

early-stage tumors, the tumor size T factor may need to be adjusted from total tumor size to only the size of the invasive component. This needs to be tested radiologically and pathologically by comparing survival according to total tumor size (GGO plus solid components by CT versus invasive versus in situ/lepidic components pathologically) compared with analysis only by the size of the solid or invasive component by CT and pathology examinations, respectively.

4. For multiple lung adenocarcinomas, comprehensive histologic subtyping can help in distinguishing intrapulmonary metastasis versus synchronous or metachronous primaries.¹⁰² The role of molecular testing in this setting is promising but needs further study.³³¹

Many of these concepts need to be tested vigorously in the next 5 years in both early- and advanced-stage lung adenocarcinoma to determine whether they are robust enough to warrant changes in the 8th Edition TNM classification.

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Aberrant Expression and Phosphorylation of 4E-BP1, a Main Target of mTOR Signaling, in Rat Mammary Carcinomas: An Association with Etiology

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Abstract. *Background/Aim:* Breast cancer is a heterogeneous disease. Animal studies indicate that this heterogeneity is caused, in part, by the type of carcinogen which causes this disease. Recently, molecular-targeted drugs, such as rapamycin, are also reported to be heterogeneous in their therapeutic effects on breast tumors. The aim of this study was to clarify the activity of mammalian target of rapamycin (mTOR), as determined by the phosphorylation status of eukaryotic translation initiation factor 4E-binding protein 1 (4E-BP1), in rat mammary carcinomas induced by several carcinogens to see if it is associated with the carcinogen species. *Materials and Methods:* The expression level of 4E-BP1 protein in its phosphorylated (Thr37/46) and unphosphorylated forms was assessed by Western blotting and immunohistochemistry in Sprague-Dawley rat mammary carcinomas induced by γ -rays, carbon ions, 1-methyl-1-nitrosourea (MNU), 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP), and γ -rays combined with MNU or PhIP, and in normal mammary glands. *Results:* 4E-BP1 was composed of at least five isoforms whose expression varied among the carcinomas. Interestingly, loss of their expression, which has not been described previously, was observed in 7 out of 56 carcinomas (13%) regardless of the carcinogen used. Phosphorylation at Thr37/46 of 4E-BP1 was detected in the largest two isoforms in most carcinomas, but in smaller isoforms in carcinomas induced by γ -rays plus PhIP.

Quantitative analysis revealed a significant decrease in phosphorylated 4E-BP1 levels in the carcinomas induced by MNU alone or MNU combined with γ -rays. Conclusion: Expression of 4E-BP1 isoforms varied among rat mammary carcinomas, their phosphorylation level being low in MNU-induced carcinomas, suggesting an association of mTOR activity with cancer etiology.

In modern industrialized societies, people are exposed to various carcinogens. Epidemiological studies and animal experiments have demonstrated that the breast is one of the organs which is highly sensitive to carcinogens, such as ionizing radiation and alkylating agents (1, 2). The rat model of mammary carcinoma is widely used for *in vivo* research as an alternative to human breast carcinomas because of its similarities to human breast cancer in pathogenesis, including its histopathology and hormone dependence (3-5). Using animal models, mammary tumors induced by ionizing radiation and chemical carcinogens have been shown to exhibit specific genomic aberrations [reviewed in (6)]. For example, it has been reported that an activating mutation in codon 12 of the H-ras gene is frequently observed in rat mammary carcinomas induced by 1-methyl-1-nitrosourea (MNU), but not in those induced by ionizing radiation (7, 8). In addition, several loss of heterozygosity (LOH) regions have been found in 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP)-induced rat mammary carcinomas (9, 10), whereas LOH is a rare event in carcinomas induced by MNU and ionizing radiation (11, 12). By microarray analysis, etiology-specific gene expression profiles have been reported for mammary carcinomas induced by several chemical carcinogens including PhIP, 7,12-dimethylbenz[a]anthracene (DMBA), 2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline, 4-aminobiphenyl and MNU (13, 14). These results suggest a difference of carcinogenic pathways in mammary carcinomas by different etiologic factors.

