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Expressions of Cell Cycle Regulators in Human Colorectal Cancer Cell Lines

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To study the altered mechanisms of cell cycle regulation in colorectal cancer, the expressions of cyclins, cyclin-dependent kinases (CDKs), CDK inhibitors, p53 and retinoblastoma (Rb) protein were analyzed by western blotting in a series of human colorectal cancer cell lines. The colorectal cancer cell lines exhibited various expression patterns of cell cycle regulators, which may reflect differences in the biological characteristics of cancer cells and in the genetic backgrounds of carcinogenesis. A correlation was found between *p53* gene alteration and *p21* expression, suggesting that *p53* gene mutation usually suppresses *p21* expression, though *p21* expression could be induced via both a *p53*-dependent and a *p53*-independent pathway in colorectal cancer. None of the cell lines studied expressed p16 protein, suggesting that inactivation of p16 may be a common alteration in colorectal cancer. Moreover, all the D-type cyclins, especially D2 and D3, were expressed at a high level in most of the cell lines. Loss of *p16* expression and increased expression of D-type cyclins promote CDK-mediated Rb phosphorylation. All of the colorectal cancer cell lines studied herein expressed Rb protein, but the growth-suppressive properties of Rb may be inactivated by the loss of *p16* expression and increased expressions of D-type cyclins. In view of the pivotal role of Rb in cell cycle regulation, loss of *p16* expression and overexpression of D-type cyclins may be critical alterations in colorectal cancer.

Key words: Cell cycle — Colorectal cancer — Cyclin — Cyclin-dependent kinase — Cyclin-dependent kinase inhibitor

The transition from G1 to S phase is a critical point in the control of the cell cycle. Cyclin-dependent kinases (CDKs) and their functional partners, the cyclins, are positive cell cycle regulators. Cyclins form complexes with CDKs, thereby activating their functions.[1,2] The D-type cyclins and cyclin E (G1 cyclins) play roles in the progression from G1 to S phase. Cyclins D1, D2 and D3 associate with CDK4 and CDK6, while cyclin E preferentially associates with CDK2.[1,2] On the other hand, the G1/S transition is negatively regulated by CDK inhibitors such as $p16^{MTS1}$,[3–5] $p15^{MTS2}$,[6] $p21^{WAF1}$ [7,8] and $p27^{KIP1}$.[9–11] The $p16^{MTS1}$ and $p15^{MTS2}$ bind to CDK4 and CDK6, preventing the activation of these CDKs by D-type cyclins.[5,6] The $p21^{WAF1}$ and $p27^{KIP1}$ bind to cyclin-CDK complexes and inhibit the activity of CDK2, CDK4 and CDK6.[7–10] A key substrate of G1 CDKs is the retinoblastoma (Rb) protein, the phosphorylation of which releases the Rb-binding transcriptional factor E2F and activates its function.[12] Many genes required for cell proliferation have an E2F binding site in their promoter.[13] The genes transcriptionally activated by E2F promote progression from G1 to S phase. Tumor suppressor gene *p53* is also known to be an important cell cycle regulator controlling the G1/S transition.[14,15] In response to DNA damage, p53 mediates cell cycle arrest in the G1 phase to allow time for DNA repair.[16,17] It was shown that p53 activates transcription of the CDK inhibitor $p21^{WAF1}$, which itself can suppress cell growth.[8]

Recent studies have shown that various types of alterations in cell cycle regulators are common in a wide variety of neoplastic diseases.[18,19] In order to gain insights into altered mechanisms of cell cycle regulation in colorectal cancer, expressions of positive and negative cell cycle regulators were studied in a series of colorectal cancer cell lines.

