Table 2 Combined effects of the *RETN* -420 C>G polymorphism and BMI on the risk of T2DM

RETN -420 C>G genotype	Body Mass Index (BMI)	
	BMI ≥ 25	BMI < 25
RETN -420 *C/C* + *C/G*	OR* = 1 (Referent)	OR* = 0.40 (0.28-0.56)
(DM/non-DM)[#]	(69/544)	(81/1,605)
RETN -420 *G/G*	OR* = 0.72 (0.36-1.46)	OR*[†] = 0.03 (0.005-0.25)
(DM/non-DM)[#]	(10/111)	(1/230)

DM, diabetes mellitus; OR, odds ratio.
*All ORs are adjusted for age and smoking status. Numbers in the parentheses indicates the 95% confidence intervals (95%CIs).
[#]DM/non-DM indicates the number of subjects with DM and that of non-DM subjects, respectively (the numbers of the subjects are described in the parentheses).
[†]OR for interaction = 0.12, *P*-value for interaction = 0.046.

promoter activity [6]; considering this, and given that the function of RETN is to increase the risk of DM by antagonizing insulin as described by some researchers [6], the risk of DM would be higher in those with *RETN* -420 *G* allele, which speculation was contrary to our observation. This discrepancy observed may stem from the fact that the function of RETN itself is still controversial, or simply from the random error caused by the relatively small sample size of the present study. Further investigation will be required to verify this association. The rs (reference SNP)-number for *RETN* -420 C>G is rs1862513, and the *C*-allele is major allele here, because it is complementary to the sequence that the genotype frequency of rs1862513 in HapMap is based on (ss14703397) in the dbSNP Build 37.3 database [21]. We confirmed that our interpretation is correct by comparing our primer-probe sequences with that of dbSNP 37.3 database.

It has been reported that the association of *RETN* -420 C>G polymorphism and T2DM may be stronger in younger-onset T2DM [22], but lack of information for the onset age of DM makes it impossible to investigate this association, which might be one potential limitation of the present study. As gender reportedly affects the association between plasma RETN level and insulin resistance, we conducted the stratified analysis by gender, which suggested the possible trend for the differential effect of *RETN* polymorphism on the risk of DM by gender, although the interaction did not reach statistical significance. Further verification on this interaction will also be needed.

Consideration of technical aspects is as follows: the statistical power for the entire study subjects of 161 DM and 2,490 non-DM was more than 90% for an OR of 2 or 0.5 with a two-sided α error of 0.05, when a genotype frequency among controls was between 30% and 70%. It was more than 50% for subjects with BMI ≥ 25 (n=79 [DM] and n=655 [non-DM]), and more than 65% for subjects with BMI < 25 (n=82 [DM] and n=1,835 [non-DM]), under the same conditions. The statistical power for this sample may be somewhat weak especially for the subgroup analyses (*e.g.*, subgroup of BMI<25) because of the low frequency of the variant allele in the Japanese population, which may require careful interpretation of the results as well as necessitate further investigation of this association in the near future. In this study, the genotype frequencies among the non-DM subjects were significantly different from the Hardy-Weinberg's equilibrium, but the absolute differences between the actual and expected frequencies were minimal (within ± 2.5%), and this departure from the Hardy-Weinberg's equilibrium may be explained by the type-I error randomly caused by the relatively large sample size. Another reason for the significant difference from Hardy-Weinberg's equilibrium in non-diabetic subjects might be speculated as a result of population stratification caused by the variation in genotype frequencies between the areas; we conducted the analyses adjusted for institutions to exclude this effect, which were not substantially different from the unadjusted results, suggesting that the variation in genotype frequencies between the areas did not affect the main results. In any case, as the absolute differences between actual and expected genotype frequencies in non-cases are minimal, the potential bias caused by this deviation from Hardy-Weinberg's equilibrium would also be substantially minimal. Adjustments for multiple comparisons may be another issue. There are also number of criticisms suggesting that correction of multiple comparisons by Bonferroni procedures is some-

times too conservative and aggravate the researchers' tendency not to conduct or present more tests [23, 24], and considering that most of the analyses in the present study were conducted under the exploratory context-mainly for the purpose of determining the best statistically fit model, we decided not to adopt these adjustments in this study.

In summary, we found significant statistical interaction between *RETN* -420 *G/G* genotype and lower BMI (BMI < 25) on the decreased risk of T2DM in Japanese, suggesting the further need for investigating the actual function of *RETN* -420 *G/G* genotype in the genesis of human metabolic disorders including DM. We should also be careful in interpretation of the present study results because of the limited sample sizes. This association observed might potentially help provide novel evidence for the individualized prevention of T2DM in humans in the future, and further investigation of this association in other ethnicities/groups or with much larger populations, as well as of the actual biological roles of RETN are also required to confirm our findings.

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Conflict of Interest

The authors declared no conflict of interests.

References

1. Alberti KG, Zimmet PZ (1998) Definition, diagnosis and classification of diabetes mellitus and its complications. Part 1: diagnosis and classification of diabetes mellitus provisional report of a WHO consultation. *Diabet Med* 15: 539-553.
2. Steppan CM, Bailey ST, Bhat S, Brown EJ, Banerjee RR, *et al.* (2001) The hormone resistin links obesity to diabetes. *Nature* 409: 307-312.
3. Lehrke M, Reilly MP, Millington SC, Iqbal N, Rader DJ, *et al.* (2004) An inflammatory cascade leading to hyperresistinemia in humans. *PLoS Med* 1: e45.
4. Cho YM, Ritchie MD, Moore JH, Park JY, Lee KU, *et al.* (2004) Multifactor-dimensionality reduction shows a two-locus interaction associated with Type 2 diabetes mellitus. *Diabetologia* 47: 549-554.
5. Conneely KN, Silander K, Scott LJ, Mohlke KL, Lazaridis KN, *et al.* (2004) Variation in the resistin gene is associated with obesity and insulin-related phenotypes in Finnish subjects. *Diabetologia* 47: 1782-1788.
6. Osawa H, Yamada K, Onuma H, Murakami A, Ochi M, *et al.* (2004) The G/G genotype of a resistin single-nucleotide polymorphism at -420 increases type 2 diabetes mellitus susceptibility by inducing promoter activity through specific binding of Sp1/3. *Am J Hum Genet* 75: 678-686.
7. Tsukahara T, Nakashima E, Watarai A, Hamada Y, Naruse K, *et al.* (2009) Polymorphism in resistin promoter region at -420 determines the serum resistin levels and may be a risk marker of stroke in Japanese type 2 diabetic patients. *Diabetes Res Clin Pract* 84: 179-186.
8. Hamajima N; J-MICC Study Group (2007) The Japan Multi-Institutional Collaborative Cohort Study (J-MICC Study) to detect gene-environment interactions for cancer. *Asian Pac J Cancer Prev* 8:317-323.
9. Wakai K, Hamajima N, Okada R, Naito M, Morita E, *et al.*; J-MICC Study Group (2010) Profile of Participants and Genotype Distributions of 108 Polymorphisms in a Cross-sectional Study to Elucidate Associations between Genotypes and Lifestyle and Clinical Factors: A Project in the Japan Multi-institutional Collaborative Cohort (J-MICC) Study. *J Epidemiol* 21:223-235.
10. Tokudome Y, Goto C, Imaeda N, Hasegawa T, Kato R, *et al.* (2005) Relative validity of a short food frequency questionnaire for assessing nutrient intake versus three-day weighed diet records in middle-aged Japanese. *J Epidemiol* 15:135-145.
11. Imaeda N, Goto C, Tokudome Y, Hirose K, Tajima K, *et al.* (2007) Reproducibility of a short food frequency questionnaire for Japanese general population.

J Epidemiol 17:100-107.
12. Greenland S, Pearl J, Robins JM (1999) Causal diagrams for epidemiologic research. *Epidemiology* 10: 37-48.
13. Lee JH, Chan JL, Yiannakouris N, Kontogianni M, Estrada E, *et al.* (2003) Circulating resistin levels are not associated with obesity or insulin resistance in humans and are not regulated by fasting or leptin administration: cross-sectional and interventional studies in normal, insulin-resistant, and diabetic subjects. *J Clin Endocrinol Metab* 88: 4848-4856.
14. Lazar MA (2007) Resistin- and Obesity-associated Metabolic Diseases. *Horm Metab Res* 39: 710-6.
15. Degawa-Yamaguchi M, Bovenkerk JE, Juliar BE, Watson W, Kerr K, *et al.* (2003) Serum Resistin (FIZZ3) Protein Is Increased in Obese Humans. *J Clin Endocrinol Metab* 88: 5452-5455.
16. Wen Y, Lu P, Dai L. Association between resistin gene -420 C/G polymorphism and the risk of type 2 diabetes mellitus: a meta-analysis. *Acta Diabetol.* 2010 Dec 29. [Epub ahead of print]
17. Hivert MF, Manning AK, McAteer JB, Dupuis J, Fox CS, *et al.* (2009) Association of variants in RETN with plasma resistin levels and diabetes-related traits in the Framingham Offspring Study. *Diabetes* 58: 750-756.
18. Onuma H, Tabara Y, Kawamura R, Tanaka T, Ohashi J, *et al.* (2010) A at single nucleotide polymorphism-358 is required for G at -420 to confer the highest plasma resistin in the general Japanese population. *PLoS One* 5: e9718.
19. Baik I, Shin C (2012) Interactions between the FTO rs9939609 polymorphism, body mass index, and lifestyle-related factors on metabolic syndrome risk. *Nutr Res Pract* 6: 78-85.
20. Park HK, Qatanani M, Briggs ER, Ahima RS, Lazar MA (2011) Inflammatory induction of human resistin causes insulin resistance in endotoxemic mice. *Diabetes* 60: 775-783.
21. dbSNP: http://www.ncbi.nlm.nih.gov/snp.
22. Ochi M, Osawa H, Hirota Y, Hara K, Tabara Y, *et al.* (2007) Frequency of the G/G genotype of resistin single nucleotide polymorphism at -420 appears to be increased in younger-onset type 2 diabetes. *Diabetes* 56: 2834-2838.
23. Kawamoto R, Tabara Y, Kohara K, Kusunoki T, Abe M, *et al.* (2012) Plasma resistin levels are associated with insulin resistance in older Japanese men from a rural village. *Metab Syndr Relat Disord* 10: 380-386.
24. Savitz DA, Olshan AF (1995) Multiple comparisons and related issues in the interpretation of epidemiologic data. *Am J Epidemiol* 142:904-908.
25. Nakagawa S (2004) A farewell to Bonferroni: the problem of low statistical power and publication bias. *Behav Ecol* 15: 1044-1045.

High susceptibility of heterozygous (+/*fa*) lean Zucker rats to 7,12-dimethylbenz(*a*)anthracene-induced mammary carcinogenesis

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Abstract. Susceptibility to 7,12-dimethylbenz(*a*)anthracene (DMBA)-induced mammary carcinogenesis was investigated in lean Zucker *(+/fa)* rats carrying one mutated leptin receptor gene and wild-type controls (+/+). Rats with both genotypes were given a single DMBA administration and divided into two groups, one group was fed on basal diet mixed with 10% corn oil and the other was fed on basal diet alone. The minimum latency period of palpable carcinomas in +/*fa* rats of both groups was 8 weeks following DMBA treatment, in contrast to the 11-12 weeks in +/+. The incidence and multiplicity of carcinomas increased or showed a tendency for increase in the early stages in +/*fa* rats of both groups as compared to the +/+ counterparts. The volumes of carcinomas showed a tendency to increase in the corn oil diet groups of both genotypes. The major histopathological phenotype of carcinomas in all groups was well-differentiated without distinct atypia (multiplicity, 0.69-1.09/rat), but moderately/poorly differentiated carcinomas with atypia were also found, predominantly in +/*fa* rats (0.09-0.21). These latter tumors were characterized by elevated ERK activity but not estrogen receptor expression. Serum leptin concentrations in +/*fa* rats at 7 weeks of age were higher than those in +/+ and were elevated by the corn oil diet; however, no obvious change was detected in other serum parameters examined. In conclusion, +/*fa* rats proved more susceptible to DMBA-induced mammary carcinogenesis than +/+ controls, and hyperleptinemia was suggested to contribute to tumor growth as well as to susceptibility to tumorigenesis and more aggressive phenotypes in Zucker lean rats.