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The mammalian target of rapamycin (mTOR) pathway controls cell growth and proliferation by modulating protein translation through phosphorylation of the eukaryotic translation initiation factor 4E-binding protein 1 (4E-BP1) and the ribosomal protein S6 kinase (15). Molecular-targeted drugs are becoming increasingly important in cancer therapy (16). Among them, rapamycin and its analogs have been a focus for their antitumor effect by inhibition of the mTOR pathway (17). These agents have shown a promising antitumor effect in several types of cancer, including breast cancer (18). However, the sensitivity to rapamycin and its analogs varies among breast tumors (19, 20) and the cell lines studied (21, 22), which is possibly due to the different expression and phosphorylation of the members of the mTOR pathway. Activation of mTOR is influenced by nutrition, such as levels of amino acid and glucose, growth factor stimulation and oxygen concentration (23, 24). Recently, signaling pathways linking to DNA damage response were reported to regulate the activity of mTOR signaling (25, 26).

In a previous study, we conducted a microarray study of rat mammary carcinomas and observed that a subset of them expressed aberrant levels of several genes involved in the mTOR pathway, including that for 4E-BP1 (27). In order to reveal mTOR activity in rat mammary carcinomas and its relation to carcinogen type, we examined the protein expression levels of 4E-BP1 in its phosphorylated and unphosphorylated forms using carcinomas induced by ionizing radiation (γ -rays and carbon ions), chemical carcinogens (MNU and PhIP) and the combination of γ -rays and MNU or PhIP.

Materials and Methods

Induction of rat mammary carcinomas. Histopathologically confirmed rat mammary carcinoma samples were used from previous animal experiments (Table I) (7, 8, 27, and an unpublished experiment). Briefly, female Sprague-Dawley rats (Clea Japan, Tokyo, Japan) were divided into 7 groups at 7 weeks of age: control, γ -irradiation, carbon ion irradiation, MNU treatment, PhIP treatment, and the combined treatment of γ -rays and MNU or PhIP. For γ -irradiation, rats were whole-body irradiated from a ^{137}Cs source (Nordion, Ottawa, Canada) at a dose rate of 0.63 Gy/min. For carbon ion irradiation, rats were irradiated using a 290 MeV/u carbon ion beam with a spread-out Bragg peak (linear energy transfer: 40-90 keV/ μm) generated from the HIMAC synchrotron at the National Institute of Radiological Sciences (NIRS), Japan. The total radiation dose was 1.0 Gy for γ -rays and 0.5 Gy for carbon ions. MNU (Nacalai Tesque, Kyoto, Japan) was injected once intraperitoneally (40 mg/kg body weight). PhIP (provided by Dr. K. Wakabayashi) was given by forced oral administration for five consecutive days, followed by two days without treatment, and then for five additional consecutive days (40 mg/kg body weight/day). In the combined treatment groups, rats were treated with MNU or PhIP at three days after γ -irradiation. Rats were fed an AIN-76A diet containing 23.5% corn oil (Clea Japan). In the present study, tumors

collected between 35 and 92 weeks of age were used. All animal experiment protocols were in accordance with the Institutional Guidelines for the Care and Use of Laboratory Animals of NIRS.

Western blotting. Mammary carcinoma and normal mammary gland tissues were minced and homogenized in lysis buffer (Cell Signaling Technology, Denver, CO, USA) containing phenylmethanesulfonyl fluoride, using a MixerMill 300 machine (Qiagen, Hilden, Germany). Protein concentrations were determined using the bicinchoninic acid method (BCA Protein Assay kit; Pierce, Rockford, IL, USA). Sample proteins were dissolved in sample buffer containing 65 mM Tris/HCl at pH 6.8, 0.1 mM sodium dodecylsulfate (SDS), 10% glycerol, 5% 2-mercaptoethanol, and 0.125 mM bromophenol blue. The samples were denatured by boiling at 100°C for 5 min before loaded onto gels. Thirty micrograms of protein was electrophoresed on 10 or 15% polyacrylamide gels containing SDS, and the proteins were transferred onto a polyvinylidene difluoride membrane (Millipore, Bedford, MA, USA). Anti-phospho-4E-BP1 (Thr37/46), anti-4E-BP1 (Cell Signaling Technology) and anti- β -actin (Sigma-Aldrich, St. Louis, MO, USA) were used as primary antibodies. Notably, Western blotting with the anti-4E-BP1 antibody detects multiple bands, representing bands generated by differential phosphorylation (28, 29). The protein bands were visualized by an alkaline phosphatase-conjugated chemiluminescence method using the ECL-Plus Western Blotting Detection system (GE Healthcare, Tokyo, Japan), and images were captured using a LAS-3000 image analyzer equipped with a charge-coupled device camera (Fujifilm, Tokyo, Japan). The intensity of the band was analyzed by using Image-Gauge software (Fujifilm). A reference sample was consistently loaded in every experiment and used for correction of possible variation among different blots. For statistical analysis, Mann-Whitney's *U*-test was performed to compare the levels of relative phosphorylated 4E-BP1 protein in the carcinomas with those in the normal mammary glands using StatMate III software (Atms, Tokyo, Japan). $P < 0.05$ was considered statistically significant.

