

## Increased Expression of Cyclin D1, Cyclin E and p21<sup>Cip1</sup> Associated with Decreased Expression of p27<sup>Kip1</sup> in Chemically Induced Rat Mammary Carcinogenesis

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We induced rat mammary tumors in 7-week-old female Sprague-Dawley rats by intragastric administration of 7,12-dimethylbenz(a)anthracene (DMBA), and analyzed by immunohistochemistry the expression of cyclin D1, cyclin E, p21<sup>Cip1</sup>, and p27<sup>Kip1</sup> in carcinomas, atypical tumors, and benign tumors as well as normal mammary glands from the control group. Proliferation status was assessed by immunohistochemistry using bromodeoxyuridine (BrdU). A sequential increase in cyclin D1-, cyclin E-, and p21<sup>Cip1</sup>-positive epithelial cells was observed from normal mammary glands, to atypical tumors, to carcinomas. In contrast, carcinomas showed a significantly lower number of epithelial cells immunoreactive to p27<sup>Kip1</sup> when compared with atypical tumors, benign tumors and normal mammary glands. The immunoreactivities of BrdU, cyclin D1, cyclin E, and p21<sup>Cip1</sup> were positively correlated, whereas that of p27<sup>Kip1</sup> appeared inversely correlated to those of the others. Reverse transcriptase-polymerase chain reaction (RT-PCR) and western blot analysis were also performed to determine the mRNA and protein levels of cyclins and cyclin-dependent kinase inhibitors in tumors and normal mammary glands. The protein levels for cyclin D1, cyclin E and p21<sup>Cip1</sup> in carcinomas and atypical tumors were significantly higher than those in benign tumors, while normal mammary glands showed negligible expression. On RT-PCR, tumors showed higher mRNA levels of cyclin D1 and cyclin E than those of normal mammary glands. Our results suggest that rat mammary carcinogenesis involves increased expression of cyclin D1, cyclin E, and p21<sup>Cip1</sup>, associated with decreased expression of p27<sup>Kip1</sup>.

Key words: Cyclin D1 — Cyclin E — p21<sup>Cip1</sup> — p27<sup>Kip1</sup> — Rat mammary carcinogenesis

DMBA, a lipophilic polycyclic aromatic hydrocarbon, is metabolized by mammary epithelial cells to polar metabolites including epoxides that may be responsible for causing DNA damage.<sup>1</sup> It is known that the administration of DMBA to virgin rats alters the differentiation of terminal end bud to alveolar bud and lobules, inducing instead the sequence of terminal end bud to intraductal proliferation and carcinoma.<sup>2,3</sup>

Cell cycle progression in mammalian cells is regulated by the interactions of cyclins, CDK and CDK inhibitors. Their functions have been analyzed in detail, especially with respect to G1 progression and the G1/S checkpoint. Cyclin D1 binds to and activates its major catalytic partners, CDK4 and CDK6, which can in turn phosphorylate the Rb tumor suppressor protein.<sup>4,5</sup> Gene rearrangement, overexpression and amplification of cyclin D1 have been

reported in a number of human tumors.<sup>6–8</sup> Recent studies have focused on the expression of this protein in animal models of carcinogenesis such as rat mammary tumors,<sup>9</sup> mouse skin tumors,<sup>10,11</sup> rat esophageal tumors,<sup>12,13</sup> and rat urinary bladder tumors.<sup>14</sup> Cyclin E binds to and activates CDK2, which also phosphorylates the Rb protein.<sup>4,5</sup> Amplification and overexpression of cyclin E have been detected in a colorectal carcinoma cell line<sup>15</sup> and breast cancer cells.<sup>16,17</sup> In addition, overexpression of cyclin E has also been found in mouse and rat mammary tumors,<sup>9,18</sup> and rat esophageal tumors.<sup>12</sup> CDKs generally inhibit the cell cycle progression by regulating cyclin-CDK complexes. The Cip/Kip family includes p21<sup>Cip1</sup>, p27<sup>Kip1</sup> and p57<sup>Kip2</sup>.<sup>19,20</sup> These proteins inhibit the kinase activities of pre-activated G1 cyclin E-CDK2, cyclin D-CDK4/6 and other cyclins. The p21<sup>Cip1</sup> gene has been shown to mediate p53 functions<sup>21</sup> and was recently reported to play a role in the differentiation of hematopoietic and hepatoma cells.<sup>22,23</sup> In addition, the overexpression of p21<sup>Cip1</sup> suppressed the proliferation of tumor cells *in vitro*, as well as tumorigenicity *in vivo*.<sup>24</sup> On the other hand, CDK complexes are primed in a noninhibitory state through the cell cycle with a single molecule of p21<sup>Cip1</sup>, and the increase in p21<sup>Cip1</sup> stoichiometry through the

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The abbreviations used are: CDK, cyclin-dependent kinase; CDKI, cyclin-dependent kinase inhibitor; DMBA, 7,12-dimethylbenz(a)anthracene; NMU, N-methyl-N-nitrosourea; BrdU, bromodeoxyuridine; RT-PCR, reverse transcriptase-polymerase chain reaction; HPRT, hypoxanthine-guanine phosphoribosyltransferase; Rb, retinoblastoma.