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Key words: mammary, carcinoma, leptin, 7,12-dimethylbenz(*a*)-anthracene, Zucker rat

Introduction

A relationship between obesity and breast cancer risk has been proposed based on epidemiological data, a positive association with increasing body mass index being found particularly in postmenopausal women (1-4). Although the underlying mechanisms have yet to be fully clarified, increased concentrations of circulating sex hormones are likely to contribute at least in part (5). In addition, circulating levels of an adipokine leptin, which is secreted mainly from adipose tissue and limits food intake and increases energy expenditure (6), was recently suggested to have a role independent of obesity indices in breast tumorigenesis (7). In estrogen receptor (ER)-positive breast cancer cells, leptin has been demonstrated to stimulate aromatase expression and cell proliferation, and both in ER-positive and -negative breast cancer cells, leptin induced transactivation of ErbB tyrosine kinase receptors, such as the epidermal growth factor receptor (EGFR) and ErbB-2 (HER2/Neu), resulting in the induction of cell proliferation and increased survival (8-10).

To investigate the effects of obesity on mammary carcinogenesis, a number of animal models, featuring inherited obesity or feeding of a high fat/calorie diet, were employed. Fatty Zucker (*fa/fa*) rats, which have autosomal recessive mutation in the leptin receptor gene (11), develop hyperinsulinemia, but blood glucose remains at normal levels (12). In addition, they demonstrate significantly increased serum triglyceride, total cholesterol and leptin levels (12,13). Lean Zucker (+/*fa* and +/+) rats, by contrast, exhibit normal appearing metabolic functions and have been utilized as controls in chemically-induced mammary carcinogenesis investigations (14-17). In a previous study, the latency period and/or the incidence of mammary carcinomas were reported to be shorter and greater, respectively, in female *fa/fa* than +/*fa* and +/+ rats treated with 7,12-dimethylbenz(*a*)anthracene (DMBA) (15,17). However, in another study, female Zucker (*fa/fa*) rats treated with *N*-methyl-*N*-nitrosourea (MNU) showed a lower incidence of mammary carcinomas compared to lean Zucker controls (+/*fa* and +/+) (14). A number of factors may contribute to the discrepancy between the DMBA- and MNU-treated rats, and it remains unclear which obesity-associated internal parameters, such as hyperin-

sulinemia, hyperleptinemia or hyperlipidemia, fundamentally affect mammary carcinogenesis.

We recently compared serum biochemical parameters between lean Zucker (+/*fa*) and (+/+) rats in combination with or without an obesity-inducing 10% corn oil diet, to clarify whether lean Zucker (+/*fa*) rats might also be more sensitive to the high fat diet than the +/+ controls (18). Serum leptin concentrations were higher in the (+/*fa*) case at 7 weeks of age (~140 pg/ml as compared to ~80 pg/ml in +/+; $P<0.01$), although the difference was significantly smaller at 12 weeks of age, and serum concentrations of other parameters including insulin, triglycerides and total cholesterol were similar between the two genotypes. In addition, both +/*fa* and +/+ rats fed basal diet mixed with 10% corn oil showed higher serum leptin levels than those fed basal diet alone, but no other parameters examined were altered by the obesity-inducing diet.

In the present study, to clarify the effects of hereditary and dietary hyperleptinemia on mammary carcinogenesis, lean Zucker (+/*fa*) rats with and without 10% corn oil feeding were utilized in a DMBA-induced mammary carcinogenesis model along with control lean Zucker (+/+) rats. In the present study, latency period and growth rates of mammary carcinomas were assessed by regular palpation, and at the termination, histopathological, immunohistochemical and western blot analyses were performed to determine expression profiles of estrogen- and intracellular signaling cascade-related proteins in the mammary carcinomas, as well as serum biochemistry for obesity-associated parameters. The data demonstrated +/*fa* rats to indeed be more susceptible to DMBA-induced mammary carcinogenesis than +/+ controls, with hyperleptinemia appearing to be partly associated with tumor growth as well as with susceptibility to tumorigenesis and a more aggressive phenotype in an estrogen-independent manner.

Materials and methods

Chemicals and animals. DMBA was purchased from Sigma Chemical (St. Louis, MO, USA) and dissolved in sesame oil at 10 mg/ml prior to administration. A total of 100 female Zucker rats (lean phenotype) at 5 weeks of age were purchased from Charles River Japan (Kanagawa, Japan) and acclimated for 1 week prior to genotyping by the method of Phillips *et al* (19). Throughout the acclimatization and experimental periods, the animals were housed at a maximum of 3 or 4 per plastic cage with white wood chips (Sankyo Laboratory Service, Tokyo, Japan) for bedding and transferred to clean cages with fresh bedding twice a week in a standard air-conditioned animal room (24±1°C, 55±5% relative humidity, 12 h light and dark cycle). All animals had free access to basal diet (CRF-1; Oriental Yeast Co., Tokyo, Japan) and tap water until the start of the experiment.

Experimental protocol. Sixty-six +/*fa* and 32 +/+ rats at 7 weeks of age received an intragastric administration of DMBA (50 mg/kg body weight) by gavage, and the animals of each genotype were then divided into basal diet (CRF-1; 357 kcal/100 g) and 10% corn oil diet (CRF-1-based, Oriental Yeast; 414 kcal/100 g) groups. The present dose level of DMBA at 50 mg/kg body weight was selected based on our previous experiments, in which palpable mammary tumors were induced at adequate incidences for detection of endogenous and exogenous tumor promoting and/or inhibitory factors in Sprague-Dawley (20) and F344 rats (21). The dietary concentration of corn oil at 10% was selected based on the previously reported effective concentrations of linoleic acid for promotion of rat mammary tumor development (22). General conditions and mortality were checked daily and body weight was measured once a week during the experimental period. The amounts of supplied and residual diet were weighted weekly in order to calculate the average daily food intake per week. Following DMBA administration, a veterinary scientist (T.I.) palpated cervix, thorax and abdomen of awake rats to detect mammary tumors once weekly. The length, width and height of each tumor were measured using a caliper and tumor volumes were calculated as follows: Volume = (length) x (width) x (height) x $\pi/6$.

For endpoints for this study, the rats were sacrificed when demonstrating over 20% decrease in body weight excluding total tumor weight and/or when symptoms of poor physical condition, such as decrease in locomotor activity, were found. Volume of mammary tumors was not considered important in this regard, since change in tumor volume was a key item for evaluation of the effects of rat genotype and corn oil diet. All remaining rats were sacrificed at 32 weeks following DMBA administration. The present study design was approved by the Animal Care and Utilization Committee of the National Institute of Health Sciences.

Necropsy and histopathology. At the end of the experimental period, blood samples were collected from the abdominal aorta of all surviving animals under ether anesthesia. Serum was separated and maintained at -80°C until use. Following euthanasia by exsanguination under ether anesthesia, animals were subjected to necropsy. Whole skins with mammary glands and tumors were removed, and the sizes of all mammary tumors were recorded. Tumor volumes were calculated in the same manner as for palpable tumors. Sections of frozen tissue of randomly selected mammary tumors of rats in all groups were prepared with liquid nitrogen and stored at -80°C until use. The remaining tumor and mammary tissues were fixed in 10% neutral buffered formalin, processed routinely to paraffin-embedded sections at 4-5 μm, and stained with hematoxylin and eosin (H&E) for histopathological analysis. Animals that died or that were sacrificed on becoming moribund were similarly necropsied and included for the sequential palpable tumor and postmortem analyses.

Immunohistochemistry. Primary antisera for the leptin receptor (goat polyclonal; Neuromics Antibodies, Edina, MN, USA; 1:1,000 dilution), smooth muscle actin (mouse clone 1A4; Dako, Glostrup, Denmark; 1:200), leptin (rabbit polyclonal; Santa Cruz Biotechnology, Santa Cruz, CA, USA; 1:200), ER α (mouse clone 6F11; Novocastra, Newcastle, UK; 1:50 or 1:500), ER β (rabbit polyclonal; Affinity BioReagents, Rockford, IL, USA; 1:100) and aromatase (rabbit polyclonal; Abcam, Cambridge, MA, USA; 1:500), were utilized for immunohistochemistry. Analyzed mammary tumors were selected from all groups, on the basis of the genotype, diet and phenotypes. Paraffin sections 5, 5, 10 and 10 carcinomas from the +/+-basal diet, +/+-corn oil diet, +/*fa*-basal diet and

+/*fa*-corn oil diet groups, respectively, were used for leptin receptor, leptin, smooth muscle actin and ER α, and frozen sections of 3, 4, 4 and 5 each were for ER β and aromatase. Antigen retrieval for paraffin sections was carried out in an autoclave for 10 min at 121°C in 10 mM citrate buffer (pH 6.0) for leptin receptor, smooth muscle actin and ER α. The streptavidin-biotin-peroxidase complex method (StreptABComplex/HRP; Dako) was used to determine the expression and localization of each antigen, and sections were lightly counterstained with hematoxylin for microscopic examination. Negative controls without primary antibody reactions were set for each antigen using serial sections. The positivities for ER α in over 1,000 mammary adenocarcinoma cells were assessed on each paraffin section to give percentage values.

Western blot analysis. Twelve mammary tumors and four normal mammary tissue samples of the +/+-basal diet, +/+-corn oil diet and +/*fa*-basal diet groups were homogenized in extraction buffer (50 mM Tris-HCl pH 7.4, 3 mM EDTA, 100 mM NaCl, 1% Tween-20, 10 mM sodium orthovanadate, 1 mM PMSF, 10 μg/ml leupeptin, 20 μg/ml aprotinin) and centrifuged at 14,000 g for 20 min. Equal amounts of protein samples (50 μg) from collected supernatants were subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) on 5-20% gradient acrylamide gels (ATTO, Tokyo, Japan), and the separated proteins were transferred to polyvinylidene difluoride membranes (Whatman, Sanford, ME, USA). Immunoblotting was performed using rabbit polyclonal antibodies against ER β (Affinity BioReagents), aromatase (Abcam), signal transducer and activator of transcription (STAT)3 and phospho-STAT3 (Thy705) (Cell Signaling Technology, Danvers, MA, USA), extracellular signal-regulated kinase (ERK)1/2 and phospho-ERK1/2 (R&D Systems, Minneapolis, MN, USA) or monoclonal antibodies against β-actin (mouse clone AC-15; Sigma), followed by exposure to peroxidase-labeled anti-rabbit or mouse polyclonal goat antibodies (Dako) and development of signals with TMB 3,3',5,5' tetramethylbenzidine (ATTO). Semi-quantitative analyses were performed using Scion Image (alpha4.0.3.0; Scion, Frederick, MD, USA).

Serum biochemistry. Concentrations of serum leptin, adiponectin, insulin and insulin-like growth factor (IGF)-I were determined for randomly selected almost half and one third of samples from +/+- and +/*fa*-groups, respectively, using rat/mouse enzyme immunoassay kits from Yanaihara Institute (Shizuoka, Japan), Adipogen (Incheon, Korea), Mercodia (Uppsala, Sweden) and R&D Systems, respectively. Other serum biochemical parameters including triglyceride, total cholesterol and glucose were measured for all samples except for those lost due to sampling error at SRL (Tokyo, Japan).

Statistical analysis. The survival rates and incidence of palpable or histopathologically defined mammary tumors were analyzed for inter-group differences by the Fisher's exact probability test. Data for body weights and multiplicity, volume and latency of mammary tumors, ER α-positivity in mammary adenocarcinoma sections, serum biochemistry and western blot analysis data were examined with the Student's or the Welch's t-test following the F-test. Significance was inferred at the 5, 1 and 0.1% levels.

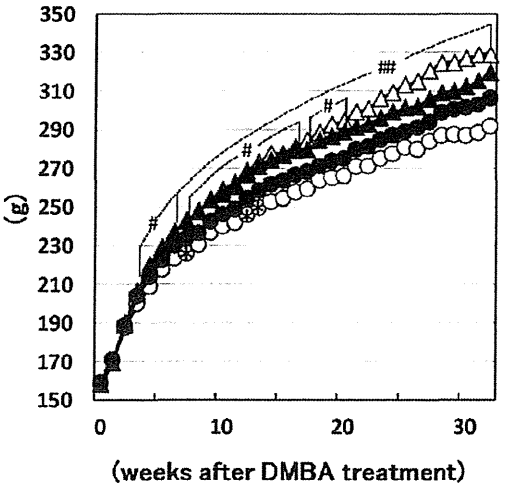

Figure 1. Body weight curves. Open circles, +/+-basal diet; open triangles, +/+-10% corn oil diet; closed circles, +/*fa*-basal diet; closed triangles, +/*fa*-10% corn oil diet. *P<0.05 vs. +/+-basal diet (difference with the genotype basis); #P<0.05, ##P<0.01 vs. +/+-basal diet or +/*fa*-basal diet (difference with the diet basis).

