thereafter, we scored the number and size of polyps and categorized them into 0.5-1.4, 1.5-2.4 or greater than or equal to 2.5 mm in diameter. We evaluated the number and size of polyps by dividing the intestine into 3 parts: the small intestine, large intestine, and total (small+large) intestine.

Determination of plasma lipid levels

Plasma levels of triglycerides (TG) and total cholesterol (T-Cho) were enzymatically measured with Triglyceride E-Test Wako and Cholesterol E-test Wako kits (Wako Pure Chemical Industries, Ltd., Osaka, Japan), respectively.

Determination of plasma adiponectin levels

Plasma high molecular weight (HMW) adiponectin (Shibayagi Co., Ltd., Gunma, Japan) levels were determined by enzyme-linked immunosorbent assay (ELISA) kits, according to the manufacturer's protocol.

Messenger RNA (mRNA) expression by quantitative reverse transcription polymerase chain reaction (RT-PCR)

Total RNA was isolated from the liver using the High Pure RNA Tissue Kit (Roche Diagnostics GmbH, Mannheim, Germany) and cDNA was amplified using the Transcriptor Universal cDNA Master Kit (Roche Diagnostics GmbH). Messenger RNA (mRNA) expression levels of peroxisome proliferator-activated receptor- α (PPAR- α) and adiponectin receptor-2 (AdipoR2) were quantified by Taqman real-time quantitative PCR using the LightCycler 480 system and LightCycler 480 Probe Master kit (Roche Diagnostics GmbH). The housekeeping gene β -actin was used for the normalization of all analyzed genes.

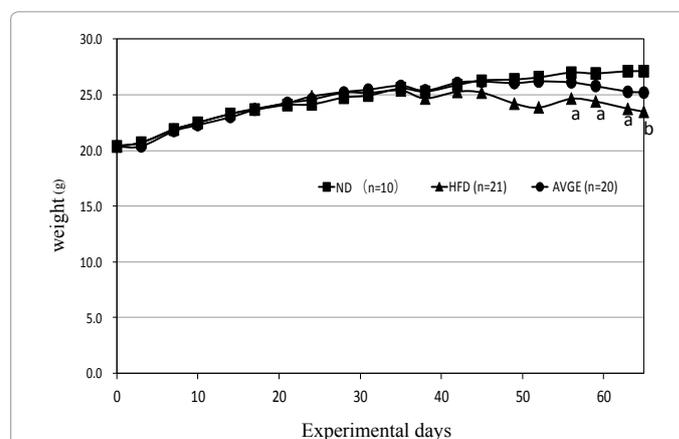


Figure 1: Changes in body weight. Body weights were significantly lower in the HFD group than in the ND group on days 56 to 65, and were slightly lower in the AVGE group.^{a,b}Significantly different from the ND group at ^a $p < 0.05$ and ^b $p < 0.01$ (Dunnett's multiple comparisons test).

Statistical analysis

Values are expressed as the mean \pm SE. The unpaired *t*-test was used to compare the ND group with the HFD group. Statistical analyses of changes in body weight, the total number of large polyps and plasma, HMW adiponectin levels were performed by a one-way analysis of variance (ANOVA) followed by the Dunnett's multiple comparisons test. Size distribution was compared with the Kruskal-Wallis test (nonparametric ANOVA) followed by the Dunn's multiple comparisons test. A correlation analysis was performed using Pearson's correlation test. These procedures were performed using InStat version 3.0 for Windows (GraphPad Software, Inc., San Diego, CA, USA). Differences were considered to be significant at $p < 0.05$.

Results

Changes in body weight and food consumption

Body weights were significantly lower in the HFD group than in the ND group from 56 days after the start of the experiment (Figure 1), and were slightly lower in the AVGE. Food consumption was not significantly different between the two groups fed HFD (data not shown). Baltgalvis et al. [9] demonstrated that a high-fat diet containing 21% fat increased body weight slightly and the total number of polyps. In the present study, we used a modified high-fat diet that provided 41% energy from fat, 39% from carbohydrates, and 19% from protein. However, body weight did not increase in the HFD group. A high-fat

diet containing 40% fat has been suggested to increase intestinal polyp formation before the onset of obesity [20].

