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Overexpression of cyclin D1 and cyclin E in 1,2-dimethylhydrazine dihydrochloride-induced rat colon carcinogenesis

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Deregulation of G1 cyclins has been reported in several human and rodent tumors including colon cancer. To investigate the expression pattern of G1 cyclins in 1,2-dimethyl-hydrazine dihydrochloride (DMH)-induced rat colon carcinogenesis, we studied the expression of cyclin D1 and cyclin E by quantitative reverse transcription-polymerase chain reaction (RT-PCR) analysis and immunohistochemistry (IHC). The mRNA level of cyclin D1 was increased 1.2-fold in adenocarcinomas but not significantly in adenomas, when compared with normal rat colonic mucosa ($p<0.05$). The cyclin E mRNA level was increased 2.7-fold in adenomas and 3.3-fold in adenocarcinomas ($p<0.05$). The PCNA mRNA level was also increased 1.9-fold in adenomas and 1.8-fold in adenocarcinomas ($p<0.05$). Immunohistochemical staining revealed exclusive nuclear staining of the neoplastic cells for cyclin D1, cyclin E and PCNA. Cyclin D1 expression was detected in 56.3% of the adenomas and in 61.5% of the adenocarcinomas examined, whereas cyclin E expression was detected in 87.5% of the adenomas and in 92.3% of the adenocarcinomas. Overall, cyclin D1, cyclin E and PCNA expression was significantly increased at both the mRNA and protein levels in normal colonic mucosa, adenomas and adenocarcinomas, but there was no significant difference in the degree of expression of these genes in adenomas and adenocarcinomas. Our results indicate that the overexpression of cyclin D1 and cyclin E may play an important role during the multistage process of rat colon carcinogenesis, at a relatively early stage, and may disturb cell-cycle control in benign adenomas, and thereafter, participate in tumor progression.

Key words: cell cycle, cyclin D1, cyclin E, colon cancer

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Introduction

Colorectal cancer in humans is one of the most common malignancies in the world [6]. Colorectal carcinogenesis is characterized by multiple genetic alterations and is preceded by a series of histopathologically recognizable precancerous lesions that progress to adenocarcinoma over a period of a year [6]. As with other tumors, cell proliferation is central to tumor progression in colorectal cancer [23] and therefore, it is essential to understand the mechanism and significance of altered cell cycle regulation.

Progression of the cell cycle is regulated by the sequential formation and degradation of multiple cyclins that bind to and stimulate the activities of a series of cyclin-dependent kinases (CDKs) [10, 29]. For example, cyclin D1 functionally forms a complex with CDK4 and CDK6, whereas cyclin E complexes with CDK2 during the G1/S phase [13, 31]. Recent studies have identified additional regulators of the cell cycle, such as $p21^{WAF1/CIP1}$, $p27^{KIP1}$, $p16^{MTS1}$ and $p15^{MTS2}$ tumor suppressor genes, which bind to the cyclin-CDK complex and inhibit kinase activities [11, 28]. Altered expression of cell cycle regulators and the subsequent deregulation of the cell cycle may be important steps in carcinogenesis and are the most consistently found events in human malignancies including colorectal cancer [5, 8]. Among the G1 cyclins, cyclin D1 and cyclin E are key regulators during the G1/S cell cycle transition, and perhaps the most important checkpoint in the mammalian cell cycle [21]. Increased expression of cyclin D1 and cyclin E has been reported in various human tumors [1, 15, 17, 18, 25, 32] and several carcinogen-induced mouse and rat tumor models [9, 16, 20, 24, 25, 27, 30, 34].

However, there has been insufficient study of the expression of cyclin D1 and cyclin E in carcinogen-induced rat colonic carcinogenesis. The purpose of this

study was to determine whether 1,2-dimethyl-hydrazine dihydrochloride (DMH)-induced rat colon tumors display altered expressions of cyclin D1 and cyclin E and to discover these alterations are linked to cell proliferative activity in this model.