Results

Survival rates, body weights and food intake. At the end of the experiment, survival rates were 94% (15/16), 81% (13/16), 85% (28/33) and 85% (28/33) in the +/+-basal diet, +/+-corn oil diet, +/*fa*-basal diet and +/*fa*-corn oil diet groups, respectively, with no significant variation among the groups. Body weight curves of each group are shown in Fig. 1. Values of the +/+-corn oil diet group were higher than those of the +/+-basal diet group from week 4 to the end of the experiment. In addition, the body weights of the +/*fa*-corn oil diet group were higher than those of the +/*fa*-basal diet group from week 8 to 20. The differences between +/+ and +/*fa* of both the basal and the corn oil diet groups were markedly smaller than those between the basal diet and corn oil diet groups in each genotype. Average food intake of the +/+-basal diet, +/+-corn oil diet, +/*fa*-basal diet and +/*fa*-corn oil diet groups were 11.8-14.2, 9.2-12.8, 11.4-14.3 and 9.7-12.6 g/rat/day, respectively, and those of the corn oil groups showed a tendency for decrease as compared to those of the basal diet groups in both genotypes.

Sequential changes in palpable mammary carcinomas. The minimum latency periods of palpable mammary carcinomas, which were histopathologically defined postmortem, were 8 weeks following DMBA administration in both the +/*fa*-basal and +/*fa*-corn oil diet groups, considerably shorter than the 11-12 weeks in the +/+-basal and +/+-corn oil diet groups (Fig. 2A). Incidence and multiplicity of palpable mammary carcinomas were increased or showed a tendency for increase in the early stages in +/*fa*-basal and +/*fa*-corn oil diet groups as compared to their +/+-counterparts, whereas their volume showed a tendency for increase in the corn oil diet groups of both +/+ and +/*fa* as compared to the basal diet groups (Fig. 2B and C).

Table I. Final incidence and multiplicity data for mammary tumors.

	+/+ genotype				+/*fa* genotype			
	Basal diet (n=16)		Corn oil diet (n=16)		Basal diet (n=33)		Corn oil diet (33)	
	Incidence (%)	Multiplicity (No./rat)	Incidence (%)	Multiplicity (No./rat)	Incidence (%)	Multiplicity (No./rat)	Incidence (%)	Multiplicity (No./rat)
Carcinoma	7 (44)	0.88±1.41[a]	8 (50)	0.69±0.79	20 (61)	1.30±1.49	19 (58)	0.94±1.27
Adenoma	1 (6)	0.06±0.25	1 (6)	0.06±0.25	0	-	1 (3)	0.03±0.17
Fibroadenoma	4 (25)	0.25±0.45	3 (19)	0.25±0.58	7 (21)	0.30±0.64	5 (15)	0.24±0.61
Fibroma	0	-	0	-	2 (6)	0.06±0.24	0	-

[a]Means ± SDs.

Table II. Final volumes of mammary tumors.

	+/+ genotype				+/*fa* genotype			
	Basal diet (n=16)		Corn oil diet (n=16)		Basal diet (n=33)		Corn oil diet (n=33)	
	No. of tumors	Volume (cm^3/tumor)	No. of tumors	Volume (cm^3/tumor)	No. of tumors	Volume (cm^3/tumor)	No. of tumors	Volume (cm^3/tumor)
Carcinoma	14	2.06±4.14	11	6.72±8.89	43	2.92±6.29	31	6.86±12.14
Adenoma	1	0.01	1	0.21	0	-	1	0.11
Fibroadenoma	4	0.26±0.23	4	18.80±31.95	10	1.49±2.54	8	4.97±13.34
Fibroma	0	-	0	-	2	35.56±50.21	0	-

Values are means ± SDs.

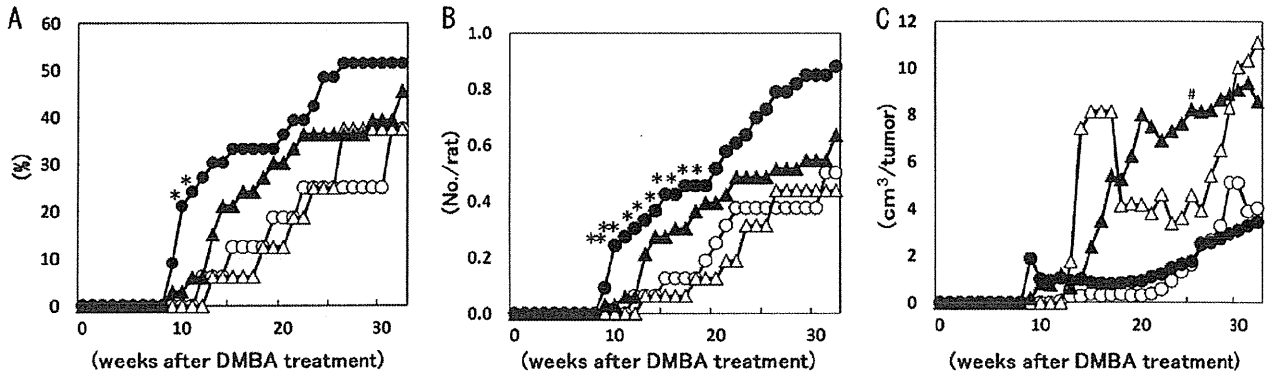

Figure 2. Sequential changes in palpable mammary carcinomas. (A) Cumulative incidence of rats with carcinomas; (B) cumulative mean number of carcinomas per rat (multiplicity); (C) cumulative mean volume of nodule/mass of carcinomas. Open circles, +/+-basal diet; open triangles, +/+-10% corn oil diet; closed circles, +/*fa*-basal diet; closed triangles, +/*fa*-10% corn oil diet. *P<0.05, **P<0.01 vs. +/+-basal diet (difference with the genotype basis); #P<0.05 vs. +/*fa*-basal diet (difference with the diet basis).

Final incidence, multiplicity and volume of mammary tumors. Incidence, multiplicity and volume findings for histopathologically defined mammary tumors are summarized in Table I. Histopathologically, mammary tumors could be classified as adenocarcinomas and benign lesions, such as adenomas, fibroadenomas and fibromas. Incidence and multiplicity of mammary carcinomas showed a tendency for increase (~1.5-fold) in the +/*fa*-basal diet group as compared with +/+-controls, but no influence on the genotype was noted in the corn oil diet groups. Furthermore, the corn oil diet showed no apparent effect on the incidence and multiplicity of mammary carcinomas in each genotype. Incidence and multi-

Table III. Distribution of sub-classified mammary carcinomas based on the morphological phenotypes among the groups.

	+/+ genotype				+/*fa* genotype			
	Basal diet (n=16)		Corn oil diet (n=16)		Basal diet (n=33)		Corn oil diet (n=33)	
	Incidence (%)	Multiplicity (No./rat)	Incidence (%)	Multiplicity (No./rat)	Incidence (%)	Multiplicity (No./rat)	Incidence (%)	Multiplicity (No./rat)
Moderately/poorly differentiated carcinoma with atypia	1 (6)	0.06±0.25[a]	0	-	6 (18)	0.21±0.48	3 (9)	0.09±0.29
Well-differentiated carcinoma without distinct atypia	7 (44)	0.81±1.22	8 (50)	0.69±0.79	16 (48)	1.09±1.49	17 (52)	0.85±1.28

[a]Means ± SDs.

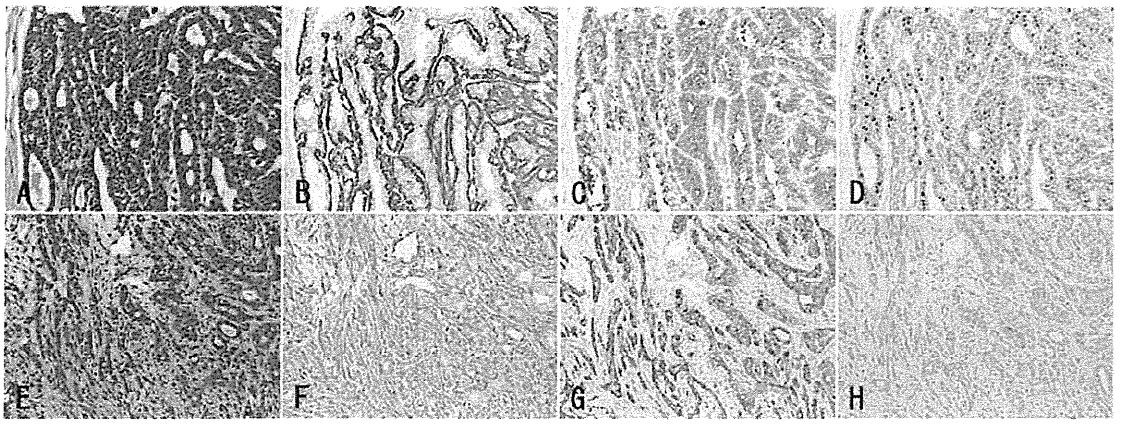

Figure 3. Histopathology and immunohistochemistry of mammary carcinomas. (A-D) A well differentiated carcinoma without distinct atypia in a +/+ rat fed basal diet; (E-H) a moderately/poorly differentiated carcinoma with atypia in a +/*fa* rat fed basal diet. (A and E) H&E. Immunohistochemistry for (B and F) α smooth muscle actin, showing cytoplasmic positivity for myoepithelial cells; (C and G) leptin receptor membranous positivity in carcinoma cells; (D and H) estrogen receptor α nuclear positivity in carcinoma cells. Original magnification, x360.

plicity of adenomas, fibroadenomas and fibromas were similar among the groups. On the other hand, volume of mammary carcinomas as well as fibroadenomas showed a tendency for increase by the corn oil diet with both +/+ and +/*fa* genotypes (Table II).

Histopathology, immunohistochemistry and western blot analysis of mammary carcinomas. Mammary adenocarcinomas found in the present experiment were mainly well differentiated without distinct nuclear atypia; however, some carcinomas showed moderately/poorly differentiated phenotypes with nuclear atypia (Fig. 3A and E). Well differentiated carcinomas showed papillotubular structures with cribriform patterns, and the tubules were generally well demarcated with α smooth muscle actin-positive myoepithelial cells (Fig. 3B). On the other hand, moderately/poorly differentiated carcinomas showed distinct invasion with small cord/glandular or scattering patterns mainly in the peripheral portion, and interstitial cell proliferation was prominent (Fig. 3F). The distribution of the sub-classified mammary carcinomas based on the morphological phenotypes among the groups is summarized in Table III. Incidence and multiplicity of moderately/poorly differentiated carcinomas with atypia showed a tendency for increase in the +/*fa*-basal diet and+/*fa*-corn oil diet groups as compared with their +/+-counterparts (Table III). The latency period of moderately/poorly differentiated carcinomas with atypia was shorter than that of well differentiated carcinomas without distinct atypia in the +/*fa*-basal diet group (Fig. 4).

To clarify expression profiles of leptin- and estrogen-related proteins in the well differentiated carcinomas without distinct atypia and moderately/poorly differentiated carcinomas with atypia, immunohistochemical and immunoblot analyses were performed. Mammary carcinomas of both phenotypes showed various expression intensities for leptin receptors (Fig. 3C and G) and leptin (data not shown), whereas the cases with atypia showed lower ER α-positivities than those without distinct atypia in the +/*fa*-basal diet and +/*fa*-corn oil diet groups (Table IV, Fig. 3D and H). For ER

Table IV. Estrogen receptor (ER) α-positivity in sub-classified mammary carcinomas based on the morphological phenotypes.

