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Original Article

A high-fat diet increases the incidence of mammary cancer in c-Ha-*ras* proto-oncogene transgenic rats

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Abstract: Mammary cancer is the most common type of cancer and the fifth most common cause of cancer-related deaths among Japanese women. The recent sharp increase in the number of women diagnosed with mammary cancer per year is thought to be associated with increased fat intake resulting from changes in the dietary habits of contemporary Japanese citizens. In this study, human c-Ha-*ras* proto-oncogene transgenic (Hras128) rats, which are highly susceptible to mammary carcinogens, were fed high- or low-fat diets to examine the relationship between fat consumption and the development of mammary cancer. Female 7-week-old Hras128 rats and wild-type littermates were administered benzo[a]pyrene. A week later, the animals were randomly assigned to high-fat or low-fat diet groups (45% or 10% of calories from fat, respectively). After 12 weeks, the rats were sacrificed and autopsied, and mammary tumors were excised and processed for microscopic observation. Mammary tumors were found in 11 of the 12 animals in the high-fat diet group and in 5 of the 12 animals in the low-fat diet group, and the numbers of mammary gland tumors per animal in these groups were 1.7 and 0.7, respectively. Notably, the observed differences in incidence and multiplicity of mammary tumors between the two groups were statistically significant. These results suggest a positive relationship between the incidence of breast cancer and high fat intake. (DOI: 10.1293/tox.2016-0052; J Toxicol Pathol 2017; 30: 145–152)

Key words: c-Ha-*ras* gene, Hras128, mammary cancer, high-fat diet

Introduction

Epidemiological studies report higher incidences of mammary cancer in North America, Australia, and Europe than in most of Africa, South Asia, and East Asia, including Japan[1]. Among US-born women, 12.4% were affected by mammary cancer in 2007, with peak incidences being observed in women between the ages of 65 and 69[2]. Notably, these numbers indicate that 1 in 8 American women will be affected by mammary cancer over the course of their lifetime. Meanwhile, there was an approximate 6% increase in the incidence of mammary cancer each year among Japanese women between 1999 and 2008[3]. Mammary cancer is now the most common cancer among Japanese women (occurring in 1 in 14 women)[3] and is the fifth most common cause of cancer-related deaths, following colorectal cancer, lung cancer, gastric cancer, and pancreatic cancer[4]. While the rate of mammary cancer diagnosis in Japanese women peaks between 45 and 49 years of age[4], the incidence of these cancers among women younger than 35-years-old has also been on the rise in recent years[5].

Excessive weight and obesity have been reported as key risk factors for the increasing incidence of mammary cancer among elderly women in the US[6, 7]. Notably, in studies of people from Japan and China (where the incidence of this disease is relatively low) who immigrated to Hawaii and California, first-generation immigrants that maintained a diet similar to that of their country of origin exhibited low incidences of mammary cancer, while individuals in the third generation, who had adapted to the local lifestyle, exhibited incidences as high as those of other US-born women. Accordingly, the incidence of mammary cancer among individuals in the second generation was intermediate, falling between those of the first and third generations[8–10]. These findings reinforce the importance of lifestyle rather than race-associated genetic predisposition in the etiology of this disease. It has been suggested that traditional Japa-

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nese dietary factors may protect against the development of mammary cancer[11], and that the recent abrupt increase in the number of Japanese women diagnosed with mammary cancer may be due to changes in the dietary habits of contemporary Japanese. Indeed, a putative association between dietary fat and mammary cancer has been reported[6, 12]; however, other studies have found no such association[13, 14]. Confounding factors include obesity and physical activity. A positive association between obesity and mammary cancer in postmenopausal women has been reported[15], and compared with lean women, obese women are more likely to die from mammary cancer following diagnosis[16]. Meanwhile, a negative association between physical activity and mammary cancer has also been suggested[17]. Regarding the relationships between weight gain and mammary cancer[15] and between obesity and mammary cancer prognosis, it has been reported that the higher the body mass index (BMI) score, the poorer the prognosis[16].