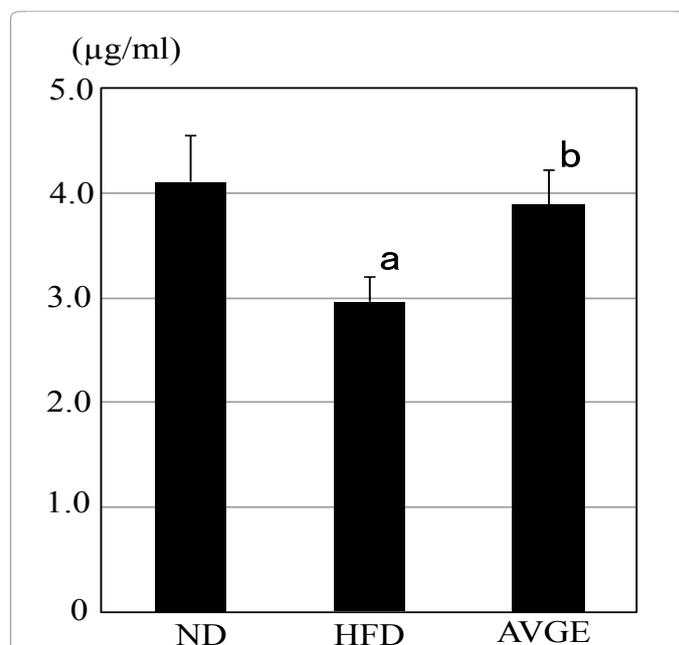
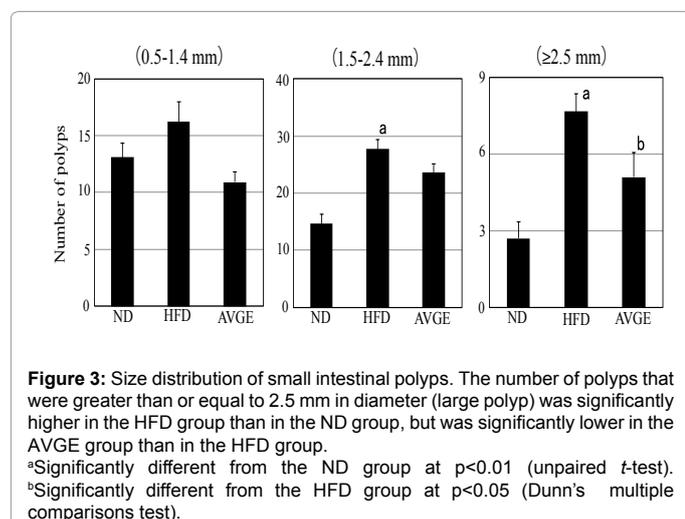
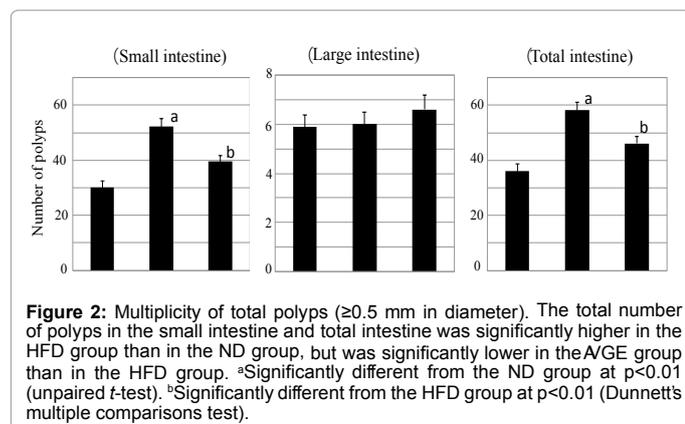
Number and size of polyps

As shown in Figure 2, the total number of polyps (≥ 0.5 mm in diameter) in the small intestine was significantly higher in the HFD group than in the ND group, but was significantly lower in the AVGE group than in the HFD group. No significant differences were observed in the total number of polyps in the large intestine among the three groups. The total number of polyps in the total intestine (small and large intestine) was significantly higher in the HFD group than in the ND group, but was significantly lower in the AVGE group than in the HFD group.