	+/+ genotype				+/*fa* genotype			
	Basal diet		Corn oil diet		Basal diet		Corn oil diet	
	No. of carcinomas examined	ER α-positivity (%)	No. of carcinomas examined	ER α-positivity (%)	No. of carcinomas examined	ER α-positivity (%)	No. of carcinomas examined	ER α-positivity (%)
Moderately/poorly differentiated carcinoma with atypia	1	1.0	0	-	7	8.9±3.9[b]	3	14.1±11.2
Well-differentiated carcinoma without distinct atypia	5	28.2±16.6[a]	6	24.2±14.2	6	36.4±15.0	10	28.9±11.4

[a]Means ± SDs; [b]P<0.01 vs. well-differentiated carcinoma without distinct atypia.

Table V. Serum biochemistry data at terminal sacrifice.

	+/+ genotype				+/*fa* genotype			
	Basal diet		Corn oil diet		Basal diet		Corn oil diet	
	No. of samples	Serum levels	No. of samples	Serum levels	No. of samples	Serum levels	No. of samples	Serum levels
Triglycerides (mg/dl)	16	340.8±138.7	15	311.5±221.5	31	392.3±312.6	31	279.6±146.5
Total cholesterol (mg/dl)	16	119.7±22.9	15	108.3±25.4	31	125.0±38.5	31	105.7±17.5[b]
Glucose (mg/dl)	16	134.6±15.9	15	145.3±15.4	31	133.7±13.3	31	141.9±17.5[b]
Leptin (pg/ml)	8	314.4±96.4	7	691.9±540.1	13	191.6±123.1	12	506.4±439.3[b]
Adiponectin (μg/ml)	8	6.7±1.4	7	7.1±3.8	12	4.0±1.0[a]	12	6.5±1.4[c]
Insulin (ng/ml)	8	2.1±0.8	7	1.5±1.0	14	1.3±0.9	13	1.5±1.1
IGF-I (ng/ml)	8	612.7±85.7	7	476.4±79.3[a]	12	609.6±178.4	12	535.3±96.6

Values are means ± SDs. [a]P<0.01 vs. +/+-basal diet group. [b]P<0.05, [c]P<0.01 vs. +/*fa*-basal diet group.

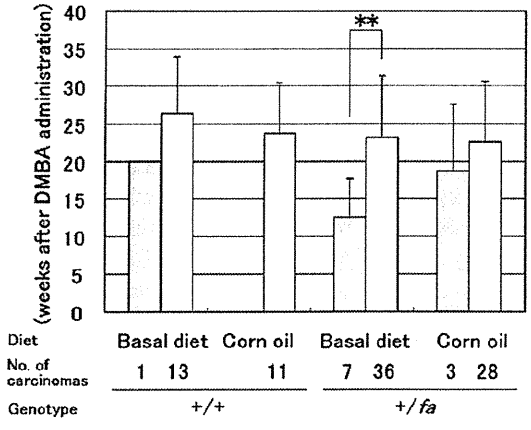

Figure 4. Latency of sub-classified mammary carcinomas based on the morphological phenotypes. Gray bars, moderately/poorly differentiated carcinoma with atypia; open bars, well-differentiated carcinoma without distinct atypia. **P<0.05.

β- and aromatase-immunohistochemistry, frozen sections of 1, 0, 3 and 3 moderately/poorly differentiated carcinomas from the +/+-basal diet, +/+-corn oil diet, +/*fa*-basal diet and +/fa-corn oil diet groups, respectively, and 2, 4, 1 and 2 well differentiated carcinomas each were used (Fig. 5A and B). Although no apparent differences in the positive intensities or positive cell ratio for ER β and aromatase were found among the combinations with two phenotypes and two diets in the immunohistochemistry, immunoblot analyses revealed a decrease in ER β expression levels in moderately/poorly differentiated carcinomas (Fig. 5C and D) and decreased expression levels of aromatase in mammary carcinomas regardless of their phenotypes and diets as compared to the normal mammary tissue (Fig. 5C and E).

To examine the relation of intracellular signaling cascades responsive to extracellular stimuli, such as growth factors or cytokines, with the mammary carcinoma phenotypes, immunoblot analyses for phosphorylation levels of ERK1/2

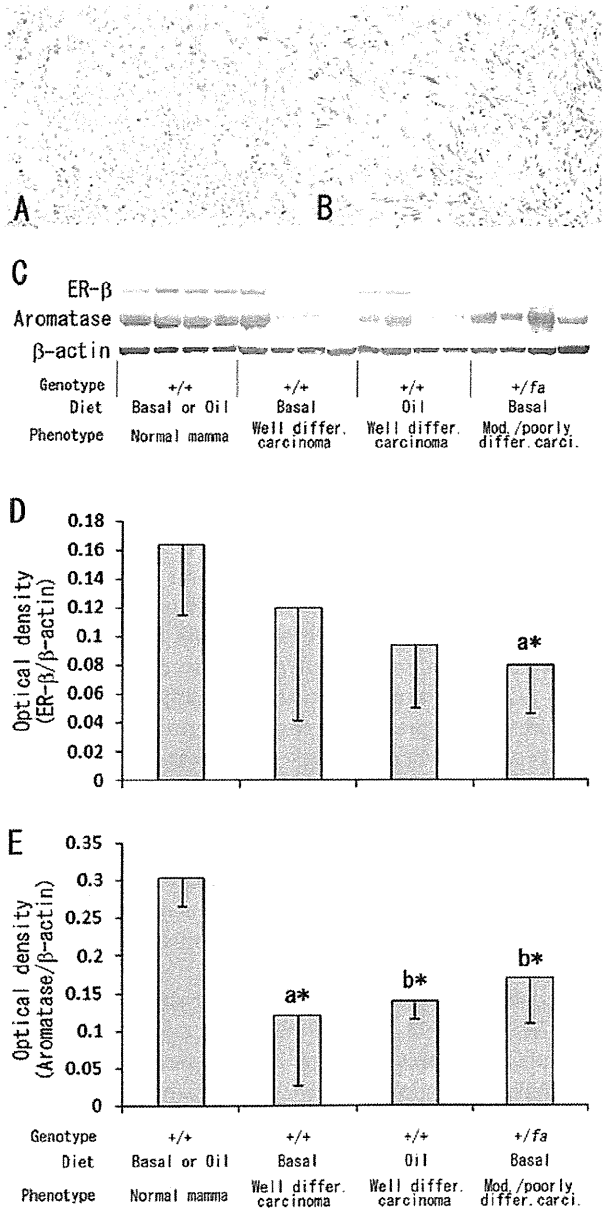

Figure 5. Expression of estrogen receptor (ER) β and aromatase in mammary carcinomas. Immunohistochemistry for (A) ER β, showing nuclear positivity in carcinoma cells of a well differentiated carcinoma in a +/+ rat fed basal diet; (B) aromatase, showing cytoplasmic positivity in presumed myoepithelial and/or mesenchymal cells of a moderately/poorly differentiated carcinoma in a +/*fa* rat fed basal diet. Western blotting for ER β and aromatase (C), and semi-quantitative optical density of ER-β (D) and aromatase (E). ^{a*}P<0.05, ^{b*}P<0.01 vs. normal mammary tissue.

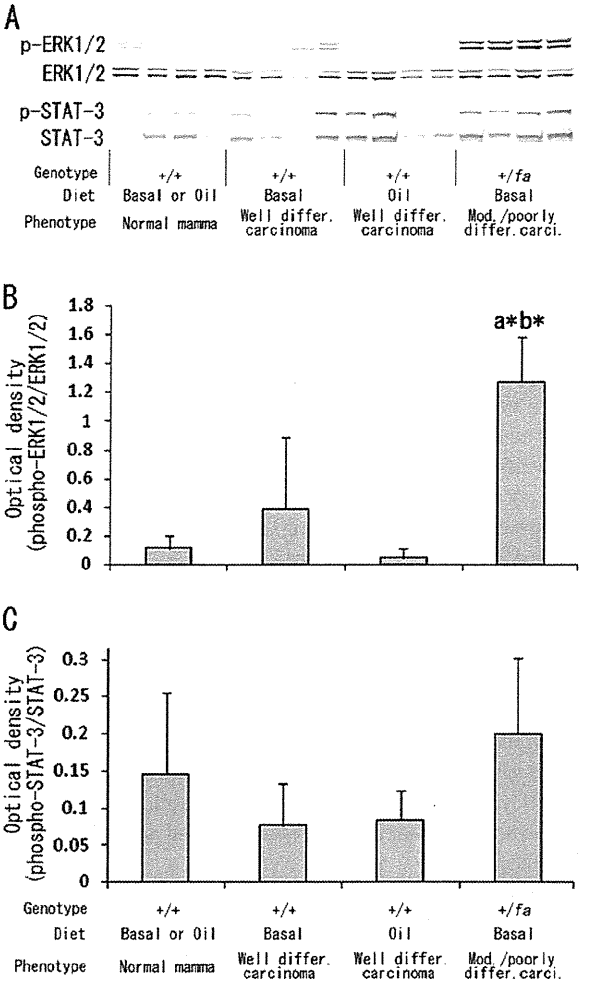

Figure 6. Phosphorylation levels of extracellular signal-regulated kinase (ERK)1/2 and signal transducer and activator of transcription (STAT)3 in mammary carcinomas. Western blotting for phospho-ERK1/2, ERK1/2, phospho-STAT3 and STAT3 (A) and semi-quantitative optical density of phospho-ERK1/2 (B) and phospho-STAT3 (C) compared to those of ERK1/2 and STAT3, respectively. ^{a*}P<0.05 vs. well differentiated carcinomas in +/+ rats fed basal diet, and ^{b*}P<0.01 vs. normal mammary tissue in +/+ rats fed basal or corn oil diet and well-differentiated carcinomas in +/+ rats fed corn oil diet.

and STAT3 were performed, and activation of the ERK1/2 signaling pathway but not STAT3 was demonstrated in moderately/poorly differentiated carcinomas with atypia as compared to normal mammary tissue and well differentiated carcinomas without distinct atypia (Fig. 6). No influence of corn oil diet was found with regard to either ERK1/2 or STAT3 activation (Fig. 6).

Serum biochemistry. Data for serum levels of triglycerides, total cholesterol and glucose at terminal sacrifice are summarized in Table V. Although triglyceride and total cholesterol levels declined or showed a tendency for decline and glucose levels were elevated by the corn oil diet in the +/*fa* genotype, no apparent change in these three parameters was observed in +/+ controls. No obvious differences in these parameters were found between the genotypes. Serum leptin levels in the +/*fa*-basal diet and the +/*fa*-corn oil diet groups were comparable to those in the +/+-counterparts, whereas corn oil diet elevated serum leptin levels in the +/*fa* genotype with a similar tendency for elevation in the +/+ genotype (Table V). Serum adiponectin levels in the +/*fa*-basal diet group were lower than in the +/+-basal diet group, and corn oil diet caused elevation only in the +/*fa* case. Serum IGF-I levels were lower in the +/+-corn oil diet than +/+-basal diet groups, but no change was observed with the +/*fa* genotype. There was no evident variation noted in serum insulin levels among the groups.

Discussion

The present DMBA-induced mammary carcinogenesis study using heterozygous (+/*fa*) and wild-type (+/+) lean Zucker rats revealed higher susceptibility of +/*fa* rats to DMBA induction of mammary tumors than +/+ rats, and also differences in histopathological phenotypes of the induced carcinomas. In particular, the latency periods of mammary carcinoma development in +/*fa* rats fed basal or corn oil diet appeared shorter than those in +/+ rats and the incidences and multiplicities of mammary carcinomas were increased or showed a tendency for increase in the early stages, with a greater percentage of more advanced cancer at the termination.

Although the body weight of +/+ and +/*fa* rats fed corn oil diet were higher than those of the rats fed basal diet, the body weight differences between +/+ and +/*fa* rats fed basal diet or corn oil diet were significantly smaller. Therefore, the short latency periods and the higher incidence and multiplicity of mammary carcinomas in the early stages in +/*fa* rats were considered not to be directly due to body weight change. On the other hand, in our preliminary study, serum leptin concentration at 7 weeks of age was ~140 pg/ml in +/*fa*, higher ($P<0.01$) than ~80 pg/ml (18). These results indicated that the increased susceptibility of +/*fa* rats to DMBA-induced mammary carcinogenesis might be at least partly associated with higher leptin levels at the initiation stage. Hyperleptinemia in juvenile stages of +/*fa* rats gradually normalized and no difference in serum leptin level was found at the terminal sacrifice between the genotypes.