MATERIALS AND METHODS

Human colorectal cancer cell lines (CaR1, SW480, HT29, Colo320, DLD1, LoVo, WiDr, Colo201) and an esophageal cancer cell line (TE1) were cultured in RPMI 1640 supplemented with 10% fetal calf serum. SW480, HT29, DLD1, WiDr and Colo201 have *p53* gene mutations, while Colo320 and LoVo have the wild-type *p53* gene.[20–22] TE1 was used as the positive control for p16 protein.[23] Cells were routinely cultured in a humidified incubator at 37°C under an atmosphere containing 5% CO_2. Total cell lysates were prepared by adding pre-warmed denaturing buffer (Tris 150 m*M*, pH 6.5, sodium dodecyl sulfate (SDS) 4%, β-mercaptoethanol 2%, glycerol 10%) directly to the exponentially growing cells.

A set with an equal amount of protein from each cell lysate was subjected to SDS-polyacrylamide gel electrophoresis (PAGE) followed by immunoblotting. The proteins were transferred from the gel onto a polyvinylidene difluoride (PVDF) membrane (Bio-Rad Laboratories,

Hercules, CA) in transfer buffer (25 m*M* Tris, 192 m*M* glycine, and 20% methanol) for 1 h. The membrane was incubated with blocking solution (10 m*M* Tris, 150 m*M* NaCl, and 3% gelatin) for 1 h at room temperature. After incubation with the primary antibody overnight at 4°C, the membrane was incubated with the secondary antibody (goat anti-mouse or anti-rabbit immunoglobulin conjugated with horseradish peroxidase; Dako) at room temperature for 30 min. An ECL chemiluminescent substrate (Amersham, Arlington Heights, IL) was applied for 2 min according to the manufacturer's instructions. Between these steps, the membrane was washed with TBS (10 m*M* Tris, 150 m*M* NaCl) and TBS-Tween (TBS with 0.1% Tween 20). The membrane was exposed to X-ray films (X-OMAT AR, Kodak). Western blotting of β-actin was performed as the control for the amount of protein applied in each sample. Densitometric scanning of the blots was performed using a Hewlett Packard Scan Jet 3c and an image analysis software program (Luminous Imager version 1.0, Aisin Cosmos R&D Co., Ltd., Kariya). The content of each protein was expressed using the densitometry score. The highest level of expression was defined as 100 (arbitrary units) for each cell cycle regulator.

Anti-β-actin mouse monoclonal antibody (mAb) was purchased from Sigma (St. Louis, MO). Anti-p53 rabbit polyclonal antibody (CM1) was purchased from Novocastra Laboratories (Newcastle, UK). Anti-Rb mAbs (G3-245 and G99-549), anti-cyclin D2 mAb (G132-43), anti-cyclin E mAb (HE12) and anti-p16 mAb (G175-1239) were purchased from Pharmingen (San Diego, CA). Anti-Rb mAb (G99-549) recognizes only the fast migrating, underphosphorylated form of Rb protein. Anti-cdk6 rabbit polyclonal antibody and anti-cyclin A mAb (BF683) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-cdk2 mAb (clone55), anti-cdk4 mAb (clone97), anti-cyclin D1 mAb (clone105), anti-cyclin D3 mAb (clone1), anti-p21 mAb (clone70) and anti-p27 mAb (clone57) were purchased from Transduction Laboratories (Lexington, KY).

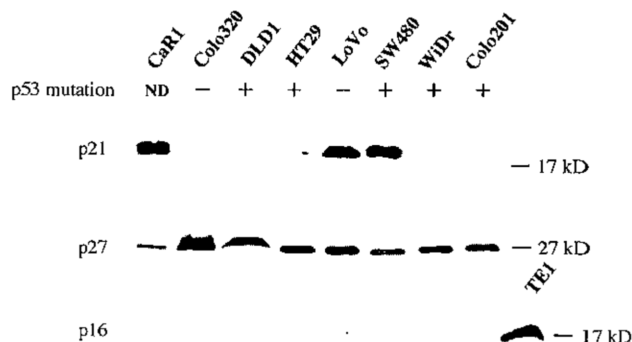

Fig. 1. Western blot analysis of CDK inhibitors (p16, p21 and p27) in colorectal cancer cell lines. An equal amount of protein was applied in each lane and analyzed as described in "Materials and Methods." The *p53* gene status is indicated in the second row. +, mutated p53 gene; −, wild-type *p53* gene; ND, not determined. An esophageal cancer cell line, TE1 was used as the positive control for p16 protein. Right ordinate, molecular weight marker size.