induction of p21<sup>Cip1</sup> promotes conversion to the inhibitory state.<sup>25)</sup> The expression of p21<sup>Cip1</sup> has been observed to be independent of proliferation in stomach<sup>26)</sup> and colon cancers,<sup>27)</sup> whereas p21<sup>Cip1</sup> positivity in breast cancers correlated with the increased proliferation.<sup>28)</sup> The expression of p21<sup>Cip1</sup> was decreased in rat esophageal<sup>29)</sup> and urinary bladder carcinogenesis,<sup>14)</sup> but there has been no report about the role for p21<sup>Cip1</sup> in carcinogen-induced rat mammary tumor. Down-regulation of p27<sup>Kip1</sup> expression has been observed in interleukin 2-induced T cell proliferation, indicating that p27<sup>Kip1</sup> may play an important role in the negative regulation of cell growth.<sup>30)</sup> In addition, p27<sup>Kip1</sup> plays an important part in repressing tumor development, as previously reported in p27<sup>Kip1</sup> knockout mice.<sup>31)</sup> However, the role of p27<sup>Kip1</sup> has not been examined in animal models of carcinogenesis.

In order to investigate the roles of the proteins related to the G1 cell cycle in mammary carcinogenesis, we analyzed the expression of cyclin D1, cyclin E, p21<sup>Cip1</sup> and p27<sup>Kip1</sup> in DMBA-induced rat mammary tumors by performing immunohistochemistry, western blot analysis and RT-PCR.

## MATERIALS AND METHODS

**Animals and tissue samples** Forty-one-day-old female Sprague-Dawley rats (Kist, Taejun, Korea) were used. Throughout the experiment, all rats were housed in a controlled environment with 12 h light/dark cycle and temperature of 22±2°C, and given a commercial diet and tap water *ad libitum*. After an acclimatization period of 1 week, rats were divided into two groups: 26 rats in the tumor-induction group received an intragastric dose of 10 mg of DMBA (Sigma Chemical Co., St Louis, MO) in 1.0 ml of sesame oil at 48 and 55 days of age, while 12 rats in the control group received the same volume of sesame oil alone. Tumor number, size, and volume were determined semiweekly, beginning 3 weeks after the second administration of DMBA. The total period of observation was 16 weeks, and rats were sacrificed at weeks 7, 10, 13, and 16. Rats were given a single intravenous injection of 0.1 mg/g body weight of BrdU (Sigma Chemical Co.) in 1.0 ml of normal saline into the tail vein 1 h before autopsy. All mammary tumors were dissected. Samples were prepared as follows: half of the tumor was fixed in 10% neutral buffered formalin for histological study and immunohistochemistry, and the other half was immediately frozen in liquid nitrogen and stored at -70°C for western blot analysis and RT-PCR. Some normal mammary glands of the control group were also treated the same way as above and stored until used.

**Pathological analysis** Mammary tumors were histologically classified into carcinoma, atypical tumor and benign tumor based on the criteria of Russo *et al.*<sup>2)</sup> Carcinomas

showed loss of the tubuloalveolar pattern of normal mammary gland, malignant cytological features of tumor cells, invasion of stroma, and prominent stromal responses. Atypical tumors showed intraductal proliferation with the absence of stromal invasion. Benign tumors showed the presence of tubuloalveolar structure mimicking fibroadenoma and tubular adenoma of human breast tumor.

**Immunohistochemistry** Serial sections of 4- $\mu$ m thickness were made and spread on poly-L-lysine-coated slides. Paraffin sections were immersed in three changes of xylene and hydrated using a graded series of alcohols. Antigen retrieval was performed routinely by immersing the sections in 0.01 M citrate buffer (pH 6.0) in a pressure cooker and autoclaving for 15 min. Sections were blocked with normal horse serum at 37°C for 30 min and then incubated with a primary antibody for 1 h at room temperature. Primary antibodies were monoclonal mouse anti-BrdU antibody (DAKO, Santa Barbara, CA) at 1:100 dilution, rabbit polyclonal anti-human cyclin D1 (Upstate Biotechnology, Inc., Lake Placid, NY) at 1:200 dilution, rabbit polyclonal anti-rat cyclin E (Santa Cruz Biotechnology, Santa Cruz, CA) at 1:100 dilution, rabbit polyclonal anti-human p21<sup>Cip1</sup> (Santa Cruz Biotechnology) at 1:200 dilution, and rabbit polyclonal anti-human p27<sup>Kip1</sup> (Santa Cruz Biotechnology) at 1:200 dilution. Staining was achieved with a DAKO LSAB+ kit (DAKO) and developed with 3,3'-diaminobenzidine tetrahydrochloride (Zymed Laboratories, Inc., San Francisco, CA). Sections were counterstained for 5 min with Meyer's hematoxylin and then mounted. Human breast cancer with intense staining for either cyclin D1, cyclin E, p21<sup>Cip1</sup>, or p27<sup>Kip1</sup> was used as a positive control. As a negative control, the primary antibodies were omitted.

**Quantitation of immunohistochemistry** Cells were considered positive for BrdU, cyclin D1, cyclin E, p21<sup>Cip1</sup>, and p27<sup>Kip1</sup> when the clear staining in the nucleus could be identified. Ten fields were randomly selected and the positive epithelial cells were counted. Labeling indices for BrdU, cyclin D1, cyclin E, p21<sup>Cip1</sup> and p27<sup>Kip1</sup> were calculated as percentage values based on the total number of examined cells. At least 1000 epithelial cells were counted throughout the entire tumor and normal mammary gland.