Materials and Methods

Animals and treatments

Six-week-old, male, Sprague-Dawley rats were purchased from Charles River Japan (Kanagawa, Japan) and maintained in a temperature (21 ± 2°C) and humidity (50 ± 3%) controlled environment with a 12 hrs light/dark illumination cycle. The rats were fed a commercial diet (Jeil Jedang, Co.) and water *ad libitum*. After a 2-week acclimatization period, one group of 50 rats was treated with DMH (Sigma, USA) by subcutaneous injection of 15 mg/Kg body weight once per week for 20 weeks. To prevent skin irritation during injection, pH of DMH was adjusted to 6.5. Twenty rats treated with saline in the same way served as controls. All animals were sacrificed at week 40 from the initiation of treatment. After both ends were ligated, the entire colons were injected with saline, cut along the longitudinal axis, and the neoplastic nodules harvested. Approximately half of the tumor tissues removed were snap frozen in liquid nitrogen and stored at -70°C until analysis. Normal colonic tissues of the control group were also harvested. For histopathology and immunohistochemistry (IHC), the remaining tumor tissues were fixed in 10% neutral phosphate-buffered formalin, routinely processed and embedded in paraffin. During 40 week exposure 3 rats died. The remaining 47 rats were examined for tumor development and 35 rats were found to have tumor (74.5% incidence). The neoplastic nodules from each mouse were classified as either adenoma or adenocarcinoma. Sixteen adenoma and 13 adenocarcinoma were used for RT-PCR analysis of cyclin D1 and cyclin E.

RNA isolation and quantitative RT-PCR analysis

Frozen tissue specimens were ground with liquid nitrogen and total cellular RNA isolated, based on the method of Chomczynski et al [4]. Two-step quantitative RT-PCR analysis was performed as previously described [19]. Two μg of total RNA was reverse transcribed into first strand cDNA in a volume of 25 μl at 37°C for 60 min using a first strand cDNA synthesis kit (Novagen, Madison, WI), and heated at 95°C for 5 min to terminate the reverse transcription reaction. Cyclin D1, cyclin E, proliferating cell nuclear antigen (PCNA) and hypoxanthine-guanine phosphoribosyltransferase (HPRT: housekeeping gene) genes were amplified from 2 μl cDNA mixtures in a final volume of 20 μl PCR reaction mixture containing, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2.5 mM $MgCl_2$, 2 mM each of dNTPs, 0.25 μM each of sense and antisense primers (Bioneer, Seoul, Korea), 1.25 U *Taq* DNA polymerase (Bioneer, Seoul, Korea) and [α-^{32}P]dCTP (3000 Ci/mmol, Amersham, Arlington Heights, IL). The PCR reactions were carried out using a Perkin-Elmer Thermocycler 9600 (Perkin-Elmer, Norwalk, CT). Reaction mixtures were first denatured at 95°C for 5 min, and amplification was performed for 35 cycles , at 95°C for 45 sec, 60°C for cyclin D1 (61°C for cyclin E, 58°C for PCNA, and 61.5°C for HPRT) for 1 min, and at 72°C for 1 min, followed by an extension for 7 min at 72°C. Primer sets for the PCR amplification of cyclin D1, cyclin E, PCNA and HPRT genes were selected based on published sequences. The PCR primer pairs used were as follows:-cyclin D1, sense, 5'-TGGAGCCCCTGAAGAAGAG-3' and antisense, 5'-AAGTGCGTTGTGCGGTAGC-3'; cyclin E, sense, 5'-CTGGCTGAATGTTTATGTCC-3' and antisense 5'-TC-TTTGCTTGGGCTTTGTCC-3'; PCNA, sense, 5'-GC-CCTCAAAGACCTCAT CAA-3' and antisense, 5'-GC-TCCCCACTCGCAGAAAAC-3'; and HPRT, sense, 5'-CGGGGGAC ATAAAAGTTAT-3' and antisense, 5'-GG-ACGCAGCAACAGACATT-3'. After running the amplified PCR products of each gene on a 1.8% agarose gel, the gels were dried at 80°C for 60 min and exposed to a Phosphoimaging plate (Fuji, Minami-Ashigara) for 3 days. After autoradiography, the imaging plate was scanned on an Image Reader BAS-2500 (Fuji, Tokyo). For quantification of the RT-PCR products, the levels of incorporated [α-^{32}P]dCTP in each band were measured with a liquid scintillation counter (Walac, OY, Finland). The radioactivity in the cyclin D1, cyclin E and PCNA band was normalized to the radioactivity of the corresponding HPRT internal control band.