Immunohistochemical staining. Immunohistochemistry was carried out on formalin-fixed, paraffin-embedded tissue sections (2- to 4- μm thick), dewaxed with *d*-limonene, and rehydrated through a series of ethanol concentrations. Antigen retrieval was performed by autoclaving the sections at 120°C for 20 min in 10 mM sodium citrate buffer (pH 6.0). To inactivate endogenous peroxidase, samples were submerged in 0.3% hydrogen peroxide/methanol for 15 min. The sections were blocked in Protein Block Serum-Free solution (Dako Cytomation, Carpinteria, CA, USA) containing 10% normal goat serum (Cedarlane Laboratories, Ontario, Canada), and then incubated with primary antibody against phospho-4E-BP1 overnight at 4°C. Nuclei were visualized with hematoxylin. In addition, hematoxylin and eosin (HE)-stained sections were made according to standard procedure.

Results and Discussion

We first examined the expression of 4E-BP1 protein in normal mammary glands and mammary carcinomas. Western blot analysis using antibody against 4E-BP1, which detected both phosphorylated and unphosphorylated proteins, revealed there to be at least five isoforms of 4E-BP1. Normal mammary glands showed fairly stable

Table I. Classification of rat mammary carcinomas.

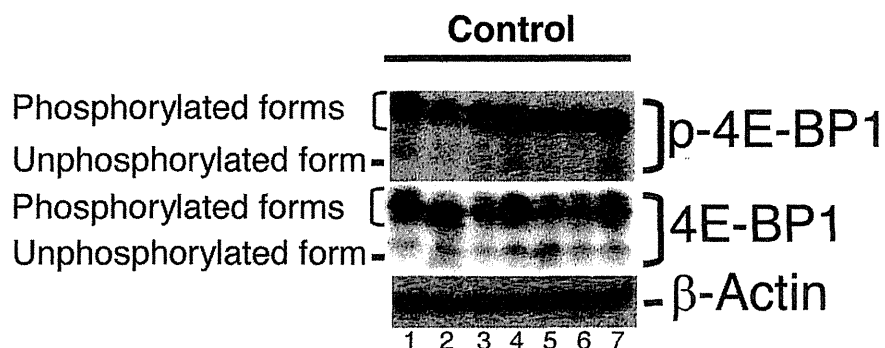
Carcinogen	Lane no.	Body weight (g)	Carcinoma weight (g)	p-4E-BP1 level ^a	
γ-Rays	8	519	10.3	1.41	
	9	558	21.5	Loss	
	10	459	5.8	1.85	
	11	558	5.9	1.16	
	12	610	40.7	0.32	
	13	420	14.8	1.28	
	14	443	0.9	1.84	
	15	330	23.4	1.12	
	16	385	25.4	0.77	
	17	433	5.4	0.99	
	Carbon ions	18	532	36.1	1.52
		19	663	0.9	1.02
		20	535	1.6	1.45
		21	433	5.5	1.63
		22	355	20.7	0.95
		23	397	35.1	1.22
		24	425	99.7	1.19
25		425	2.3	2.53	
26		447	0.8	0.28	
27		385	67.5	0.07	
MNU		28	342	14.0	Loss
		29	562	4.1	0.64
		30	351	31.6	Loss
		31	449	2.2	0.79
		32	567	173.5	0.78
		33	587	4.5	1.06
		34	566	6.7	0.87
	35	562	9.7	Loss	
	36	558	3.5	0.77	
	37	573	28.0	0.88	
	γ-Rays + MNU	38	429	3.1	0.39
		39	373	2.4	0.44
		40	429	1.2	0.60
		41	518	3.0	0.75
		42	793	14.8	0.66
		43	585	27.2	0.65
		44	562	34.7	0.44
45		562	19.2	0.66	
46		416	28.1	0.62	
47		518	14.5	0.60	
PhIP		48	536	4.2	1.20
		49	641	16.2	0.86
		50	565	0.5	0.91
		51	453	1.3	1.07
		52	737	20.9	0.96
		53	827	7.4	Loss
		54	671	1.9	0.40
	γ-Rays + PhIP	55	438	9.1	2.13
		56	528	3.9	1.36
		57	682	2.8	1.60
		58	682	14.1	Loss
		59	633	2.0	2.04
		60	438	7.9	1.79
		61	586	2.5	0.89
		62	650	183.0	0.30
		63	638	2.0	0.86
		64	682	0.8	Loss