**Western blot analysis** Eight carcinomas, 4 atypical tumors, and 4 benign tumors, as well as normal mammary glands of the control group, were analyzed. Frozen tissue from each tumor and from normal mammary glands was sonicated according to Sgambato *et al.*<sup>9)</sup> The supernatants were assayed for protein content by the Bradford method and stored at -70°C until used. Fifty micrograms of protein extracts was run in a NuPAGE 4-12% Bis-Tris polyacrylamide gel (NOVEX, San Diego, CA) and electroblotted onto a nitrocellulose membrane (BioRad, Richmond, CA) according to the manufacturer's instructions. The blots were then blocked overnight at 4°C and incu-

bated for 2 h at room temperature with the same primary antibodies used for immunohistochemistry at 1:500 dilution. After having been washed with TBS-0.05% Tween 20, the blots were incubated for 45 min at room temperature with the secondary antibody (DAKO) at 1:1000 dilution, washed again, and developed with western blotting chemiluminescence luminol reagent (Santa Cruz Biotechnology) for X-ray film exposure.

**RNA isolation and RT-PCR analysis** Using a High Pure RNA Tissue kit (Boehringer Mannheim, Mannheim, Germany), total cellular RNA was isolated from previously frozen tissues of 8 carcinomas, 4 atypical tumors, and 4 benign tumors, as well as normal mammary glands, according to the manufacturer's instructions. RNA samples were fractionated on formaldehyde gels to check the integrity of 18S and 28S rRNA. RNA concentrations were determined by measuring absorbance at 260 nm and 280 nm on a spectrophotometer. Using a Titan One-tube RT-PCR System (Boehringer Mannheim), reverse transcription of 1 µg of total RNA was performed at 50°C for 30 min and subsequently PCR was carried out using a Perkin-Elmer DNA Thermocycler 2400 (Perkin-Elmer, Norwalk, CT). A housekeeping gene, hypoxanthine-guanine phosphoribosyltransferase was used as an internal control. PCR was carried out by running 40 cycles of denaturation at 94°C for 30 s, annealing for 30 s (at 45°C for cyclin D1 and cyclin E, and at 48°C for HPRT) and extension at 68°C for 40 s, followed by further incubation at 68°C for 7 min. The sequences of primers are listed in Table I.<sup>12, 14)</sup>

**Statistical analysis** The significance of differences between groups was evaluated by use of the Kruskal Wallis test and Mann-Whitney *U* test, and the Pearson correlation coefficient was used to test the strength of association between two parameters, except for BrdU. The significance of correlations between BrdU and the other parameters was evaluated by linear regression. *P*<0.05 was considered significant.

**RESULTS**

**Incidence of tumors and immunohistochemical stain of BrdU** Two tumors, measuring 7.5 and 11 mm in the larg-

est diameter, were found at the 5th week in two rats of the DMBA-treated group. By the end of week 16, 80 mammary tumors had developed in 23 out of the 26 DMBA-treated rats (mean number±standard deviation; 3.08±2.13), but none was found in the control group. Tumors were classified histologically as 47 carcinomas, 25 atypical tumors and 8 benign tumors.

As can be seen in Fig. 1, A and B, as well as Table II, there is a sequential increase in the BrdU-labeled epithelial cells from normal mammary gland, to atypical tumors, to carcinomas (*P*<0.05).

**Expression of cyclin D1 and cyclin E in rat mammary glands and tumors** Nuclear staining for cyclin D1 and cyclin E was observed mainly in the epithelial cells of tumors and normal mammary gland, and slightly in stromal spindle cells, but not in adipose tissue. The number of cyclin D1-positive epithelial cells in carcinomas, was significantly the highest, while the increases in atypical tumors and benign tumors were less pronounced, and the number in normal mammary glands of the control group was the lowest (*P*<0.05) (Fig. 1, C and D; Table II). Western blot analysis showed higher expression of cyclin D1 in carcinomas and atypical tumors compared with benign tumors, while the normal mammary gland was negative (Fig. 2). RT-PCR, however, revealed no significant difference among the histologic types of tumors, although cyclin D1 mRNA appeared scarce in the normal mammary glands (Fig. 3).

A sequential increase was observed in the number of cyclin E-positive epithelial cells from normal mammary gland, to atypical tumors, to carcinomas (*P*<0.05) (Fig. 1, E and F; Table II). Several isoforms of cyclin E which ranged between 28 and 51 kD, were detected on western blots, although the 51 kD isoform was predominant in carcinomas and atypical tumors (Fig. 2). Benign tumors expressed similarly several isoforms of cyclin E, and in one case the 30 kD isoform was predominant (Fig. 2, lane 7 of cyclin E). The expression of 51 kD isoforms in carcinomas and atypical tumors was higher than that in benign tumors, while normal mammary glands showed minimal expression (Fig. 2). Carcinomas showed higher expression of cyclin E mRNA compared with atypical tumors and benign tumors, while normal mammary glands showed insignificant expression (Fig. 3).

