Deregulation of the Cyclin D1/Cdk4 Retinoblastoma Pathway in Rat Mammary Gland Carcinomas Induced by the Food-derived Carcinogen 2-Amino-1-methyl-6-phenylimidazo[4,5-b]pyridine

Cuningping Qiu, Liang Shan, Minshu Yu, and Elizabeth G. Snyderwine

Chemical Carcinogenesis Section, Laboratory of Experimental Carcinogenesis, Center for Cancer Research, National Cancer Institute, National Health of Institutes, Bethesda, Maryland 20892

Abstract

2-Amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) is a suspected human breast carcinogen found in cooked meat that induces mammary gland cancer in rats. By real time PCR analysis, PhIP-induced rat mammary gland carcinomas showed statistically higher expression of the G1-S cyclin D1 (5-fold) and its kinase partner cyclin-dependent kinase (Cdk)-4 (37-fold) in comparison with normal mammary gland, whereas cyclin D2, cyclin D3, and Cdk6 were not statistically changed. Amplification of cyclin D1 was observed by real time PCR in 24% of carcinomas (15 of 63). Only 1 of 47 carcinomas showed Cdk4 amplification. By Western blotting, the level of phospho-Rb was >2-fold higher in carcinomas than in normal mammary gland. By immunohistochemical analysis, cyclin D1, Cdk4, and phospho-Rb nuclear protein expression was 5.7-, 3.9-, and 2.3-fold higher, respectively, in carcinomas than in normal mammary gland, whereas the expression of cyclin D2, cyclin D3, and Cdk6 was similar. Among carcinomas, Cdk4 and phospho-Rb levels were positively correlated with cell proliferation. Previous studies by this laboratory indicated that these carcinomas harbor a high frequency of H-ras mutations. The H-ras pathway is linked to the cell cycle via cyclin D1. The results from the current study implicate cyclin D1/Cdk4, phospho-Rb as a central pathway in PhIP-induced rat mammary gland carcinogenesis.

Introduction

Proliferation and progression of the cell cycle in mammalian cells is regulated in an orderly manner by a subset of cell cycle genes that encode cyclin-dependent kinases (Cdk), and their regulatory proteins the cyclins and Cdk inhibitors (1, 2). D-type cyclins are synthesized as cells enter G1 from quiescence (G0) and are responsible for initiating cell cycle progression. The D-type cyclins consist of a family of three members, D1, D2, and D3, that are expressed in an overlapping and redundant manner depending on cell type (2). In conjunction with their partner kinases Cdk4 and Cdk6, these cyclins link mitogenic signals to cell cycle progression. Cyclin D-Cdk4/6 initiates a signaling cascade involving inactivating the retinoblastoma protein via phosphorylation, thereby overcoming the retinoblastoma-mediated blockade of G1 and facilitating G1-S progression.

Cancer is increasingly viewed as a cell cycle disease (2). Substantial evidence indicates that alteration in cyclin D1 plays a crucial role in mammary gland carcinogenesis. In human breast cancer, cyclin D1 is frequently amplified and overexpressed (3). Transgenic mice engineered to overexpress cyclin D1 in the mammary gland develop mammary gland cancer suggesting a causative role for this protein (4). Less is known about cyclins D2 and D3 with regard to breast cancer. In transgenic models, up-regulation of cyclin D2 is associated with inhibition of lobuloalveolar development during pregnancy and accelerated involution (5). Human breast cancers have been reported to show lower expression of cyclin D2 as a consequence of promoter hypermethylation (6). Cyclin D3 has been reported to be overexpressed in higher grade invasive ductal carcinomas and to be associated with metastatic potential (7). There is growing evidence that the D-type cyclins each have distinct functions in the mammary gland (8). For example, recent studies have indicated that in mammary epithelial cells, the Ras pathway is wired to the cell cycle specifically via cyclin D1 and not cyclin D2 or D3 (9). In contrast, other proteins such as c-Myc appear to also link to the cell cycle machinery via cyclin D2.