Histopathologically, adenocarcinomas in +/*fa* rats were more likely to present characteristic features such as moderate/poor differentiation, nuclear atypia, prominent interstitial cell proliferation and low ER α positivity. Expression levels of aromatase were decreased in mammary carcinomas regardless of the phenotype as compared to normal mammary tissue. On the other hand, leptin receptor and leptin were expressed with various intensities and no distinct differences were found between carcinomas with and without atypia. Although the cause of the lowered ER α protein expression in the moderately/poorly differentiated carcinomas with atypia is not clear, one possibility is that mammary epithelial cells of +/*fa* rats were initiated under conditions without estrogen-dependence but with close dependence on other growth factors, such as leptin or EGFR (10,23). Activation of the mitogen-activated protein kinase (MAPK) system has been demonstrated in moderately/poorly differentiated carcinomas with atypia. Thus, Thordarson *et al* (24) reported that *N*-methyl-*N*-nitrosourea (MNU)-induced mammary carcinomas in ovariectomized Sprague-Dawley rats showed a more aggressive phenotype with a significant increase in MAPK activity (phosphorylation) as compared to carcinomas in intact rats, suggesting a relationship between loss of estrogen-dependence and growth. Also, in an estrogen-non-responsive human breast cancer cell line, MAPK activity was found to be increased as compared to the original estrogen-dependent sample, suggesting that increased activity of MAPK may contribute to the estrogen non-responsive growth phenotype (25).

Epidemiologically, breast cancer rates among pre-and perimenopausal ages are reported to be higher among US-born Chinese than those born in foreign countries, and similar findings were found in Filipina women as well, to the extent that contemporary rates may equal or exceed those of non-Hispanic Whites, indicating that becoming acculturated to the western lifestyle might be a breast cancer risk factor to some younger Asian women (26). Plasma leptin levels were demonstrated to be twice as high in US-born South Asian (India, Bangladesh, Sri Lanka) women aged 18-30 years than in European women (27), presumably related to the increasing rate of breast cancer. In addition, in certain Asian countries, such as India and Singapore, breast cancer patients present at a younger age, with more advanced stage and fewer estrogen-ER-positive tumors, as compared to western countries (28,29). Therefore, we propose that the present DMBA-induced mammary carcinogenesis in +/*fa* lean Zucker rats may be a useful model of increasing breast cancer in younger Asian women.

A further significant finding of the present DMBA-induced mammary carcinogenesis study in +/*fa* and +/+ rats with and without 10% corn oil diet is that elevation of serum leptin level may contribute to the growth of mammary tumors. In our preliminary study, corn oil diet, similarly prepared as in the present study, significantly elevated serum leptin concentrations of 12-week-old +/+ and +/*fa* rats as compared to basal diet, as also confirmed in the present study. These data are consistent with the previous reports of overexpression of leptin and its receptor in human breast cancer cases (30,31), and in *in vitro* studies revealing that leptin can stimulate breast cancer cell proliferation (23,32). From epidemiological studies, it is well recognized that obesity increases the risk of breast cancer in postmenopausal women, with a suggested association with menstrual and reproductive factors (33) or higher circulating levels of leptin. However, the mechanisms have yet to be fully elucidated (34,35).

In conclusion, +/*fa* rats in the present study proved more susceptible to DMBA-induced mammary carcinogenesis than +/+ controls, and this might be at least partly related to the higher leptin levels in the early stages. Corn oil diet possibly contributed to the growth of mammary tumors via elevated serum leptin levels. In addition, an aggressive phenotype of carcinoma, in which MAPK cascade but not estrogen signaling was activated, was found predominantly in +/*fa* rats. Further studies are required to examine the mechanisms of MAPK activation for mammary carcinogenesis in +/*fa* rats.

Acknowledgements

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References

1. van den Brandt PA, Spiegelman D, Yaun SS, *et al*: Pooled analysis of prospective cohort studies on height, weight, and breast cancer risk. Am J Epidemiol 152: 514-527, 2000.

2. Lahmann PH, Hoffmann K, Allen N, *et al*: Body size and breast cancer risk: findings from the European Prospective Investigation into Cancer and Nutrition (EPIC). Int J Cancer 111: 762-771, 2004.
3. Reeves GK, Pirie K, Beral V, Green J, Spencer E and Bull D: Cancer incidence and mortality in relation to body mass index in the Million Women Study: cohort study. BMJ 335: 1134, 2007.
4. Iwasaki M, Otani T, Inoue M, Sasazuki S and Tsugane S: Body size and risk for breast cancer in relation to estrogen and progesterone receptor status in Japan. Ann Epidemiol 17: 304-312, 2007.
5. Key TJ, Appleby PN, Reeves GK, *et al*: Body mass index, serum sex hormones, and breast cancer risk in postmenopausal women. J Natl Cancer Inst 95: 1218-1226, 2003.
6. Auwerx J and Staels B: Leptin. Lancet 351: 737-742, 1998.
7. Wu MH, Chou YC, Chou WY, *et al*: Circulating levels of leptin, adiposity and breast cancer risk. Br J Cancer 100: 578-582, 2009.
8. Soma D, Kitayama J, Yamashita H, Miyato H, Ishikawa M and Nagawa H: Leptin augments proliferation of breast cancer cells via transactivation of HER2. J Surg Res 149: 9-14, 2008.
9. Saxena NK, Taliaferro-Smith L, Knight BB, *et al*: Bidirectional crosstalk between leptin and insulin-like growth factor-I signaling promotes invasion and migration of breast cancer cells via transactivation of epidermal growth factor receptor. Cancer Res 68: 9712-9722, 2008.
10. Cirillo D, Rachiglio AM, la Montagna R, Giordano A and Normanno N: Leptin signaling in breast cancer: an overview. J Cell Biochem 105: 956-964, 2008.
11. Chua SC Jr, Chung WK, Wu-Peng XS, *et al*: Phenotypes of mouse diabetes and rat fatty due to mutations in the OB (leptin) receptor. Science 271: 994-996, 1996.
12. Bray GA: The Zucker-fatty rat: a review. Fed Proc 36: 148-153, 1977.
13. Rayner DV, Dalgliesh GD, Duncan JS, Hardie LJ, Hoggard N and Trayhurn P: Postnatal development of the ob gene system: elevated leptin levels in suckling fa/fa rats. Am J Physiol 273: R446-R450, 1997.
14. Lee WM, Lu S, Medline A and Archer MC: Susceptibility of lean and obese Zucker rats to tumorigenesis induced by *N*-methyl-*N*-nitrosourea. Cancer Lett 162: 155-160, 2001.
15. Hakkak R, Holley AW, Macleod SL, *et al*: Obesity promotes 7,12-dimethylbenz(a)anthracene-induced mammary tumor development in female zucker rats. Breast Cancer Res 7: R627-R633, 2005.
16. Hakkak R, MacLeod S, Shaaf S, *et al*: Obesity increases the incidence of 7,12-dimethylbenz(*a*)anthracene-induced mammary tumors in an ovariectomized Zucker rat model. Int J Oncol 30: 557-563, 2007.
17. de Assis S, Wang M, Goel S, Foxworth A, Helferich W and Hilakivi-Clarke L: Excessive weight gain during pregnancy increases carcinogen-induced mammary tumorigenesis in Sprague-Dawley and lean and obese Zucker rats. J Nutr 136: 998-1004, 2006.
18. Cho YM, Imai T, Takami S, Ogawa K and Nishikawa A: Female heterozygous (*+/fa*) Zucker rats as a novel leptin-related mammary carcinogenesis model. J Toxicol Sci 37: 1025-1034, 2012.
19. Phillips MS, Liu Q, Hammond HA, *et al*: Leptin receptor missense mutation in the fatty Zucker rat. Nat Genet 13: 18-19, 1996.
20. Imai T, Cho YM, Hasumura M and Hirose M: Enhancement by acrylamide of *N*-methyl-*N*-nitrosourea-induced rat mammary tumor development-possible application for a model to detect co-modifiers of carcinogenesis. Cancer Lett 230: 25-32, 2005.
21. Cho YM, Imai T, Hasumura M and Hirose M: Lack of enhancement of susceptibility to mammary and thyroid carcinogenesis in rats exposed to DMBA and DHPN following prepubertal iodine deficiency. Cancer Sci 97: 1031-1036, 2006.
22. Ip C, Carter CA and Ip MM: Requirement of essential fatty acid for mammary tumorigenesis in the rat. Cancer Res 45: 1997-2001, 1985.
23. Mauro L, Catalano S, Bossi G, *et al*: Evidences that leptin up-regulates E-cadherin expression in breast cancer: effects on tumor growth and progression. Cancer Res 67: 3412-3421, 2007.
24. Thordarson G, Lee AV, McCarty M, *et al*: Growth and characterization of *N*-methyl-*N*-nitrosourea-induced mammary tumors in intact and ovariectomized rats. Carcinogenesis 22: 2039-2047, 2001.
25. Coutts AS and Murphy LC: Elevated mitogen-activated protein kinase activity in estrogen-non responsive human breast cancer cells. Cancer Res 58: 4071-4074, 1998.
26. Gomez SL, Quach T, Horn-Ross PL, *et al*: Hidden breast cancer disparities in Asian women: disaggregating incidence rates by ethnicity and migrant status. Am J Public Health 100 (Suppl 1): S125-S131, 2010.
27. Kalhan R, Puthawala K, Agarwal S, Amini SB and Kalhan SC: Altered lipid profile, leptin, insulin, and anthropometry in offspring of South Asian immigrants in the United States. Metabolism 50: 1197-1202, 2001.
28. Ghumare SS and Cunningham JE: Breast cancer trends in Indian residents and emigrants portend an emerging epidemic for India. Asian Pac J Cancer Prev 8: 507-512, 2007.
29. Lim SE, Back M, Quek E, Iau P, Putti T and Wong JE: Clinical observations from a breast cancer registry in Asian women. World J Surg 31: 1387-1392, 2007.
30. Ishikawa M, Kitayama J and Nagawa H: Enhanced expression of leptin and leptin receptor (OB-R) in human breast cancer. Clin Cancer Res 10: 4325-4331, 2004.
31. Garofalo C, Koda M, Cascio S, *et al*: Increased expression of leptin and the leptin receptor as a marker of breast cancer progression: possible role of obesity-related stimuli. Clin Cancer Res 12: 1447-1453, 2006.
32. Ray A, Nkhata KJ and Cleary MP: Effects of leptin on human breast cancer cell lines in relationship to estrogen receptor and HER2 status. Int J Oncol 30: 1499-1509, 2007.
33. Iwasaki M, Otani T, Inoue M, Sasazuki S and Tsugane S: Role and impact of menstrual and reproductive factors on breast cancer risk in Japan. Eur J Cancer Prev 16: 116-123, 2007.
34. Grossmann ME, Ray A, Nkhata KJ, *et al*: Obesity and breast cancer: status of leptin and adiponectin in pathological processes. Cancer Metastasis Rev 29: 641-653, 2010.
35. Hjartaker A, Langseth H and Weiderpass E: Obesity and diabetes epidemics: cancer repercussions. Adv Exp Med Biol 630: 72-93, 2008.

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Original Article

Female heterozygous (+/*fa*) Zucker rats as a novel leptin-related mammary carcinogenesis model

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ABSTRACT — The homozygous mutant fatty Zucker rat (*fa/fa*) is the prominent model for the research of obesity, one of the most well-known risk factor of postmenopausal mammary cancer. But the usage as a mammary gland carcinogenesis model is considered to be restricted due to the hypoplasia of mammary gland. In the present study, to find the validity of heterozygous mutant (+/*fa*) lean Zucker rats as a new leptin-related mammary carcinogenesis model, we examined whether the number of terminal end buds of mammary gland, the serum biochemistry, leptin concentration in serum and adipose tissue are changed in 7-week-old female +/+, +/*fa* and *fa/fa* rats, and whether these changes and leptin, TNF-α and VEGF mRNA expression in adipose tissue of +/+ and +/*fa* rats are influenced by 10% corn oil diet for 5 weeks. We confirmed that mild hyperleptinemia was more pronounced in 7-week-old +/*fa* as compared with wild type (+/+) and hypoplasia of mammary glands characterized by fewer numbers of terminal end buds in *fa/fa* was not observed in +/*fa*. With 10% corn oil diet, leptin mRNA expression in adipose tissue showed increasing tendency both in +/*fa* and +/+. Comparing with +/+, adipose tissue in +/*fa* treated with 10% corn oil diet was found to be significantly increased in the concentration of leptin protein and tended to be elevated expression of TNF-α mRNA. These results suggest that +/*fa* with 10% corn oil diet may be a useful model for investigation of the participation of leptin and TNF-α in mammary gland carcinogenesis.

