JSHC
Japan Society of
Histochemistry and Cytochemistry
Established 1959

Acta Histochem. Cytochem. 54 (2): 73–78, 2021
doi: 10.1267/ahc.20-00029

Note

Supplementation with Fermented Barley Extract Prevents Mammary Epithelial Cell Invasion in an Early Breast Cancer Model

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Received October 26, 2020; accepted January 28, 2021; published online February 27, 2021

Diet-based prevention of malignant transformation contributes to the maintenance of quality of life by avoiding a battle against cancer. Invasion is one of the features of malignant breast cancer, and the prevention of invasion may reduce breast cancer malignancy. A recently established early breast cancer model system showed mammary ductal dysplasia with invasion in mice. This study utilized the model system and investigated the effect of fermented barley extract (FBE), a food material. The elastic fiber layer is the outermost layer of the mammary duct. A reduction in the elastic fiber layer was observed in the mammary glands of the model system, whereas supplementation with 8% FBE containing water prevented this reduction. Moreover, we found that FBE supplementation prevented mammary epithelial cell invasion. Based on our findings, FBE might be a candidate material for a diet-based prevention of early breast cancer invasion.

Key words: breast cancer, elastic fiber layer, fermented barley extract, invasion, mammary ductal dysplasia

I. Introduction

The mammary duct has a mammary epithelial cell layer, myoepithelial cell layer, basement membrane, fibrous stroma and elastic fiber layer. The mammary epithelial cell layer is the innermost luminal layer. Mammary epithelial cells and myoepithelial cells form the biphasic pattern. A basement membrane exists between the myoepithelial cell layer and the fibrous stroma. The elastic fiber layer is the outermost layer. If mammary epithelial cells undergo malignant transformation, the cells invade to the outside of the myoepithelial cell layer and basement membrane [10].

Invasion is considered to be one of the early steps of metastasis [4]. Because breast cancer metastasis causes death, the prevention of invasion may reduce breast cancer mortality. To address this, identification of a novel material that can prevent invasion is required.

In precancerous lesions and early breast cancer, mammary ductal dysplasia is observed [10]. Recently, a mouse model system to induce early-stage breast cancer has been reported [9]. In the model system, dysplasia is induced by consecutive daily injection of estradiol (E2) for 30 days in scid mice, which have a DNA repair deficiency. Some dysplastic ducts have invasive mammary epithelial cells, suggesting that this model system is useful to study the prevention of invasion.

Fermented soy foods reduce breast cancer risk [11]. This suggests that supplementation of fermented material(s) has the potential to prevent breast tumorigenesis and progression without identification of the active components. Fermented barley extract (FBE) can be obtained from *shochu* distillery by-products (SDB). In rodent studies, FBE supplementation reduced liver damage caused by chronic alcohol consumption [2] and lipopolysaccharide-induced inflammation [3]. Urinary uric acid excretion was

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increased in hyperuricemic rats by FBE supplementation [6]. FBEP, an Amberlite FPX66-purified fraction of FBE [5], was shown to be safe and to lower serum uric acid in humans [8]. These studies suggest that FBE has unrevealed physiological effects on health. Based on this notion, this study explores the effect of FBE in an early breast cancer model system.

II. Materials and Methods

Mouse experiments

Mice were maintained under specific pathogen free conditions. Scid mice (female, 6~8 weeks old) were purchased from CLEA Japan (Tokyo, Japan). In each experimental group, 5 mice were used for histological staining and *in vivo* zymography, 6 mice were used for fluorescent immunostaining (total 11 mice). Intraperitoneal injection of PBS (control) or E2 (6 μg/day) was performed with a 30G needle for 30 days. FBE was dissolved in autoclaved reverse osmosis-treated water. FBE-containing water (8% w/w) was supplied and was renewed every day. The middle part of 4th mammary glands was isolated from 11~13 weeks old mice euthanized by cervical dislocation. The animal experiments were approved by the Animal Research Committee of Kyoto University, numbers MedKyo17554 and MedKyo18321. All animals were maintained according to the Guide for the Care and Use of Laboratory Animals (National Institute of Health Publication).