Chemically induced rat mammary gland cancer provides a useful model system for human breast cancer (10). Deregulation of the cell cycle including overexpression of cyclin D1 and Cdk4 has been observed in mammary gland cancers induced by the experimental carcinogens 7,12-dimethylbenz[a]anthracene and N-methyl-N-nitrosourea (11, 12). Through cDNA microarray analysis, cyclin D1 has also been found to be elevated in rat mammary gland carcinomas induced by PhIP, a carcinogen found in the human diet (13, 14). In addition, PhIP-induced carcinomas were shown to harbor a high frequency of H-ras mutations, suggesting that the H-ras pathway is critical in PhIP-induced rat mammary gland carcinogenesis (15). In light of the necessity of cyclin D1 in H-ras-mediated mammary gland carcinogenesis, the current study tests the hypothesis that the dominant pathway for PhIP-induced carcinogenesis and cell cycle deregulation specifically involves cyclin D1, rather than cyclin D2 or D3. We further examine the levels of the cyclin D partner kinases and the phospho-Rb protein. The findings from this study provide evidence for deregulation of a major signaling pathway in PhIP-induced rat mammary gland cancer.

Materials and Methods

Mammary Gland Samples. Archival samples of mammary gland carcinomas from PhIP-treated female Sprague Dawley rats stored frozen at −80°C and as formalin-fixed paraffin embedded samples at 4°C were used (14, 16). Normal mammary gland tissue was obtained from archival samples from control rats. Histopathology was evaluated in H&E-stained sections as described (16). Tubulopapillary carcinomas, the major type of carcinoma induced by PhIP in this rat model, were used in this study.

DNA, RNA, and Protein Isolation. Genomic DNA from carcinomas and normal mammary gland were isolated from frozen tissue by phenol-chloroform extraction as described previously (17). Total RNA was isolated using TRIzol extraction reagent (Invitrogen Life Technologies, Inc., Carlsbad, CA) according to the protocol provided by Life Technologies, Inc. DNA and RNA concentration and purity was determined spectrophotometrically by absorbance at 260 and 280 nm. RNA was stored as aliquots at −80°C, and each aliquot was thawed only once and used immediately. For Western blotting,
tissues were sonicated and protein was isolated according to the method of Sgambato et al. (12). A fatty layer (particularly from control mammary gland) that formed on top of the homogenate after a 30-min incubation on ice and centrifugation at 4°C was discarded. The remaining supernatant was assayed for protein concentration with the bicinchoninic acid protein assay kit (Pierce, Rockford, IL) with BSA as a reference standard and used for Western blotting. Adipose tissue is the major stromal contaminant of control virgin rat mammary gland, constituting 70% of the gland by weight (18). Fat was effectively removed during RNA/DNA isolation by phenol-chloroform extraction and from protein samples by chilling the samples as described above. When nucleic acid was isolated from mammary glands from virgin (adipose-rich) and pregnant (epithelial cell-enriched), adipose tissue contributed little to the yield of RNA or DNA (data not shown). Specifically, the yield of nucleic acid appeared to largely reflect the density of epithelial cells.

Quantitative Real Time PCR Analysis. Gene expression was determined by quantitative real time PCR analysis on the ABI Prism 7900 Sequence Detection System (PE Applied Biosystems, Foster City, CA) as described in detail previously (19). Primers and probes3 for the quantitative real time PCR analysis were designed using Primer Express Software V2.0 (Applied Biosystems). The TaqMan rodent glyceraldehyde-3-phosphate dehydrogenase primer set (Applied Biosystems) was used as the reference control for normalization. Rodent glyceraldehyde-3-phosphate dehydrogenase was chosen as the reference because it was consistently and reproducibly expressed in all samples. Amplification of cyclin D1 was detected to largely reflect the density of epithelial cells.


delta \Delta C_T = \delta C_T (tumor) - \delta C_T (control)

Relative expression = 2^{-\Delta \Delta C_T}

For statistical analysis, Student’s t test compared \Delta C_T of the tumor to the \Delta C_T of the control tissue. The range for the relative expression was derived from the SE of the \Delta C_T values and calculated as the exponential function of -\Delta \Delta C_T + SE and -\Delta \Delta C_T - SE.