Key words: Leptin, Tumor models, Mammary cancer

INTRODUCTION

General obesity is an important risk factor of mammary cancer in postmenopausal women, and central obesity was further reported to increase mammary cancer risk in premenopausal as well as postmenopausal populations (Calle and Thun, 2004; Phillips *et al.*, 1996; Schaffler *et al.*, 2007). The mechanisms involved remain largely unclear, but it is suggested that various bioactive factors synthesized by adipose tissue might exert tumor-stimulatory effects on the mammary gland epithelium (Caldefie-Chezet *et al.*, 2005; Housa *et al.*, 2006). One principal bioactive substance produced by adipocytes is leptin (Anubhuti and Arora, 2008), a 167-aminoacid peptide hormone encoded by the obesity gene (*ob*), which is secreted and plays important roles in regulating food intake and energy expenditure through binding to specific receptors (OB-R) (Anubhuti and Arora, 2008). Leptin also controls other common physiological processes such as immune responses, cell differentiation, proliferation and angiogenesis (Zhang *et al.*, 2005). Furthermore, several evidences suggest that leptin could be involved in tumorigenesis as a mitogenic, transforming or migration factor, especially active in the development of mammary, colorectal and prostate cancers (Garofalo and Surmacz, 2006; Hu *et al.*, 2002; Rouet-Benzineb *et al.*, 2004; Somasundar *et al.*, 2004).

Both leptin and OB-R appear to be significantly over-expressed in human mammary cancer tissue relative to non-cancer epithelium (Ishikawa *et al.*, 2004). In addition, higher expression of OB-R protein has been demonstrated in estrogen receptor α (ERα)-positive human mammary carcinoma cells MCF-7 and T47D than ERα-negative carcinoma cells MDA-MB-231 and MDA-MB 435 (Garofalo *et al.*, 2004). Leptin stimulates estrogen production through enhanced aromatase mRNA expres-

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sion, protein content and enzymatic activity in MCF-7, via AP-1 (Catalano *et al.*, 2003). In general, elevated lifetime estrogen exposure is considered a major risk factor for mammary cancer in human (Key *et al.*, 2002). Leptin signaling is also reported to play an important role in the growth of mammary cancers through promotion of the expression of vascular endothelial growth factor (VEGF)/vascular endothelial growth factor receptor type 2 (VEGFT2) (Rene Gonzalez *et al.*, 2009). Moreover, its synthesis is influenced most notably by tumor necrosis factor-α (TNF-α) (Zhang *et al.*, 2000), insulin (Cusin *et al.*, 1995) and reproductive hormones (Machinal-Quelin *et al.*, 2002), all of which have been associated with mammary gland neoplastic processes. For example, there is evidence that hyperinsulinemia promotes mammary cancer progression through leptin-dependent mechanisms (Bartella *et al.*, 2008; Garofalo *et al.*, 2006). Estrogen regulates leptin productions in rats and humans subjects *in vivo* (Alonso *et al.*, 2007; Shimizu *et al.*, 1997).

TNF-α is a multifunctional cytokine that plays important roles in diverse cellular events such as immune function, cell survival, proliferation, differentiation, and death (Wang and Lin, 2008). Administration of TNF-α increased leptin mRNA and protein levels in adipose tissue of hamsters (Grunfeld *et al.*, 1996). Adipose tissues of the obese *db/db, ob/ob*, *tub/tub* mice, and the *fa/fa* Zucker rat expressed high levels of TNF-α mRNA and circulating plasma levels of TNF-α protein significantly elevated in *db/db* mice (Hotamisligil *et al.*, 1993). TNF-α, a proinflammatory cytokine, has been shown to be synthesized and secreted from macrophage as well as adipocyte (Kern *et al.*, 1995; Weisberg *et al.*, 2003), which may be involved in inflammation-associated carcinogenesis (Balkwill, 2009).

In animal models, a higher body weight is linked with increased incidences of both spontaneous and chemically induced mammary tumors (Haseman *et al.*, 1994; Waxler *et al.*, 1953; Wolff *et al.*, 1982). Zucker rats with a homogeneous spontaneous mutation in the leptin receptor gene (*fa/fa*) (Phillips *et al.*, 1996) are known to be obese, hyperphagic and hyperinsulinemic (Bray, 1977). In contrast, lean Zucker (+/*fa* or +/+) rats show almost normal metabolic functions, and have been used as controls in various types of physiochemical and pathological experiments (Bray, 1977). The Zucker rat has been recognized as a superior model to investigate effects of obesity on chronic disease development, including cancer (Bray, 1977; de Assis *et al.*, 2006; Hakkak *et al.*, 2007), but its utility for investigations of mammary carcinogenesis is limited due to scant epithelial development in mature mammary glands of obese as compared with lean counterparts (Hu *et al.*, 2002). Since it was reported that young heterozygous lean Zucker (+/*fa*) rats demonstrate a number of differences from wild type lean Zucker (+/+) rats, e.g., higher body weights, fat cell size, inguinal fat pad weights, pad-to-body weight ratios, serum cholesterol, adipose tissue lipoprotein lipase and glycerol-3-phosphate dehydrogenase, hepatic and adipose tissue 6-phosphogluconate dehydrogenase activities and serum leptin levels (1.6 and 0.9 ng/ml in +/*fa* and +/+, respectively, (Cleary and Phillips, 1999)) (Cleary *et al.*, 1999; Heo *et al.*, 2002; Phillips and Cleary, 1994; Truett *et al.*, 1995; Zhang *et al.*, 1997), we here investigated whether they might provide the basis for a leptin-related mammary carcinogenesis model. Two independent experiments were performed. In experiment 1, serum biochemistry, histological characteristics of mammary glands and leptin levels of serum and adipose tissue in 7-week-old female +/*fa* lean Zucker rats were compared with those of *fa/fa* and +/+ siblings. In experiment 2, we tested whether 10% corn oil diet affects serum biochemistry and histological characteristics of mammary glands as well as leptin, TNF-α and VEGF mRNA expression in adipose tissue of female +/*fa* and +/+ lean Zucker rats.

MATERIALS AND METHODS

Animals

Homozygous obese (*fa/fa*), heterozygous lean (+/*fa*) and wild type (+/+) female Zucker rats at 6 weeks of age were purchased from Charles River Japan (Kanagawa, Japan). They were housed in clear polycarbonate cages with heat-treated white wood chips for bedding (Sankyo Laboratory Service, Tokyo, Japan) in an air conditioned room (24 ± 1°C, 55 ± 5% relative humidity, 12 hr light and dark cycle) and given basal diet (CRF-1, Oriental Yeast, Tokyo, Japan) and tap water *ad libitum*. The composition of the basal diet is 22.4% crude protein, 5.7% crude fat, 6.6% crude ash, 3.1% crude fiber, 7.8% moisture content and 54.5% nitrogen-free extract and the calorie of cereal-based diet is 359 kcal/100 g. The present study design was approved by the Animals Care and Utilization Committee of the National Institute of Health Sciences.

Genotyping

The animals were divided into each genotype group on the basis of genotyping, as described previously (Phillips *et al.*, 1996). For polymerase chain reaction (PCR) amplification of DNA sequences encoding leptin receptor isoform, digested 0.5 mm tail samples were amplified with the primers 5'-GTTTGCGTATGGAAGTCACAG-3' and

5'-ACCAGCAGAGATGTATCCGAG3' at the annealing temperature of 67°C for 30 cycles. The PCR products were incubated with *Msp*I for 1 hr at 37°C to indicate the presence of the mutation-derived restriction site in Zucker rat genomic DNA.

Experiment 1

Female Zucker rats (+/+, n = 8; +/*fa*, n = 16; *fa*/*fa*, n = 6) at 7-weeks of age were weighed and sacrificed without overnight fasting and blood samples were collected from the abdominal aorta under ether anesthesia for serum biochemistry and leptin and insulin enzyme assays. Serum biochemistry measurements of glucose, triglyceride (TG), total cholesterol (T-Cho) and a double antibody radioimmunoassay for estradiol were performed at SRL (Tokyo, Japan). Leptin levels in serum and homogenates of adipose tissue that carefully excluded their mammary gland from the one side of an inguinal fat pad and serum insulin levels were measured with an enzyme-linked immunosorbent assay (ELISA) kit, YK050 (Yanaihara Institute, Shizuoka, Japan) and by rat insulin ELISA (Mercodia AB, Uppsala, Sweden), respectively, according to the manufacturer's instructions. After macroscopic observation of abdominal viscera, subcutis and inguinal fat pads, liver and remaining inguinal fat pads containing mammary gland tissue were removed and fixed in 10% neutral buffered formalin for routine preparation of paraffin-embedded sections, then, hematoxylin and eosin (H.E.) staining and immunohistochemical analysis were performed. Livers were weighed before processing. The other side of inguinal fat pad in each animal was used for whole-mount preparation.

Experiment 2

Female Zucker rats at 7-weeks of age were fed basal (+/+, n = 5; +/*fa*, n = 6) or basal diet + 10% corn oil (+/+, n = 14; +/*fa*, n = 15) for 5 weeks and then sacrificed without overnight fasting in the same manner with experimental 1. In addition to the measured items in experiment 1 except serum estradiol concentration and total number of TEB in whole-mount preparation, expression of mRNAs for leptin, tumor necrosis factor-α (TNF-α), vascular endothelial growth factor A (VEGFA) and aromatase in inguinal adipose tissue was analyzed using real-time reverse transcription (RT)-PCR.

Mammary gland whole mounts and quantification

The whole-mount preparation protocol was a modification of previously described procedures (You *et al.*, 2002). Freshly dissected inguinal fat pad containing mammary glands tissues were placed flat between a pair of glass slides and fixed in 10% formalin for 24 hr, then dehydrated with 70, 95 and 100% ethanol (about 3 hr in each). The samples were defatted in acetone for approximately 12 hr and rehydrated in diluted ethanol solution ranging from 100 to 40% for 3 hr each. The samples were stained with 0.005% toluidine blue for 30 min and then dehydrated again in ethanol solution. The tissue pieces were then finally immersed in xylene for approximately 6 hr and mounted on glass slides with a mounting agent. The total numbers of terminal end buds (TEB) were counted from the distal portions of the mammary gland under a stereomicroscope, according to the criteria of Russo (Russo *et al.*, 1990).

Immunohistochemistry

The streptavidin-biotin peroxidase complex method (StreptABComplex/HRP, DAKO, Glostrup, Denmark) was used to determine the expression and localization of leptin and leptin receptors in mammary glands and inguinal adipose tissue of Zucker rats at 7 and 12 weeks of age. Polyclonal antibodies against leptin (Ob) were purchased from Santa Cruz Biotechnology (A-20; Santa Cruz, CA, USA) and used at a dilution of 1/100. A polyclonal antibody against the leptin receptor (OB-R) recognizing both wild and mutant forms was accessed from Neuromics Antibodies (Edina, MN, USA) and used at 1/1000. Antigen retrieval was performed in an autoclave for 10 min at 121°C in 10 mM citrate buffer (pH 6.0) for leptin receptors. Sections were lightly counterstained with hematoxylin for microscopic examination. Negative controls without primary antibodies were included for each antigen using serial sections.