MNU: 1-Methyl-1-nitrosourea; PhIP: 2-amino-1-methyl-6-phenylimidazo-[4,5-b]pyridine. ^aRelative p-4E-BP1 level expressed in arbitrary units of the Western blotting experiment as described in the Materials and Methods. The mean value of normal mammary glands was 1.09 (SE=0.058, n=7).

expression of the largest two isoforms of 4E-BP1 (Figure 1A). In contrast, mammary carcinomas induced by ionizing radiation and chemical carcinogens showed varying expression of 4E-BP1 proteins (Figure 1B). For example, a subset of carcinomas induced by γ-rays expressed the largest two isoforms (lane 14); another subset, the second largest two (lanes 8, 10, 13 and 16); and still another, the third largest (lane 12); one carcinoma expressed a trace amount of the second largest isoform (lane 15). Expression of the smallest two isoforms was occasionally observed in carcinomas induced by MNU (lanes 29, 31, 32 and 36) and γ-rays plus MNU (lanes 38, 42 and 46). In addition, we observed a loss of 4E-BP1 protein from some carcinoma samples (*e.g.* lanes 9, 28, and 53 in Figure 1B), which was the first observation for the tumors.

4E-BP1 is a repressor of translation initiation (15). Activation of mTOR phosphorylates Thr37/46 of 4E-BP1, which inactivates the function of the protein. On its phosphorylation, eukaryotic translation initiation factor 4E (eIF4E), the rate-limiting translation initiation factor, is released from 4E-BP1 and assembles with other translation initiation factors to initiate protein translation. When 4E-BP1 is hypophosphorylated, it binds to and inhibits eIF4E (15). Therefore, in those carcinomas with loss of 4E-BP1 protein, eIF4E is considered to be released and contribute to protein translation, regardless of the activation of mTOR. It has been reported that reduced expression of 4E-BP1 is associated with enhanced cell proliferation and survival (30), implying an aggressive phenotype of these tumors without 4E-BP1. On the other hand, the antibody against phospho-4E-BP1 reacted mainly with large isoforms (Figure 1). Exceptionally, strong signals indicating a small isoform with phosphorylation at Thr37/46 were detected in one carbon ion-induced carcinoma (lane 25) and four carcinomas induced by a combination of γ-rays and PhIP (lanes 55, 57, 59 and 60). These isoforms appeared to be characteristic of PhIP-induced carcinomas. Interestingly, large isoforms of 4E-BP1 in three carcinomas (lanes 12, 27 and 62) were not phosphorylated at Thr37/46, suggesting inactivation of mTOR. Possibly, other kinases are implicated, such as cyclin-dependent kinase 1, ataxia telangiectasia mutated, phosphoinositide-3-kinase/v-akt murine thymoma viral oncogene homolog and extracellular signal-regulated kinases 1 and 2 (31-36). Moreover, we noticed that the anti-phospho-4E-BP1 antibody detected immunoreactivity in some carcinomas even when the expression of 4E-BP1 was almost undetectable (*e.g.* lanes 15, 43 and 61 in Figure 1B). This observation may be due to some cross-reactivity of the anti-phospho-Thr37/46 antibody against other 4E-BP family members, 4E-BP2 and 4E-BP3 (15, 37). Still, this possible cross-reactivity may also reflect the overall levels of the 4E-BP family members and, hence, mTOR activity, since all 4E-BP family members are known to be regulated similarly (15).