Immunohistochemical staining

Immunohistochemical staining was performed to detect the degree of cyclin D1, cyclin E and PCNA expression on replicate sections of the selected neoplastic tissues used for RT-PCR analysis. Tissue sections were placed on Probe-On slides (Fisher scientific, Pittsburgh, PA), deparaffinized and rehydrated. After inhibiting endogenous peroxidase activity with methanol containing 3% H_2O_2, tissue sections were heated in 10 mM sodium citrate (pH 6.0) in a pressure cooker for 6 min for antigen retrieval. After blocking non-specific binding by treating the slides with 10 % normal goat serum at 37°C for 60 min, the slides were incubated at 4°C overnight with commercially available antibodies to cyclin D1 (mouse monoclonal; Santa Cruz Biotech., Santa Cruz, CA), cyclin E (rabbit polyclonal; Santa Cruz Biotech., Santa Cruz, CA) and PCNA (mouse monoclonal; Novocatra, Newcastle, UK) at 1 : 100, 1 : 100 and 1 : 200 dilutions, respectively. After washing, the sections were incubated with biotinylated goat anti-mouse IgG or goat anti-rabbit IgG (Vector Lab, Burlingame, CA) at 37°C for 60 min. Sections were then

washed and incubated with Streptavidin (DAKO, Copenhagen, Denmark) at 37°C for 60 min. 3,3-diaminobenzidine was used as a chromogen to show the antigen and sections were counterstained with Harris hematoxylin. Negative control tissues were prepared in the same manner as that described above, except for the omission of primary antibodies and the substitution of an isotype-matched but irrelevant antibody.

Results

Quantitative RT-PCR analysis of cyclin D1, cyclin E and PCNA mRNA expression

Quantitative RT-PCR analysis of the tissue samples using primers specific for cyclin D1, cyclin E, PCNA and HPRT revealed product bands of the expected size. The results of autoradiography are shown in Figure 1. The width and intensity of the cyclin D1, cyclin E and PCNA bands were markedly increased from those normal mucosa (Fig. 1A) in both adenomas (Fig. 1B) and adenocarcinomas (Fig. 1C). The mRNA levels of cyclin D1, cyclin E and PCNA in each stage, as quantified by measuring the radioactivity of each band, are shown in Figure 2. The mRNA levels of cyclin D1 (Fig. 2A), cyclin E (Fig. 2B) and PCNA (Fig. 2C) were all significantly increased in the tumor tissues compared with normal colon tissues. The mRNA level of cyclin D1 was increased 1.2-fold in adenocarcinomas ($p<0.05$) but in adenomas it was not significantly increased. Cyclin E mRNA was increased 2.7-fold in adenomas and 3.3-fold in adenocarcinomas ($p<0.05$) compared to normal mucosa. The proliferative activity of the tumor cells as determined by PCNA mRNA level was also increased, 1.9-fold in adenomas and 1.8-fold in adenocarcinomas ($p<0.05$), respectively. However, there were no significant differences in the cyclin D1, cyclin E and PCNA mRNA expression levels of adenomas and

