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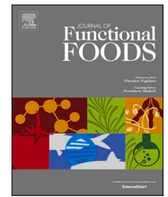

Prevention of tumor progression in inflammation-related carcinogenesis by anti-inflammatory and anti-mutagenic effects brought about by ingesting fermented brown rice and rice bran with *Aspergillus oryzae* (FBRA)

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ABSTRACT

Fermented brown rice and rice bran with *Aspergillus oryzae* (FBRA) has shown chemopreventive effectiveness against inflammation-related carcinogenesis, by inhibiting inflammation. We found that *Aspergillus oryzae* is the main component of the anti-inflammatory effect of FBRA and that the intestinal flora is involved. Mice fed FBRA were found to have a lower tumor-forming rate than mice fed the basic diet. Tumor cell lines obtained from basic diet-fed mice metastasized to distant organs. By contrast, those from FBRA-fed mice acquired reduced metastatic capacity. Micronucleus testing detected somatic mutations in tumor cell lines directly established from primary tumors; these decreased in a dose-dependent manner in FBRA-fed mice. To determine whether FBRA ingestion induced systemic anti-mutagenicity, micronucleus testing was performed using reticulocytes in mice injected with typical mutagens. Mutations induced by these mutagens were suppressed in FBRA-fed mice. These results indicate that anti-inflammation and anti-mutagenicity induced by FBRA intake suppressed the inflammation-related carcinogenic process.

1. Introduction

According to a large international epidemiological study on cancer survival (CONCORD-3) based on personal records of 37 million patients diagnosed with cancer, cancer diagnosis and treatment have improved over the last two decades, and cancer survival has improved (Allemani et al., 2018). Additionally, survival rates of deadly cancers such as liver, pancreas, and lungs have increased by up to 5% (Allemani et al., 2018). The main cause of decreased cancer survival, accounting for approximately 90% of cancer-related deaths (Lambert et al., 2017), is distant metastases rather than growth of the primary tumor. Most patients with advanced metastatic disease experience incurable terminal illness, even with current treatment regimens. While metastasis is the greatest risk for cancer patients, few compounds have been reported to effectively prevent it. This is largely because few animal models consistently mimic the entire carcinogenic process from the development of a primary tumor to the metastasis of tumor cells.

The clinical course of human tumors can be divided into three stages: precancerous lesions, primary tumors, and distant metastases (Yokota & Sugimura, 1993). Precancerous lesions include dysplasia, hyperplasia, leukoplakia, and adenoma; however, some tumors do not originate from these characteristic precancerous lesions. The initiated cells of the precancerous lesion grow in a clonal manner, having the advantage of selective growth due to genetic alterations (Yokota & Sugimura, 1993).

Abbreviations: FBRA, fermented brown rice and rice bran with *Aspergillus oryzae*; QR-32, a regressive mouse fibrosarcoma cells; QRsP-11, tumorigenic cell line derived from QR-32 cells by inflammation; MNRETs, micronucleated reticulocytes.

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When the initiated cells continue to proliferate or are further affected by carcinogenesis-accelerating factors such as inflammation (Greten & Grivennikov, 2019; Okada et al., 2021; Okada, 2014) that promote cell proliferation, they irreversibly transition into tumorigenic converted cells, with additional genetic alterations, and form primary tumors. During the expansion of converted cells in primary tumors, additional genetic alterations accumulate in some cells, generating new malignant clones with a more aggressive phenotype (Kay et al., 2019). The new aggressive clones (progressed cells) highly proliferative and invasive tumor cells, form metastases (Greten & Grivennikov, 2019; Yokota & Sugimura, 1993). Based on the presence of close genetic relationships between primary tumors and their metastatic tumors, recent genomic studies have substantiated the fact that metastatic cells are the descendants of a minor subpopulation of the converted cells present in the primary tumor (Ding et al., 2010; Naxerova & Jain, 2015; Yachida et al., 2010; Yates et al., 2015).

To mimic the carcinogenic process that occurs in humans, we used precancerous-like cells obtained from mouse cells. We have established a regressive clonal QR-32 cell line from clonal and tumorigenic fibrosarcoma cells, BMT-11 cl-9, by exposing them *in vitro* to quercetin, a mutagen/carcinogen (Ishikawa, Okada, et al., 1987; Ishikawa, Hosokawa, et al., 1987). QR-32 cells were non-tumorigenic and non-metastatic in normal syngeneic C57BL/6 mice, whereas they grew progressively in immunosuppressed hosts; we determined that their regression was mediated by host immunity (Okada et al., 1990). Since the parental BMT-11 cl-9 cells are derived from those induced by the chemical carcinogen 3-methylcholanthrene, QR-32 cells can be considered initiated cells (Ishikawa, Hosokawa, et al., 1987). The cell characteristics not only show genetically similar alterations to the initiated cells, but also mimic the *in vivo* phenotype of precancerous lesion. We have developed methods to suppress carcinogenesis and tumor progression using a mouse model that mimics the human carcinogenesis process (Ohnishi et al., 1996; Okada, Shionoya, et al., 2006; Okada et al., 2021; Okada, 2002, 2014; Onuma et al., 2009, 2011, 2015).

By assuming QR-32 cells as precancerous-like cells, we have revealed that inflammation is a potential carcinogenesis-accelerating intrinsic factor by developing an inflammation-related carcinogenesis model (Okada, 2014). Using this model, we clarified the following points: i) Inflammatory cells are involved in carcinogenesis (Okada et al., 1992). ii) Carcinogenesis can be suppressed by removing inflammatory cells, especially activated neutrophils from inflamed lesions (Tazawa et al., 2003). iii) Genotoxic reactive nitric oxide (Okada, Tazawa, et al., 2006), reactive oxygen species (Okada, Kobayashi, et al., 2006), and reactive sulfur species (Okada, 2019) derived from inflammatory cells are involved in carcinogenesis. Using an inflammation-related carcinogenesis model, we revealed that FBRA has chemopreventive effects on the carcinogenesis (Onuma et al., 2015). In our previous study, we showed that FBRA ingestion significantly suppressed the frequency of inflammation-related carcinogenesis in QR-32 cells (primary tumor formation) due to suppression of inflammatory cell infiltration into inflamed sites (Onuma et al., 2015). However, whether FBRA suppresses the malignant progression of tumor cells acquired in growing tumors has not been investigated.

The purpose of this study was to determine the potential inhibitory effect of FBRA on malignant progression of tumor cells, namely the acquisition of metastatic potential in growing primary tumors. We have herein reported that FBRA ingestion prevented the acquisition of tumor cell metastasis through suppression of inflammatory-cell infiltration and somatic mutations, both of which are critical factors that accelerate carcinogenesis and tumor progression (Greten & Grivennikov, 2019; Kay et al., 2019; Okada et al., 2021; Okada, 2014).

2. Materials and methods

2.1. Chemicals

Bleomycin sulfate, busulfan, cyclophosphamide hydrate, and doxorubicin were purchased from Nippon Kayaku (Tokyo, Japan), Otsuka Pharmaceutical (Tokyo, Japan), Shionogi (Osaka, Japan), and Sandoz K. K. (Tokyo, Japan), respectively. Tobacco-specific nitrosamine, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) was obtained from Toronto Research Chemicals (M325750; Toronto, CA), and a carcinogenic heterocyclic amine, 2-amino-3-methylimidazo[4,5-b]pyridine (PhIP hydrochloride), was obtained from Fujifilm Wako Pure Chemical (163–15951; Osaka, Japan).

2.2. Cell lines and culture conditions

The origin and characteristics of the QR-32 cell lines have been described previously (Ishikawa, Okada, et al., 1987). Briefly, BMT-11, a transplantable fibrosarcoma, was induced in a C57BL/6 mouse by 3-methyl-cholanthrene, and a tumorigenic clone, BMT-11 cl-9, was subsequently isolated by limiting dilution (Ishikawa, Hosokawa, et al., 1987). BMT-11 cl-9 cells were exposed to quercetin *in vitro*, which gave rise to several random subclones (Ishikawa, Okada, et al., 1987). The QR-32 cells and QRsP tumor cell lines were maintained in Eagle's minimum essential medium (05900; Nissui Pharmaceutical, Tokyo, Japan) supplemented with 8% fetal bovine serum (F7524; Sigma-Aldrich, St. Louis, MO), sodium pyruvate, non-essential amino acids, and L-glutamine. The cell lines were maintained at 37 °C in a humidified 5% CO_2/95% air mixture.