Transgenic rats carrying the human c-Ha-*ras* proto-oncogene (Hras128)[18] are highly susceptible to mammary carcinogens and can therefore be used as a medium-term bioassay model[19, 20]. The majority of mammary cancers that develop in Hras128 rats are papillotubular carcinomas with the same histologic type as mammary cancers that develop in humans[20], rendering the Hras128 rat an appropriate animal model of human mammary cancer. In this study, we therefore examined the effect of high- and low-fat diets on mammary cancer incidence and pathology in carcinogen (benzo[a]pyrene, B[a]P)-exposed Hras128 rats to further investigate the possible link between fat intake and the development of mammary cancer.

Materials and Methods

Chemicals and diets

B[a]P was purchased from Sigma-Aldrich Corporation (St. Louis, MO, USA). Two diets, a 45 kcal% fat diet (high fat diet; D12451) and 10 kcal% fat diet (D12450B) containing fat derived from lard (39.5% and 4.4% of total energy, respectively) and soybean oil (5.5% of total energy for each), were purchased from Rodent Diets, Inc. (New Brunswick, NJ, USA). The basal diet (Oriental MF) was purchased from Oriental Yeast Co., Ltd. (Tokyo, Japan).

Animals

Female Hras128 rats[18] (n = 24; 5-week-old) and female wild-type (n = 24; 5-week-old) Sprague-Dawley littermates were purchased from CLEA Japan, Inc. (Tokyo, Japan). Animals were housed two to three per cage at the Hyogo University Animal Study Facilities under specific pathogen-free conditions, with a temperature of 22 ± 2°C, a relative humidity of 55 ± 10%, and a 12-h light/12-h dark cycle. Animals underwent a 1-week quarantine and acclimation period before the beginning of the experiment. During this period, basal diet and water were available *ad libitum*. All experiments were approved by the Institutional Committee for Ethics of Animal Experimentation (No. A10002; May 25th, 2010) of Hyogo University. Experiments were conducted according to the Guidelines for Animal Experiments in Hyogo University, Japan, promulgated by the committee and were designed to follow the principles of replacement, refinement, and reduction of animal testing (3Rs). It should also be noted that the study protocol was approved prior to the committee prohibiting the use of ether anesthesia for animals[21].

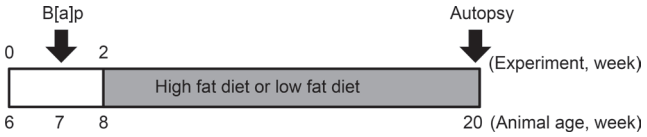

Fig. 1. Schematic of the experimental protocol. B[a]p was administered by gastric intubation 1 week into the experiment, and rats were sacrificed at 20 weeks of age. The duration of the experiment was 14 weeks.

Experimental protocol

The experimental protocol is depicted in Fig. 1. After the quarantine/acclimation period, at which point the animals were 6 weeks old, Hras128 and wild-type rats were randomly divided into high- and low-fat diet groups (i.e., four experimental groups). The animals were allowed *ad libitum* access to the basal diet for the first 2 weeks of the experiment. After 1 week, B[a]P dissolved in olive oil as previously described[20] was administered to all animals (50 mg/kg) by gastric intubation. One week after B[a]P administration, the animals were placed on either the high- or low-fat diet. The diet and drinking water were available *ad libitum*. Body weights were measured at least once a week. All rats were sacrificed at 20 weeks of age under deep ether anesthesia. Blood was collected from the abdominal aorta before autopsy under deep anesthesia. The autopsies in this study were performed according to a protocol that was approved in 2010, when ether anesthesia was still acceptable. One wild-type rat in the low-fat diet group died before the scheduled sacrifice from causes unrelated to the experiment and was therefore excluded from analysis. All Hras128 and wild-type rats were handled in exactly the same manner.