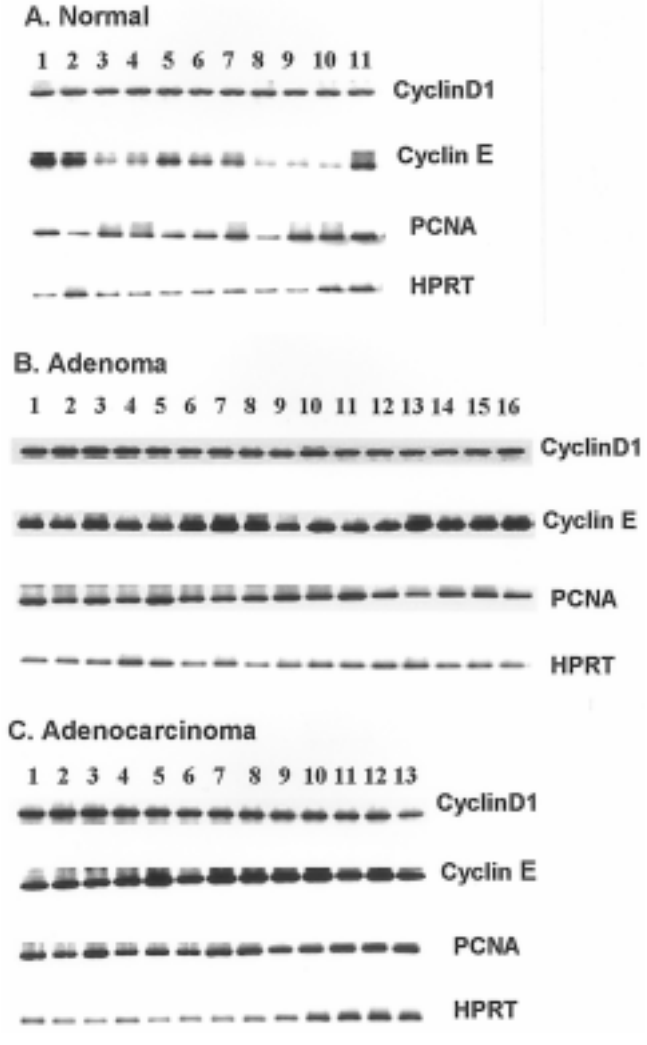

Fig. 1. RT-PCR analysis of cyclin D1, cyclin E and PCNA mRNA levels using HPRT as an internal control in the rat colon carcinogenesis model. (A) Normal colorectal mucosa from the control group. (B) adenomas harvested from DMH-treated rats. (C) adenocarcinomas harvested from DMH-treated rats.

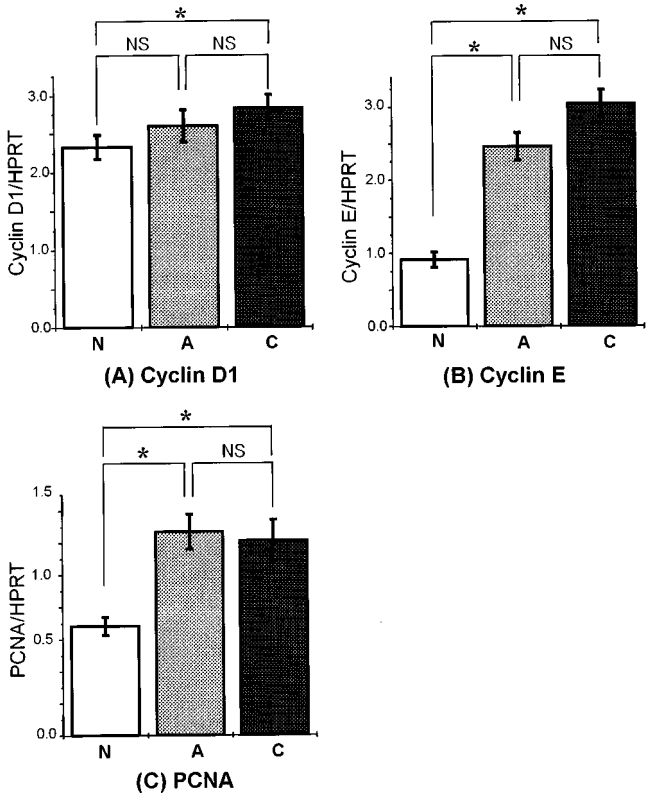

Fig. 2. Quantitation of cyclin D1, cyclin E and PCNA mRNA expression by quantitative RT-PCR analysis. Bars represent levels of incorporation of [α-^{32}P]dCTP in cyclin D1, cyclin E and PCNA PCR products after normalization to HPRT, by measuring the radioactivity (c.p.m.) of each band in Figure 1. Results quoted are the mean ± SE of each group of tissues. mRNA levels of (A) cyclin D1, (B) cyclin E and (C) PCNA. NS, not significant. *, $P<0.05$. N, normal colonic mucosa. A, colonic adenomas. C, colonic adenocarcinomas.

adenocarcinomas ($p<0.05$, Fig. 2).

Immunohistochemical analysis of cyclin D1, cyclin E and PCNA protein expression

Before IHC, neoplastic nodules were examined microscopically and classified as normal mucosa, adenomas or adenocarcinomas, respectively. Sixteen adenomas and 13 adenocarcinomas from different rats and 10 normal colonic mucosa were selected for immunohistochemical study.