Preparation of fermented barley extract

The preparation of FBE was as follows: Barley-SDB, provided by Sanwa Shurui (Oita, Japan), was prepared using pearled barley (barley pearled to approximately 65% of its initial weight) as the raw material. To produce steamed barley, pearled barley was allowed to absorb water until a 40% w/w water content was achieved, steamed for 40 min, and subsequently cooled to 40°C. Barley-*koji* was produced by inoculating the steamed barley with white *koji* mold (*Aspergillus luchuensis mut. kawachii*) (1 kg/ton of barley), which was maintained at 38°C and 95% relative humidity for 24 hr with subsequent incubation at 32°C and 92% relative humidity for 20 hr. The seed mash was prepared with 3.6 kL water, 30 liters of *shochu*-yeast (*Saccharomyces cerevisiae*) preculture (3×10^8 cfu/mL) and 3 tons of barley-*koji*. The seed mash was fermented for 5 days at 25°C. The main mash was prepared by adding 7 tons of steamed barley and 11.4 kL of water to the seed mash. The main mash was fermented for 11 days at 25°C. After fermentation, the main mash was subjected to single batch distillation to obtain 10 kL of unrefined barley-*shochu* and 15 kL of barley-SDB. Barley-SDB was filtered with a stainless-steel mesh net (1 mm). The extracted liquid was then filtered using a ceramic filter (porosity 0.2 μm) to remove solid residues, such as plant cell walls and microbial cells. Subsequently, the aqueous solution was freeze-dried and mixed with 50% (w/w) water-soluble dextrin (Pinedex 100TM; Matsutani Chemical Industry Co., Ltd., Hyogo, Japan) as an excipient.

Immunostaining

Ten μm cryosection of mammary gland was dried, fixed with 4% paraformaldehyde (PFA) in PBS for 3 min at room-temperature and washed with PBS containing 0.05% Tween-20 (PBS-T) 3 times. The specimen was incubated with blocking solution (PBS containing 5% goat serum) for 1 hr at room-temperature and washed with PBS-T. Primary antibody incubation was performed with anti-CK8 antibody (Developmental Studies Hybridoma Bank, TROMA-I, Iowa City, IA, USA, 1/200 dilution) and anti-CK5 antibody (Abcam, ab75869, Cambridge, UK, 1/200 dilution) for overnight at 4°C. These primary antibodies were used in the previous study [9]. The anti-CK8 antibody immunoreacted to mammary epithelial cells, the inner cells of mammary ducts (positive control), and not reacted to other cells in mammary gland (negative control). The anti-CK5 antibody immunoreacted myoepithelial cells, which are p63-positive outer cells of mammary ducts (positive control), and not reacted to other cells in mammary gland (negative control) [9]. The specimen was washed with PBS-T 3 times and incubated with the secondary antibodies, goat anti-rat IgG conjugated to Alexa Fluor 488 (Cell Signaling Technology, 4416S, Danvers, MA, USA, 1/1000 dilution) and goat anti-rabbit IgG antibody conjugated to Alexa Fluor 546 (Life Technologies, A11010, Carlsbad, CA, 1/1000 dilution), for 1 hr at room-temperature. After secondary antibody incubation, the specimen was washed with PBS, counterstained with Hoechst 33342 (Dojindo, 346-07951, Kamimashiki, Japan, 1/500 dilution) in PBS, washed with PBS, dried and mounted with Fluoromount-G (SouthernBiotech, 0100-01, Birmingham, AL, USA).

Histological criteria for mouse mammary ductal dysplasia

Images were collected with the all-in-one microscope BZ-9000 (Keyence, Osaka, Japan) and BZ-II Viewer software (Keyence). Because terminal end buds have no or thin stromal cell layer, images of mammary ducts with stromal cell layer were collected. The criteria of dysplasia to investigate dysplasia formation were the loss of biphasic mammary epithelial and myoepithelial patterns and/or the loss of the lumen structure. Mammary ductal dysplasia with the disruption of myoepithelial cell layer was defined as invasive dysplasia, because the previous study showed that CK8-positive mammary ductal dysplasia with the disruption of CK5-positive myoepithelial cell layer exhibited the disruption of the basement membrane [9]. Dysplasia without the disruption of myoepithelial cell layer was defined as non-invasive dysplasia.

In situ zymography

Isolated mammary gland was fixed with 4% PFA in PBS for 15 min at 4°C. Ten μm cryosection of mammary gland was dried for 1 hr at room-temperature, washed

with PBS for 5 min, and rinsed with water. Dried section was incubated with the reaction mixture (50 μg/mL dye-quenched (DQ)-gelatin (Thermo Fisher Scientific, D12054, Waltham, MA), 50 mM Tris-HCl pH7.5, 150 mM NaCl, 5 mM $CaCl_2$) for 1 hr at room-temperature. The section was rinsed with PBS, fixed with 4% PFA for 15 min at room-temperature and washed with PBS for 5 min 3 times. The section was counterstained with Hoechst 33342, washed with PBS for 5 min, rinsed with water, dried and mounted with Fluoromount-G.