Pathological evaluation

Mammary gland tumors were removed, counted, measured, and fixed in 10% neutral buffered formalin solution. Mammary tissues and tumors were individually embedded in paraffin, sliced to a thickness of 4 micrometers, and stained with hematoxylin and eosin (H&E) for microscopic examination. The heart, lung, liver, and kidney were collected and visually inspected for the presence of any abnormalities, placed in 10% neutral buffered formalin solution, and processed for histopathological examination. All tissues were processed in an identical manner.

Immunohistochemical evaluation

Tissue sections were deparaffinized in xylene, hydrated in a graded ethanol series, and treated according to the

instructions in a VECTASTAIN Elite ABC Rabbit IgG Kit (Vector Laboratories, Burlingame, CA, USA). The nuclei were counterstained with hematoxylin. Tissues were stained with anti-estrogen receptor rabbit polyclonal IgG (diluted 1:50; MC-20, Santa Cruz Biotechnology, Inc., Dallas, TX, USA), anti-progesterone receptor rabbit polyclonal IgG (diluted 1:50; C-19, Santa Cruz Biotechnology, Inc.), and anti-Ki-67 rabbit monoclonal antibody (diluted 1:100; clone no. 30-9, Ventana, Tucson, AZ, USA). All immunohistochemical staining procedures were performed in accordance with the VECTASTAIN protocol. The stain was developed using DAB stain (Vector Laboratories).

Measurement of the biochemical parameters of the blood

The biochemical parameters of the blood were measured using a Hitachi 7080 automatic biochemistry analyzer (Hitachi Ltd., Tokyo, Japan), according to the manufacturer's recommendations.

Statistical analysis

Values are reported as means ± standard deviations (SD) or standard errors (SE), as indicated. Differences in tumor incidences between groups were evaluated by Pearson's chi-square test using the Microsoft Excel 2010 software, while differences in mean body weights and blood biochemical parameters were analyzed by one-way and two-way analysis of variance (ANOVA), respectively, using IBM SPSS Statistics version 22.0 (IBM Corp., Armonk, NY, USA). For all analyses, $p<0.05$ was considered statistically significant.

Results

Body weights of Hras128 and wild-type rats fed different diets post B[a]P exposure

No significant differences in weight gain were noted for Hras128 rats fed high- or low-fat diets, although the weight gain tended to be slightly higher in the high-fat diet group (Fig. 2). Similarly, no significant differences in weight gain were seen for wild-type rats fed high- and low-fat diets. However, the body weights of Hras128 rats in the high-fat diet group were significantly higher than those of wild-type rats fed the high-fat diet when the animals were between 9 and 20 weeks old. Similarly, the Hras128 rats in the low-fat diet group exhibited significantly greater body weights than the wild-type rats in the low-fat diet group at 8 and 11 weeks of age.

Blood parameters of Hras128 and wild-type rats fed different diets post B[a]P exposure

The results of the blood biochemical testing are presented in Table 1. Alanine transaminase levels (IU/L) and nonesterified fatty acid levels (mEq/L) were significantly lower in the Hras128 high-fat diet group than in the wild-type high-fat diet group ($p=0.02$ and $p=0.04$, respectively). No significant differences in any other blood biochemical parameters were observed between the groups.