2.3. Animals and diet

Female C57BL/6 mice (5 weeks-old) were obtained from Nippon SLC (Hamamatsu, Japan) and maintained under specific-pathogen-free conditions with light–dark cycles (12 h each) at 23 ± 3 °C and 50 ± 10% humidity at the Institute for Animal Experimentation, Tottori University. The animals were used after one week of acclimation. Fungal body of *Aspergillus oryzae* was harvested after culturing in Czapek-Dox minimal medium (C1551-250G; Merck, Osaka, Japan) containing 3% sucrose, washed with a sterile water, and was processed to a freeze-dried powder with a starch as a dispersant. Rice bran was obtained from Matsubara Beikoku (Sapporo, Japan).

The manufacturing process for FBRA has been previously reported (Kuno et al., 2015). Namely, the process contains two major steps of fermentation. First, brown rice and rice bran are steamed to make a fermentation base, which is then fermented by *Aspergillus oryzae* for approximately 24 h. After that, it is post-fermented (aging) for another 24 h. The final product (FBRA) was used for research.

Mice were fed a basic diet (MF; Oriental Yeast, Tokyo, Japan) alone, or MF supplemented with FBRA. The mice were divided randomly into three groups: basic diet (MF), MF with 5% FBRA, and MF with 10% FBRA. Diets containing FBRA or its constituents were available *ad libitum* starting 2 days before implantation and throughout the experiment. All experimental procedures and protocols were approved by the Institutional Animal Care and Use Committee of Tottori University (Permit No. 19-Y-37) and were performed in accordance with the approved guidelines.

2.4. Antibiotic treatment

The method of eradicating mouse intestinal microbiota with antibiotics was used a slight modification of previous reports (Alhabbab et al., 2015; Han et al., 2015). Broad-spectrum antibiotic treatment was supplied for 3 weeks in drinking water containing ampicillin sodium (016–23301; 1 g/L), vancomycin hydrochloride (222–01303; 0.5 g/L), neomycin sulfate (146–08871; 1 g/L), and metronidazole (132–18061;

1 g/L) purchased from Fujifilm Wako Pure Chemical. All antibiotic solutions were sterilized by 0.2 μm membrane filtration and made daily.

Fresh cecal contents were collected, suspended in sterile saline, spread on glass slides (S011120; Matsunami Glass, Osaka, Japan) and dried. Gram stain was performed using Nissui Favor G gram stain (Nissui Pharmaceutical). Fecal bacteria were photographed under a microscope (DM500; Leica Camara Japan, Tokyo, Japan) using a Leica ICC50 W camera.

2.5. Gelatin sponge infiltrating cell count and inflammation-related carcinogenesis model

The detailed procedures have been described previously (Okada, Shionoya, et al., 2006). Briefly, for the number of gelatin sponge infiltrating cells, a sponge (10 × 5 × 3 mm, Astellas Pharma, Tokyo, Japan) was inserted subcutaneously into mice, taken out 5 days later, and treated with 0.2% collagenase to dissolve the sponge. Infiltrated inflammatory cells were collected and counted using a hemocytometer.

For the inflammation-related carcinogenesis model, after mice were anesthetized a piece of gelatin sponge was inserted subcutaneously, and 1×10^5 QR-32 cells were immediately injected into the inserted sponge (Okada et al., 1992) (Fig. 1A).

2.6. Evaluation of primary tumor malignancy

Subcutaneously grown tumors were aseptically removed to establish individual culture cell lines after mechanical disaggregation with scissors (Fig. 1B). Each tumor cell line was injected intravenously (1×10^6 cells) into normal mice. On day 28, the mice were sacrificed and metastatic nodules at the surface of the lungs or other organs were counted macroscopically (Fig. 1C).

2.7. In vitro mutagenicity

Micronucleus staining with acridine orange was performed according to a previous report (Hayashi et al., 1983), with slight modifications. Cells from each cell line were allowed to spread on glass coverslips (C022221; Matsunami Glass) in 6-well plates (3516; Corning Japan, Tokyo, Japan; Fig. 1B). QR-32 cells were treated with or without doxorubicin for 6 h, then switched to normal maintenance medium and incubated for an additional 48 h. The cells were then fixed with cold methanol for 5 min and stained with acridine orange (17503; 0.1 mg/mL; AAT Bioquest, Sunnyvale, CA) for 5 min. Micronuclei were counted over 200 cells using a microscope (BZ-X710; Keyence, Osaka, Japan) with a maximum excitation of 502 nm and a maximum emission of 525 nm.

2.8. In vivo mutagenicity

The *in vivo* micronucleus test was performed according to a previously published method (Matsumoto, Takahashi, et al., 2017; Matsumoto, Nishikawa, et al., 2017). The *in vivo* mutagenicity experiments were approved by the Experimental Animal Research Committee of Kyoto Pharmaceutical University (Permit No. 17-031). Briefly, the FBRA-containing diets were made available *ad libitum* starting 2 days before implantation and throughout the experiment. Mutagenic substances such as bleomycin (5 mg/kg), busulfan (10 mg/kg), cyclophosphamide hydrate (40 mg/kg), doxorubicin (5 mg/kg), or PhIP hydrochloride (50 mg/kg) were injected intraperitoneally into mice once. NNK (100 mg/kg) was intraperitoneally injected twice at 24-h intervals. Further, 5 μL of blood was collected from the tail vein before (0 h) and 24, 48, and 72 h after injection of the mutagen (Fig. 1D). Micronuclei were scored among approximately 1000 reticulocytes per slide at ×400 magnification using a fluorescent microscope (Olympus, Tokyo, Japan) equipped with a blue excitation filter set (440–470 nm) and a barrier filter at 510 nm (B-2A; Nikon, Tokyo, Japan).

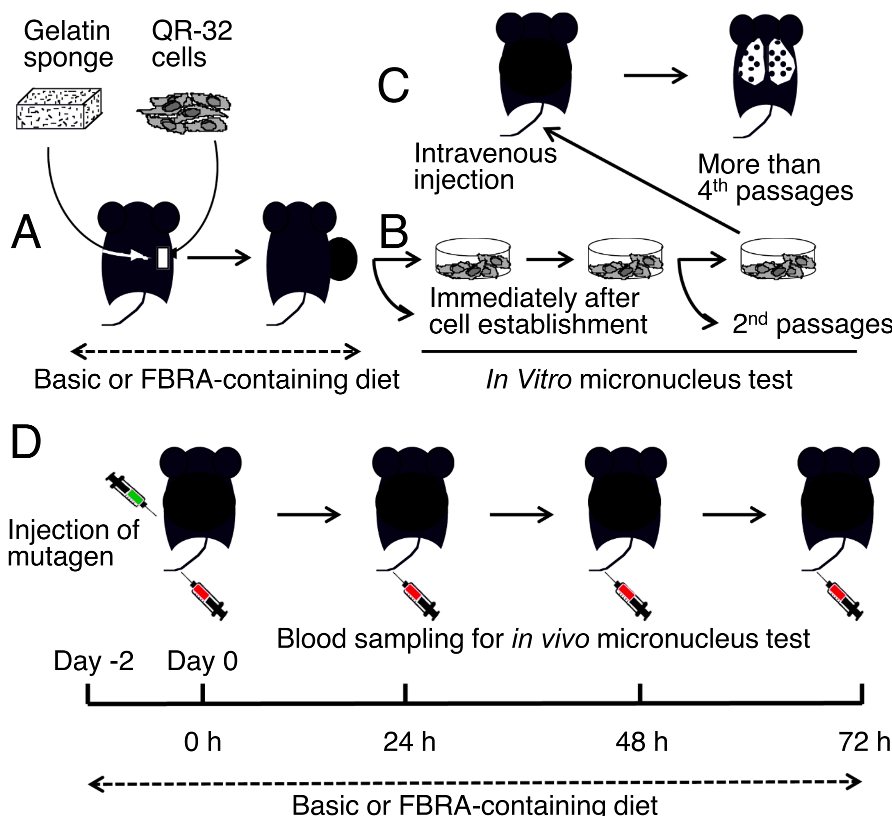

Fig. 1. Schematic Illustrations of Experimental Models of Inflammation-related Carcinogenesis and Tumor Progression, and Micronucleus Tests to Assess Somatic Mutation. (A) Regressive QR-32 cells (1×10^5) were injected into a pre-inserted piece of a foreign body, gelatin sponge, in the subcutaneous space; these grew lethally in the mice. On day 28, the mice were sacrificed to establish culture cell lines from the resultant tumors. (B) Mutations arising in the primary tumor were determined by micronucleated tumor cells immediately after establishment from subcutaneously growing tumors, and in tumor cells of the second passage, under routine culture conditions. (C) The tumor cell lines that had been passaged at least four times were then implanted intravenously (1×10^6 cells) into mice to examine their metastatic ability. (D) Systemically induced mutations were evaluated based on the micronucleus formation in peripheral blood reticulocytes after intraperitoneal administration of various mutagenic substances.