**Expression of p21<sup>Cip1</sup> and p27<sup>Kip1</sup> in rat mammary glands and tumors** Nuclear staining for p21<sup>Cip1</sup> was observed mainly in the epithelial cells of tumors, whereas the epithelial cells of normal mammary gland, adipose tissue and stromal spindle cells did not show any staining. The number of p21<sup>Cip1</sup>-positive epithelial cell in carcinomas was significantly the highest and that in atypical tumors and benign tumors was less pronounced, while that in the normal mammary glands was the lowest (*P*<0.05) (Fig. 4, A and B; Table III). p21<sup>Cip1</sup> protein level in carci-

Table I. Sense and Antisense Primers for RT-PCR

Genes	Sequence (5'-3')	Amplified size (bp)	Reference
Cyclin D1	TGGAGCCCCTGAAGAAGAG AAGTGCCTTGTGCGGTAGC	420	12
Cyclin E	CTGGCTGAATGTTTATGTCC TCTTTGCTTGGGCTTTGTCC	380	12
HPRT	CGGGGGACATAAAAGTTAT CCACTTTCGCTGATGACAC	300	

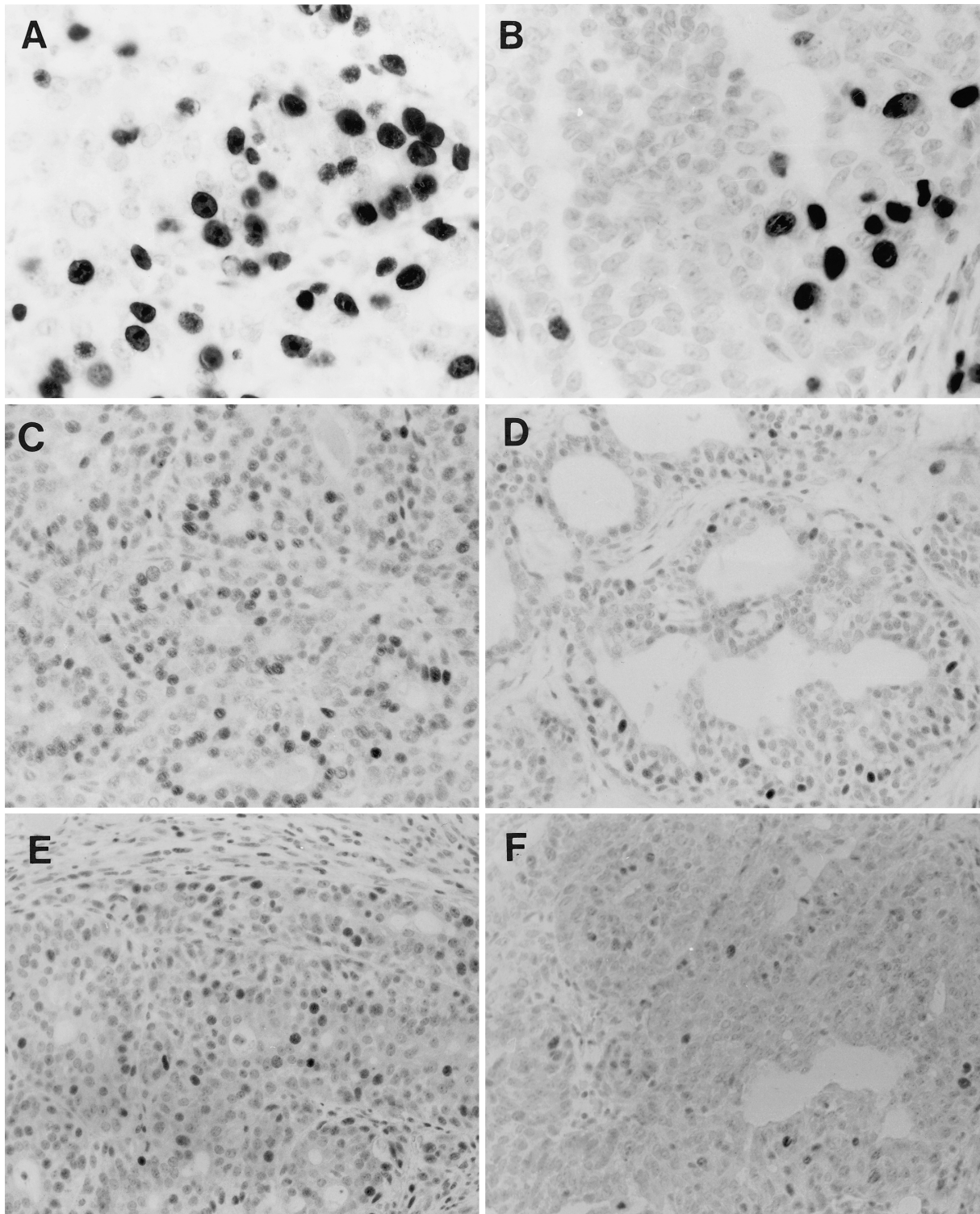


Fig. 1. Immunohistochemical staining of BrdU, cyclin D1, and cyclin E in rat mammary tumors. A, B, BrdU; C, D, cyclin D1; E, F, cyclin E. BrdU-labeled tumor cells in invasive carcinomas (A) are increased compared with atypical tumors (B). Cyclin D1- and cyclin E-positive tumor cells are increased in invasive carcinomas (C, E) compared with atypical tumors (D, F). Magnification: A and B,  $\times 400$ ; C-F,  $\times 200$ .

nomas was also higher than that in atypical tumors and benign tumors, but that in normal mammary glands was negligible (Fig. 2).