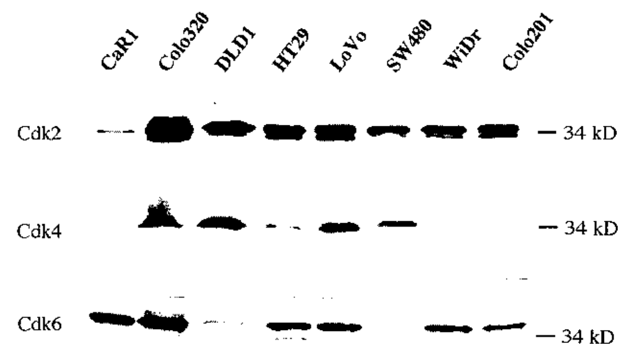

Fig. 2. Western blot analysis of CDKs (CDK2, 4 and 6) in colorectal cancer cell lines. An equal amount of protein was applied in each lane and analyzed as described in "Materials and Methods." Right ordinate, molecular weight marker size.

RESULTS

The results of western blotting are shown in Figs. 1–4. Densitometric scanning was performed on each group of blots. Fig. 5 shows the results of quantification of cell cycle regulators. None of the colorectal cancer cell lines studied expressed p16 protein, while all expressed p27 protein. p21 was expressed in half of the cell lines. *p53* expression was detected in all cell lines except LoVo. Most of the colorectal cancer cell lines with *p53* gene mutations expressed high levels of p53 protein and low or undetectable levels of p21 protein. Increased *p21* expression was found in LoVo with the wild-type *p53* gene and in SW480 with a mutated *p53* gene. All of the cell lines expressed Rb protein, while the fast-migrating form of Rb was detectable in half of the cell lines.

The colorectal cancer cell lines showed various expression patterns of CDK inhibitors, CDKs and cyclins. Most of the cell lines expressed high levels of D-type cyclins. Cyclin D2 or D3 overexpression was more frequently observed than cyclin D1 overexpression. Overexpression of cyclin A or E was found in some of the cell lines. We found that CDK2 expression paralleled that of p27. No correlation was found between the expressions of CDKs and those of cyclins.

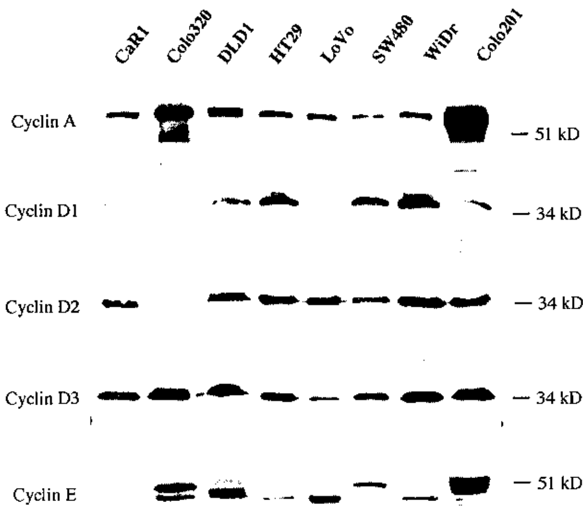

Fig. 3. Western blot analysis of cyclins (cyclin A, D1, D2, D3 and E) in colorectal cancer cell lines. An equal amount of protein was applied in each lane and analyzed as described in "Materials and Methods." Right ordinate, molecular weight marker size.

