〈Original article〉

Erythrocyte polyamine levels are greater than triglyceride levels as markers of intestinal polyp formation in Min mice

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Summary Multiple intestinal neoplasia (Min) mice are a model of familial adenomatous polyposis coli (FAP). Min mice spontaneously develop intestinal polyps, mainly in the small intestine, and, as such, are regarded as an excellent animal model for investigating the effects of chemopreventive compounds on colon tumorigenesis. Serum lipid levels are higher in Min mice, and a relationship has been reported between serum lipid levels and intestinal polyp formation. In this study, we analyzed erythrocyte polyamine levels in 12- and 19-week-old Min mice. At 19-weeks, erythrocyte polyamine and triglyceride levels were significantly higher in Min mice than in wild-type mice, but the rates of increases in erythrocyte polyamine levels were higher than that in triglycerides levels. At 12-weeks, the erythrocyte polyamine levels were significantly higher in Min mice than in wild-type mice. However, triglyceride levels were not significantly higher. Thus, the determination of erythrocyte polyamine levels with age are greater than triglyceride levels as markers of intestinal polyp formation in Min mice.

Key words: Multiple intestinal neoplasia (Min) mice, Intestinal polyps, Triglycerides, Erythrocyte polyamine

1. Introduction

Multiple intestinal neoplasia (Min) mice were originally identified by Moser et al.[1] Min mice have a heterozygous mutation in the tumor suppressor gene, adenomatous polyposis coli (*Apc*). The *APC* gene is mutated in familial adenomatous polyposis (FAP)[2]. Min mice spontaneously develop intestinal polyps, similar to humans, but mainly in the small intestine[2]. Polyps form as early as approximately 4 weeks of age[3]. At 23-26 weeks of age, approximately 100 polyps develop in the small intestine, at which time these mice become moribund and die due to severe anemia and apparent intestinal obstruction[4]. Therefore, Min mice are regarded as an excellent animal model for investigating the effects of chemopreventive compounds on colon tumorigenesis[5]. Serum levels of triglycerides are higher in Min mice than in wild-type mice. Moreover, mRNA levels of lipoprotein lipase, which catalyzes the hydrolysis of triglycerides, are markedly lower in the liver and

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small intestine[6]. Niho et al. previously reported that pioglitazone, a peroxisome proliferator-activated receptor (PPAR) γ agonist, suppressed serum lipid levels and intestinal polyp formation[7], and demonstrated that indomethacin, a known general cyclooxygenase inhibitor, exerted the same effects in Min mice[8]. These findings suggest that a relationship exists between serum lipid levels and intestinal polyp formation in Min mice. In our previous study, we had measured plasma triglyceride levels in order to determine whether chemopreventive compounds inhibit intestinal polyp formation using Min mice[9,10].

Polyamines (putrescine, spermidine, and spermine) are low-molecular-weight biogenic polycationic amines that exist in all living cells. Polyamines are closely involved in many aspects of cell growth and proliferation. Russell was the first to show that urinary polyamine levels increased in several cancer patients, and suggested that these levels had potential as a useful marker for cancer[11]. Increases in erythrocyte polyamine levels were subsequently demonstrated in cancer patients and animals[12]. The greater utility of erythrocyte polyamines over urinary polyamines as cancer markers has been reported[13,14,15]. We also confirmed the usefulness of erythrocyte polyamine levels in mice in which duodenal tumorigenesis was induced using *N*-ethyl-*N*'-nitro-*N*-nitrosoguanidine[16].

In the present study, we measured erythrocyte polyamine levels in Min mice, and compared the usefulness of erythrocyte polyamine levels and plasma triglyceride levels as markers of intestinal polyp formation.

2. Materials and methods

2.1. Animals

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 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.[17] These mice were maintained under the management of laboratory animals in the Nanakuri Laboratory of Animal Models for Human Diseases, Fujita Health University. They were kept in groups of two or three in plastic cages on woodchip bedding in an animal facility controlled at a temperature of 23±5°C, 60±5% humidity, and with a 12-h light/dark cycle. Mice were fed the normal diet MF (Oriental Yeast Co., Ltd., Tokyo, Japan). The care and use of animals was according to the Regulations for the Management of Laboratory Animals at Fujita Health University, which is accredited by the Japanese Association of Laboratory Animals Facilities of Public and Private Universities (JALAP). Experimental protocols were approved by the Institutional Animal Care and Use Committee of Fujita Health University.