A Normal mammary glands



B Mammary carcinomas

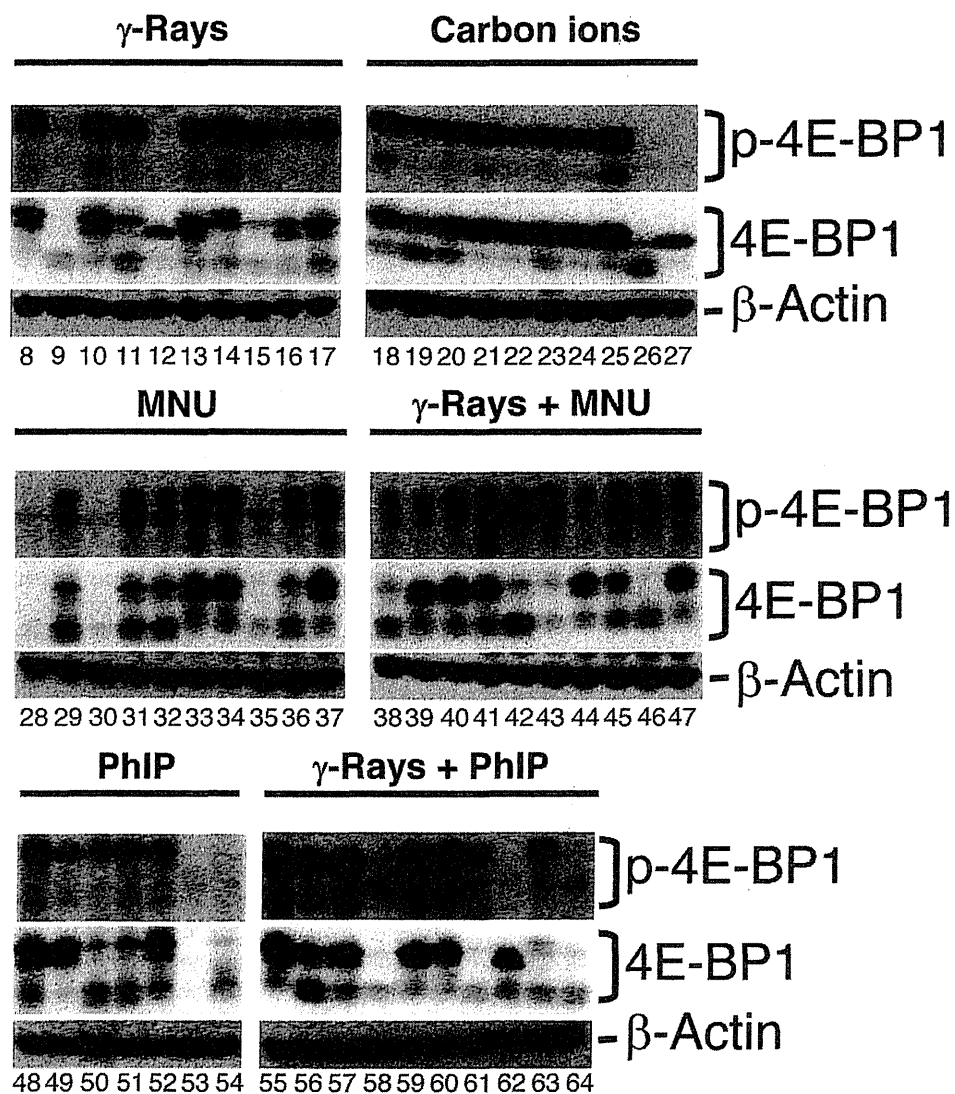


Figure 1. The expression of 4E-BP1 protein in normal mammary gland and mammary carcinomas induced by ionizing radiation and chemical carcinogens in rats. A: Normal mammary glands; B: mammary carcinomas. Antibodies against phospho-4E-BP1 (Thr37/46), total 4E-BP1, and β -actin (as a loading control) were used. Of note, Western blotting with the anti-phospho-4E-BP1 (Thr37/46) and 4E-BP1 antibodies detect multiple bands (indicated on the left in panel A), representing bands generated by differential phosphorylation.