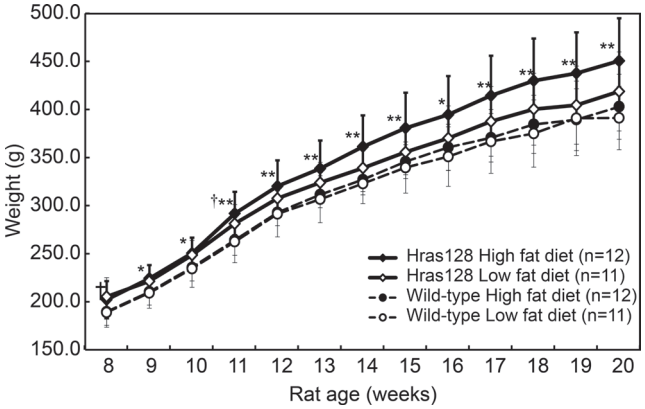

Fig. 2. Comparison of the body weights of Hras128 and wild-type rats fed different diets. Values are presented as means ± standard deviations. The body weights of the Hras128 rats in the high-fat diet group were significantly greater (*$p<0.05$; **$p<0.01$) than those of the wild-type rats fed the high-fat diet at most time points. The body weights of the Hras128 rats in the low-fat diet group were significantly greater (†$p<0.05$) than those of the wild-type rats fed the low fat-diet at two time points, as indicated.

Incidence of mammary tumors in Hras128 and wild-type rats fed different diets post B[a]P exposure

In total, 11 of the 12 Hras128 rats (91.7%) in the high-fat group exhibited mammary tumors, while only 5 of the 12 Hras128 rats (41.7%) in the low-fat diet group exhibited tumor formation (Table 2). This difference was statistically significant ($p<0.03$). In addition, the number of individual mammary tumors per animal was significantly higher in the high-fat diet group than in the low-fat diet group (1.7 vs. 0.7, respectively; $p<0.05$). Conversely, no mammary tumors were found in the wild-type rats fed either the high- or low-fat diet (Table 2).

Mammary cancer pathology vs. diet – macroscopic observations

Examples of macroscopic observations at autopsy are shown in Fig. 3. Collectively, we detected 20 tumors (maximum ~3.5 cm in diameter) in the mammary glands of Hras128 rats fed the high-fat diet and 8 tumors (~2.0 cm in diameter) in the mammary glands of Hras128 rats fed low-fat diets (Table 2). No macroscopic lesions were apparent in the mammary glands of wild-type rats.

Mammary cancer pathology vs. diet – microscopic observations

We subsequently analyzed mammary tissues harvested from the four treatment groups using histologic and immunohistochemical methods. Figures 4–6 contain representative micrographs indicative of our findings. Invasive ductal carcinomas were visible in tumors of the high- and low-fat diet Hras128 rats (Fig. 4, left). In contrast, no microscopic

Table 1. Biochemical Blood Parameters of Hras128 and Wild-type Rats Fed High- and Low-fat Diets

	High-fat diet		Low-fat diet	
	Hras128	Wild-type	Hras128	Wild-type
TP[a] (g/dL)	7.0 ± 0.4	7.4 ± 0.5	7.0 ± 0.5	7.3 ± 0.6
Alb (g/dL)	5.3 ± 0.3	5.5 ± 0.2	5.2 ± 0.3	5.3 ± 0.5
AST (IU/L)	68.8 ± 14.6	81.8 ± 18.1	83.2 ± 23.2	97.4 ± 86.6
ALT (IU/L)	23.0 ± 2.4*	29.2 ± 4.7	28.0 ± 7.2	27.6 ± 12.7
TL (mg/dL)	545.8 ± 173.0	596.5 ± 121.3	649.4 ± 465.0	1167.0 ± 742.5
TCHO (mg/dL)	76.0 ± 10.3	87.0 ± 12.6	89.8 ± 29.2	106.8 ± 20.7
HDL (mg/dL)	23.7 ± 2.9	25.2 ± 2.3	23.6 ± 5.5	27.8 ± 4.7
LDL (mg/dL)	7.8 ± 1.2	10.0 ± 2.8	9.4 ± 4.0	11.8 ± 8.1
TG (mg/dL)	224.2 ± 159.7	232.5 ± 82.5	287.8 ± 390.9	712.4 ± 697.7
PL (mg/dL)	197.7 ± 23.8	224.5 ± 27.6	217.4 ± 66.7	288.6 ± 68.8
NEFA (μEq/L)	1139.8 ± 349.5*	1778.0 ± 554.1	1710.4 ± 1019.9	1402.8 ± 667.2
BUN (mg/dL)	22.9 ± 5.6	21.7 ± 3.8	18.4 ± 5.1	24.4 ± 8.3
Cr (mg/dL)	0.41 ± 0.04	0.42 ± 0.03	0.40 ± 0.05	0.40 ± 0.04