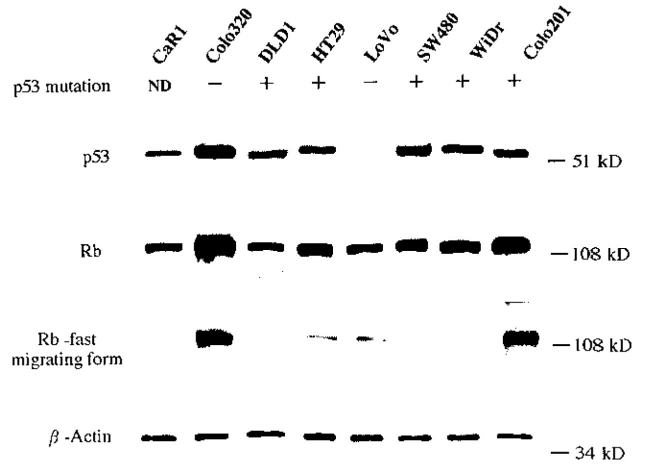

Fig. 4. Western blot analysis of p53 and Rb in colorectal cancer cell lines. An equal amount of protein was applied in each lane and analyzed as described in "Materials and Methods." Western blotting of β-actin was performed as a control for the amount of protein. The fast-migrating form of Rb protein corresponds to an underphosphorylated Rb protein. The *p53* gene status is indicated in the second row. +, mutated *p53* gene; −, wild-type *p53* gene; ND, not determined. Right ordinate, molecular weight marker size.