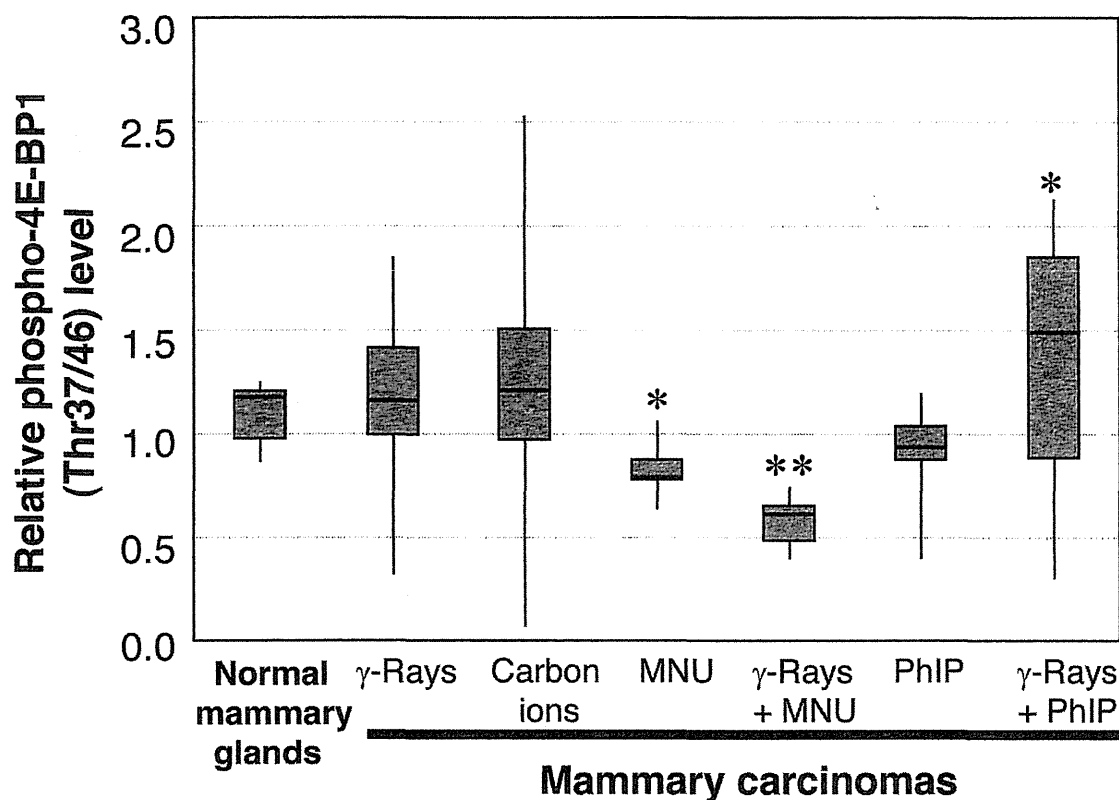


Figure 2. Relative phospho-4E-BP1 (Thr37/46) levels in a panel of rat mammary carcinomas induced by ionizing radiation and chemical carcinogens, as well as in normal mammary glands. To calculate the relative levels of 4E-BP1 protein phosphorylated at Thr37/46, signal intensities of bands were corrected by that of the reference sample. The median values are indicated with a horizontal line in the boxes. The boxes contain the values between the 25th and 75th percentiles. The whiskers extend to the highest and the lowest values. * $p < 0.05$ and ** $p < 0.001$ compared to normal mammary glands.

In order to quantify the activity of mTOR, the relative levels of phospho-4E-BP1 (Thr36/46) were determined for the carcinomas, excluding those with 4E-BP1 protein loss. A quantitative analysis indicated that relative levels of phospho-4E-BP1 protein were significantly decreased in carcinomas induced by MNU and by the combined treatment with γ -rays and MNU (Figure 2). This result was unexpected because carcinomas induced by these agents are known to frequently harbor an activating mutation of the *H-ras* gene (7, 38), which is generally known to stimulate the mTOR signaling pathway (39).

We then examined expression of phospho-4E-BP1 immunohistochemically in mammary carcinoma tissues. Nuclear and cytoplasmic localization of phosphorylated 4E-BP1 was detected in luminal epithelial cells of mammary carcinomas, except for those with loss of 4E-BP1 (representative results are shown in Figure 3). Within a carcinoma, the expression of phospho-4E-BP1 protein was heterogeneous, with positive and negative populations for phospho-4E-BP1, although proliferating cell nuclear antigen expression was observed homogeneously. Mitotic figures

were observed in phospho-4E-BP1-positive cells. It is reported that nuclear phospho-4E-BP1 expression is related to clinicopathological characteristics, including tumor size and lymph node metastasis (40-42). However, we were not able to associate the level of phosphorylation of 4E-BP1 with the size of carcinomas (Table I).