Statistical analyses

Student's *t*-test was used for statistical analyses. The elastic fiber layer index was obtained by dividing the resorcin-fuchsin positive area by the area of the duct and multiplying by 100. $P < 0.05$ was considered to be statistically significant.

III. Results and Discussion

FBE does not enhance E2-induced dysplasia formation in scid mice

The effect of FBE on early stage breast cancer remains unknown. During breast tumorigenesis and progression, mammary ductal dysplasia is observed [10]. We therefore investigated the effect of FBE in the mammary ductal dysplasia model in *in vivo* experiments involving daily intraperitoneal injection of E2 into scid mice for 30 days [9]. Eight percent FBE-containing water was supplied from day 0 to day 30. The FBE-containing water was renewed every day. The means ± standard deviations of the body weights of PBS-injected, E2-injected and E2-injected FBE-supplied mice were 20.38 g ± 1.56, 20.69 g ± 1.39 and 20.55 g ± 1.21. Not significant change in body weights in FBE-supplied mice suggests that FBE is not toxic.

To observe the morphologies of the mammary ducts, we performed hematoxylin and eosin (HE) staining. We observed normal mammary ducts in PBS-administered mice (Fig. 1A). In E2-administered mice, some ducts exhibited dysplasia (Fig. 1A). E2-administered FBE-supplied mice also had dysplastic ducts (Fig. 1A).

We investigated the frequencies of dysplasia formation. The frequencies of dysplasia were increased by E2 administration (Fig. 1B). The values of dysplasia frequencies were comparable between E2-administerd mice with and without FBE supplementation (Fig. 1B), suggesting that FBE did not enhance dysplasia formation.

FBE maintains elastic fiber layers in breast tissue

Elastic fibers exist in the outermost region of the mammary duct. HE staining cannot visualize elastic fibers. To stain these fibers, we performed Elastica van Gieson (EVG) staining. In EVG staining, resorcin-fuchsin stains elastic fibers black-purple. We observed elastic fiber layers in PBS-administered mice (Fig. 2A). A reduction in resorcin-fuchsin-stained fibers was observed in E2-administered mice (Fig. 2A). In E2-administered FBE-supplied mice, a normal elastic fiber layer was observed, although mammary ductal dysplasia was formed (Fig. 2A).

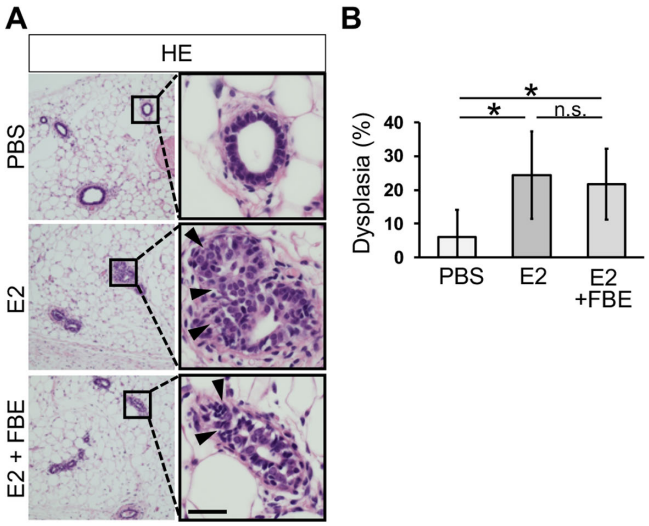

Fig. 1. E2 administration induced mammary ductal dysplasia. (**A**) HE staining images of mammary tissues are shown. Arrowheads indicate dysplasia. Bar = 30 μm. (**B**) The graph shows the frequencies of dysplasia formation (n = 5 mice). *: $P < 0.05$, n,s,: not significant. Error bars indicate the standard deviation.

To quantify the elastic fiber layer, we measured the area of a duct (Fig. 2B blue) and the resorcin-fuchsin-positive area of the duct (Fig. 2B, yellow). The elastic fiber layer index was defined by the ratio of the resorcin-fuchsin positive area to the area of the duct (Fig. 2B, formula). The results of the quantification showed that the elastic fiber layer was significantly reduced by E2 administration (Fig. 2C). The elastic fiber layer index of FBE-supplied mice was comparable with that of PBS controls (Fig. 2C). These results suggest that FBE supplementation maintains the elastic fiber layers of mammary ducts in E2-administered scid mice.