Real-time RT-PCR

Quantitative real-time RT-PCR using an ABI Prism 7000 sequence detection system (Applied Biosystems Japan, Tokyo, Japan) was performed for leptin (*Lep*), TNF-α (*Tnf*), VEGFA (*Vegfa*) and aromatase (*Cyp19a1*). One microgram aliquots of total RNA isolated from inguinal fat pads of all rats and from ovaries and livers of basal diet +/+ as positive and negative control of aromatase, respectively, in experiment 2 using Isogen™ (Nippon Gene, Tokyo, Japan) were applied to RT with a High-Capacity cDNA Archive Kit (Applied Biosystems Japan, Tokyo, Japan) in a 100 μl total reaction volume. For real-time PCR analysis, ABI Assays-on-Demand™ TaqMan probe and primer sets from Applied Biosystems (available at https://products.appliedbiosystems.com/ab/en/US/adirect/ab?cmd=catNavigate2&catID=601267/) were employed. Real-time PCR was performed in a 50-μl reaction volume using the TaqMan probe detection

system (Applied Biosystems Japan) with specific primers, the corresponding TaqMan™ MGB probes (FAM™ dye labeled) and RT products. For the quantification of expression data, a standard curve method and normalization with a housekeeping gene, hypoxanthine-guanine phosphoribosyltransferase were applied.

Statistical analysis

Variance in data was checked for homogeneity by Bartlett's procedure. When the data were homogeneous, one-way analysis of variance for homogeneity (ANOVA) was used. In the heterogeneous cases, the Kruskal Wallis test was applied. When statistically significant differences were indicated, the Dunnett's multiple test was employed for comparisons between groups; in experiment 1, among all groups; in experiment 2, +/+ basal diet vs. +/+ 10% corn oil, +/+ basal diet vs. +/*fa* basal diet, +/+ 10% corn oil vs. +/*fa* 10% corn oil and +/*fa* basal diet vs. +/*fa* 10% corn oil. Value are presented as means ± standard deviations or standard error. p values of less than 0.05 were considered to be statistically significant. .

RESULTS

Experiment 1

In female fatty (*fa/fa*) Zucker rats at 7-weeks old of age, body weights and absolute and relative liver weights were higher ($p < 0.05$ or 0.01) than those of lean +/+ and/or +/*fa* rats (Table 1) and excess accumulation of adipose tissue in abdominal viscera, subcutis and inguinal fat pads at necropsy and increased storage of hepatocellular glycogen on histopathology were observed. There were higher ($p < 0.05$ or 0.01) concentrations of serum TG, T-Cho and insulin in *fa/fa* rats than in +/+ and/or +/*fa* rats, but not of glucose and estradiol (Table 2). No difference of body and liver weights and serum T-Cho, TG and insulin values were observed between +/+ and +/*fa* rats. Leptin concentrations in serum and adipose tissue were higher ($p < 0.05$ or 0.01) in *fa/fa* rats than +/+ and +/*fa* rats, and those of serum were also higher in +/*fa* rats ($p < 0.05$) than in +/+ rats (Table 3).

With inguinal mammary gland whole mounts, poorly developed tissue characterized by thinner ducts and immature glands and lower ($p < 0.01$) numbers of TEB (Fig. 1) was observed in female fatty (*fa/fa*) Zucker rats at 7-week old of age, as compared with lean +/+ and +/*fa* rats. Immunohistochemical analysis revealed that adipocytes in inguinal fat pad express leptin and its intensity was increased in hypertrophied adipocytes of *fa/fa* rats as compared with +/+ and +/*fa* (data not shown). Ductal and glandular epithelium of mammary gland of all genotypes showed positive reaction to an anti-leptin receptor antibody that recognized both wild and mutant form (data not shown).

Table 1. Experiment 1; body and liver weights

Genotype	N	Body weight (g)	Absolute liver weight (g)	Relative liver weight (g/100g b.w.)
+/+	8	152 ± 3[a]	7.1 ± 0.4	4.7 ± 0.2
+/*fa*	16	153 ± 8	7.2 ± 1.0	4.7 ± 0.5
fa/fa	6	222 ± 9**,##	11.5 ± 1.3**,##	5.2 ± 0.4#

N: No. of animals. [a]: Mean ± S.D.
**: Significantly different from +/+ at $p < 0.01$ (Dunnett's multiple test).
#,##: Significantly different from +/*fa* at $p < 0.05$ and 0.01, respectively (Dunnett's multiple test).

Table 2. Experiment 1; serum biochemistry

Genotype	N	Glucose (mg/dl)	Triglyceride (mg/dl)	Total cholesterol (mg/dl)	Insulin (ug/l)	Estradiol (pg/ml)
+/+	8	142 ± 7[a]	153 ± 44	90 ± 11	1.2 ± 0.3	34 ± 30[b]
+/*fa*	16	149 ± 28	165 ± 50	87 ± 7	1.4 ± 0.6	24 ± 13[c]
fa/fa	6	155 ± 40	302 ± 134#	129 ± 14**,##	6.8 ± 6.0**,##	19 ± 10

N: No. of animals. [a]: Mean ± S.D. [b]: n = 7 [c]: n = 15
**: Significantly different from +/+ at $p < 0.01$ (Dunnett's multiple test)
#,##: Significantly different from +/*fa* at $p < 0.05$ and 0.01, respectively (Dunnett's multiple test)

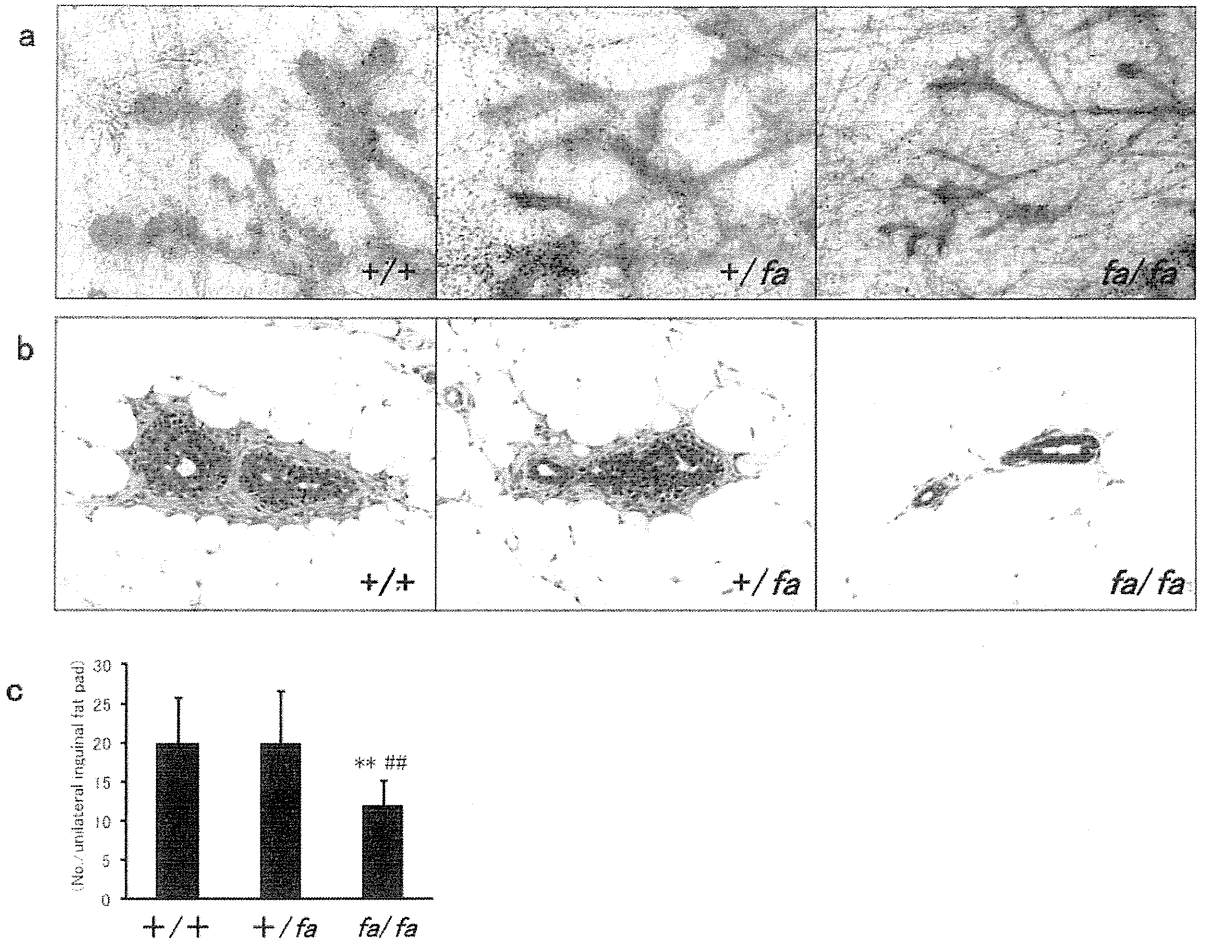

Fig. 1. Experiment 1; representative whole-mount preparation (a, toluidine blue staining, original magnification x40), histology (b, HE staining, original magnification x200) and numbers of terminal end buds (TEBs) (c) of mammary tissue of 7-week-old female Zucker rats. Poorly developed tissue characterized by thinner ducts and immature glands and lower numbers of TEB was noted in female fatty (*fa/fa*, n = 6) Zucker rats at 7-week old of age, comparing with +/+ (n = 8) and +/*fa* (n = 16) rats. **: Significantly different from +/+ at $p < 0.01$. ##: Significantly different from +/*fa* at $p < 0.01$.

Table 3. Experiment 1; leptin levels in serum and adipose tissue

Genotype	N	Serum leptin (ng/ml)	Adipose tissue leptin (ng/g)
+/+	8	0.08 ± 0.02[a]	1.3 ± 0.6
+/*fa*	16	0.14 ± 0.05*	1.6 ± 1.0
fa/fa	6	1.24 ± 0.13**,#	8.5 ± 1.6**,##

N: No. of animals. [a]: Mean ± S.D.

*, **: Significantly different from +/+ at $p < 0.05$ and 0.01, respectively (Dunnett's multiple test)

#, ##: Significantly different from +/*fa* at $p < 0.05$ and 0.01, respectively (Dunnett's multiple test)

Experiment 2

In +/*fa* rats significantly higher final body weight ($p < 0.01$) was shown in rats fed 10% corn oil when compared with rats fed basal diet (Table 4). In +/*fa* rats fed 10% corn oil, absolute and relative liver weight were significantly lower than those of +/+ ($p < 0.05$ or 0.01, Table 4). No histopathological differences in liver and mammary glands were observed between +/+ and +/*fa* rats fed basal or 10% corn oil mixed diet for 5 weeks (data not shown). In serum biochemistry, glucose concentration ($p < 0.01$) showed significantly lower values in +/*fa* rats as compared to +/+, but TG showed lower values without statis-

Table 4. Experiment 2; final body and liver weights

Genotype and diet		N	Body weight (g)	Liver weight (g)	Relative liver weight (g/100g b.w.)
+/+	Basal diet	5	229 ± 10[a]	8.2 ± 1.0	3.6 ± 0.3
	10% Corn oil diet	6	233 ± 12	8.4 ± 0.7	3.6 ± 0.2
+/*fa*	Basal diet	14	222 ± 10	7.7 ± 0.6	3.5 ± 0.2
	10% Corn oil diet	15	235 ± 9[ss]	7.8 ± 0.2[##]	3.3 ± 0.1[#]

N: No. of animals. [a]: Mean ± S.D.
[#,##]: Significantly different from +/+ 10% Corn oil diet at $p < 0.05$ and 0.01, respectively (Dunnett's multiple test)
[ss]: Significantly different from +/*fa* basal diet at $p < 0.01$ (Dunnett's multiple test)

Table 5. Experiment 2; serum biochemistry

Genotype and diet		N	Glucose (mg/dl)	Triglyceride (mg/dl)	Total cholesterol (mg/dl)	Insulin (ug/l)
+/+	Basal diet	5	179 ± 24[a]	346 ± 90	92 ± 20	1.9 ± 1.1
	10% Corn oil diet	6	172 ± 15	233 ± 77	98 ± 6	1.9 ± 0.8
+/*fa*	Basal diet	14	153 ± 13**	239 ± 100	86 ± 11	2.0 ± 0.9
	10% Corn oil diet	15	162 ± 15	176 ± 85	84 ± 10	1.7 ± 0.9

N: No. of animals. [a]: Mean ± S.D.
**: Significantly different from +/+ basal diet at $p < 0.01$ (Dunnett's multiple test)

Table 6. Experiment 2; leptin levels in serum and adipose tissue

Genotype and diet		N	Serum leptin (ng/ml)	Adipose tissue leptin (ng/g)
+/+	Basal diet	5	0.2 ± 0.1[a]	3.8 ± 1.0
	10% Corn oil diet	6	0.5 ± 0.3	2.6 ± 0.7
+/*fa*	Basal diet	14	0.3 ± 0.2	4.7 ± 2.2
	10% Corn oil diet	15	0.5 ± 0.3*	7.7 ± 1.5*,[##]

N: No. of animals. [a]: Mean ± S.D.
*: Significantly different from +/*fa* basal diet at $p < 0.05$ (Dunnett's multiple test)
[##]: Significantly different from +/+ 10% Corn oil diet at $p < 0.01$ (Dunnett's multiple test)

tical significance in +/*fa* rats with and without 10% corn oil mixed feeding as compared to their +/+ counterparts (Table 5). Insulin concentrations showed similar values among all the groups, but T-Cho concentration showed lower in +/*fa* than +/+ with 10% corn oil feeding (Table 5). Without 10% corn oil feeding, serum and adipose tissue leptin levels in +/*fa* showed a non-significant tendency for elevation than in +/+. With 10% corn oil feeding, serum and adipose tissue leptin levels were significantly increased in +/*fa* than in +/*fa* with basal diet ($p < 0.05$). Moreover, 10% corn oil feeding increased adipose tissue leptin level in +/*fa* than in +/+ ($p < 0.01$, Table 6).

