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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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.

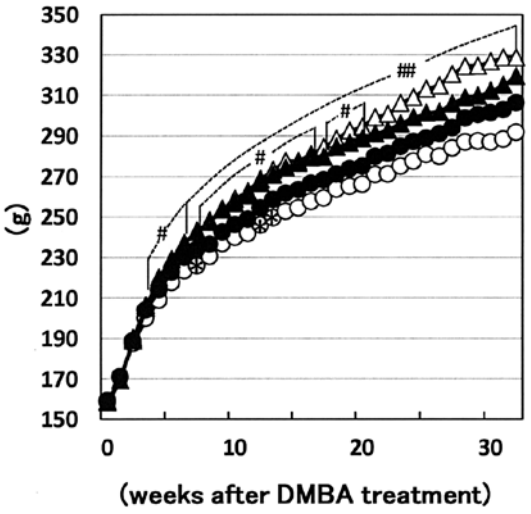

Figure 1. Body weight curves. Open circles, +/+-basal diet; open triangles, +/+-10% corn oil diet; closed circles, +/*fa*-basal diet; closed triangles, +/*fa*-10% corn oil diet. *P<0.05 vs. +/+-basal diet (difference with the genotype basis); #P<0.05, ##P<0.01 vs. +/+-basal diet or +/*fa*-basal diet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

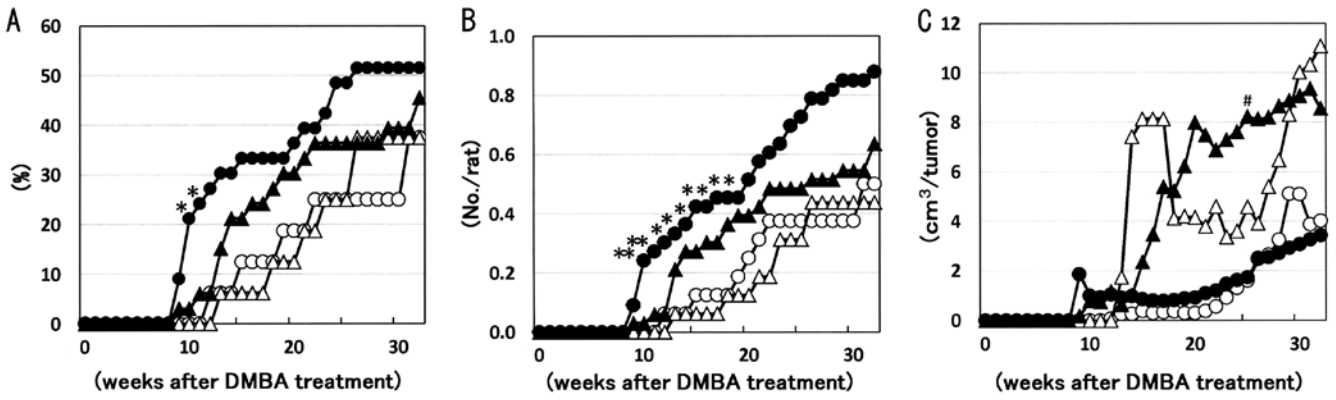

Figure 2. Sequential changes in palpable mammary carcinomas. (A) Cumulative incidence of rats with carcinomas; (B) cumulative mean number of carcinomas per rat (multiplicity); (C) cumulative mean volume of nodule/mass of carcinomas. Open circles, +/+-basal diet; open triangles, +/+-10% corn oil diet; 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.