FBE prevents mammary epithelial cell invasion

Some E2-induced mammary ductal dysplasia exhibited epithelial cell invasion in scid mice [9]. Maintenance of elastic fiber layer in FBE-supplied mice (Fig. 2) suggests that FBE supplementation prevented breast cancer progression from non-invasive to invasive. In mammary ductal dysplasia with invasion, the myoepithelial cell layer is disrupted. To investigate the occurrence of invasion, we immunostained with antibodies against CK8, a mammary epithelial cell marker, and CK5, a myoepithelial cell marker. In PBS-administered mice, normal mammary ducts were observed (Fig. 3A). Invasive mammary epithelial cells were observed in E2-administered mice (Fig. 3A). In dysplastic ducts with invasion, the CK5-positive cell layer was disrupted, and some CK8-positive cells invaded to the outside of the duct (Fig. 3A, arrowheads). In FBE-supplied mice, most dysplastic ducts did not exhibit inva-

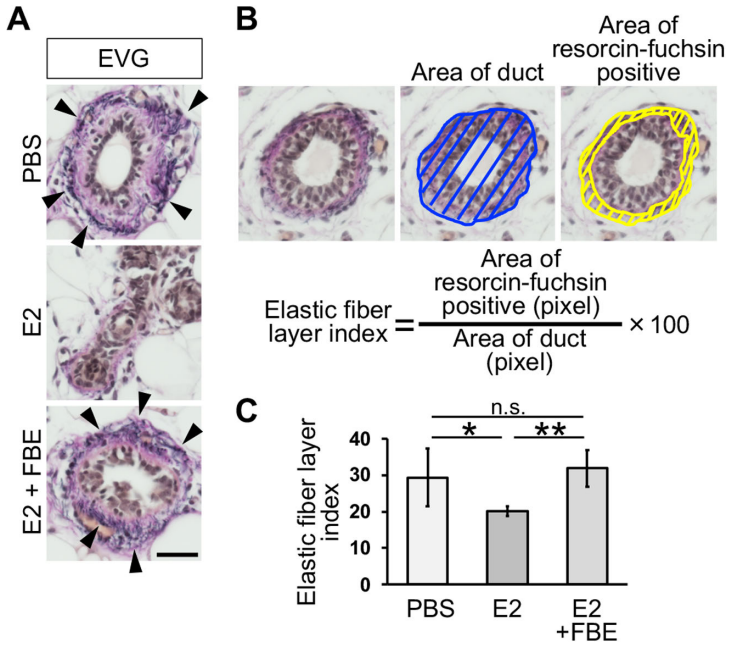

Fig. 2. FBE supplementation maintained the elastic fiber layer. (**A**) EVG staining images are shown. Arrowheads indicate resorcin-fuchsin-stained fibers. For simplicity, not all resorcin-fuchsin-stained fibers are indicated. Bar = 30 μm. (**B**) Quantification of the elastic fiber layer is shown. (**C**) The graph shows the results of elastic fiber layer quantification (n = 5 mice). *: $P < 0.05$, **: $P < 0.01$, n,s,: not significant. Error bars indicate the standard deviation.