2.2. Experimental design

In experiment, 47 Min mice (26 males and 21 females) and 30 wild-type mice (C57BL/6J-*Apc*$^{+/+}$, 15 males and 15 females) were maintained until 19 weeks. Swamy et al.[18] previously reported that 19-week-old Min mice were likely to die from severe anemia or intestinal obstruction. These mice were anesthetized using an intraperitoneal injection of Nembutal, exsanguinated via the heart into heparin-coated syringes, and carefully autopsied. After sacrifice, the small intestine and large intestine were removed from each mouse. Blood was used to measure erythrocyte polyamine levels and plasma triglyceride levels, while the intestines were used to evaluate polyp formation. The number of polyps was determined according to the procedure described by 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 (diameter) of polyps. In addition, male Min mice and wild-type mice (n=6 each) were maintained until 12 weeks of age. The experimental procedure was the same as that described above.

2.3. Determination of erythrocyte polyamine levels

Sample preparation

Heparinized blood samples were centrifuged at 4400 × g at 4°C for 20 min. After the removal of plasma and the buffy coat layer, two volumes of water were added to packed erythrocytes, which were then and hemolyzed by vortex-mixing for 30 seconds. The same volume of ice-cold perchloric acid (100 g/L) with water was added, and the mixture was incubated on ice overnight to precipitate proteins. It was then centrifuged for 20 min at 17400 × g. The pH of the supernatant was adjusted to 7 by adding 1.4 mol/L potassium hydroxide and then left to stand at -20°C for 3 hours. The supernatant was centrifuged and the liquid phase was evaporated to dryness. The residue was dissolved in the same amount of 0.1 mol/L HCl with packed erythrocytes and 10 μL was injected into the HPLC system.

Apparatus and chromatographic conditions

The HPLC system consisted of a Waters 600E pump and system controller (Waters Corporation, Milford, MA, USA), Rheodyne injection valve equipped with a 20-μL sample loop (Rheodyne Inc., Cotati, CA, USA), Intelligent Column Oven CO-1565 (Japan Spectroscopic Co., Ltd. Tokyo, Japan), and Intelligent Spectrofluorometer 821-FP (Japan Spectroscopic Co., Ltd. Tokyo, Japan).

Erythrocyte polyamines were assayed using a slightly modified version of the method described by Löser et al.[20] Briefly, the elution procedure was performed using two mobile phases. Solvent A was 0.1 mol/L sodium acetate containing 0.01 mol/L sodium octanesulphonate, while solvent B was 0.2 mol/L sodium containing acetonitrile (10:3, v/v) and 0.01 mol/L sodium octanesulphonate with the following gradient conditions: 50% B at 0 min, 85% B at 15 min, 100% B at 7.5 min, 100% B at 15 min, and 50% B at 16 min. The flow rate was 1.5 mL/min and the temperature of the column oven was 35°C. The separation column was a TSK-gel ODS-100V (4.6 mm I.D. × 250 mm, 5 μm particle size, TOSOH Corporation, Tokyo, Japan). After post-column derivatization with *o*-phthalaldehyde, fluorescence intensity was measured at 455 nm after excitation at 345 nm.

Determination of plasma triglyceride levels

Plasma triglyceride levels were enzymatically measured with the Triglyceride E-Test Wako kit (Wako Pure Chemical Industries, Ltd., Osaka, Japan), according to the manufacturer's protocol.

2.4. Statistical analysis

Values are expressed as the mean ± SE. Statistical analyses of plasma triglyceride levels and erythrocyte polyamine levels were performed using the unpaired *t*-test. A correlation analysis was performed using Pearson's correlation test. These procedures were performed with InStat version 3.0 for Windows (Graph Pad Software, Inc., San Diego, CA, USA).

3. Results

3.1. Number of polyps in 19-week-old Min mice

The total number of polyps was 50.2 ± 2.8. Of these, the number of polyps equal to or greater than 1.5 mm in diameter was 29.5 ± 2.5. The 1.5 mm diameter was an appropriate value that indicated follow-up assessment of polyp growth with age in Min mice[21].

3.2. Plasma triglyceride levels at 19-weeks

As shown in Figure 1, plasma triglyceride levels were significantly higher (by approximately 4-fold) in Min mice than in wild-type mice.