[a]Abbreviations: TP, total protein; Alb, albumin; AST, aspartate transaminase; ALT, alanine transaminase; TL, total lipids; TCHO, cholesterol; HDL, high-density lipoprotein; LDL, low-density lipoprotein; TG, triglycerides; PL, phospholipids; NEFA, nonesterified fatty acids; BUN, blood urea nitrogen; Cr, creatinine. *p<0.05, Hras128 vs. wild-type rats on high-fat diets. No significant differences between Hras128 rats fed the high-fat diets vs. Hras128 rats fed the low-fat diet.

Table 2. Incidences of Mammary Cancer among Hras128 and Wild-type Rats Fed High- and Low-fat Diets

		Hras128	Wild-type
High-fat diet	(Total)	11 (n = 12)	0 (n = 12)
	(Incidence rate)	91.7%*	0%
	(Tumors/rat)	1.7*	0
Low-fat diet	(Total)	5 (n = 12)	0 (n = 11)
	(Incidence rate)	41.7%	0
	(Tumors/rat)	0.7	0

*p<0.05 for the cancer incidences among Hras128 rats fed high- vs. low-fat diets. The total number of tumors was 20 for the high-fat diet Hras128 group and 8 for the low-fat diet Hras128 group.

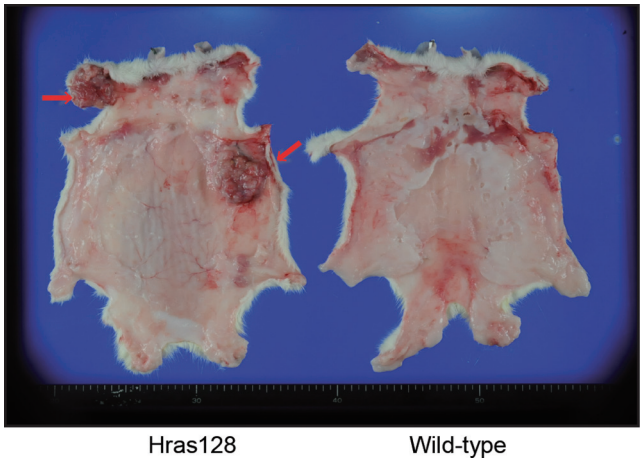

Fig. 3. Macroscopic observations at autopsy of Hras128 and wild-type rats. Tumors observed in the mammary tissue of an Hras128 rat (left) are indicated by arrows. No tumors were evident in wild-type rats (right). The scale is indicated at the bottom of the figure (cm).

lesions were apparent in the mammary glands of any of the wild-type rats (Fig. 4, right). Mammary tumors that developed in the Hras128 high- and low-fat diet animals exhibited elevated expression of estrogen and progesterone receptors, as visualized using specific anti-estrogen receptor and anti-progesterone receptor antibodies (Fig. 5). These tissues also contained large numbers of Ki-67-positive cells (Fig. 6).

Discussion

Treatment with 7,12-dimethylhenz[a]anthracene (DMBA) is a well-characterized method for inducing mammary carcinogenesis in rats, and it has been referred to as the Huggins breast cancer model[22, 23]. In this study, however, we selected B[a]P, a compound found in tobacco smoke, as a carcinogen, as administration of this compound was previously found to induce 100% carcinogenesis in Hras128 rats[20].