In summary, we clarified that the expression level of 4E-BP1 protein and its phosphorylated forms varied dramatically among rat mammary carcinomas, associated with the etiology. These findings provide a basis for diagnosis of breast cancer for molecular-targeted therapy with mTOR inhibitors and contribute to understanding the mechanism of the resistance to such a therapy.

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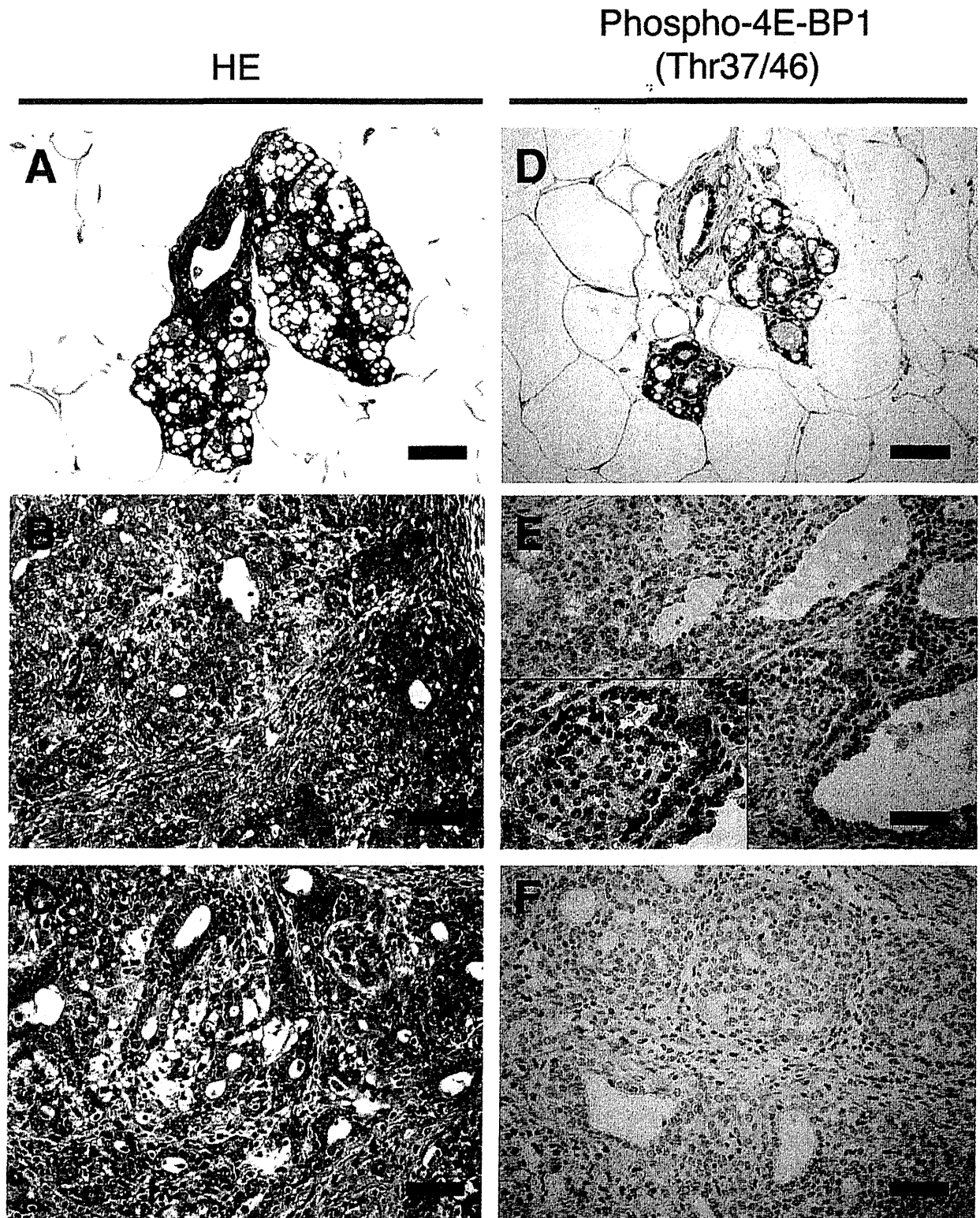