Nuclear staining for p27<sup>Kip1</sup> was observed in the epithelial cells of tumors and normal mammary gland as well as adipose tissue and stromal lymphocytes. In contrast, carci-

Table II. Labeling Indices of BrdU, Cyclin D1 and Cyclin E in Tumors and the Normal Mammary Glands of the Control Group

Histologic type	Number of tested tissues	BrdU <sup>d)</sup>	Cyclin D1 <sup>d)</sup>	Cyclin E <sup>d)</sup>
Carcinomas	21	56.91±17.90 <sup>a)</sup>	79.44±11.93 <sup>a)</sup>	45.91±18.19 <sup>a)</sup>
Atypical tumors	12	17.13±13.98 <sup>b)</sup>	61.22±11.17 <sup>b)</sup>	12.48±8.11 <sup>b)</sup>
Benign tumors	3	4.33±4.59	53.43±2.95 <sup>c)</sup>	4.30±7.10
Normal	10	1.11±1.61	5.90±3.44	2.08±2.53

a) *P*<0.05, in comparison with atypical tumors, benign tumors and normal mammary glands of the control group.

b, c) *P*<0.05, in comparison with normal mammary glands of the control group.

d) Percent.

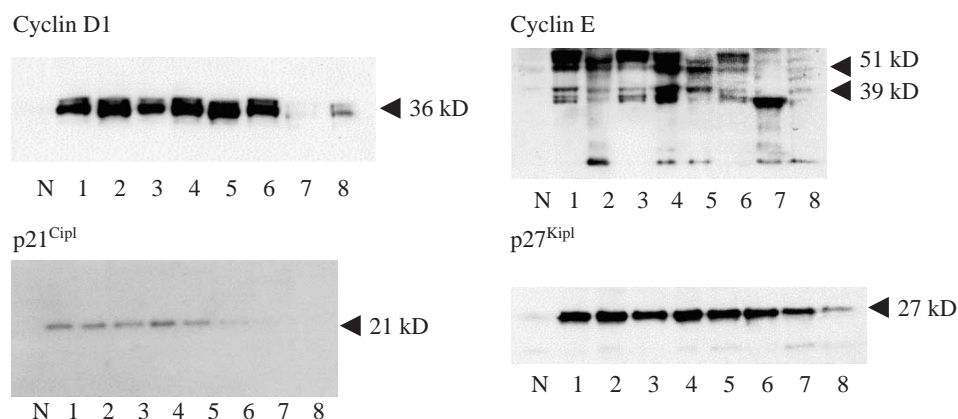


Fig. 2. Western blot analysis of cyclin D1, cyclin E, p21<sup>Cip1</sup> and p27<sup>Kip1</sup> in normal mammary gland and tumors. Fifty micrograms of protein extracts was used for electrophoresis. Lane N, normal mammary gland; lanes 1–4, carcinomas; lanes 5 and 6, atypical tumors; lanes 7 and 8, benign tumors. The numbers on the right indicate molecular weights in kD.

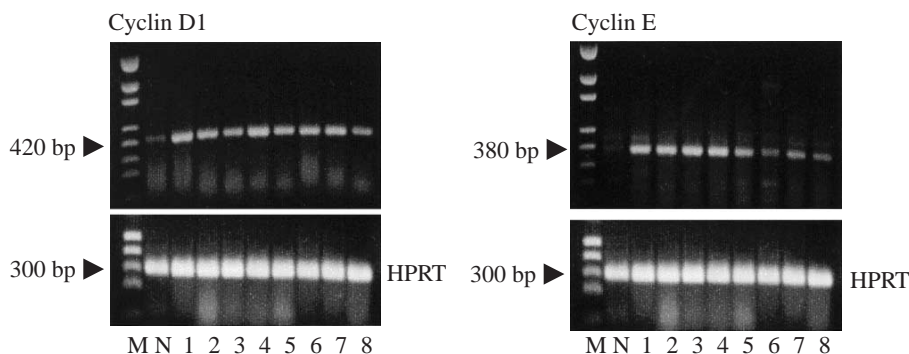


Fig. 3. The mRNA levels of cyclin D1 and cyclin E in rat mammary tumors and normal mammary gland. Top, RT-PCR products of cyclin D1 and cyclin E; bottom, RT-PCR of the internal control, HPRT. Lane M, 100-bp DNA size marker; lane N, normal mammary gland; lanes 1–4, carcinomas; lanes 5 and 6, atypical tumors; lanes 7 and 8, benign tumors.