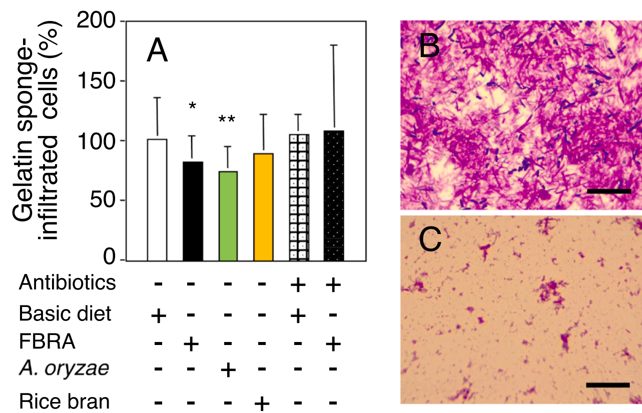

Fig. 2. *Aspergillus oryzae* is the Main Component that Suppresses Infiltration of Inflammatory Cells Caused by Ingestion of FBRA. Five days after gelatin sponge insertion into subcutaneous space of mice, inflammatory cells infiltrated into gelatin sponge were collected and counted after digestion of collagenase. (A) Diets containing FBRA or its constituents had been fed *ad libitum* starting 2 days before the insertion and throughout the experiment. Data represent the sum of five independent experiments (without antibiotics groups; total number of mice used in each group: $n = 30$) and the sum of two independent experiments with similar results (with antibiotics group; total number of mice used in each group: $n = 8–10$). The significance of the difference in the number of infiltrated inflammatory cells into gelatin sponge was calculated by Student's *t*-test. $^*p < 0.05$, $^{**}p < 0.001$ compared to basic diet. (B) Gram stain of intestinal bacteria in mice fed with basic diet. Scale bar, 20 μm. (C) Some mice were given broad-spectrum antibiotic water, containing 1 g/L ampicillin, 1 g/L neomycin, 0.5 g/L vancomycin, and 1 g/L metronidazole, from 3 weeks prior to gelatin sponge insertion until the end of the experiment. Typical gram stain of intestinal bacteria of mice treated with antibiotics. Scale bar, 20 μm.

2.9. Statistical analysis

The differences in tumor development and metastatic incidences were calculated by Fisher's exact probability test. The number of lung metastatic nodules was calculated by Welch's *t*-test and the difference in the number of infiltrated inflammatory cells was calculated by Student's *t*-test. The differences in micronucleated frequency were analyzed by the Welch's *t*-test and Student's *t*-test. For statistical comparisons, the significance was set at $p < 0.05$.

3. Results

3.1. Aspergillus oryzae is a major component that suppresses inflammatory cell infiltration caused by FBRA ingestion

FBRA have been reported to prevent inflammation-related carcinogenesis in mice by inhibiting the infiltration of inflammatory cells (Onuma et al., 2015). We examined which component of FBRA is effective in suppressing inflammatory exudation. A mixed laboratory basic diet of *Aspergillus oryzae* or rice bran, which is the main component of FBRA, was prepared to be equal to the amount of the diet containing 10% of FBRA. A prophlogistic gelatin sponge was inserted subcutaneous space of each diet-fed mice to compare the total number of infiltrated inflammatory cells. In contrast to the basic diet, FBRA administration suppressed the infiltration of inflammatory cells into the sponge. This inhibitory effect was brought about by *Aspergillus oryzae*, but not by rice bran (Fig. 2A).

Next, we investigated the effects of intestinal flora on inflammatory infiltration induced by sponge. Many bacteria were present in mice prior to antibiotic administration (Fig. 2B), but gram-positive bacteria were almost depleted after 3 weeks of administration of 4 antibiotics (ampicillin, neomycin, vancomycin, and metronidazole) (Fig. 2C). Depletion of these intestinal microbiota abolished the anti-inflammatory effects of FBRA (Fig. 2A). However, the microbiota did not affect inflammatory cell infiltration in mice fed a basic diet (Fig. 2A). These results reveal that *Aspergillus oryzae* is the main component of the infiltration inhibitory effect of FBRA ingestion and that the intestinal microbiota may be involved.

3.2. Inhibition of inflammation-related carcinogenesis by FBRA ingestion

We reaffirmed that the inhibitory effect of FBRA on an inflammation-related carcinogenesis (Table 1). As a result, regressive QR-32 cells co-implanted with a gelatin sponge grew lethally in 16 out of 22 mice that were administered the basic diet (73%), 9 out of 22 mice administered the 5% FBRA diet (41%), and 6 out of 22 mice administered the 10% FBRA diet (27%). Tumor formation was significantly reduced in the FBRA-fed mice as compared to mice fed the basic diet (Table 1).

3.3. Inhibition of acquisition of metastatic ability of tumors growing in FBRA-fed mice

We then examined how FBRA diets affected the acquisition of malignancy of tumors grown in mice. *In vitro* cell lines were established from tumors that grew in mice fed a basic diet, 5% FBRA, or 10% FBRA, and were named QRsP, QRsP-F5, and QRsP-F10 tumor lines, respectively (Table 2). Arabic numerals refer to individual mice with established tumors.

Table 2 shows the metastatic ability of the tumor cell lines obtained from established tumors. Intravenous injection of QRsP-F5 and QRsP-F10 tumor cell lines resulted in lung metastases to 7 out of 27 lungs (26%) and 2 out of 18 lungs (11%), respectively. By contrast, QRsP tumor cell lines metastasized in 37 out of 48 lungs (77%). The acquisition of lung metastatic potential was significantly suppressed in both the QRsP-F5 tumor cell lines and the QRsP-F10 tumor cell lines compared to that in the QRsP tumor cell lines. Moreover, QRsP-F5 and QRsP-F10 tumor cell lines tended to suppress extrapulmonary metastasis; particularly, tumors that grew in 10% FBRA-fed mice exhibited significantly reduced acquisition of distant metastatic ability (Table 2). These results showed that the acquisition of lung and extrapulmonary metastatic ability was significantly suppressed in tumors growing in FBRA-fed mice.

Table 1
Inhibition of inflammation-related carcinogenesis by ingestion of FBRA.

Treatment[a]	Gelatin sponge implantation[b]	QR-32 cells injection[c]	No. of mice with tumor/no. of mice tested	*p* value[d]
None	–	+	0/20 (0%)	
None	+	+	16/22 (73%)	
5% FBRA	+	+	9/22 (41%)	$p = 0.033$
10% FBRA	+	+	6/22 (27%)	$p = 0.003$

a. Basic or FBRA-containing diet was administered *ad libitum* from 2 days before co-implantation and continued throughout the experiment.
b. A small incision was made in the right flank of mice, a piece of gelatin sponge (10 × 5 × 3 mm) was inserted, and the wound was closed with clips.
c. QR-32 cells (1×10^5) were injected into the pre-inserted gelatin sponge.
d. Incidences of tumor development were calculated by Fisher's exact probability test as compared to basic diet-fed mice co-implanted QR-32 cells with gelatin sponge.

Table 2
Inhibition of acquisition of metastatic ability of tumors growing in FBRA-fed mice.

	Lung-colonizing ability[b]		Other metastasis sites	
Cell line[a]	No. of mice with lung metastasis/no. of mice tested	No. of lung metastatic nodules	No. of mice with other metastases/no. of mice tested	Sites (Incidence)[g]
QRsP-1	3/3	5, 9, 34	0/3	None
QRsP-2	3/3	13, 44, 106	1/3	O (1/3)
QRsP-3	2/3	0, 2, 68	1/3	O (1/3)
QRsP-4	3/3	33, 95, 122	2/3	O (2/3), AT (1/3)
QRsP-5	2/3	0, 15, 57	1/3	ALN (1/3)
QRsP-6	2/3	0, 28, 92	0/3	None
QRsP-7	2/3	0, 57, 66	0/3	None
QRsP-8	0/3	0, 0, 0	1/3	O (1/3), AT (1/3)
QRsP-9	3/3	1, 1, 7	0/3	None
QRsP-10	2/3	0, 5, 8	1/3	O (1/3), AT (1/3)
QRsP-11	2/3	0, 1, 1	0/3	None
QRsP-12	2/3	0, 1, 5	0/3	None
QRsP-13	2/3	0, 1, 12	2/3	O (1/3), L (1/3)
QRsP-14	3/3	3, 27, 30	1/3	ALN (1/3)
QRsP-15	3/3	1, 2, 13	0/3	None
QRsP-16	3/3	2, 23, 46	0/3	None
Total	37/48		10/48	
QRsP-F5-1	2/3	0, 1, 1	0/3	None
QRsP-F5-2	0/3	0, 0, 0	0/3	None
QRsP-F5-3	2/3	0, 1, 5	1/3	O (1/3)
QRsP-F5-4	0/3	0, 0, 0	0/3	None
QRsP-F5-5	0/3	0, 0, 0	0/3	None
QRsP-F5-6	0/3	0, 0, 0	0/3	None
QRsP-F5-7	1/3	0, 0, 5	1/3	O (1/3), AT (1/3)
QRsP-F5-8	1/3	0, 0, 31	1/3	ALN (1/3)
QRsP-F5-9	1/3	0, 0, 2	0/3	None
Total	7/27[c]		3/27[d]	
QRsP-F10-1	1/3	0, 0, 2	0/3	None
QRsP-F10-2	0/3	0, 0, 0	0/3	None
QRsP-F10-3	0/3	0, 0, 0	0/3	None
QRsP-F10-4	0/3	0, 0, 0	0/3	None
QRsP-F10-5	1/3	0, 0, 3	0/3	None
QRsP-F10-6	0/3	0, 0, 0	0/3	None
Total	2/18[e]		0/18[f]	