For measurement of gene amplification, rat genomic DNA (6750-bp; BD Clontech, Palo Alto, CA) was used in standard curve construction. Rat Alb and Aqp genes were selected as reference genes to normalize the quantity of genomic DNA. Rat Alb and Aqp are located in regions of chromosomes 14p and 11q, respectively, that show no apparent gain or loss in PhIP-induced rat mammary gland carcinomas by comparative genomic hybridization analysis (20). The cyclin D1 primer set used to measure gene amplification was identical with that used to measure relative mRNA expression. Data from standard curves with a correlation coefficient of <0.99 were discounted. The relative gene copy number was evaluated from the \Delta C_T of the target gene and the a reference gene. By subtracting the \Delta C_T of the reference gene from the \Delta C_T of the target gene, the \Delta C_T values for the target gene was determined. Amplification was routinely confirmed by using both reference genes. Each \Delta C_T in a carcinoma was subsequently compared with the mean \Delta C_T of normal mammary gland samples. A reduction of the \Delta C_T by one cycle represented a 2-fold increase in copy number. A target gene in carcinomas was considered to be amplified if copy number was at least 2-fold higher than the value in normal mammary gland.

Immunohistochemical Assay. Primary antibodies for phospho-Rb (sc-16671), cyclin D2 (34B1–3), and CDK4 (sc-260-G) were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Cdk6 (DCS83) and cyclin D3 (DCS22) were from Cell Signaling Tech. (Beverly, MA). Cyclin D1 (clone DOS-6) and PCNA (M0879) antibodies were obtained from DAKO ( Carpinteria, CA). Immunohistochemical staining was conducted using the Universal DAKO LSAB2 System. Briefly, 5-μm tissue sections were deparaffinized and rehydrated in xylene and different concentrations of ethanol. The endogenous peroxidase activity was blocked by incubation with 0.25% hydrogen peroxide. The slides were incubated sequentially with primary antibodies (1:50) overnight at 4°C and then with horseradish peroxidase-labeled secondary antibody for 2 h at room temperature. Negative controls were run without the primary antibody incubation. Sections were developed using 3-aminop-9-ethylcarbazole substrate and counterstained with Mayer’s hematoxylin. For quantification of immunohistochemical stained cells, 1000 cells in 4 high-power fields (>x400) were counted. Cells with brown nuclear staining were scored as positive, and the percentage of stained cells was calculated.

Western Blot. Protein extract (35 μg) from normal mammary glands and PhIP-induced carcinomas were run on NovaT 10% Tris-glycine gels (Invitrogen Life Technology, Carlsbad, CA) and blotted onto polyvinylidene difluoride membrane (Invitrogen Life Technology) according to the manufacturer’s protocol. The blots were then blocked by nonfat milk for 1 h, incubated with 1:1000 dilutions of the phospho-Rb antibody (No. 9308S; Cell Signaling Technology, Inc., Beverly, MA) or actin (sc-1616; Santa Cruz Biotechnology), respectively, for 2 h at room temperature. After incubation with secondary antibody (SC-2005; Santa Cruz Biotechnology) at a 1:2000 dilution, the membranes were developed by ECL Western blotting detection reagents (Amersham Biosciences Corp., Piscataway, NJ) for X-ray film exposure. Protein bands were scanned and recorded accordingly. The intensity of the bands was quantified by the ImageQuant 5.1 software and normalized by actin before statistical analysis.

Statistical Analysis. Statistical analysis including Student’s t test (one or two tailed) and Spearman rank order correlation was conducted where indicated, using SigmaStat Statistical Software, version 2.0 (Jandel, San Rafael, CA). Statistical significance was assumed at P < 0.05.

Results

mRNA Expression and Gene Amplification in PhIP-induced Rat Mammary Gland Carcinomas. By real time PCR analysis, cyclin D1 but not cyclins D2 and D3 showed significantly higher relative expression in carcinomas than did normal mammary gland (Table 1). The relative expression of cyclin D1 was 5-fold higher in carcinomas than in normal mammary gland. Although cyclin D2 expression in carcinomas was slightly elevated and cyclin D3 expression was slightly reduced in comparison with normal mammary gland, the differences were not statistically significant. The relative expressions of Cdk4 and Cdk6 were also examined. The relative expression of Cdk4 was 37-fold higher in carcinomas than in normal mammary gland, whereas the expression of Cdk6 was not statistically different.