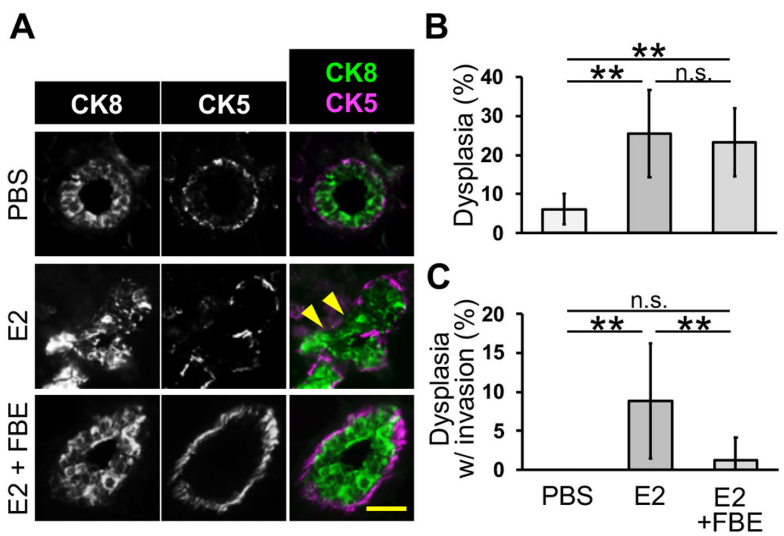

Fig. 3. FBE supplementation prevented mammary epithelial cell invasion in E2-administered scid mice. (**A**) Immunostaining was performed to visualize CK8, a mammary epithelial cell marker, and CK5, a myoepithelial cell marker. Arrowheads indicate mammary epithelial cell invasion. Bar = 30 μm. (**B, C**) The frequencies of dysplasia (**B**) and dysplasia with invasion (**C**) are indicated (n = 6 mice). **: $P < 0.01$, n,s,: not significant. Error bars indicate the standard deviation.

sion (Fig. 3A).

We quantified the frequencies of dysplasia with and without invasion in immunostained samples. The frequencies of dysplasia formation were increased in E2-administered mice with and without FBE supplementation (Fig. 3B), which is consistent with the results of HE staining, as shown in Fig. 1B. We investigated the frequencies of dysplasia with invasion only. The results showed that E2 administration increased the frequency of dysplasia with invasion, and this increase was not observed in FBE-supplied mice (Fig. 3C).

Matrix metalloproteinases (MMPs) degrades extracellular matrix, which supports cancer cell invasion. To investigate MMP activity, we performed *in situ* zymography with DQ-gelatin. Weak MMP activity was observed in the mammary cells of PBS-administered mice (Fig. 4).

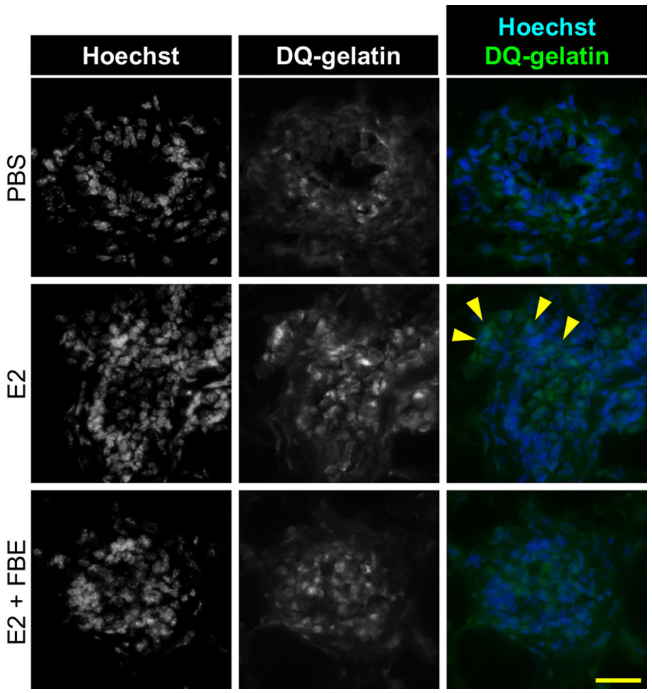

Fig. 4. FBE supplementation reduced the MMP activity of mammary cells in E2-administrared mice. MMP activity was visualized by *in situ* zymography with DQ-gelatin. Typical images are shown. Arrowheads indicate increased MMP activity. Bar = 30 μm.

Increased MMP activity was observed in the cells of the dysplastic mammary ducts of E2-administered mice. Cells expanded to extraductal area exhibited MMP activity (Fig. 4, arrowheads). In E2-administered FBE-supplied mice, MMP activity was weak, although a small number of cells showed MMP activity (Fig. 4). Although *in situ* zymography is not quantitative, these results suggest that FBE prevents epithelial cell invasion with MMP-mediated extracellular matrix degradation in the early breast cancer model.

This study demonstrated that food material(s) have the potential to prevent the progression of noninvasive to invasive breast cancer in a mouse model. Previous studies supplied 10% FBE containing food to mice [5, 7]. Given that 20 g mouse takes 4~5 g of food in one day [1], feeding 10% FBE containing food would administer 0.40~0.50 g FBE in one day. FBE used in this study is easy to solve in water. Given that 20 g mouse drinks 5~6 mL of water in one day [1], supplementation of 8% FBE containing water would administer 0.40~0.48 g FBE in one day. We therefore supplied 8% FBE in this study and observed the prevention effect of mammary epithelial cell invasion.