a. Cultured cell lines established from tumors developed in each mouse as shown in Table 1 were examined.
b. Cells from each cell line (1×10^6) were intravenously injected into mice. The mice were sacrificed 28 days later, and metastatic nodules at the surface of the lung were counted macroscopically.
c–f. Incidences of lung metastasis and extrapulmonary metastases were calculated by Fisher's exact probability test as compared to QRsP tumor cells. c; $p = 3.826E{-}7$, d; $p = 0.230$, e; $p = 1.459E{-}6$, f; $p = 0.031$.
g. The abbreviations used are ALN, abdominal lymph node; AT, ascites tumor; L, liver and O, ovary.

Table 3 summarizes the details of lung metastases shown in Tables 2. The number of metastatic lung nodules was significantly suppressed in both QRsP-F5 and QRsP-F10 tumor cell lines compared to QRsP tumor cell lines. The rate of reduction, the mean numbers of metastatic nodules, and their range also tended to be less aggressive in tumors growing in FBRA-fed mice.

3.4. Suppression of somatic mutation in tumors grown in FBRA-fed mice

Mutations are known to be critical factors in tumor development and the acceleration of tumor malignancies (Kay et al., 2019). Therefore, we investigated the mutation frequency observed in tumors that grew in mice receiving FBRA. Micronucleus tests were used to detect somatic mutations in tumor cells.

First, a typical iatrogenic mutagen, doxorubicin, was added to the QR-32 cells to evaluate whether the micronucleus test could detect mutagenicity in our experimental model. Addition of doxorubicin to the culture medium induced micronuclei in QR-32 cells in a dose-dependent manner (Fig. 3A). Typical micronucleus images are shown in Fig. 3C-E.

We then compared the frequency of mutations occurring in the tumor tissues. To measure mutations formed during tumor growth, we used tumor cells that immediately separated from growing tumors *in vivo* (Fig. 1B). As shown in Fig. 4A, the frequency of micronucleus formation in tumor tissues obtained immediately after establishment was significantly reduced in mice fed the FBRA-containing diet compared to those fed a basic diet. FBRA intake was found to reduce mutations in tumor tissue. The number of micronuclei formed in tumor cells were analyzed. Tumors grown in FBRA-fed mice tended to have fewer micronuclei but were not significantly different from tumors grown in mice basic diet-fed mice (Fig. 4B). The reduced number of micronuclei formed in the tumor cells obtained from FBRA-fed mice almost disappeared after two subcultures of the established tumor cell lines (Fig. 4C).

3.5. Inhibition of systemically induced somatic mutations by ingestion of FBRA

Further, we investigated whether systemic anti-mutagenicity was induced in FBRA-fed mice using micronucleated reticulocytes (MNRET) in peripheral blood. As mutagenic anti-tumor agents, the alkylating agents busulfan (BUS, Fig. 5A) and cyclophosphamide (CY, Fig. 5B), and the antibiotic anti-tumor agents doxorubicin (DXR, Fig. 5C) and bleomycin (BLM, Fig. 5D) were used. As mutagenic carcinogens in the living environment, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK, Fig. 5E) present in tobacco smoke and 2-amino-3-methylimidazo[4,5-*b*] pyridine (PhIP, Fig. 5F), a heterocyclic amine contained in cooked foods (Tanaka et al., 2005) was used.

The frequency of MNRET formation induced by six chemical mutagens significantly suppressed somatic mutations occurring in mice fed 5% or 10% FBRA compared to mice fed a basic diet. Suppression of

Table 3
Inhibition effect of FBRA ingestion on acquisition of lung metastatic ability of tumor cells derived from inflammation-related carcinogenesis.

		Lung-colonizing ability[b]				
Cell lines[a]	No. of cell lines examined	Total no. of mice with lung metastasis/no. of mice tested	No. of metastatic nodules	Percent reduction	Mean value	Range
QRsP	16	37/48	21.6 ± 31.6	0	5	0–122
QRsP-F5	9	7/27[c]	1.7 ± 6.0[e]	92	0	0–31
QRsP-F10	6	2/18[d]	0.3 ± 0.8[f]	99	0	0–3

a. The detailed results of Tables 2 are summarized.
b. Cells from each cell line (1×10^6) were intravenously injected into the mice. The mice were sacrificed 28 days later, and metastatic nodules at the surface of the lung were counted macroscopically. The incidence of lung metastases was summarized.
c,d. Incidence of lung metastases was calculated by Fisher's exact probability test compared to the QRsP group. c; $p = 3.826E{-}5$, d; $p = 1.459E{-}6$.
e,f. The number of lung metastatic nodules was calculated by Welch's *t*-test compared to the QRsP group. e; $p = 9.305E{-}5$, f; $p = 2.576E{-}5$. Data are mean ± s.d.

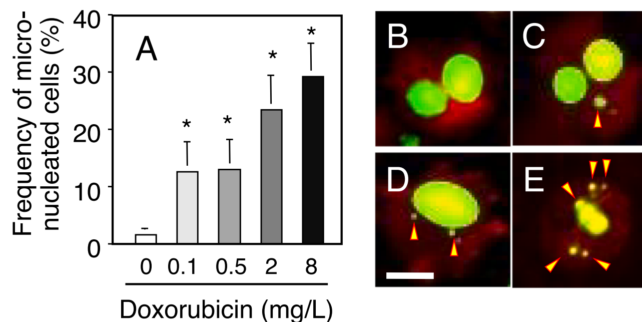

Fig. 3. Doxorubicin-induced Micronucleated Cells in QR-32 Cells QR-32 cells were treated *in vitro* for 6 h with or without doxorubicin, then switched to normal maintenance medium and incubated for an additional 48 h. The cells were then stained with acridine orange. Typical data was presented from three independent experiments with similar results (A). The micronucleated cell frequency was calculated by Welch's *t*-test. Data are mean ± s.d. $*p < 0.001$ compared to the incidence of untreated cells. Typical micronucleated cells found among QR-32 cells: no micronucleated cells (B); one micronucleated cells (C); two micronucleated cells (D); and five micronucleated cells (E).

induced mutations was found to be dependent on dietary FBRA content. *In vivo* systemic anti-mutagenicity following ingestion of FBRA was demonstrated regardless of the type of chemical or environmental mutagenic substance (Fig. 5).

4. Discussion

This study revealed that ingestion of FBRA prevented the metastatic properties acquired during inflammation-related carcinogenesis. We previously reported that FBRA intake suppressed tumor development in inflammation-induced carcinogenesis by suppressing the exudation of inflammatory cells into inflamed lesion (Onuma et al., 2015). The model used in these studies has already shown that inflammation facilitates not only the formation of primary tumors, but also the malignant progression of tumor cells, and especially the acquisition of metastatic potential (Okada et al., 1992; Tazawa et al., 2003). It has been reported that infiltrated inflammatory cells promote the carcinogenic process of this model by generating gaseous genotoxic reactive species (Okada, Kobayashi, et al., 2006; Okada, Tazawa, et al., 2006; Okada, 2019). Activated inflammatory cells release genotoxic reactive substances that may injure the surrounding cells. Surviving damaged cells proliferate under the influence of soluble growth factors released from inflamed lesions. These inflammatory environments lead to accumulation of DNA damage/genetic alterations, mutation fixation, and accelerating carcinogenic processes (Greten & Grivennikov, 2019; Kay et al., 2019; Okada et al., 2021). The inflammatory environment can generally be understood as a "carcinogenic niche" (Okada et al., 2021; Okada, 2014), and this study showed that FBRA ingestion chemopreventively regulates the carcinogenic niche against the acquisition of metastatic potential of tumor cells.