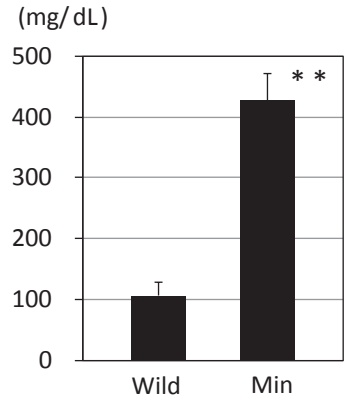

Fig. 1 Plasma triglyceride levels at 19-weeks
* * p<0.01 (*vs* Wild-type mice ; unpaired *t*-test)

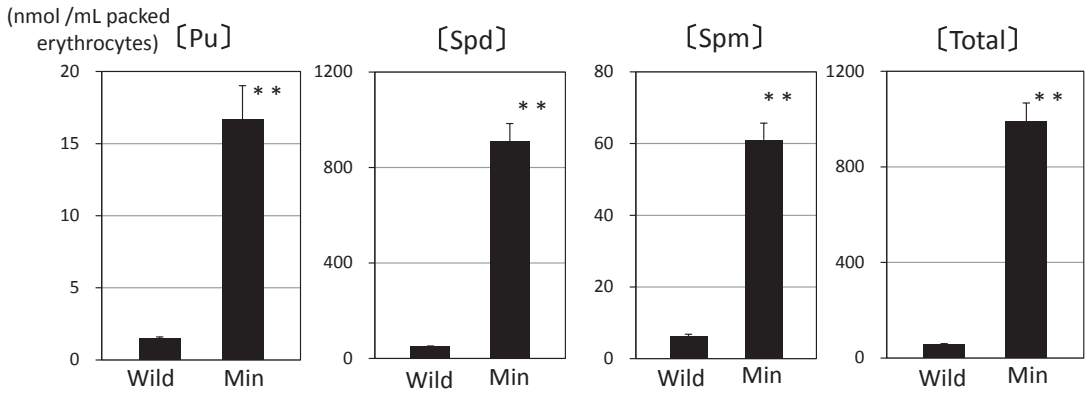

Fig.2 Erythrocyte polyamine levels at 19-weeks
Abbreviations: Pu, Putrescine; Spd, Spermidine; Spm, Spermine; Total, Total polyamine (putrescine + spermidine + spermine)
* * p<0.01 (*vs* Wild-type mice ; unpaired *t*-test)

3.3. Erythrocyte polyamine levels at 19-weeks

Figure 2 shows that the erythrocyte polyamines putrescine, spermidine, and spermine and total polyamine (putrescine + spermidine + spermine) levels were significantly higher (by approximately 11-fold, 18-fold, 10-fold, and 17-fold, respectively) in Min mice than in wild-type mice.

3.4. Relationship between the number of polyps and plasma triglyceride levels at 19-weeks

The relationship between the numbers of polyps and plasma triglyceride levels was shown in Figure 3. A positive correlation was observed between the number of polyps and plasma triglyceride levels ($r=0.49$, $p<0.001$).

3.5. Relationships between the number of polyps and erythrocyte polyamine levels at 19-weeks

The results in Figure 4 show relationships between the number of polyps and each polyamine and total polyamine levels. Positive correlations were observed for all data sets (putrescine; $r=0.54$, $p<0.0001$, spermidine; $r=0.46$, $p<0.005$, spermine; $r=0.40$, $p<0.01$, total; $r=0.46$, $p<0.005$).

3.6. Number of polyps in 12-week-old Min mice

The total number of polyps in 12-week-old Min mice (52.5 ± 8.7) was approximately the same as that in 19-week-old Min mice. On the other hand, the number of polyps equal to or greater than 1.5 mm in diameter (11.5 ± 3.1) was significantly lower than that at 19-weeks ($p<0.01$).

3.7. Plasma triglyceride levels at 12-weeks

As shown in Figure 5, plasma triglyceride levels were approximately 1.2-fold in 12-week-old Min mice than in wild-type mice.