Estrogen and progesterone receptors are known mammary cancer hormone receptors. Here, we detected expression of both receptors in >95% of the Hras128 tested (Fig. 5), and there were no significant differences in the expression of these proteins among the rats fed the high-fat and low-fat diets. These data indicate that the mammary cancer induced in this study is hormone dependent and therefore equivalent to that induced in the Huggins model. Furthermore, there were no significant differences in the Ki-67 indices (Fig. 6) of the Hras128 rats given the high-fat (22.8 ± 7.5%; n = 5) and low-fat (20.6 ± 8.2%; n = 5) diets (data not shown). This combination of a high Ki-67 index and positive estrogen receptor expression indicates that the cancer induced in this study was also equivalent to human luminal B breast cancer[24].

There was a predictable difference in breast cancer occurrence between the Hras128 and wild-type Sprague-

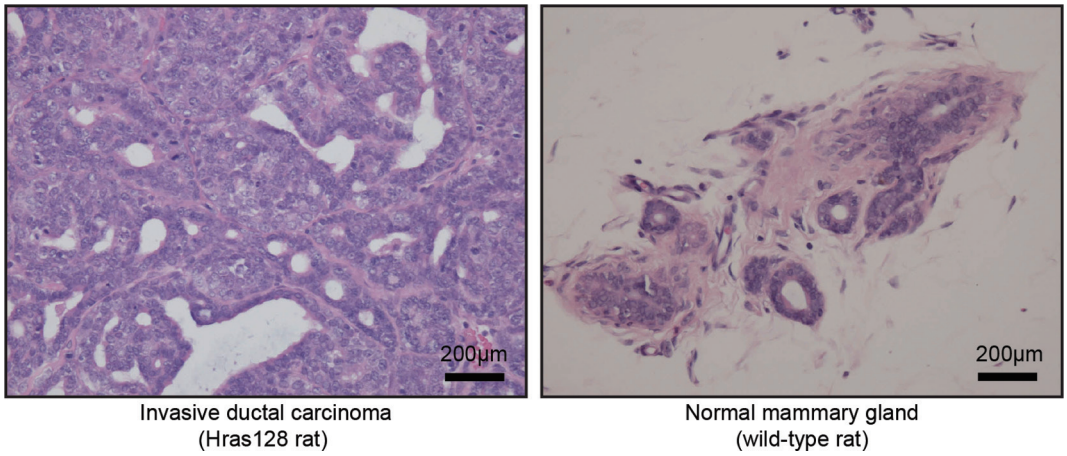

Fig. 4. Light micrographs of mammary tissues harvested from Hras128 and wild-type rats. (Left) Image of an invasive ductal carcinoma harvested from an Hras128 rat mammary tumor. (Right) Image of normal mammary tissue harvested from a wild-type rat. Hematoxylin and eosin (200× magnification).