Intriguingly, anti-mutagenicity was induced in FBRA-fed mice. There are two explanations for the reduction in somatic mutations in tumors. First, the suppression of inflammatory exudation by FBRA results in an

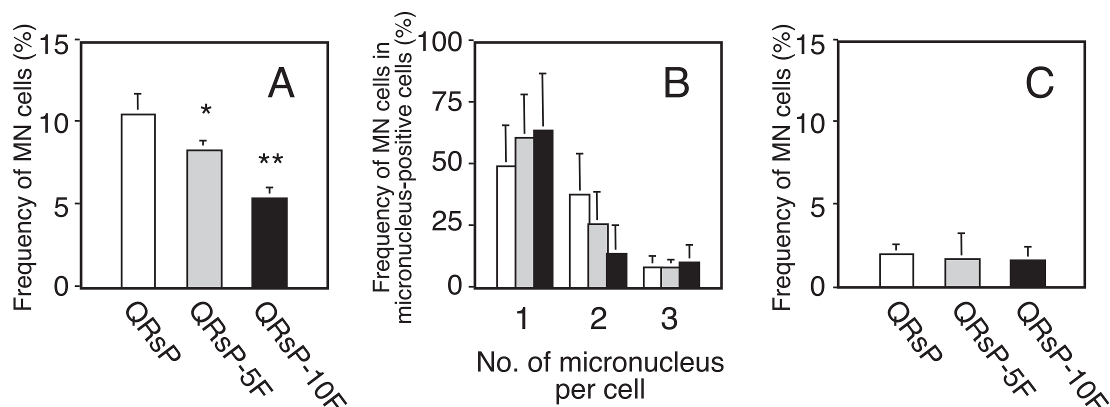

Fig. 4. Reduced Somatic Mutations in Tumor Tissues of FBRA-fed Mice. Mice were fed a basic diet (open bar), 5% FBRA (gray bar), and 10% FBRA (closed bar) diet *ad libitum*, starting 2 days before implantation and throughout the experiment. Micronuclei were counted over 200 cells per cell line. Typical data was presented from at least two independent experiments with similar results. The micronucleated cell frequency was calculated by Student's *t*-test. Data are mean ± s.d. (A) Subcutaneously grown tumors were aseptically removed and used to establish individual culture cell lines. Immediately after mechanical disaggregation of the tumor with scissors, the established tumor cells were immediately subjected to micronucleus (MN) formation analysis. $*p < 0.05$, $**p < 0.001$, compared to the frequency of MN observed in cell lines established from tumors grown in non-treated mice (QRsP tumor cell lines) ($n = 4$). (B) The same sample analyzed in A was used to measure the number of MN formed per tumor cell. Not significant as compared to the frequency of QRsP tumor cell lines ($n = 4$). (C) The frequency of MN formation in tumor cells was analyzed after two *in vitro* passages of the cell lines used in A. Not significant as compared to the frequency of QRsP tumor cell lines ($n = 4$).

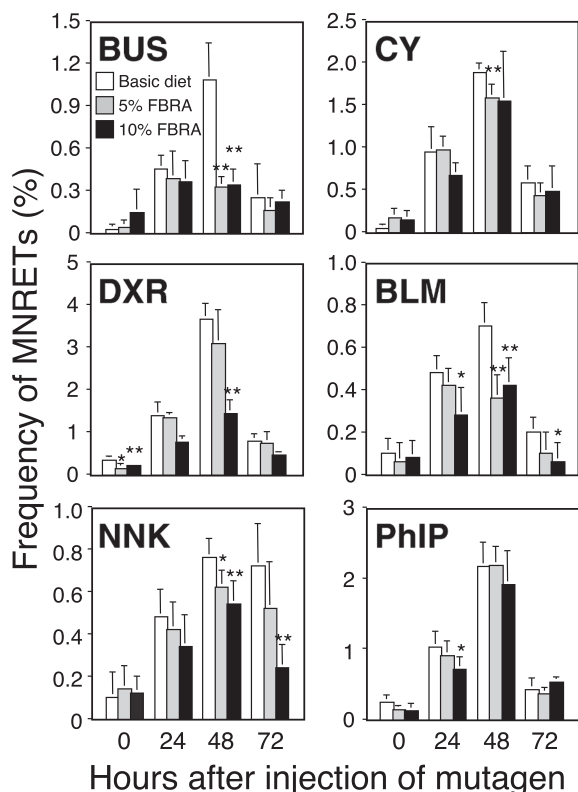

Fig. 5. Inhibition of Systemically Induced Somatic Mutations by Ingestion of FBRA. Ingestion of the basic diet (open bar), 5% FBRA-containing diet (gray bar), or 10% FBRA-containing diet (closed bar) was started 2 days before injection of the mutagens and continued throughout the experiment. Busulfan (BUS, 10 mg/kg, $n = 5$), cyclophosphamide (CY, 40 mg/kg, $n = 6$), doxorubicin (DXR, 5 mg/kg, $n = 5$), bleomycin (BLM, 5 mg/kg, $n = 5$), 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK, 100 mg/kg, $n = 5$), and 2-amino-3-methylimidazo[4,5-b]pyridine (PhIP, 50 mg/kg, $n = 6$) were injected intraperitoneally into mice to induce micronuclei. Tail vein blood was collected from mice, and micronucleated reticulocytes (MNRETs) were scored. Approximately 1,000 reticulocytes per mouse were counted. Typical data was presented from two to three independent experiments with similar results. The micronucleated cell frequency was calculated by Student's *t*-test. $^{*}p < 0.05$, $^{**}p < 0.01$, compared to the frequency of MNRETs observed in reticulocytes obtained from mice fed a basic diet. Data are mean ± s.d.

absolute reduction in the quantity of genotoxic reactive substances released from infiltrate inflammatory cells. Second, FBRA intake itself induces systemic anti-mutagenicity, suppressing mutations formed in tumors. The simultaneous action of FBRA in suppressing inflammatory exudation and induction of anti-mutagenicity is thought to suppress the acquisition of metastatic potential during the growth process of the primary tumor. Using *in vitro* and *in vivo* micronucleus tests, we have shown for the first time that FBRA intake induces systemic anti-mutagenicity against multiple chemical mutagens. There are three fundamental mechanisms that induce somatic mutations (Soria-Valles et al., 2017). The first occurs when intrinsic and/or extrinsic genotoxic stimuli are excessively generated or exposed to the cells. Intrinsic stimuli include genotoxic reactive substances (reactive nitric oxide and oxygen species), deamination reactions, and replication errors. Extrinsic stimuli include ultraviolet radiation, ionizing radiation, and environmental genotoxic chemicals (Helleday et al., 2014). The second mechanism occurs when the DNA repair systems, such as DNA damage response and the cell cycle arrest, do not work (Chatzinikolaou et al., 2014). The third occurs when cells that cannot fix DNA defects are not eliminated by apoptosis or enter cell senescence (Soria-Valles et al., 2017). FBRA contains several antioxidants and is thought to have the potential to scavenge intrinsic genotoxic substances (Esaki et al., 1999; Kuno et al., 2006). We will examine by which of the three reasons FBRA intake suppresses systemic somatic mutations.

The main anti-inflammatory effect of FBRA was determined to be *Aspergillus oryzae*. The prebiotics/probiotics effects and anti-inflammatory function of *Aspergillus oryzae*'s are observed across species. Feeding *Aspergillus oryzae* to ectothermic flies (*Drosophila melanogaster*) improves survival against heat stress through inhibition of inflammation (Kaufman et al., 2021). This phenomenon has also been validated in mammals by the fact that feeding *Aspergillus oryzae* to lactating cows experiencing heat stress reduces several inflammatory markers (Kaufman et al., 2021). Dietary intake of *Aspergillus oryzae* have been reported to reduce the expression of inflammation-mediated molecules such as endothelial adhesion molecules (VCAM-1 and ICAM-1) (Ham et al., 2019), cyclooxygenase-2 (Ham et al., 2019), interleukin-1ß (Chuang et al., 2019; Hermes et al., 2011), interleukin-6 (Kaufman et al., 2021), inducible nitric oxide synthases (Chuang et al., 2019), interferon-γ (Chuang et al., 2019), lipopolysaccharide-binding protein (Kaufman et al., 2021), serum amyloid A (Kaufman et al., 2021), and NF-κB (Chuang et al., 2019). The anti-inflammatory function of *Aspergillus oryzae* intake is recognized as common because it is found in species such as flies (Kaufman et al., 2021), chickens (Chuang et al., 2019), mice (Ham et al., 2019), pigs (Hermes et al., 2011), and cattle (Kaufman et al., 2021).