Immunoreactivity for cyclin D1, cyclin E and PCNA was confined predominantly to the nuclei of the neoplastic cells (Fig. 3, C-H). Normal colonic mucosa showed only weak to undetectable staining for cyclin D1 and cyclin E, whereas positively stained cells for PCNA were primarily detected in the basal layer of normal colonic mucosa. The number and distribution of cyclin D1- and cyclin E-positive cells in both adenomas and adenocarcinomas was generally variable and heterogeneous, whereas PCNA was diffusely positive, and irrespective of cyclin D1 and cyclin E positivity. PCNA protein was expressed in almost all the tumor cells of the adenomas and adenocarcinomas examined, but the topological distribution of PCNA-positive cells was not colocalized with that of cyclin D1- and cyclin E-positive cells (Fig. 3, C-H). Cyclin D1 immunoreactivity was noted in 9/16 (56.3%) of the adenomas and in 8/13 (61.5%) of the adenocarcinomas examined (Fig. 3, E and F). Cyclin E expression was detected in 14/16 (87.5%) of the adenomas and in 12/13 (92.3%) of the adenocarcinomas (Fig. 3, G and H). Although the staining intensity of cyclin D1 and cyclin E was variable among the cases studied, the degree of immunoreactivity was generally weak in adenomas and relatively strong in adenocarcinomas.

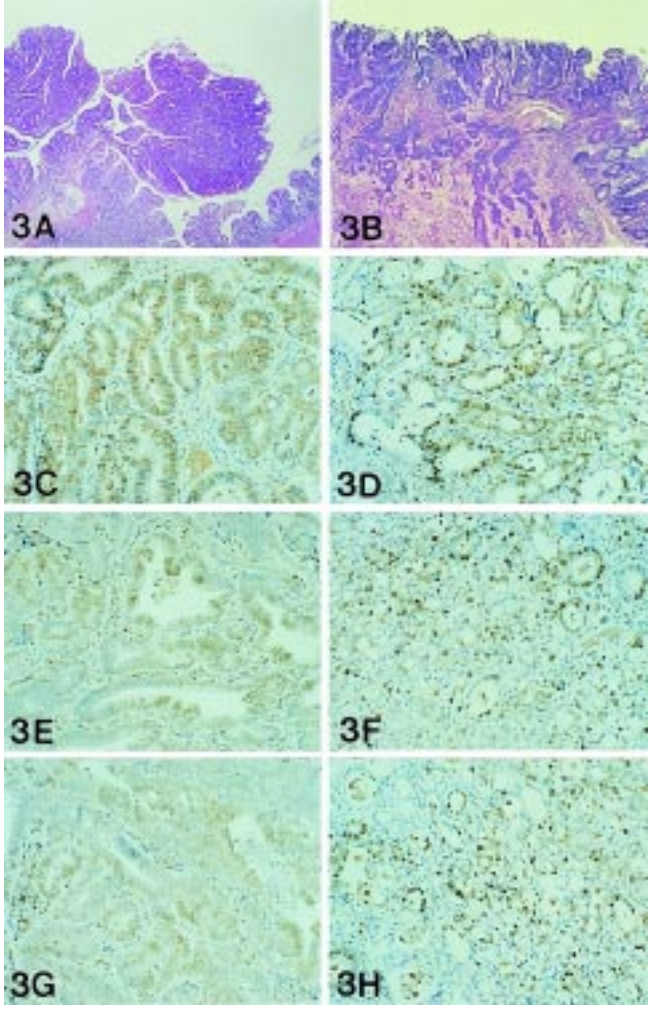

Fig. 3. Topologic distributions of PCNA, cyclin D1 and cyclin E in DMH-induced rat colonic adenoma (A, C, E, G) and adenocarcinoma (B, D, F, H). (A, B) H&E staining. (C, D) IHC of PCNA. (E, F) IHC of cyclin D1. (G, H) IHC of cyclin E. Exclusive nuclear staining of PCNA, cyclin D1 and cyclin E was observed. PCNA positive nuclei were confined to the highly proliferative regions, but the topological distribution of PCNA-positive nuclei often did not colocalized with those of cyclin D1- and cyclin E-positive nuclei. Magnification: A, B, × 50; C-H, × 200.