3.8. Erythrocyte polyamine levels at 12-weeks

Erythrocyte polyamine levels in 12-week-old Min mice were shown in Figure 6. Spermidine and total polyamine levels were significantly higher (by approximately 3.7-fold and 3.4-fold, respectively, $p<0.05$) in Min mice than in wild-type mice. Putrescine and spermine levels were approximately 2.1-fold and 1.8-fold higher, respectively, in Min

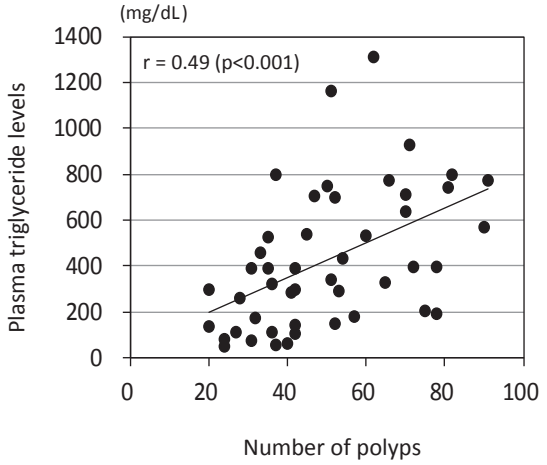

Fig. 3 Relationship between the number of polyps and plasma triglyceride levels at 19-weeks

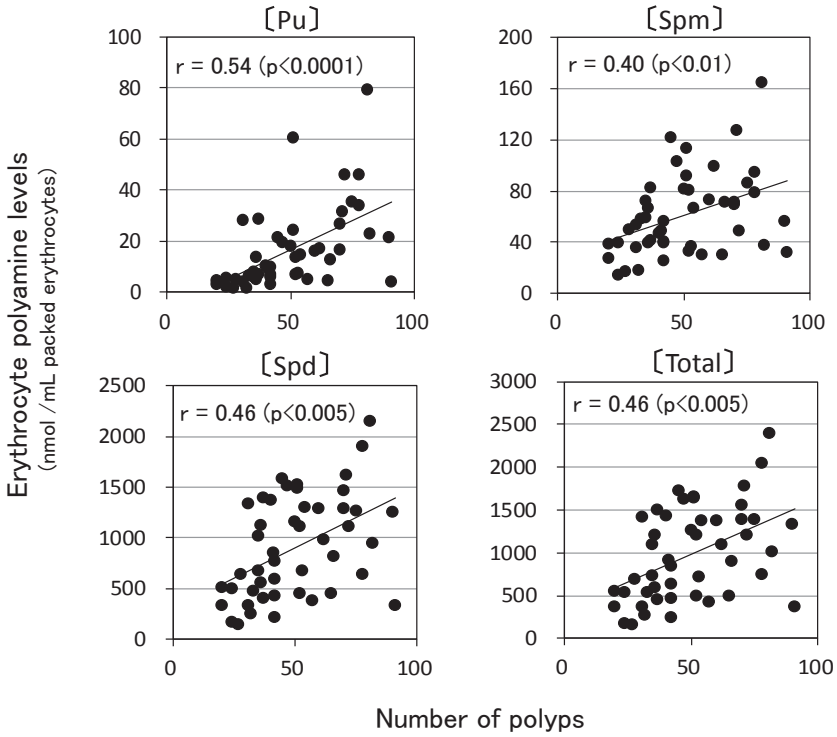

Fig. 4 Relationship between the number of polyps and erythrocyte polyamine levels at 19-weeks

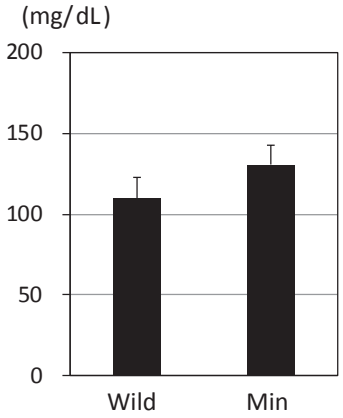

Fig. 5 Plasma triglyceride levels at 12-weeks

mice than wild-type mice. However, they were not statistically significant.

4. Discussion

Min mice are an animal model for human FAP[2], and, thus, regarded as an excellent model for investigating the effects of chemopreventive compounds in a genetic model of intestinal cancer[5]. Niho et al. demonstrated that triglyceride and free fatty acid levels increased with age in Min mice[6] and