It is also found that the anti-inflammatory effect of FBRA depends on the intestinal bacteria. Incubation of fecal slurry with FBRA increased number of bifidobacterial and generated organic acid suggesting that FBRA functions as a prebiotic (Nemoto et al., 2011). The organic acids have beneficial functions for the host, including immunomodulatory effects on inflammation (Neish, 2009; Topping & Clifton, 2001; Wächtershäuser & Stein, 2000). It is necessary to examine whether the intestinal bacteria responsible for the anti-inflammatory effect caused by FBRA intake are bifidobacteria alone or a combination of multiple intestinal bacteria.

It has been reported that ingestion of FBRA suppresses experimental carcinogenesis, such as spontaneous carcinogenesis (Kuno et al., 2018), chemical carcinogenesis (Katayama et al., 2003; Katyama et al., 2002; Kuno et al., 2004, 2006; Long et al., 2007; Phutthaphadoong, Yamada, Hirata, & Tomita et al., 2009; Tomita et al., 2008), carcinogenesis in genetically modified animals (Kuno et al., 2016; Phutthaphadoong, Yamada, Hirata, & Tomita et al., 2010), and carcinogenesis after benign/regressive tumor cell transplantation (Onuma et al., 2015). There are a wide variety of target organs in which carcinogenesis is suppressed by FBRA intake, including the oral cavity (Long et al., 2007), lungs (Phutthaphadoong, Yamada, Hirata, & Tomita et al., 2009), esophagus (Kuno et al., 2004), stomach (Tomita et al., 2008), liver (Katayama et al., 2003), pancreas (Kuno et al., 2015), large intestine (Katyama et al., 2002; Phutthaphadoong, Yamada, Hirata, & Tomita et al., 2010), bladder (Kuno et al., 2006), and prostate (Kuno et al., 2016), as well as development of lymphoma (Kuno et al., 2018), and tumorigenic conversion of regressive fibrosarcoma cells (Onuma et al., 2015). More importantly, the action of FBRA has been shown to be effective across species, as it has a common carcinogenic inhibitory effect even when used in mice (Kuno et al., 2006, 2018; Onuma et al., 2015; Phutthaphadoong, Yamada, Hirata, & Tomita et al., 2009, 2010), rats (Katayama et al., 2003; Katyama et al., 2002; Kuno et al., 2004, 2016; Long et al., 2007; Tomita et al., 2008), and hamsters (Kuno et al., 2015). It has been reported that ingestion of FBRA may inhibit the growth of tumor cells (Phutthaphadoong, Yamada, Hirata, & Tomita et al., 2009), suppress the expression of cytochrome P450 2A5 (Cyp2a5) which activates carcinogenic compounds (Phutthaphadoong, Yamada, Hirata, & Tomita et al., 2009), and suppress inflammation-related gene expressions (Onuma et al., 2015). To date, however, why FBRA intake has a wide range of carcinogenesis-suppressive functions is not conclusively explained. This study suggested that FBRA intake may suppress inflammation and mutagenicity, which are both involved in generally accelerating the carcinogenic process (Greten & Grivennikov, 2019; Kay

et al., 2019; Okada et al., 2021; Okada, 2014).

Our next interest is whether the anti-inflammatory and anti-mutagenic effects induced by FRRA ingestion are independent phenomena, or whether anti-mutagenicity is caused as a result of the anti-inflammatory effects. The above-mentioned action of FBRA cannot be determined in experiments using a live animal in which an inflammatory reaction is always present and induced. For that purpose, we are currently working on an *in vitro* reconstruction experimental system using FBRA-treated mouse tissue that can eliminate or add to the involvement of inflammatory cells.

5. Conclusions

Oral ingestion of fermented brown rice and rice bran with *Aspergillus oryzae* (FBRA) in mice has a chemopreventive effect on both inflammation-related tumor development and progression (metastasis). The suppression of carcinogenic processes associated with inflammation is due to the anti-inflammatory and anti-mutagenic effects brought about by ingestion of FBRA. *Aspergillus oryzae* is the main component of FBRA's anti-inflammatory effect, and it has been clarified that the intestinal flora is involved. We also found that systemic somatic mutations induced by iatrogenic, environmental, and food-derived mutagenicity were all suppressed in FBRA-fed mice. We herein report that FBRA ingestion prevented the tumor development and acquisition of metastatic potential through suppression of inflammatory cell infiltration and somatic mutations, both of which are critical factors in accelerating carcinogenesis and tumor progression.

Ethical statement

All *in vivo* experimental procedures and protocols were approved by the Institutional Animal Care and Use Committee of Tottori University (Permit No. 19-Y-37) and were performed in accordance with the approved guidelines. The *in vivo* mutagenicity experiments were approved by the Experimental Animal Research Committee of Kyoto Pharmaceutical University (Permit No. 17–031).

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CRediT authorship contribution statement

Hideyuki Nemoto: Conceptualization, Validation, Formal analysis, Investigation, Writing – original draft. **Marina Otake:** Investigation. **Takahiro Matsumoto:** Formal analysis, Investigation. **Runa Izutsu:** Validation, Investigation. **Jumond P Jehung:** Validation, Investigation. **Keisuke Goto:** Investigation. **Mitsuhiko Osaki:** Conceptualization, Formal analysis, Writing – review & editing. **Masafumi Mayama:** Conceptualization, Supervision. **Masataka Shikanai:** Conceptualization, Supervision. **Hiroshi Kobayashi:** Conceptualization, Supervision. **Tetsushi Watanabe:** Conceptualization, Formal analysis, Supervision. **Futoshi Okada:** Conceptualization, Formal analysis, Writing – review & editing, Supervision, Project administration.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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References

Alhabbab, R., Blair, P., Elgueta, R., Stolarczyk, E., Marks, E., Becker, P. D., Ratnasothy, K., Smyth, L., Safinia, N., Sharif-Paghaleh, E., O'Connell, S., Noelle, R. J., Lord, G. M., Howard, J. K., Spencer, J., Lechler, R. I., & Lombardi, G. (2015). Diversity of gut microflora is required for the generation of B cell with regulatory properties in a skin graft model. *Scientific Reports, 5*, 11554. https://doi.org/10.1038/srep11554

Allemani, C., Matsuda, T., Carlo, V. D., Harewood, R., Matz, M., Nikšić, M., Bonaventure, A., Valkov, M., Johnson, C. J., Esteve, J., Ogunbiyi, O. J., Silva, G. A. E., Chen, W.-Q., Eser, S., Engholm, G., Stiller, C. A., Monnereau, A., Woods, R. R., Visser, O., Coleman, M. P., … CONCORD Working Group. (2018). Global surveillance of trends in cancer survival 2000–14 (CONCORD-3): Analysis of individual records for 37 513 025 patients diagnosed with one of 18 cancers from 322 population-based registries in 71 countries. *Lancet, 391*, 1023–1075. https://doi.org/10.1016/S0140-6736(17)33326-3

Chatzinikolaou, G., Karakasilioti, I., & Garinis, G. A. (2014). DNA damage and innate immunity: Links and trade-offs. *Trends Immunology, 35*, 429–435. https://doi.org/10.1016/j.it.2014.06.003

Chuang, W. Y., Lin, W. C., Hsieh, Y. C., Huang, C. M., Chang, S. C., & Lee, T.-T. (2019). Evaluation of the combined use of Saccharomyces cerevisiae and Aspergillus oryzae with phytase fermentation products on growth, inflammatory, and intestinal morphology in broilers. *Animals, 9*, 1051. https://doi.org/10.3390/ani9121051

Ding, L., Ellis, M. J., Li, S., Larson, D. E., Chen, K., Wallis, J. W., Harris, C. C., McLellan, M. D., Fulton, R. S., Fulton, L. L., Abbott, R. M., Hoog, J., Dooling, D. J., Koboldt, D. C., Schmidt, H., Kalicki, J., Zhang, Q., Chen, L., Lin, L., … Mardis, E. R. (2010). Genome remodelling in a basal-like breast cancer metastasis and xenograft. *Nature, 464*, 999–1005. https://doi.org/10.1038/nature08989

Esaki, H., Kawakishi, S., Morimitsu, Y., & Osawa, T. (1999). New potent antioxidative o-dihydroxyisoflavones in fermented Japanese soybean products. *Bioscience Biotechnology and Biochemistry, 63*, 1637–1639. https://doi.org/10.1271/bbb.63.1637