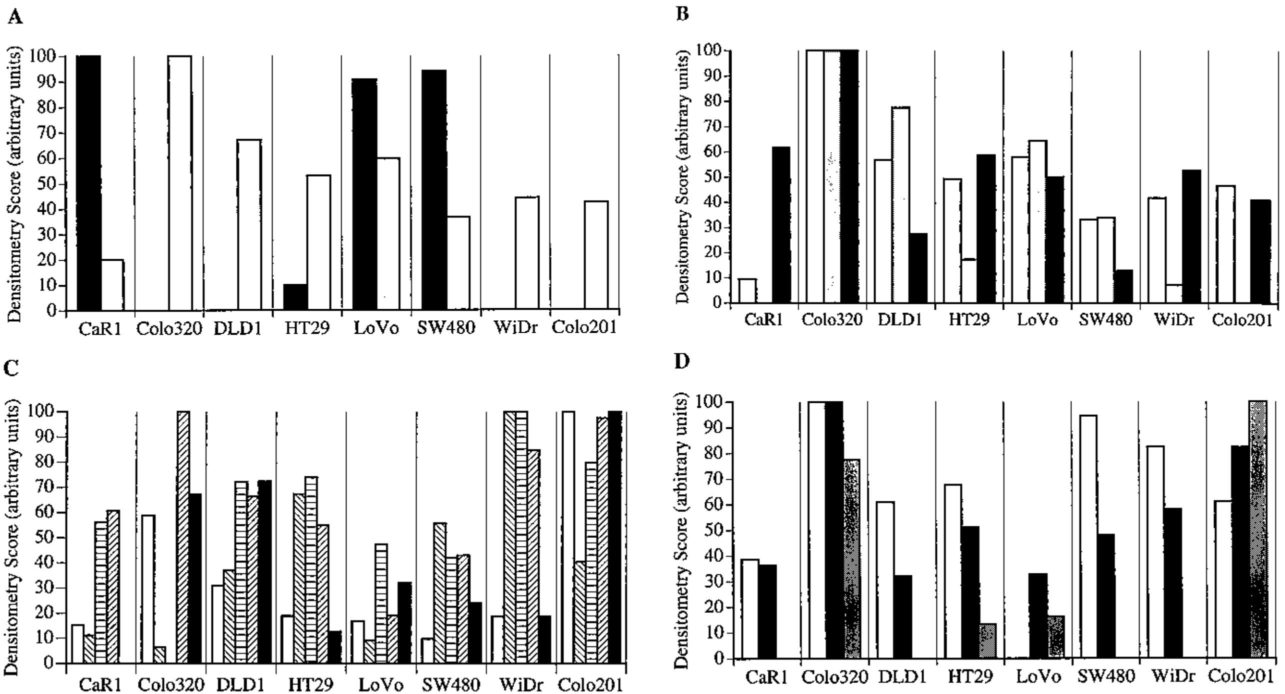

Fig. 5. Quantification of the expressions of cell cycle regulators (A, CDK inhibitors, ■ p21, □ p27; B, CDKs, □ CDK2, ▨ CDK4, ■ CDK6; C, cyclins, □ cyclin A, ▧ cyclin D1, ▤ cyclin D2, ▨ cyclin D3, ■ cyclin E; D, □ p53, ■ Rb, ▨ fast-migrating form of Rb) in colorectal cancer cell lines. Densitometric scanning was performed in the series of blots from each cell cycle regulator. The highest level of expression was defined as 100 (arbitrary units) in each set of blots. Fast-migrating form of Rb protein corresponds to an underphosphorylated Rb protein.

DISCUSSION

None of the colorectal cancer cell lines studied expressed p16 protein, suggesting that inactivation of p16 may be a common alteration in colorectal carcinogenesis. Homozygous deletions and point mutations of p16 have been described in a variety of neoplastic diseases.[3, 4, 23–26] However, *p16* gene alterations are rare in some types of tumors, including colorectal cancer,[26,27] and are less frequent in primary tumors than in established cell lines.[28, 29] A recent study showed that *de novo* methylation of 5′ CpG islands in the p16 promoter region suppresses the transcription of p16, resulting in loss of *p16* expression.[30] This aberrant methylation was found to be common in colorectal, lung, breast and bladder cancers.[27, 30, 31] Therefore, the loss of *p16* expression in our series of colorectal cancers may be due to methylation of the 5′ CpG islands of p16.

Loss of *p16* expression promotes CDK4-mediated Rb phosphorylation, which inactivates Rb functions and promotes cell cycle progression.[32, 33] Moreover, increased expression of D-type cyclins can suppress Rb functions by activating CDK4 and CDK6.[1, 2] Gene amplification and subsequent overexpression of cyclin D1 have been reported in a variety of carcinomas.[34–36] Our results show that all the D-type cyclins, especially D2 and D3, were expressed at high levels in most of the colorectal cancer cell lines. All of the colorectal cancer cell lines studied herein expressed Rb protein, but the growth-suppressive properties of Rb may be inactivated by the loss of *p16* expression and increased expressions of D-type cyclins. Even for other types of cancer, Rb inactivation may be an important alteration. Rb-positive lung cancers reportedly express little or no p16, while Rb-negative cancers show abundant *p16* expression.[37] Moreover, cyclin D1 overexpression was more frequently found in Rb-positive than in Rb-negative tumors of the lung and esophagus.[38, 39] As a means of inactivating Rb function, cancer cells apparently acquire one of the following alterations; loss of *p16* expression, increased expressions of D-type cyclins or loss of Rb expression.[31, 37, 40]

Tumor suppressor gene *p53* is one of the most frequently mutated genes in various forms of malignancy.[14, 32] Wild-type but not mutant p53 activates p21, which mediates G1 arrest of the cell cycle and suppresses cell growth.[7, 8] However, it has been shown that p21 can also be activated in a p53-independent manner.[33, 34] We have shown that most colorectal cancer cell lines with a mutated *p53* gene expressed low or undetectable levels of p21 protein. On the other hand, increased *p21* expression was found in LoVo with the wild-type *p53* gene and SW480 with a mutated *p53* gene. *p53* gene status appeared to be a determinant of *p21* expression in most cases, but *p21* expression could be induced via both a p53-dependent and a p53-independent pathway in colorectal cancer.

The colorectal cancer cell lines exhibited various expression patterns of CDKs, CDK inhibitors and cyclins. *p27* expression was associated with CDK2 expression, but no other correlations were apparent, as far as studied by western blotting. We have confirmed that altered expression of cell cycle regulators is a common finding in colorectal cancer cell lines. Distinct cell cycle regulator expression patterns may reflect differences in the biological characteristics of cancer cells and in the genetic backgrounds acquired during the process of malignant transformation. In view of the pivotal role of Rb in cell cycle regulation, loss of *p16* expression and overexpression of D-type cyclins may be critical alterations in colorectal cancer.

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