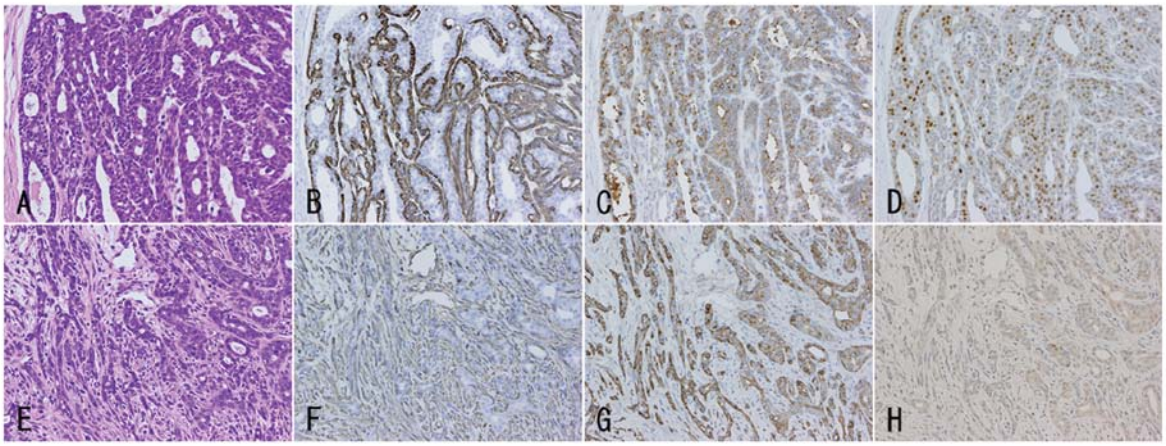

Figure 3. Histopathology and immunohistochemistry of mammary carcinomas. (A-D) A well differentiated carcinoma without distinct atypia in a +/+ rat fed basal diet; (E-H) a moderately/poorly differentiated carcinoma with atypia in a +/*fa* rat fed basal diet. (A and E) H&E. Immunohistochemistry for (B 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.

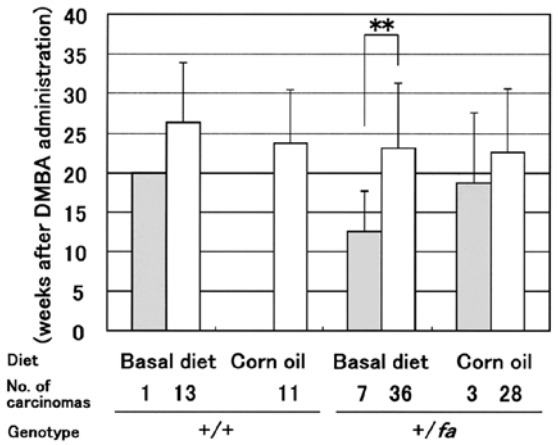

Figure 4. Latency of sub-classified mammary carcinomas based on the morphological phenotypes. Gray bars, moderately/poorly differentiated carcinoma with atypia; open bars, well-differentiated carcinoma without distinct atypia. **P<0.05.