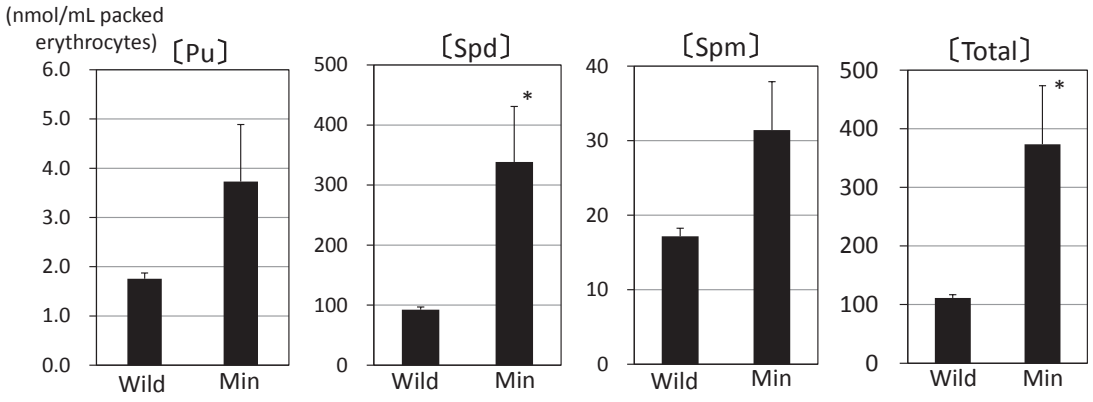

Fig. 6 Erythrocyte polyamine levels at 12-weeks
* p<0.05 (*vs* Wild-type mice ; unpaired *t*-test)

that reductions in serum lipid levels suppressed intestinal polyp formation[8]. Hence, the level of one type of lipid, triglyceride, is an important measure in experiments using Min mice.

In the present study, we showed that triglyceride levels were significantly higher (approximately 4-fold) in 19-week-old Min mice than in wild-type mice of the same age. On the other hand, the levels of the erythrocyte polyamines, namely putrescine, spermidine, spermine and total polyamine levels were also significantly higher (by approximately 11-fold, 18-fold, 10-fold, and 17-fold, respectively) in Min mice than in wild-type mice. The rates of increases in all polyamine levels were higher than that in triglyceride levels. Additionally, relationships between the number of polyps and erythrocyte putrescine levels were stronger than that for triglycerides levels ($p<0.0001$).

We also compared triglyceride and erythrocyte polyamine levels in 12-week-old Min mice with those in wild-type mice of the same age. The results obtained showed that triglyceride levels were slightly higher (not significant), whereas erythrocyte polyamine, namely spermidine, and total polyamine levels were significantly higher in Min mice than in wild-type mice. Putrescine and spermine levels were also increased. Erdman et al.[22] reported that the content of polyamines in the small intestinal tissue was already higher in at 65-day-old Min mice than in normal littermates. Soda[23] showed that increases in blood and urine polyamine levels reflected enhancements in the synthesis of polyamines in cancer tissues.

In conclusion, we herein analyzed plasma levels of triglycerides and erythrocyte polyamine levels in order to compare their usefulness as markers of intestinal polyp formation in 12- and 19-week-old Min mice. At 19-weeks, both were significantly higher in Min mice than in wild-type mice. However, the rates of increase observed in erythrocyte polyamine levels were higher than that in triglyceride levels. At 12-weeks, erythrocyte polyamine levels were significantly higher in Min mice than in wild-type mice, whereas triglyceride levels were not. Thus, investigation on changes in erythrocyte polyamine levels with age may be useful for assessing intestinal polyp formation in Min mice.

Conflict of interests

The authors declare no conflict of interests.

Acknowledgements

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References

1. Moser AR, Pitot HC, Dove WF: A dominant mutation that predisposes to multiple intestinal neoplasia in the mouse. Science, 247: 322-324, 1990.
2. Moser AR, Luongo C, Gould KA, McNeley MK, Shoemaker AR, Dove WF: *Apc*^{Min}: a mouse model for intestinal and mammary tumorigenesis. Eur J Cancer, 31A: 1061-1064, 1995.
3. Puppa MJ, White JP, Sato S, Cairns M, Baynes JW, Carson JA: Gut barrier dysfunction in the *Apc*$^{Min/+}$ mouse model of colon cancer cachexia. Biochim Biophys Acta, 1812: 1601-1606, 2011.
4. Reitmair AH, Cai J-C, Bjerknes M, et al.: MSH2 deficiency contributes to accelerated APC-mediated intestinal tumorigenesis. Cancer Res, 56: 2922-2926, 1996.
5. Mutanen M, Pajari A-M, Päivärinta E, et al.: Berries as chemopreventive dietary constituents – a mechanistic approach with the ApcMin/+ mouse. Asia Pac J Clin Nutr, 17: 123-125, 2008.
6. Niho N, Takahashi M, Kitamura T, et al.: Concomitant suppression of hyperlipidemia and intestinal polyp formation in *Apc*-decifient mice by peroxisome proliferator-activated receptor ligands. Cancer Res, 63: 6090-6095, 2003.
7. Niho N, Takahashi M, Shoji Y, et al.: Dose-dependent suppression of hyperlipidemia and intestinal polyp formation in Min mice by pioglitazone, a PPARγ ligand. Cancer Sci, 94: 960-964, 2003.
8. Niho N, Mutoh M, Komiya M, Ohta T, Sugimura T, Wakabayashi K: Improvement of hyperlipidemia by indomethacin in Min mice. Int J Cancer, 121: 1665-1669, 2007.
9. Chihara T, Shimpo K, Beppu H, et al.: Reduction of intestinal polyp formation in Min mice fed a high-fat diet with *Aloe vera* gel extract. Asian Pac J Cancer Prev, 14: 4435-4440, 2013.
10. Chihara T, Shimpo K, Beppu H, et al.: Dietary administration of *Aloe vera* gel extract inhibits