Figure 3. Immunohistochemistry of normal mammary glands and mammary carcinomas for phosphorylated 4E-BP1 (Thr37/46) expression. Histological images of normal mammary glands (A, D) and mammary carcinomas with (B, E) and without (C, F) phospho-4E-BP1 positivity. Samples were stained with HE (A, B, C) or immunostained with phospho-4E-BP1 (Thr37/46) (D, E, F). Original magnification was $\times 40$. Higher magnification ($\times 100$) is shown in the inset figure. Arrows indicate mitotic cells. Scale bar, 50 μm .

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Dysregulation of Gene Expression in the Artificial Human Trisomy Cells of Chromosome 8 Associated with Transformed Cell Phenotypes

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Abstract

A change in chromosome number, known as aneuploidy, is a common characteristic of cancer. Aneuploidy disrupts gene expression in human cancer cells and immortalized human epithelial cells, but not in normal human cells. However, the relationship between aneuploidy and cancer remains unclear. To study the effects of aneuploidy in normal human cells, we generated artificial cells of human primary fibroblast having three chromosome 8 (trisomy 8 cells) by using microcell-mediated chromosome transfer technique. In addition to decreased proliferation, the trisomy 8 cells lost contact inhibition and repopulated after exhibiting senescence-like characteristics that are typical of transformed cells. Furthermore, the trisomy 8 cells exhibited chromosome instability, and the overall gene expression profile based on microarray analyses was significantly different from that of diploid human primary fibroblasts. Our data suggest that aneuploidy, even a single chromosome gain, can be introduced into normal human cells and causes, in some cases, a partial cancer phenotype due to a disruption in overall gene expression.

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Introduction

During cell division, errors in chromosomal segregation result in the loss or gain of chromosomes in daughter cells, which is referred to as aneuploidy. An extra or missing chromosome, known as trisomy and monosomy, respectively, is observed in people with developmental disabilities, mental retardation, and cancer. In addition, various types of cancer have karyotypes with complex numerical aberrations [1–3]. Although increasing evidence that aneuploidy is a hallmark of cancer, the causal relationship between aneuploidy and tumorigenesis remains unclear.

The addition of a single chromosome has been reported to have various transcriptional effects in several cell types [4–6]. An increase in the average transcriptional activity of a trisomic chromosome has been observed in trisomic primary mouse cells, human trisomic colorectal cancer cells, immortalized trisomic mammary epithelial cells, and acute myeloid leukemia (AML) cells (with an additional chromosome 8; derived from an AML patient). One chromosome affected not only the gene expression levels on the trisomic chromosome but also a large number of genes on other diploid chromosomes [5]. Apoptosis-regulating genes were significantly down regulated in AML cells containing an additional chromosome 8 that were derived from an AML patient [6].

Despite increased information about the characteristics of aneuploid human cells, the data reported thus far have been obtained from immortalized or cancer-derived cells. Normal human cells have a limited life span and ultimately enter a non-dividing state called senescence [7,8]. It remains unclear whether aneuploidy renders cells immortal or if immortalization induces aneuploidy in cells. To determine the role(s) that aneuploidy plays in human cancer, it will be indispensable to design artificial aneuploid model cells derived from normal human cells.

Trisomy is a simple model of aneuploidy with the gain of a single chromosome. Trisomy of chromosome 8 is the most commonly observed trisomic chromosomal aberration, as has been demonstrated in fibroblastic/myofibroblastic tumors (Fig. S1) [9–12]. In this study, we used normal human diploid embryonic cells (HE35) as a donor for chromosome transfer, and chromosome 8 was chosen as the introduction chromosome. We succeeded in isolation of multiple clones that chromosome 8 became trisomic (trisomy 8 cells). All of the trisomy 8 cells expressed transformed cell-like phenotypes, such as a loss of contact inhibition, regrowth after senescence, and chromosome instability. The overall gene expression profile, determined by microarray analysis, was significantly different from that of the diploid HE35 cells. Our results suggest that aneuploidy is a key factor in tumorigenesis, as demonstrated by disrupted gene expression.