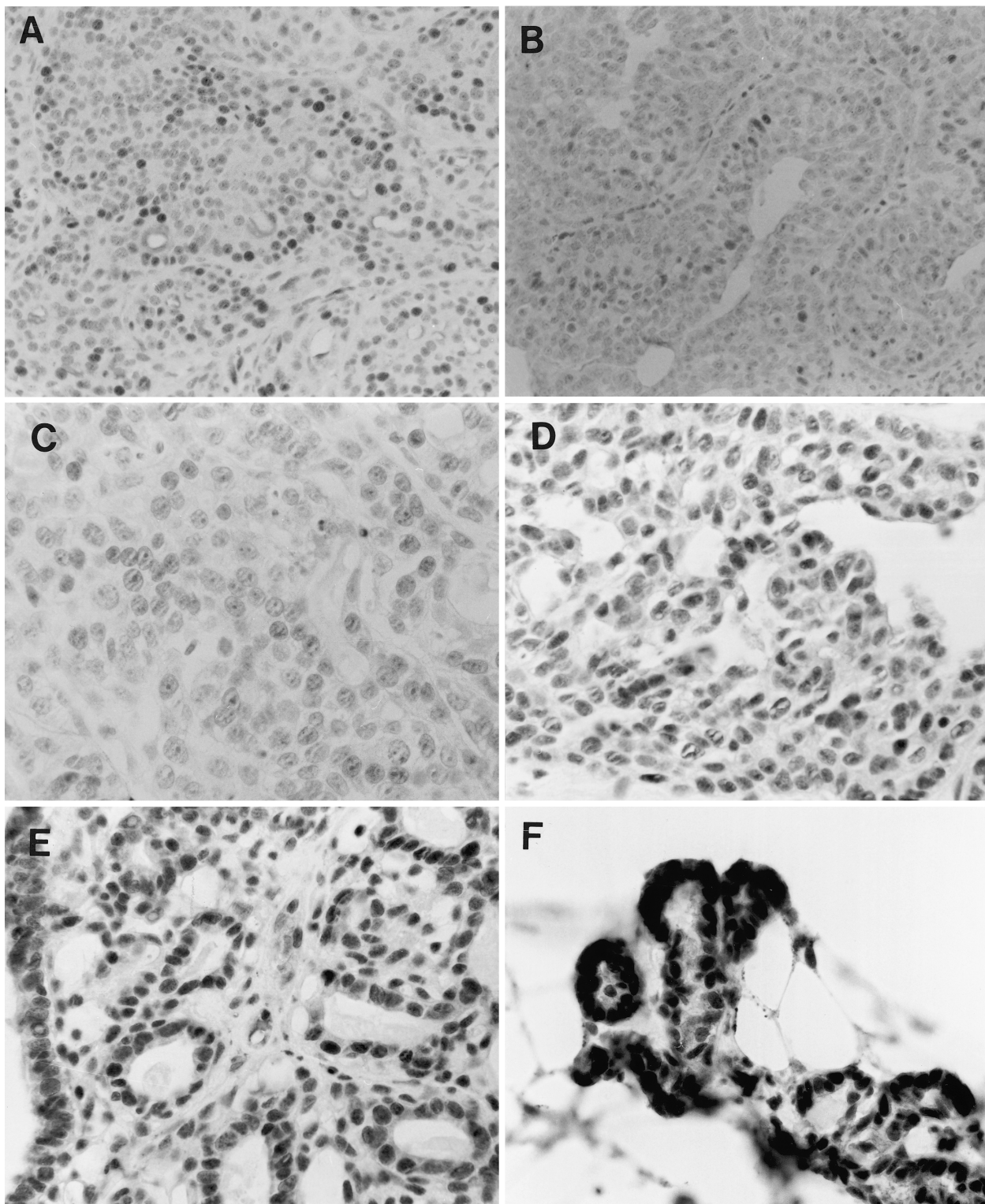


Fig. 4. Immunohistochemical staining of p21<sup>Cip1</sup> and p27<sup>Kip1</sup> in rat mammary tumors and normal mammary gland. A and B, p21<sup>Cip1</sup>; C–F, p27<sup>Kip1</sup>. p21<sup>Cip1</sup>-positive tumor cells are increased in invasive carcinomas (A) compared with atypical tumors (B), whereas p27<sup>Kip1</sup>-positive tumor cells are decreased in invasive carcinomas (C) compared with atypical tumors (D), benign tumors (E) and the normal mammary gland of the control group (F). Magnification: A and B, ×200; C–F, ×400.

Table III. Labeling Indices of p21<sup>Cip1</sup> and p27<sup>Kip1</sup> in Tumors and the Normal Mammary Glands of the Control Group

Histologic type	Number of tested tissue	p21 <sup>Cip1 d)</sup>	p27 <sup>Kip1 d)</sup>
Carcinomas	21	49.73±21.61 <sup>a)</sup>	84.05±12.25 <sup>a)</sup>
Atypical tumors	12	19.93±6.87 <sup>b)</sup>	97.68±1.02
Benign tumors	3	9.87±10.44 <sup>c)</sup>	98.65±0.64
Normal	10	0	97.59±2.34

a) *P*<0.05, in comparison with atypical tumors, benign tumors and normal mammary glands of the control group.

b, c) *P*<0.05, in comparison with normal mammary glands of the control group.

d) Percent.