With real-time RT-PCR analysis in adipose tissue, increased tendencies for leptin and TNF-α mRNA expression and opposite decrease of VEGFA were observed with the 10% corn oil diet in both genotypes (Fig. 2). Regarding TNF-α mRNA expression, +/*fa* rats showed higher tendencies regardless of the diet (Fig. 2b). Expression of aromatase mRNA was detected in ovaries as a positive control, but not in adipose tissue and livers of either +/*fa* and +/+ rats (data not shown).

DISCUSSION

The present investigation of female heterozygous lean Zucker rats in comparison with *fa*/*fa* and +/+ animals pointed to the potential of heterozygous lean Zucker rat as a possible new leptin-related mammary carcinogenesis

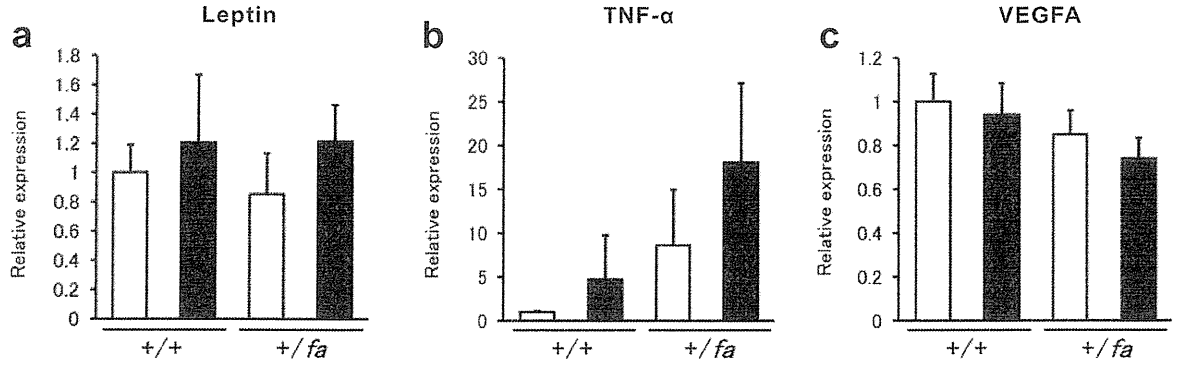

Fig. 2. Experiment 2; leptin (a), TNF-α (b) and VEGFA (c) mRNA expression in adipose tissue of female Zucker rats fed 10% corn oil for 5 weeks. Tendencies for slight increase of leptin and TNF-α expression and decrease of VEGFA were observed on feeding the 10% corn oil diet with both genotypes, but TNF-α expression showed higher tendencies in +/*fa* rats regardless of the diet. mRNA expression was normalized to the expression level of a housekeeping gene, hypoxanthine-guanine phosphoribosyltransferase. Basal diet = open bar, 10% corn oil = closed bar. n = 3-5 (basal diet, +/+), 4-6 (10% corn oil, +/+), 10-14 (basal diet, +/*fa*), 14-15 (10% corn oil, +/*fa*). Values were set at 1 in +/+ basal diet group and expressed as mean ± S.E. relative values.

model. While fatty *fa/fa* rats show dramatically high values for serum insulin, and serum and adipose tissue leptin, they have only few TEBs at around 7 weeks of age, when rats are reported to be most sensitive to carcinogens targeting the mammary gland (Russo *et al.*, 1979). In contrast, lean +/*fa* rats feature normal mammary gland development. Corn oil-supplemented diet increased the serum leptin level. Interestingly, the increase of the leptin level in adipose tissue by 10% corn-oil diet was only observed in +/*fa* rat. TNF-α mRNA expression in+/*fa* was higher than in +/+ and further increased with corn-oil diet. All these results suggested female lean +/*fa* rats may be a potential model for investigation of mammary carcinogenesis in which leptin and TNF-α are the major related factors.

Epidemiologically mammary cancer has been shown to be associated with obesity in postmenopausal women (Calle and Thun, 2004) and hyperleptinemia is also recognized as a risk factor (Wu *et al.*, 2009). Fatty Zucker rats with hyperleptinemia have been widely used as animal obesity model which mimics human obesity and the metabolic syndrome, and recently mammary gland carcinogenicity was investigated with 7,12-dimethylbenz(*a*) anthracene (DMBA) or *N*-methyl-*N*-nitrosourea (MNU)-treated Zucker rat models. Hakkak *et al.* (2005, 2007) reported that DMBA administration by gavage at 50 days old at 65 mg/kg body weight caused more mammary tumors in female obese Zucker (*fa/fa*) rats than their lean (+/*fa* or +/+) siblings. In this model, it is uncertain which obese-related parameters, e.g., hyperinsulinemia, hyperleptinemia or hyperlipidemia, affected the mammary carcinogenesis. In contrast, Lee *et al.* (2001) indicated that no increase in susceptibility with MNU at doses of 37.5 or 20 mg/kg body weight administered to fifty-day-old female lean (+/*fa* or +/+) or obese Zucker (*fa/fa*) rats. The controversial results may be partially due to the dose of administered carcinogen based on the body weight and poor development of mammary glands with low numbers of TEBs in homozygous obese Zucker rats, as shown in Fig. 1. Scant epithelial development in mammary glands were also known in non-transgenic genetically obese leptin-deficient (*ob/ob*) and genetically obese leptin receptor-deficient (*db/db*) mice as compared with their lean counterparts (Hu *et al.*, 2002). Impaired development of mammary glands have been described in transgenic *TGF-α/ob/ob* mice (Cleary *et al.*, 2003) and high fat diet-dependent nulliparous nonpregnant obese mice (Kamikawa *et al.*, 2009). Leptin-dependent inhibition of cell proliferation has been reported in noncancerous mouse mammary epithelial cell line (Baratta *et al.*, 2003; Motta *et al.*, 2007).

It has been also reported that the reason for the poorly developed mammary gland in obese might be abnormal endogenous steroid production rather than hyperleptinemia (Marin Bivens and Olster, 1997). *fa/fa* zucker rats also show delayed vaginal opening, subsequent abnormal estrous cyclicity, undeveloped uteri and lack of deciduomata formation (Saiduddin *et al.*, 1973) as well as

abnormal estrous cycles (Marin Bivens and Olster, 1997). These facts suggest that young *fa/fa* zucker rats may have disadvantage as a mammary carcinogenesis model in aspect s of abnormal development of mammary glands and hormone environment. Therefore, the model in which the level of leptin can be effectively controlled by some exogenous factor such as diet might be a better one. In the present study, the increase of adipose tissue leptin by corn-oil diet was more evident in +/*fa* than +/+. Maher *et al.* (1996) also showed that adipose tissue leptin levels was significantly higher in fat pads of +/*fa* compared to wild type rats and +/*fa* rats fed high-fat diet showed an additional two-fold increase in leptin levels compared to wild type rats on the same diet.

It was reported that adipose tissues of the obese *fa/fa* Zucker rat expressed high level of TNF-α mRNA as compared to lean +/+ or +/*fa* (Hotamisligil *et al.*, 1993). In the present study expression of TNF-α mRNA in the adipose tissue tended to be higher in +/*fa* Zucker rats than +/+ and further increased by the 10% corn oil diet, but there was no statistical significances presumably due to small number of +/+ and/or wide variability. TNF-α stimulates the release of preformed leptin from human mature adipocytes and differentiated preadipocytes (Zhang *et al.*, 2000), and has the ability to promote tumor progression and cancer cell dissemination (Montesano *et al.*, 2005). TNF-α is synthesized and secreted from macrophage as well as adipocyte (Kern *et al.*, 1995; Weisberg *et al.*, 2003). Mammary glands of a diet induced obese mouse model harbored more infiltrating macrophages (Kamikawa *et al.*, 2009), while in the present study, increased macrophage infiltration was not observed in +/*fa* with or without the 10% corn oil diet (data not shown). Taken together, our results suggest that TNF-α expression may be stimulated by leptin-rich adipocyte rather than macrophage around mammary glands in +/*fa* and further by the 10% corn oil diet to promote leptin secretion and mammary carcinogenesis. There were no significant differences in VEGFA mRNA expression adipose tissue between our +/*fa* and +/+ rats and links with leptin gene expression are not clear (Hausman and Richardson, 2004).

Expression level of leptin mRNA in adipose tissue of 12-week-old +/*fa* rats did not show statistically significant differences from those of +/+ rats (Fig. 2a), while that of leptin protein in adipose tissue of +/*fa* fed with 10% corn oil was significantly higher than those of +/+ rats (Table 6). Our present data suggested that differences in translation efficiency, stability and efficient usage of leptin protein might be related to inconsistency of leptin mRNA expression and protein levels in +/*fa* rats. In addition, the adipose tissue leptin level in 12 week-old females +/*fa* is over 3 times higher than those of 7-week-old +/+ in the present study. A previous study indicated that adipose tissue mRNA levels for leptin were higher in +/*fa* rats than +/+ rats at 10-days of age (Zhang *et al.*, 1997). Leptin level in serum and leptin mRNA expression in adipose tissue in Wistar rats was increased with age (Oliver *et al.*, 2001).

Fasting serum glucose, TG and insulin were not changed in +/*fa* compared to +/+ in many studies (Phillips and Cleary, 1994; Schwarzer *et al.*, 1997; Zhang *et al.*, 1997). Glucose and TG concentration showed lower values with and without statistical significance, respectively, in experiment 2, but not in experiment 1. Conflicting results may be partially explained by that animals were sacrificed without overnight fasting, because serum glucose and TG levels are easily affected by food consumption. On the other hand, T-Cho concentration showed lower in +/*fa* than +/+ with 10% corn oil, which may be due to cholesterol elimination promoted by increased leptin (VanPatten *et al.*, 2001). Period of 5 weeks for diet fat supplementation is apparently shorter than that necessary for mammary carcinogenesis, but it is considered enough to examine the effects of high fat diet on the factors related to mammary carcinogenesis (Flachs *et al.*, 2006).

In conclusion, these results suggests that +/*fa* rats may be a useful model for investigation of mammary carcinogenesis in which leptin and TNF-α are the major related factors.

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REFERENCES

Alonso, A., Fernandez, R., Moreno, M., Ordonez, P., Diaz, F. and Gonzalez, C. (2007): Leptin and its receptor are controlled by 17beta-estradiol in peripheral tissues of ovariectomized rats. Exp. Biol. Med. (Maywood), **232**, 542-549.

Anubhuti and Arora, S. (2008): Leptin and its metabolic interactions: an update. Diabetes. Obes. Metab., **10**, 973-993.

Balkwill, F. (2009): Tumour necrosis factor and cancer. Nat. Rev. Cancer, **9**, 361-371.

Baratta, M., Grolli, S. and Tamanini, C. (2003): Effect of leptin in proliferating and differentiated HC11 mouse mammary cells. Regul. Pept., **113**, 101-107.

Bartella, V., Cascio, S., Fiorio, E., Auriemma, A., Russo, A. and Surmacz, E. (2008): Insulin-dependent leptin expression in breast cancer cells. Cancer Res., **68**, 4919-4927.