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

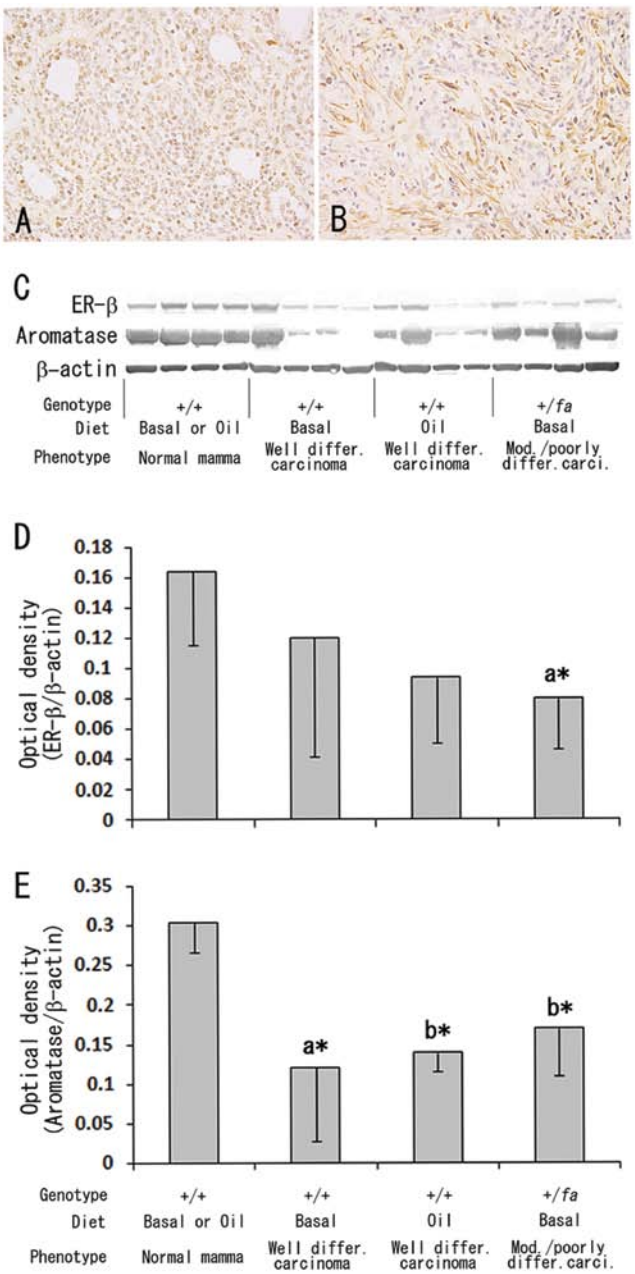

Figure 5. Expression of estrogen receptor (ER) β and aromatase in mammary carcinomas. Immunohistochemistry for (A) ER β, showing nuclear positivity in carcinoma cells of a well differentiated carcinoma in a +/+ rat fed basal diet; (B) aromatase, showing cytoplasmic positivity in presumed myoepithelial 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.

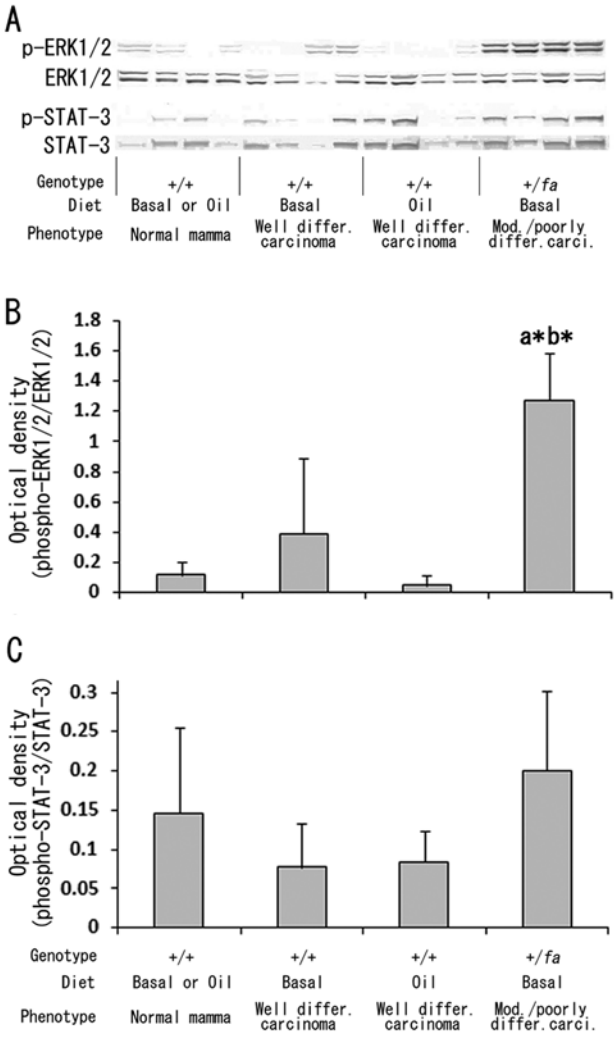

Figure 6. Phosphorylation levels of extracellular signal-regulated kinase (ERK)1/2 and signal transducer and activator of transcription (STAT)3 in mammary carcinomas. Western blotting for phospho-ERK1/2, ERK1/2, phospho-STAT3 and STAT3 (A) and semi-quantitative optical density of phospho-ERK1/2 (B) and 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.

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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.