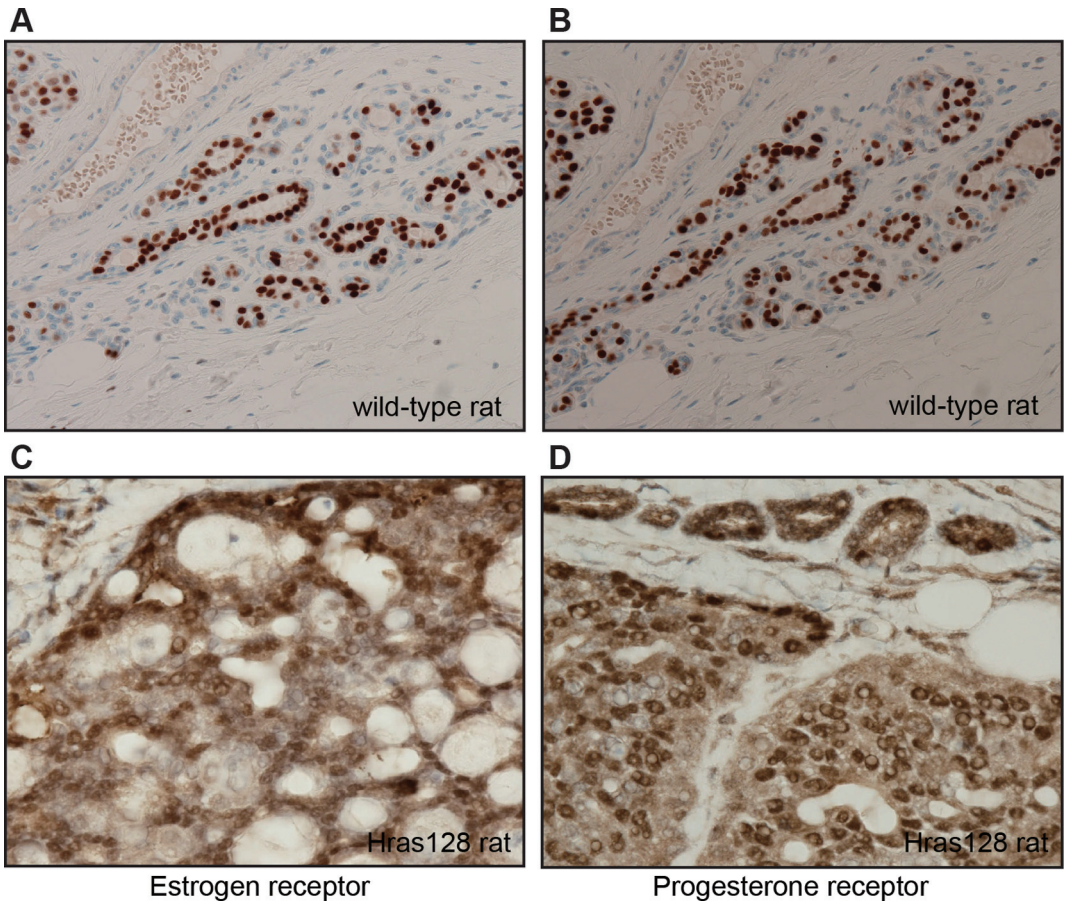

Fig. 5. Immunohistochemical staining of estrogen and progesterone receptors in mammary tissues harvested from Hras128 and wild-type rats. Expressions of estrogen (panel A) and progesterone (panel B) receptor are shown in mammary glands of wild-type rats fed a high-fat diet. High expressions of estrogen (panel C) and progesterone (panel D) receptors are shown in mammary tumors of Hras128 rats fed a high-fat diet. Immunohistochemical staining for estrogen and progesterone receptors with DAB stain (200× magnification).

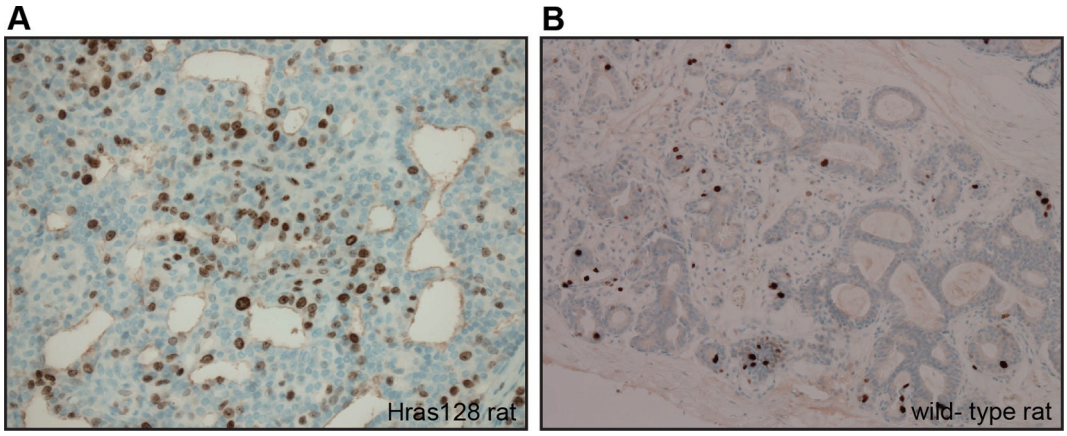

Fig. 6. Immunohistochemical staining of mammary tissues harvested from Hras128 and wild-type rats for Ki67-positive cells. (Panel A) Mammary tumors that developed in Hras128 rats fed a high-fat diet contained high numbers of Ki-67-positive cells. (Panel B) Stained wild-type rat fed a high–fat diet.