Discussion

The deregulation of cell cycle regulators is one of the most common events in tumor development. Numerous studies have indicated that G1 cyclins are frequently deregulated in various human malignancies including breast [26], lung [17], gastric [1], urinary bladder [15] and colorectal cancers [18, 32]. Similar findings have been reported in rodent tumor models, such as, mouse and rat mammary tumors [25, 27], mouse skin carcinogenesis [24, 34], rat bladder carcinogenesis [16] and rat esophageal carcinogenesis [30, 33]. Recently, Otori et al. [20] reported that the overexpression of cyclin D1 occurs early in rat colon tumor induced by azoxymethane. However, this work studied cyclin D1 expression only at the protein level by IHC, not at the mRNA level, and did not investigate the expression status of other important G1 cell cycle regulators, such as cyclin E. Thus, in the present study, we analyzed the expression pattern of cyclin D1 and cyclin E at the protein and mRNA levels and compared their expressions with the expression of PCNA.

In the present study, we observed significantly increased expressions of cyclin D1 and cyclin E mRNA in both adenomas and adenocarcinomas, as compared with normal colonic tissues (Fig. 1 and 2). Immunohistochemical findings also revealed that the expressions of cyclin D1 and cyclin E were increased in both adenomas and adenocarcinomas, but that it is undetectable in normal colonic mucosa, indicating that the degree of induction of these proteins during carcinogenesis may be related to oncogenic transformation. However, there was no

significant difference in either the mRNA levels or protein expressions of cyclin D1 and cyclin E in adenomas and adenocarcinomas. These results suggest that once the tumor has been established at the adenoma stage, there is no need for further expression of these proteins for malignant transformation. Therefore, the overexpression of these genes may be involved in the development and progression of colorectal adenocarcinomas and seems to be an early event during the multistage carcinogenesis of rat colon tumor. Similar results were also found in rat esophageal tumor [30] and in human colorectal carcinogenesis [2].

One recent study has shown that PCNA, a marker for cell proliferation, is maximally elevated in the late G1 and S phases of proliferating cells [14]. Furthermore, it has also been reported that the degree of PCNA expression generally correlates well with the mitotic activity of the neoplastic cells and the grade of tumor [7]. Thus, we compared cyclin D1 and cyclin E mRNA levels with tissue PCNA in the same stage. We also compared the topologic distributions of cyclin D1 and cyclin E with that of PCNA by IHC. The present study revealed that the topologic distributions of cyclin D1- and cyclin E-immunoreactive cells did not correspond to PCNA-immunoreactive cells in either adenomas or adenocarcinomas (Fig. 3). These findings suggest that there was no specific association between the overexpression of cyclin D1 or cyclin E with PCNA, and that cyclin D1 and cyclin E overexpression occurred independently of PCNA. These results also suggest that the overexpression of either cyclin D1 or cyclin E is not a mere consequence of cellular proliferative activity, but rather represents a true difference between the normal and tumorous states. Our findings are consistent with several previous reports showing that no simple correlation was observed between cyclin D1 and PCNA expression, nor was there a correlation between cyclin E and PCNA expression [3, 12, 16, 25, 33]. Since the overexpressions of cyclin D1 and cyclin E have been shown to cause abnormalities in growth control and cell cycle progression, the increased expression of PCNA in our study is probably a consequence of these events. In addition, no association was found between the overexpressions of cyclin D1 and cyclin E, suggesting that multiple independent mechanisms of cell cycle deregulation may be present during colonic carcinogenesis in our model.

So far, several studies have been performed to investigate the possiblity of using cyclin D1 and cyclin E overexpression as a prognostic factor for tumors [1, 2, 15, 18, 22] but the results have been conflicting. In gastric, urinary bladder, and breast tumors, cyclin D1 and cyclin E overexpression correlates highly with tumor clinical and pathologic parameters [1, 15, 22], whereas other studies have failed to find any correlation between cyclin D1 and cyclin E overexpression and the clinicopathologic factors of colorectal cancer [2, 18]. Further investigation is needed to determine whether the altered expressions of cyclin D1 and cyclin E can be used as an independent prognostic markers in an animal colorectal carcinogenesis model that is relevant to human colorectal carcinoma.

In conclusion, our findings indicate that the overexpression of cyclin D1 and cyclin E may play an important role during the early progression of DMH-induced rat colon carcinogenesis deregulating cell cycle control at the in benign adenoma stage, and thereafter, participating in tumor progression. Furthermore, our results also suggest that the overexpressions of cyclin D1 and cyclin E occur independently of PCNA expressions, and therefore, that the overexpression of these genes is not just a secondary phenomenon following cell proliferation.

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