Greten, F. R., & Grivennikov, S. I. (2019). Inflammation and cancer: Triggers, mechanisms, and consequences. *Immunity, 51*, 27–41. https://doi.org/10.1016/j.immuni.2019.06.025

Ham, Y. M., Song, H. S., Kwon, J. E., Jeon, H., Baek, H. J., Kim, C. W., Yoon, W.-J., Choung, E. S., & Kang, S. C. (2019). Effects of fermented Sorghum bicolor L. Moench extract on inflammation and thickness in a vascular cell and atherosclerotic mice model. *Journal of Natural Medicine, 73*, 34–46. https://doi.org/10.1007/s11418-018-1231-9

Han, D., Walsh, M. C., Kim, K. S., Hong, S.-W., Lee, J., Yi, J., Rivas, G., Surh, C. D., & Choi, Y. (2015). Microbiota-independent ameliorative effects of antibiotics on spontaneous Th2-associated pathology of the small intestine. *PLoS ONE, 10*, Article e0118795. https://doi.org/10.1371/journal.pone.0118795

Hayashi, M., Sofuni, T., & Ishidate, M., Jr. (1983). An application of Acridine Orange fluorescent staining to the micronucleus test. *Mutation Research, 120*, 241–247. https://doi.org/10.1016/0165-7992(83)90096-9

Helleday, T., Eshtad, S., & Nik-Zainal, S. (2014). Mechanisms underlying mutational signatures in human cancers. *Nature Reviews Genetics, 15*, 585–598. https://doi.org/10.1038/nrg3729

Hermes, R. G., Manzanilla, E. G., Martín-Orúe, S. M., Pérez, J. F., & Klasing, K. C. (2011). Influence of dietary ingredients on in vitro inflammatory response of intestinal porcine epithelial cells challenged by an enterotoxigenic Escherichia coli (K88). *Comparative Immunology Microbiology & Infectious Diseases, 34*, 479–488. https://doi.org/10.1016/j.cimid.2011.08.006

Ishikawa, M., Okada, F., Hamada, J., Hosokawa, M., & Kobayashi, H. (1987). Changes in the tumorigenic and metastatic properties of tumor cells treated with quercetin or 5-azacytidine. *International Journal of Cancer, 39*, 338–342. https://doi.org/10.1002/ijc.2910390312

Ishikawa, M., Hosokawa, M., Oh-hara, N., Niho, Y., & Kobayashi, H. (1987). Marked granulocytosis in C57BL/6 mice bearing a transplanted BMT-11 fibrosarcoma. *Journal of National Cancer Institute, 78*, 567–571 [PMID: 3469467].

Katayama, M., Sugie, S., Yoshimi, N., Yamada, Y., Sakata, K., Qiao, Z., Iwasaki, T., Kobayashi, H., & Mori, H. (2003). Preventive effect of fermented brown rice and rice bran on diethylnitrosoamine and phenobarbital-induced hepatocarcinogenesis in male F344 rats. *Oncology Reports, 10*, 875–880 [PMID: 12792738].

Katyama, M., Yoshimi, N., Yamada, Y., Sakata, K., Kuno, T., Yoshida, K., Qiao, Z., Vihn, P. Q., Iwasaki, T., Kobayashi, H., & Mori, H. (2002). Preventive effect of fermented brown rice and rice bran against colon carcinogenesis in male F344 rats. *Oncology Reports, 9*, 817–822 [PMID: 12066215].

Kaufman, J. D., Seidler, Y., Bailey, H. R., Whitacre, L., Bargo, F., Lüersen, K., Rimbach, G., Pighetti, G. M., Ipharraguerre, I. R., & Ríus, A. G. (2021). A postbiotic from Aspergillus oryzae attenuates the impact of heat stress in ectothermic and endothermic organisms. *Scientific Reports, 11*, 6407. https://doi.org/10.1038/s41598-021-85707-3

Kay, J., Thadhani, E., Samson, L., & Engelward, B. (2019). Inflammation-induced DNA damage, mutations and cancer. *DNA Repair, 83*, Article 102673. https://doi.org/10.1016/j.dnarep.2019.102673

Kuno, T., Hirose, Y., Hata, K., Kato, K., Qiang, S. H., Kitaori, N., Hara, A., Iwasaki, T., Yoshimura, T., Wada, K., Kobayashi, H., & Mori, H. (2004). Preventive effect of fermented brown rice and rice bran on N-nitrosomethylbenzylamine-induced esophageal tumorigenesis in rats. *International Journal of Oncology, 25*, 1809–1815 [PMID: 15547721].

Kuno, T., Hirose, Y., Yamada, Y., Hata, K., Qiang, S. H., Asano, N., Oyama, T., Zhi, H., Iwasaki, T., Kobayashi, H., & Mori, H. (2006). Chemoprevention of mouse urinary bladder carcinogenesis by fermented brown rice and rice bran. *Oncology Reports, 15*, 533–538 [PMID: 16465408].

Kuno, T., Takahashi, S., Tomita, H., Hisamatsu, K., Hara, A., Hirata, A., Kobayashi, H., & Mori, H. (2015). Preventive effects of fermented brown rice and rice bran against N–nitrosobis (2–oxopropyl) amine–induced pancreatic tumorigenesis in male hamsters. *Oncology Letters, 10*, 3377–3384. https://doi.org/10.3892/ol.2015.3809

Kuno, T., Nagano, A., Mori, Y., Kato, H., Nagayasu, Y., Naiki-Ito, A., Suzuki, S., Mori, H., & Takahashi, S. (2016). Preventive effects of fermented brown rice and rice bran against prostate carcinogenesis in TRAP Rats. *Nutrients, 8*, 421. https://doi.org/10.3390/nu8070421

Kuno, T., Kato, H., Naiki-Ito, A., Suzuki, S., Tanaka, T., Takahashi, S., & Mori, H. (2018). Preventive effects of fermented brown rice and rice bran on spontaneous lymphomagenesis in AKR/NSlc female mice. *Asian Pacific Journal of Cancer Prevention, 19*, 3217–3223. https://doi.org/10.31557/APJCP.2018.19.11.3217

Lambert, A. W., Pattabiraman, D. R., & Weinberg, R. A. (2017). Emerging biological principles of metastasis. *Cell, 168*, 670–691. https://doi.org/10.1016/j.cell.2016.11.037

Long, N. K., Makita, H., Yamashita, T., Toida, M., Kato, K., Hatakeyama, D., & Shibata, T. (2007). Chemopreventive effect of fermented brown rice and rice bran on 4-nitroquinoline 1-oxide-induced oral carcinogenesis in rats. *Oncology Reports, 17*, 879–885 [PMID: 17342331].

Matsumoto, T., Takahashi, K., Kanayama, S., Nakano, Y., Imai, H., Kibi, M., Imahori, D., Hasei, T., & Watanabe, T. (2017). Structures of antimutagenic constituents in the peels of citrus limon. *Journal of Natural Medicine, 71*, 735–744. https://doi.org/10.1007/s11418-017-1108-3

Matsumoto, T., Nishikawa, T., Furukawa, A., Itano, S., Tamura, Y., Hasei, T., & Watanabe, T. (2017). Antimutagenic effects of polymethoxy flavonoids of citrus unshiu. *Natural Product Communications, 12*, 23–26 [PMID: 30549817].

Naxerova, K., & Jain, R. K. (2015). Using tumour phylogenetics to identify the roots of metastasis in humans. *Nature Review Clinical Oncology, 12*, 258–272. https://doi.org/10.1038/nrclinonc.2014.238

Nemoto, H., Ikata, K., Arimochi, H., Iwasaki, T., Ohnishi, Y., Kuwahara, T., & Kataoka, K. (2011). Effects of fermented brown rice on the intestinal environments in healthy adult. *Journal of Medical Investigation, 58*, 235–245. https://doi.org/10.2152/jmi.58.235

Neish, A. S. (2009). Microbes in gastrointestinal health and disease. *Gastroenterology, 136*, 65–80. https://doi.org/10.1053/j.gastro.2008.10.080

Ohnishi, Y., Fujii, H., Kimura, F., Mishima, T., Murata, J., Tazawa, K., Fujimaki, M., Okada, F., Hosokawa, M., & Saiki, I. (1996). Inhibitory effect of a traditional Chinese medicine, Juzen-taiho-to, on progressive growth of weakly malignant clone cells derived from murine fibrosarcoma. *Japanese Journal of Cancer Research, 87*, 1039–1044. https://doi.org/10.1111/j.1349-7006.1996.tb03107.x

Okada, F., Hosokawa, M., Hasegawa, J., Ishikawa, M., Chiba, I., Nakamura, Y., & Kobayashi, H. (1990). Regression mechanisms of mouse fibrosarcoma cells after in vitro exposure to quercetin: Diminution of tumorigenicity with a corresponding decrease in the production of prostaglandin E2. *Cancer Immunology Immunotherapy, 31*, 358–364. https://doi.org/10.1007/BF01741407. PMID: 2386981.