intestinal polyp formation in Min mice fed a high-fat diet. Pharm Anal Acta, 6: 1000340, 2015.

11. Russell DH: Increased polyamine concentrations in the urine of human cancer patients. Nat New Biol, 233: 144-145, 1971.
12. Takami H and Nishioka K: Raised polyamines in erythrocytes from melanoma-bearing mice and patients with solid tumours. Br J Cancer, 41: 751-756, 1980.
13. Fujita K, Nagatsu T, Maruta K, Ito M, Senba H, Miki K: Urinary putrescine, spermidine, and spermine in human blood and solid cancers and in an experimental gastric tumor of rats. Cancer Res, 36: 1320-1324, 1976.
14. Shimpo K, Kawai K, Ogawa T, et al.: Erythrocyte polyamine analysis method and its clinical application [Jpn]. Rinsho Byori, 59: 157-166. 1984.
15. Edited by Imahori K, Suzuki F, Suzuki O, Bachrach U: Nagatsu T, Shimpo K, Kawai K, Shinzato M, Ito S, Matsui T, Nakamura K, Kitagawa Y, Hirano M, Ito M, Fujita K: Acetylpolyamines in urine and polyamines in erythrocytes as biochemical markers of cancer, Polyamines: Basic and Clinical Aspects, 349-356, VNU Science Press, The Netherlands, (1985)
16. Chihara T, Shimpo K, Shinzato M, et al.: Inhibition of *N*-ethyl-*N*'-nitro-*N*-nitrosoguanidine-induced duodenal tumorigenesis in mice by whole-leaf *Aloe arborescens* Miller var. *natalensis* Berger. Asian Pac J Cancer Prev, 1: 283-288, 2000.
17. Jacoby RF, Marshall DJ, Newton MA, et al.: Chemoprevention of spontaneous intestinal adenomas in the *Apc*^{Min} mouse model by the nonsteroidal anti-inflammatory drug piroxicam. Cancer Res, 56: 710-714, 1996.
18. Swamy MV, Patlolla JMR, Steele VE, Kopelovich L, Reddy BS, Rao CV: Chemoprevention of familial adenomatous polyposis by low doses of atorvastatin and celecoxib given individually and in combination to APC^{Min} mice. Cancer Res, 66: 7370-7377, 2006.
19. Ushida Y, Sekine K, Kuhara T, Takasuka N, Iigo M, Tsuda H: Inhibitory effects of bovine lactoferrin on intestinal polyposis in the Apc^{Min} mouse. Cancer Lett, 134: 141-145, 1998.
20. Löser C, Wunderlich U, Fölsch UR: Reversed-phase liquid chromatographic separation and simultaneous fluorimetric detection of polyamines and their monoacetyl derivatives in human and animal urine, serum and tissue samples: An improved, rapid and sensitive method for routine application. J Chromatogr, 430: 249-262, 1988.
21. Ju J, Hong J, Zhou J-N, et al.: Inhibition of intestinal tumorigenesis in *Apc* $^{min/+}$ mice by (-)-epigallocatechin-3-gallate, the major catechin in green tea. Cancer Res, 65: 10623-10631, 2005.
22. Erdman SH, Ignatenko NA, Powell MB, et al.: APC-dependent changes in expression of genes influencing polyamine metabolism, and consequences for gastrointestinal carcinogenesis, in the *Min* mouse. Carcinogenesis, 20: 1709-1713, 1999.
23. Soda K: The mechanisms by which polyamines accelerate tumor spread. J Exp Clin Cancer Res, 30: 95, 2011.