Table IV. Correlation between BrdU Labeling Indices and Other Parameters by Using Linear Regression

Variable	β	Standard error	<i>P</i> value
Cyclin D1	0.23	0.09	0.02
Cyclin E	0.42	0.13	0.003
p21 <sup>Cip1</sup>	0.31	0.12	0.01
p27 <sup>Kip1</sup>	-0.52	0.19	0.01

Table V. Correlation between Labeling Indices of Cyclin D1, Cyclin E, p21<sup>Cip1</sup> and p27<sup>Kip1</sup>

	Cyclin D1 <sup>a)</sup>	Cyclin E <sup>a)</sup>	p21 <sup>Cip1 a)</sup>	p27 <sup>Kip1 b)</sup>
Cyclin D1	1	0.69	0.77	-0.41
Cyclin E	0.69	1	0.79	-0.60
p21 <sup>Cip1</sup>	0.77	0.79	1	-0.43
p27 <sup>Kip1</sup>	-0.41	-0.60	-0.43	1

a) Positive correlation among cyclin D1, cyclin E, and p21<sup>Cip1</sup> (*P*<0.05).

b) p27<sup>Kip1</sup> was negatively correlated with cyclin D1, cyclin E and p21<sup>Cip1</sup> (*P*<0.05).

nomas showed a significantly lower number of epithelial cells immunoreactive to p27<sup>Kip1</sup> when compared with atypical tumors, benign tumors and normal mammary glands (*P*<0.05) (Fig. 4, C–F; Table III). Western blot analysis, in contrast to the immunohistochemical results, showed higher expression of p27<sup>Kip1</sup> in carcinomas and atypical tumors than in benign tumors, while normal mammary glands showed negligible expression (Fig. 2).

**Correlation and topological distribution of immunohistochemical staining for BrdU, cyclin D1, cyclin E, p21<sup>Cip1</sup>, and p27<sup>Kip1</sup>** The number of BrdU-labeled epithelial cells was positively correlated with that of cyclin D1, cyclin E, and p21<sup>Cip1</sup> but negatively with that of p27<sup>Kip1</sup> (*P*<0.05) (Table IV). In addition, the immunoreactivities of BrdU, cyclin D1, cyclin E, and p21<sup>Cip1</sup> were positively correlated, while that of p27<sup>Kip1</sup> appeared inversely correlated to the others (*P*<0.05) (Table V). In addition, the

topological staining patterns of BrdU, cyclin D1, cyclin E, and p21<sup>Cip1</sup> were correlated with each other, as assessed by staining of consecutive tissue sections (data not shown). On the other hand, that of p27<sup>Kip1</sup> was not always concordant with the others, that is, p27<sup>Kip1</sup> was expressed concurrently with BrdU, cyclin D1, cyclin E, and p21<sup>Cip1</sup> in some areas, as well as independently in other areas (data not shown).

## DISCUSSION

In the present study, increased expression of cyclin D1 and cyclin E was observed in DMBA-induced rat mammary tumors when compared with the normal mammary glands of the control group. Overexpression of cyclin D1 in transgenic mice resulted in hyperplasia and tumors of the mammary epithelium<sup>32)</sup> and rat mammary tumors induced by NMU showed a 10- to 15-fold increase in the level of cyclin D1 and a 1.5- to 2-fold increase in the level of cyclin E.<sup>9)</sup> In mouse mammary tumorigenesis, the expression level of normal cyclin E (p50) was positively correlated with the tumorigenic potentials of different hyperplasia cell lines.<sup>33)</sup> Therefore, the overexpression of cyclin D1 and cyclin E appears to play a role in mammary carcinogenesis of rodents. The present study revealed a sequential increase in the cyclin D1- and cyclin E-positive epithelial cells from normal mammary gland, to atypical tumors, to carcinomas. Thus, the increased expression of cyclin D1 and cyclin E appears to occur relatively early during DMBA-induced rat mammary carcinogenesis and to be associated with tumor development. Similar findings have been reported for cyclin D1 and cyclin E in mouse mammary tumor development,<sup>33)</sup> mouse skin carcinogenesis,<sup>10, 11)</sup> rat esophageal carcinogenesis,<sup>12, 13)</sup> and rat bladder carcinogenesis.<sup>14)</sup>

Overexpression of cyclin D1 has been associated with DNA amplification in some, but not all, human cancers<sup>34, 35)</sup>; however, increased expression of cyclin D1 was observed in mouse skin tumors induced by DMBA/12-*O*-tetradecanoylphorbol-13-acetate<sup>10)</sup> and rat mam-

mary tumors induced by NMU<sup>9)</sup> without gene amplification of cyclin D1. These observations suggest that other molecular mechanisms may, in part, account for the elevated expression of cyclin D1. Filmus *et al.*<sup>36)</sup> reported that activated *ras* induced significant overexpression of cyclin D1 in epithelial cells derived from normal rat intestine and mouse mammary gland. DMBA initiation is characterized by a specific mutation at codon 61 of the *Ha-ras* gene<sup>37)</sup> that leads to consistent activation of its signal transduction pathway. These findings point to *ras* activation as a possible mechanism of cyclin D1 overexpression in the present study. The percentage of cyclin D1-positive epithelial cells was higher in carcinomas than in atypical tumors and benign tumors, even though there was no significant difference in the mRNA level of cyclin D1, indicating that the cyclin D1 protein levels in rat mammary tumors may be posttranscriptionally regulated. In one of the breast carcinoma cell lines, cyclin D1 was overexpressed, because its mRNA was stabilized in consequence of truncation at the 3'-nontranslated region.<sup>38)</sup> Overexpression of cyclin E has been associated with DNA amplification in a human breast cancer cell line.<sup>16)</sup> However, in rat mammary tumors induced by NMU,<sup>9)</sup> increased expression of cyclin E was observed without amplification of the *cyclin E* gene. Other molecular changes relevant to altered cyclin E expression remain to be determined.