The size distribution of small intestinal polyps was shown in Figure 3. No significant differences were observed in the number of polyps that were 0.5 to 1.4 mm in diameter among the three groups. The number of polyps that were 1.5 to 2.4 mm in diameter was significantly higher in the HFD group than in the ND group, while no significant differences were observed between the AVGE group and ND group. The number of polyps that were greater than or equal to 2.5 mm in diameter (large polyp) was significantly higher in the HFD group than in the ND group, but was significantly lower in the AVGE group than in the HFD group. No significant differences were noted in the respective number of polyps of the three categorized sizes in the large intestine between the HFD group and AVGE group.

Plasma TG and T-Cho levels

Plasma TG levels (HFD group: 348.6 ± 44.7 mg/dL vs. ND group: 119.2 ± 28.2 mg/dL, $p < 0.01$) were significantly higher in the HFD group than in the ND group. However, a slight decrease was noted in the AVGE group (317.8 ± 43.7 mg/dL). Plasma T-Cho levels (HFD



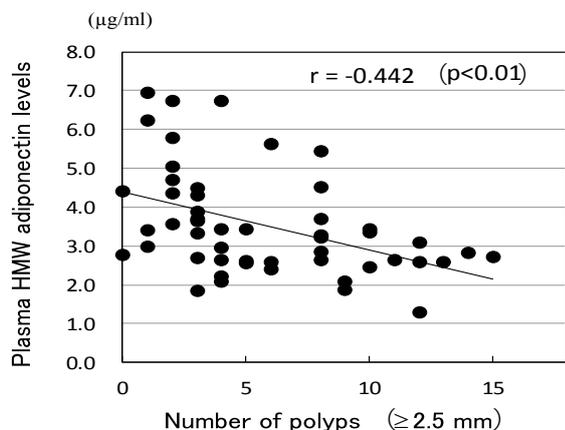


Figure 5: Relationships between the number of large polyps (≥ 2.5 mm) and plasma HMW adiponectin levels in the small intestine. A negative correlation was observed between the number of large polyps (≥ 2.5 mm in diameter) and plasma HMW adiponectin levels in the small intestine.

group: 113.4 ± 6.9 mg/dL vs. ND group: 92.7 ± 3.7 mg/dL, $p < 0.01$) were also significantly higher in the HFD group than in the ND group. On the other hand, a slight decrease was observed in the AVGE group (104.3 ± 9.5 mg/dL).

Plasma HMW adiponectin levels

As shown in Figure 4, HMW adiponectin levels were significantly lower in the HFD group than in the ND group. On the other hand, the levels in the AVGE group were significantly ($p < 0.05$) higher than those in the HFD group. As shown in Figure 5, we also confirmed that a negative correlation existed between the number of large polyps (≥ 2.5 mm in diameter) and plasma HMW adiponectin levels in the small intestine ($r = -0.442$, $p < 0.01$).

AdipoR2 and PPAR- α mRNA expression levels in the liver

AdipoR2 mRNA expression levels (HFD group: 0.58-fold, $p < 0.01$; AVGE group: 0.62-fold, $p < 0.01$ vs. ND group) were significantly lower in the HFD and AVGE groups than in the ND group. No significant differences were observed in PPAR- α mRNA expression levels (HFD group: 0.89-fold, AVGE group: 0.97-fold, vs. ND group) between the three groups.

Discussion

In the present study, we showed that the total number of polyps (≥ 0.5 mm in diameter) in the small intestine and total (small and large) intestine was significantly lower in the AVGE group than in the HFD group. We also showed that the number of large polyps (≥ 2.5 mm in diameter) in the small intestine was lower in the AVGE group than in the HFD group. Niho et al. [7] reported that NO-1886, 4-[(4-bromo-2-cyanophenyl)carbamoyl]benzylphosphonate, which increases LPL mRNA and protein levels, decreased serum TG and T-Cho levels as well as intestinal polyp formation in Min mice fed the AIN-76A basal diet. In this experiment, plasma TG and T-Cho levels in the AVGE group were not significantly lower than those in the HFD group. Nomaguchi et al. [4] demonstrated that two kinds of *Aloe*-sterols, lophenol and cycloartanol, which were orally administered for 12 weeks, reduced serum TG and T-Cho levels in a diet-induced obesity mouse model fed a HFD. However, lophenol and cycloartanol was orally administered at a daily dose of 1 μ g in their experiment. In our study, mice ingested *Aloe*-sterols at a daily dose of 0.2 μ g when computed from food

consumption. We speculated that the differences observed in TG and T-Cho levels depended on the dose and duration of the administration protocol.