Cyclin D1 and D3, the two genes showing increased expression at the message level, were further examined for amplification in rat mammary gland carcinomas. Amplification of cyclin D1 was detected by real time PCR analysis as a reduction in the threshold cycle required to generate the product at equal levels of DNA template

Table 1. Relative mRNA expression in PhIP-induced rat mammary gland carcinomas compared with normal mammary gland

<table>
<thead>
<tr>
<th>Genes</th>
<th>Normal gland</th>
<th>Carcinomas</th>
</tr>
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<tbody>
<tr>
<td>Cyclin D1</td>
<td>1</td>
<td>5.0b</td>
</tr>
<tr>
<td></td>
<td>(0.7–1.5)</td>
<td>(3.7–6.8)</td>
</tr>
<tr>
<td>Cyclin D2</td>
<td>1</td>
<td>2.0</td>
</tr>
<tr>
<td></td>
<td>(0.4–2.3)</td>
<td>(0.7–2.7)</td>
</tr>
<tr>
<td>Cyclin D3</td>
<td>1</td>
<td>0.4</td>
</tr>
<tr>
<td></td>
<td>(0.3–0.8)</td>
<td>(0.2–0.7)</td>
</tr>
<tr>
<td>Cdk4</td>
<td>37.4a</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(0.6–1.6)</td>
<td>(25.8–54.1)</td>
</tr>
<tr>
<td>Cdk6</td>
<td>0.8</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(0.7–1.4)</td>
<td>(0.6–1.1)</td>
</tr>
</tbody>
</table>

a Values are determined by real time PCR and calculated as described in “Materials and Methods” using the comparative Ct method. Range (in parentheses) is derived from the standard error of Ct. Each normal mammary gland mRNA sample represents a pool of 2–3 glands from separate rats. Carcinomas were examined individually. Number (n) of samples examined is as indicated.

b Values are significantly different from normal mammary gland glands compared with carcinomas, Student’s t test, P < 0.05.
Approximately 24% of PhIP-induced carcinomas (15 of 63) showed cyclin D1 gene amplification. Among the carcinomas showing amplification, the increase in copy number ranged from 2- to 4-fold. Carcinomas with amplification showed increased expression of cyclin D1 at the message level. But cyclin D1 expression was also consistently elevated among samples without amplification, and there was no statistically significant difference in cyclin D1 mRNA expression in carcinomas with and without amplification. Only 1 of 47 carcinomas showed amplification of Cdk4.

**Analysis of Phospho-Rb Levels by Western Blotting.** Cyclin D/Cdk4 contributes to the phosphorylation of retinoblastoma in G1. By Western blot analysis, PhIP-induced carcinomas showed higher levels of phospho-Rb than did normal mammary gland tissues (Fig. 2). After quantifying band intensities from Western blots, the level of phospho-Rb was determined to be on average 2-fold higher in carcinomas than in normal mammary gland, a difference that was statistically significant.

**Immunohistochemical Analysis of PhIP-induced Rat Mammary Gland Carcinomas.** By immunostaining, phospho-Rb was detected in the nuclei of both carcinomas and normal mammary gland (Fig. 3). The percentage of phospho-Rb-positive stained cells was ∼2.3-fold higher in carcinomas than in normal mammary gland. All 41 carcinomas examined showed conspicuous staining of phospho-Rb, suggesting that there was no loss of Rb expression by mutation or gene deletion. The expressions of cyclin D1 and Cdk4, also detected immunohistochemically in nuclei, were 5.7- and 3.9-fold higher, respectively, in carcinomas than in normal mammary gland. Accordingly, cellular proliferation was also statistically higher in carcinomas than in normal mammary gland as measured by PCNA immunostaining. In contrast, immunostaining of cyclin D2, cyclin D3, and Cdk6 was very weak in both carcinomas and normal mammary gland, with <4% of cells showing positive staining (immunostaining not shown). No statistical differences were observed in the level of expression of these proteins between carcinomas and normal mammary gland. By Spearman rank order correlation analysis of the immunohistochemistry data, a statistically significant positive correlation was observed between PCNA and Cdk4 expression (correlation coefficient, 0.35; \( P = 0.03; n = 41 \) carcinomas) and between PCNA and phospho-Rb expression (correlation coefficient, 0.39; \( P = 0.012; n = 41 \) carcinomas). However, cyclin D1 was not statistically correlated with PCNA (correlation coefficient, 0.11; \( P = 0.48; n = 41 \)).

**Discussion**

PhIP is a suspected human mammary gland carcinogen that is found in the diet in cooked meat (21, 22). PhIP induces mammary gland cancer in rats and has been linked to a higher risk of breast cancer among women consuming well-done cooked meat (23). Knowledge of the molecular alterations induced by PhIP in the rat model is expected to provide insight into the mechanisms of carcinogenesis of this compound.