Several isoforms of cyclin E were detected in this study, as previously reported in human breast cancers<sup>16, 17, 39)</sup> and mouse mammary tumor development.<sup>33)</sup> In addition, the alterations increased with increasing grade and stage of cancers.<sup>16, 17, 39)</sup> The cause of the alterations was suggested to be gene amplification<sup>15, 16)</sup> or alternative splicing of cyclin E mRNA.<sup>40)</sup> Low-molecular-weight isoforms of cyclin E, except for the 51 kD isoform, were reported to lack any CDK2-associated kinase activity,<sup>33, 40)</sup> which partly explained the lower BrdU-labeling index of benign tumors when compared with carcinomas and atypical tumors in the present study.

The expression of p21<sup>Cip1</sup> protein was increased in DMBA-induced rat mammary tumors when compared with normal mammary glands, and a sequential increase was also observed in the p21<sup>Cip1</sup>-positive epithelial cells from normal mammary gland, to atypical tumors, to carcinomas. This result is at variance with some experimental models<sup>24, 41)</sup> showing the suppression of tumor cells through increased expression of p21<sup>Cip1</sup> mRNA and protein. But similar findings to ours were observed in human breast carcinomas, whose p21<sup>Cip1</sup> expression was associated with high nuclear grade and poor tubule formation,<sup>28, 42)</sup> as well as in *in situ* carcinoma.<sup>28)</sup> The lack of an inverse association between p21<sup>Cip1</sup> expression and BrdU-labeling index is difficult to explain, but is not an uncommon phenomenon, as seen in several carcinomas of stomach,<sup>26)</sup> colon<sup>27)</sup> and breast.<sup>42)</sup> Moreover, a positive cor-

relation was reported in breast carcinomas,<sup>28)</sup> as in this study. However, this apparent paradox may be explained by the observation that p21<sup>Cip1</sup> promotes the association of CDK4 with cyclin D1 and at lower concentrations exerts a positive influence on cell cycle progression.<sup>43)</sup>

*p53* mutation, in contrast to *Ha-ras* mutation, did not seem to be prerequisite for carcinogenesis in chemically induced rat mammary tumors.<sup>44)</sup> p21<sup>Cip1</sup> has recently been proposed as a mediator of cyclin D1 induction by the wild-type p53 protein,<sup>45)</sup> and the overexpression of cyclin D1 in human fibroblasts is associated with increased expression of p21<sup>Cip1</sup> protein.<sup>46)</sup> These findings are consistent with the positive correlation between the percentage of p21<sup>Cip1</sup>-positive and cyclin D1-positive epithelial cells in the present study.

Western blotting and immunohistochemical results for the expression level of p27<sup>Kip1</sup> were discordant in tumors and normal mammary glands of the control group. Stromal lymphocytes showed nuclear staining for p27<sup>Kip1</sup> and the number of stromal lymphocytes in carcinomas and atypical tumors was more elevated than that in benign tumors and normal mammary gland. Thus, discordance between western blotting and immunohistochemical results in tumors could be caused by stromal expression of p27<sup>Kip1</sup> that had been disregarded in immunohistochemistry. Moreover, within the normal mammary gland, only a small fraction of the cells are mammary epithelial cells and the remainder are adipose tissue, whereas the tumor samples contain mainly epithelial tumor cells. Therefore, the discrepancy in the results could also be due to the difference between normal mammary glands and tumors in the relative amounts of epithelial and adipose tissues. The number of epithelial cells immunoreactive to p27<sup>Kip1</sup> was significantly lower in carcinomas when compared with atypical tumors, benign tumors and the normal mammary glands. Similar findings have been observed in human pituitary tumors<sup>47)</sup> and squamous neoplastic lesion of the oral cavity.<sup>48)</sup> Thus, it is speculated that the decreased expression of p27<sup>Kip1</sup> contributes to the progression of rat mammary tumors. p27<sup>Kip1</sup> may have important roles in the regulation of cell proliferation because *p27<sup>Kip1</sup>* knockout mice show multiorgan hyperplasia,<sup>31)</sup> which supports the inverse relationship between p27<sup>Kip1</sup> expression and BrdU-labeling index in the present study, as also seen in oral squamous neoplastic lesion<sup>48)</sup> and lymphomas.<sup>49)</sup> However, an inconsistent relationship between p27<sup>Kip1</sup> protein level and cell proliferation has been noted in breast cancers<sup>50)</sup> and colorectal tumors.<sup>51)</sup>

Some areas of rat mammary tumors showed concurrent expression of BrdU, cyclin D1, cyclin E, p21<sup>Cip1</sup>, and p27<sup>Kip1</sup>. Overexpression of cyclin D1<sup>51, 52)</sup> and cyclin E<sup>53)</sup> in some cell systems and tumors has been reported to be associated with the expression level of p27<sup>Kip1</sup>. Weinstein *et al.* postulated the existence of a feedback inhibitory



loop between cyclin D or cyclin E and p27<sup>Kip1</sup>, whose function is to maintain homeostatic control between positive- and negative-acting factors involved in cell cycle progression and perhaps to prevent potentially toxic effects of excessive cyclin D1/CDK or cyclin E/CDK kinase activity.<sup>52,53</sup> These findings led us to hypothesize that one of the most important factors determining cell proliferation is the balance between the two opposing regulators of cell proliferation, cyclin and CDKs.

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