Dawley rats included in this study. While spontaneous development of breast cancer in wild-type Sprague-Dawley rats typically occurs after a year or more, breast cancer can develop in as early as 15 days after carcinogen injection in Hras128 rats[25].

Previous studies have reported that body weight and alcohol consumption are risk factors for developing mammary cancer[13, 14, 26, 27]. Each of these studies noted, however, that a clear association between diet or specific dietary components and this cancer has yet to be established. Meanwhile, physical activity has been identified as another factor that may inhibit the development of mammary cancer[13, 17, 27]. Notably, a recent prospective study involving a large heterogeneous population of European women reported that a high-fat diet increased the risk of developing mammary cancer[12]. Moreover, a Westernized diet has been identified as a factor that might be associated with the observed increase in the prevalence of a variety of cancers[17], including mammary cancer[9]. Nevertheless, it is difficult to establish a definitive link between specific dietary components and mammary cancer from this study given the small sample size.

Animal models are widely used in risk assessment of potential carcinogens. Advantages of animal models include controlled experimental conditions and the opportunity for carrying out mechanistic studies. Therefore, animal models of mammary cancer could potentially increase our understanding of the etiology of mammary cancer, the most common cancer in women worldwide, and facilitate the development of prevention strategies. Mammary cancer developed in the Hras128 rat model is histologically similar to human mammary cancer. Using this model, we found that, following administration of B[a]P, rats fed a high-fat diet developed significantly more mammary tumors than those fed a low-fat diet. Importantly, this increased incidence of mammary cancer was not caused by obesity, as there were no significant differences in the body weights of animals in the two groups. Likewise, there were no significant differences in blood parameters among the Hras128 rats in the high-fat and low-fat diet groups, indicating that the same degree of health was maintained in each animal.

The tumors observed in the B[a]P-fed rats were estrogen receptor and progesterone receptor positive and contained high numbers of Ki-67-positive cells. Notably, these findings are consistent with those of a recent study, which reported that a high intake of saturated fats was associated with an increased risk for developing estrogen receptor- and progesterone receptor-positive breast cancer in humans[12]. Meanwhile, compared with normal mammary glands harvested from untreated Sprague-Dawley rats[28], the wild-type rats evaluated in this study exhibited higher rates of estrogen receptor expression, which was likely due to the administration of B[a]P.

The fact that Hras128 rats can develop mammary tumors over the course of several weeks renders this animal model very useful for investigating the mechanisms by which a high-fat diet promotes mammary cancer development. For example, it was reported that a high-fat diet increases the expression of leptin and tumor necrosis factor (TNF)-α within the mammary adipose tissue of Zucker rats heterozygous for a mutation in the leptin receptor gene (+/*fa*) and that this effect might play a role in the development of mammary cancer[29].

Watanabe reported that the observed increase in the occurrence of breast cancer among Japanese women was due to the change in dietary habits from traditional Japanese to Western style. Notably, components of the traditional Japanese diet, such as miso (fermented soy bean paste) soup, may prevent breast cancer[30]. In fact, long-term administration of miso was suggested to prevent mammary tumor development in Sprague-Dawley rats[31, 32]. Accordingly, based on the data reported in our study, future animal experiments should be conducted to continue to explore the putative link between high-fat diets and breast cancer, as well as dietary interventions for the prevention and treatment of breast cancer.

In conclusion, the present study provides evidence of

a positive association between the consumption of a high-fat diet and the rate of mammary cancer development in Hras128 rats. These findings comprise an important step towards understanding the dietary risk factors underlying the development of this cancer and the development of informed preventative breast cancer strategies.

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Disclosure of Potential Conflicts of Interest: The authors declare that there are no conflicts of interest.

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