G1-S deregulation involving aberrant expression of cyclin D1, down-regulation of p16, or inactivation of Rb is common in carcinogenesis (2). However, relatively little is known regarding alterations in the G1-S phase of the cell cycle in PhIP-induced rat mammary gland cancer.
gland carcinomas. One prior study indicated that p16, although not down-regulated in PhIP-induced carcinomas, showed negligible expression in cell lines established from the carcinomas (24). In addition, using cDNA microarray analysis, an elevated expression of cyclin D1 was detected in PhIP-induced carcinomas (13, 14). Herein we confirmed the overexpression of cyclin D1 in PhIP-induced carcinomas using real time PCR and immunohistochemistry and further show that the cyclin D1/Cdk4-retinoblastoma pathway is specifically deregulated in these carcinomas. Of the three D-type genes, only cyclin D1 showed statistically increased expression by real time PCR. Furthermore, Cdk4, but not Cdk6, showed highly elevated expression at the message level. By immunohistochemistry, the level of expression of cyclin D2, cyclin D3, and Cdk6 in carcinomas was low and not significantly different from normal mammary gland. However, protein expression of cyclin D1 and Cdk4 detected immunohistochemically in carcinomas was on average 4–6-fold higher than in normal mammary gland. Cyclin D1/Cdk4 is known to phosphorylate Rb, facilitating cell cycle progression. In conjunction with higher expression of cyclin D1/Cdk4, the level of phospho-Rb was significantly elevated in carcinomas. In further support for the specific deregulation of cyclin D1/Cdk4-retinoblastoma pathway in PhIP-induced carcinomas, we observed a positive correlation between the levels of Cdk4 or phospho-Rb with elevated cellular proliferation as detected by PCNA immunostaining in a bank of 41 PhIP-induced carcinomas. PhIP-induced carcinomas show a high frequency of H-ras mutations (15) concomitant with elevated cyclin D1 expression, but these carcinomas show no statistically significant alterations in cyclin D2 or D3 expression. These findings are consistent with the notion that an H-ras-cyclin D1/Cdk4-Rb pathway is a predominant route for cell cycle deregulation in PhIP-induced rat mammary gland carcinogenesis.

It is further notable that several other genes that regulate the expression of cyclin D1 at the level of either transcription or translation also show altered expression in PhIP-induced carcinomas. Stat5A, a gene found to be overexpressed in these carcinomas (14), transac-
tivates the cyclin D1 promoter and appears to mediate its transcriptional regulation (28). In addition, platelet-derived growth factor and PI-3 kinase (p85α-regulatory subunit) are overexpressed in PhIP-induced rat mammary gland carcinomas (14, 19). Both platelet-derived growth factor and PI-3 kinase activate the cyclin D1 promoter (29). In addition, cyclin D1 is also regulated posttranslationally by PI-3 kinase/Akt (protein kinase B) pathway (30, 31). Thus in PhIP-induced rat mammary gland carcinomas, there appears to be a convergence of several potentially deregulated pathways on cyclin D1, which is likely to lead to an increased expression of cyclin D1, increased inactivation of Rb by phosphorylation and acceleration through G1-S. These possibilities further support the critical role of cyclin D1 in PhIP-induced rat mammary gland cancer.

There are parallels between human breast cancers and PhIP-induced rat mammary gland cancers with regard to cyclin D1 alteration. The frequency of cyclin D1 amplification is very similar between PhIP-induced rat mammary gland cancers and human breast cancers. In humans, cyclin D1 amplification frequency has been reported to be 10–16% with some studies reporting a frequency upwards of 24%, similar to the rat (3). Furthermore, as was seen in PhIP-induced rat mammary gland cancers, the majority of human breast cancers (upwards of ~80%) overexpress cyclin D1. In humans, high levels of cyclin D1 are associated with estrogen-responsive and well-differentiated breast carcinomas, which are also typical of rat mammary gland cancers (3, 8, 10). Because PhIP-induced rat mammary gland cancer shows a specific alteration in cyclin D1 and resemblance to the human disease, this rat model may be useful for studying the effectiveness of anti-cyclin D1 therapy.

Acknowledgments

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References

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