Okada, F., Hosokawa, M., Hamada, J.-I., Hasegawa, J., Kato, M., Mizutani, M., Ren, J., Takeichi, N., & Kobayashi, H. (1992). Malignant progression of a mouse fibrosarcoma by host cells reactive to a foreign body (gelatin sponge). *British Journal of Cancer, 66*, 635–639. https://doi.org/10.1038/bjc.1992.329

Okada, F. (2002). Inflammation and free radicals in tumor development and progression. *Redox Report, 7*, 357–368. https://doi.org/10.1179/135100002125001135

Okada, F., Shionoya, H., Kobayashi, M., Kobayashi, T., Tazawa, H., Onuma, K., Iuchi, Y., Matsubara, N., Ijichi, T., Dugas, B., & Hosokawa, M. (2006). Prevention of inflammation-mediated acquisition of metastatic properties of benign mouse fibrosarcoma cells by administration of an orally available superoxide dismutase. *British Journal of Cancer, 94*, 854–862. https://doi.org/10.1038/sj.bjc.6603016

Okada, F., Tazawa, H., Kobayashi, T., Kobayashi, M., & Hosokawa, M. (2006). Involvement of reactive nitrogen oxides for acquisition of metastatic properties of benign tumors in a model of inflammation-based tumor progression. *Nitric Oxide, 14*, 122–129. https://doi.org/10.1016/j.niox.2005.06.009

Okada, F., Kobayashi, M., Tanaka, H., Kobayashi, T., Tazawa, H., Iuchi, Y., Onuma, K., Hosokawa, M., Dinauer, M. C., & Hunt, N. H. (2006). The role of nicotinamide adenine dinucleotide phosphate oxidase-derived reactive oxygen species in the acquisition of metastatic ability of tumor cells. *American Journal of Pathology, 169*, 294–302. https://doi.org/10.2353/ajpath.2006.060073

Okada, F. (2014). Inflammation-related carcinogenesis: Current findings in epidemiological trends, causes and mechanisms. *Yonago Acta Medica, 57*, 65–72 [PMID: 25324587; PMCID: PMC4198572].

Okada, F. (2019, September). Gaseous molecules as an endogenous factor in the inflammation-related carcinogenesis model. In *Symposium session presentation at the meeting of the Persulfide Conference 2019, Proceedings of the 1st International Conference on Persulfide and Sulfur Metabolism in Biology and Medicine, Sendai, Japan* (pp. 76–77).

Okada, F., Izutsu, R., Goto, K., & Osaki, M. (2021). Inflammation-related carcinogenesis: Lessons from animal models to clinical aspects. *Cancers, 13*, 921. https://doi.org/10.3390/cancers13040921

Onuma, K., Sato, Y., Ogawara, S., Shirasawa, N., Kobayashi, M., Yoshitake, J., Yoshimura, T., Iigo, M., Fujii, J., & Okada, F. (2009). Nano-scaled particles of titanium dioxide convert benign mouse fibrosarcoma cells into aggressive tumor cells. *American Journal of Pathology, 175*, 2171–2183. https://doi.org/10.2353/ajpath.2009.080900

Onuma, K., Suenaga, Y., Sakaki, R., Yoshitome, S., Sato, Y., Ogawara, S., Suzuki, S., Kuramitsu, Y., Yokoyama, H., Murakami, A., Hamada, J., Nicolson, G. L., Kobayashi, M., Fujii, J., & Okada, F. (2011). Development of a quantitative bioassay to assess preventive compounds against inflammation-based carcinogenesis. *Nitric Oxide, 25*, 183–194. https://doi.org/10.1016/j.niox.2011.02.003

Onuma, K., Kanda, Y., Suzuki-Ikeda, S., Sakaki, R., Nonomura, T., Kobayashi, M., Osaki, M., Shikanai, M., Kobayashi, H., & Okada, F. (2015). Fermented brown rice and rice bran with Aspergillus oryzae (FBRA) prevents inflammation-related carcinogenesis in mice, by inhibiting infiltration of inflammatory cells. *Nutrients, 7*, 10237–10250. https://doi.org/10.3390/nu7125531

Phutthaphadoong, S., Yamada, Y., Hirata, A., Tomita, H., Taguchi, A., Hara, A., Limtrakul, P. N., Iwasaki, T., Kobayashi, H., & Mori, H. (2009). Chemopreventive effects of fermented brown rice and rice bran against 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone-induced lung tumorigenesis in female A/J mice. *Oncology Reports, 21*, 321–327 [PMID: 19148502].

Phutthaphadoong, S., Yamada, Y., Hirata, A., Tomita, H., Hara, A., Limtrakul, P., Iwasaki, T., Kobayashi, H., & Mori, H. (2010). Chemopreventive effect of fermented brown rice and rice bran (FBRA) on the inflammation-related colorectal carcinogenesis in $Apc^{Min/+}$ mice. *Oncology Reports, 23*, 53–59 [PMID: 19956864].

Soria-Valles, C., López-Soto, A., Osorio, F. G., & López-Otín, C. (2017). Immune and inflammatory responses to DNA damage in cancer and aging. *Mechanisms of Ageing and Development, 165*, 10–16. https://doi.org/10.1016/j.mad.2016.10.004

Tanaka, T., Suzuki, R., Kohno, H., Sugie, S., Takahashi, M., & Wakabayashi, K. (2005). Colonic adenocarcinomas rapidly induced by the combined treatment with 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine and dextran sodium sulfate in male ICR mice possess b-catenin gene mutations and increases immunoreactivity for b-catenin, cyclooxygenase-2 and inducible nitric oxide synthase. *Carcinogenesis, 26*, 229–238. https://doi.org/10.1093/carcin/bgh292

Tazawa, H., Okada, F., Kobayashi, T., Tada, M., Mori, Y., Une, Y., Sendo, F., Kobayashi, M., & Hosokawa, M. (2003). Infiltration of neutrophils is required for acquisition of metastatic phenotype of benign murine fibrosarcoma cells: Implication of inflammation-associated carcinogenesis and tumor progression. *American Journal of Pathology, 163*, 2221–2232. https://doi.org/10.1016/S0002-9440(10)63580-8

Tomita, H., Kuno, T., Yamada, Y., Oyama, T., Asano, N., Miyazaki, Y., Baba, S., Taguchi, A., Hara, A., Iwasaki, T., Kobayashi, H., & Mori, H. (2008). Preventive effect of fermented brown rice and rice bran on N-methyl-N'-nitro-N-nitrosoguanidine-induced gastric carcinogenesis in rats. *Oncology Reports, 19*, 11–15 [PMID: 18097570].

Topping, D. L., & Clifton, P. M. (2001). Short-chain fatty acids and human colonic function: Roles of resistant starch and nonstarch polysaccharides. *Physiological Reviews, 81*, 1031–1064. https://doi.org/10.1152/physrev.2001.81.3.1031

Wächtershäuser, A., & Stein, J. (2000). Rationale for the luminal provision of butyrate in intestinal diseases. *European Journal of Nutrition, 39*, 164–171. https://doi.org/10.1007/s003940070020

Yachida, S., Jones, S., Bozic, I., Antal, T., Leary, R., Fu, B., Kamiyama, M., Hruban, R. H., Eshleman, J. R., Nowak, M. A., Velculescu, V. E., Kinzler, K. W., Vogelstein, B., & Iacobuzio-Donahue, C. A. (2010). Distant metastasis occurs late during the genetic evolution of pancreatic cancer. *Nature, 467*, 1114–1117. https://doi.org/10.1038/nature09515

Yates, L. R., Gerstung, M., Knappskog, S., Desmedt, C., Gundem, G., Van Loo, P., Aas, T., Alexandrov, L. B., Larsimont, D., Davies, H., Li, Y., Ju, Y. S., Ramakrishna, M., Haugland, H. K., Lilleng, P. K., Nik-Zainal, S., McLaren, S., Butler, A., Martin, S., … Campbell, P. J. (2015). Subclonal diversification of primary breast cancer revealed by multiregion sequencing. *Nature Medicine, 21*, 751–759. https://doi.org/10.1038/nm.3886

Yokota, J., & Sugimura, T. (1993). Multiple steps in carcinogenesis involving alterations of multiple tumor suppressor genes. *FASEB Journal, 7*, 920–925. https://doi.org/10.1096/fasebj.7.10.8344488