The results of the present study showed that the decreased levels of HMW adiponectin by HFD was significantly inhibited by AVGE. Misawa et al. [21] also reported that serum HMW adiponectin concentrations in Zucker diabetic fatty rats fed HFD were slightly increased by 44 consecutive days of the oral administration of lophenol and cycloartanol. Adiponectin is an adipocyte-specific secretory protein that has been implicated in metabolic syndrome, type 2 diabetes mellitus, obesity, and arteriosclerosis [22]. Particularly, plasma adiponectin levels were shown to be reduced in obese human with the accumulation of visceral fat [13]. In the present study, however, weight loss in the HFD group was attributed to a polyp burden, and not a reduction in visceral fat. Indeed, body weight in the HFD group was significantly lower than that in the ND group. However, the total number of polyps in the HFD group was not decreased; it actually increased.

Adiponectin circulates in serum in at least 3 forms: a low-molecular weight (LMW) trimer, middle-molecular weight (MMW) hexamer and high-molecular weight (HMW) multimer adiponectin [23]. In these forms, the HMW multimer form was shown to be the active form of this protein [24]. Pajvani et al. [25] reported that HMW adiponectin correlated with metabolic disorders. Liu et al. [26] also demonstrated that HMW adiponectin was the main physiological determinant of the metabolic effects of adiponectin. Therefore, we analyzed plasma HMW adiponectin levels. Azoxymethane-induced colorectal carcinogenesis was previously shown to be enhanced in adiponectin-deficient mice fed a HFD [27]. They also demonstrated that colon epithelial cell proliferative activity in adiponectin-deficient mice fed a HFD was increased. Mutoh et al. [28] found that adiponectin-deficient Min mice fed a ND had a higher total number of polyps in the total intestine. Furthermore, the suppression of the growth of intestinal adenomas in Min mice by the exogenous administration of adiponectin was previously reported [29]. In the present study, a negative correlation was observed between HMW adiponectin levels and the number of large polyps (≥ 2.5 mm in diameter) in the small intestine. Otani et al. [29] also reported that the number of polyps that were greater than or equal to 2 mm in diameter in the small intestine was decreased by the administration of adiponectin. HMW adiponectin is thought to be involved in reducing the size of large polyps in the small intestine. Furthermore, we demonstrated that cell proliferation in the large intestines of wild mice fed the same AVGE diet for 6 weeks was decreased (Chihara et al., unpublished data). By combining our results with the above findings, HMW adiponectin, which was ameliorated by AVGE, appeared to decrease the number of large polyps by inhibiting cell proliferation in Min mice fed a HFD. The main components in AVGE that ameliorated HMW adiponectin may be *Aloe*-sterol. However, HMW-adiponectin, which was ameliorated by AVGE, did not affect the mRNA expression levels of AdipoR2 and PPAR- α in the liver. Nomaguchi et al. [4] demonstrated that the oral administration of lophenol and cycloartanol for 4 weeks increased liver PPAR- α mRNA expression levels in a diet-induced obesity mouse model fed a HFD. We speculated that the difference observed in PPAR- α mRNA levels depended on the dose administered.

On the other hand, Min mice are commonly known as a model of cancer cachexia [30]. The severe weight loss associated with cancer

cachexia was previously reported following normal weight loss at 15-18 weeks of age [30]. In the present study, weight reductions in the AVGE group were not significant. Therefore, AVGE may have attenuated the progression of cancer cachexia.

In conclusion, the dietary administration of AVGE reduced the total number of polyps in the total and small intestine and the number of large polyps in the small intestine. AVGE also ameliorated plasma HMW adiponectin levels, which is consistent with previous findings. Plasma TG and T-Chol levels remains unchanged. Furthermore, the mRNA expression levels of AdipoR2 and PPAR- α were not increased in the liver. By combining our results with other findings, the inhibition of cell proliferation by HMW adiponectin was suggested as one of the mechanisms underlying the suppression of intestinal polyp formation. We are now analyzing the mRNA expression levels of lipid metabolism genes in the liver to clarify the precise mechanism responsible for this suppression.

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