During cancer invasion, MMP degrades extracellular matrix. Because MMP activity is regulated by tissue inhibitor of metalloprotainases (TIMPs), and there are various MMP and TIMP genes, detection of MMP activity *in situ* is demanded to assess invasion rather than staining MMP-related molecules. We therefore performed *in situ* zymography. We observed MMP activity in dysplastic mammary duct of E2-administered scid mice, whereas in the mammary glands of E2-administered FBE-supplied mice, increase in MMP activity was not observed. This result suggests that FBE-supplementation prevents MMP activity, or changes the expression of MMP-related molecules, such as MMPs and TIMPs, or FBE blocks transformation from noninvasive to invasive cells.

In the mammary glands of E2-administered mice, we observed reduced elastic fiber, which contains elastin. MMP degrades components of extracellular matrix including elastin. It is suggested that maintenance of elastic fiber layer in the mammary glands of E2-administered FBE-supplied mice was due to prevention of increase in MMP activity.

Although it remains unclear the details of how FBE inhibits mammary epithelial cell invasion, this study showed the novel effect of FBE in breast cancer invasion in a mouse model.

IV. Conflicts of Interest

JI was employee of Kyoto University's Sponsored Research Program funded by Taiho Pharmaceutical Co., Ltd. AN and HH are employees of Sanwa Shurui Co., Ltd. MT received research funding from Taiho Pharmaceutical Co., Ltd. The funding source had no role in the study design, experiment, analysis, interpretation or writing the manuscript.

V. Acknowledgments

We thank the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by the University of Iowa. The manuscript was proofread by *American Journal Experts*. Financial support was provided by Taiho Pharmaceutical Co., Ltd. and JSPS KAKENHI Grant Number JP19K07665.

VI. References

1. Bachmanov, A. A., Reed, D. R., Beauchamp, G. K. and Tordoff, M. G. (2002) Food intake, water intake, and drinking spout side preference of 28 mouse strains. *Behav. Genet.* 32; 435–443.
2. Giriwono, E. P., Hashimoto, T., Ohsaki, Y., Shirakawa, H., Hokazono, H. and Komai, M. (2010) Effect of fermented barley attenuates chronic alcohol induced liver damage by increasing antioxidative activities. *Food Res. Int.* 43; 118–124.
3. Giriwono, P. E., Shirakawa, H., Hokazono, H., Goto, T. and Komai, M. (2011) Fermented barley extract supplementation maintained antioxidative defense suppressing lipopolysaccharide-induced inflammatory liver injury in rats. *Biosci. Biotechnol. Biochem.* 75; 1971–1976.
4. Gupta, G. P. and Massagué, J. (2006) Cancer metastasis: building a framework. *Cell* 127; 679–695.
5. Hokazono, H., Omori, T., Suzuki, H. and Ono, K. (2009) Effects of Fermented Barley Extract on Antioxidant Status in Mice. *Food Sci. Technol. Res.* 15; 599–604.
6. Hokazono, H., Omori, T. and Ono, K. (2010) Anti-hyperuricemic Effect of Fermented Barley Extract is Associated with Increased Urinary Uric Acid Excretion. *Food Sci. Technol. Res.* 16; 295–304.
7. Hokazono, H., Omori, T. and Ono, K. (2010) Effects of single and combined administration of fermented barley extract and gamma-aminobutyric acid on the development of atopic dermatitis in NC/Nga mice. *Biosci. Biotechnol. Biochem.* 74; 135–139.
8. Hokazono, H., Omori, T., Yamamoto, T., Akaoka, I. and Ono, K. (2010) Effects of a fermented barley extract on subjects with slightly high serum uric acid or mild hyperuricemia. *Biosci. Biotechnol. Biochem.* 74; 828–834.
9. Itou, J., Takahashi, R., Sasanuma, H., Tsuda, M., Morimoto, S., Matsumoto, Y., *et al.* (2020) Estrogen Induces Mammary Ductal Dysplasia via the Upregulation of Myc Expression in a DNA-Repair-Deficient Condition. *iScience* 23; 100821.
10. Sgroi, D. C. (2010) Preinvasive breast cancer. *Annu. Rev. Pathol.* 5; 193–221.
11. Yamamoto, S., Sobue, T., Kobayashi, M., Sasaki, S. and Tsugane, S. (2003) Soy, isoflavones, and breast cancer risk in Japan. *J. Natl. Cancer Inst.* 95; 906–913.