Tumorigenesis and Neoplastic Progression

Regulation of Skp2-p27 Axis by the Cdh1/Anaphase-Promoting Complex Pathway in Colorectal Tumorigenesis

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Abrogated entry into S phase is a common hallmark of cancer cells. Skp2, a subunit of ubiquitin ligase, is critical for regulating the G₁/S transition. Uncontrolled Skp2 activity is detected frequently in human tumors, often correlated with poor prognosis. Current studies have suggested that the regulation of Skp2 turnover is mediated by another critical ubiquitin ligase, the anaphase-promoting complex (APC), in association with its substrate-specific factor Cdh1. To dissect the potential role of Cdh1/APC in tumorigenesis through the degradation of Skp2, we analyzed the Cdh1/APC-Skp2-p27 axis in colorectal tumorigenesis using a human tumor array and biochemical analyses. Our results show that the percentage of Cdh1- and p27-positive samples in colon cancer tissues was significantly lower than that in adjacent nonmalignant tissue. Conversely, the percentage of Skp2-positive colon cancer samples was significantly higher than that in normal tissue. Furthermore, results from clinicopathological analysis revealed that elevated Cdh1 expression was associated with lower histological grade tumors. In addition, depletion of Cdh1 by RNA interference in nonmalignant colon cells resulted in increased cellular proliferation, whereas knockdown of Skp2 significantly suppressed cancer cell growth. Our result suggests a pathological correlation between Skp2 and Cdh1/APC in colorectal cancer. Thus, Cdh1 may function as a component in tumor suppression via proteolysis of Skp2 in colorectal tumorigenesis and may serve as a prognostic marker in colon cancer patients. (*Am J Pathol* 2008, 173:217–228; DOI: 10.2353/ajpath.2008.070957)

S phase kinase-associated protein 2 (Skp2) acts as the substrate-specific factor for the Skp1-Cul1-F-box (SCF) complex that catalyzes ubiquitylation of multiple functional protein for degradation, including p27, p21, Rb, and p53.^{1–3} Dysregulated regulation of Skp2 is often detected in human tumor tissue, which is correlated with poor prognosis. The well understood role of Skp2 is to govern the transition from G₁ to S phase through degradation of p27 thereby regulating cyclin E/CDK2.^{2,3} Both Skp2 transcripts and protein oscillate during the cell cycle.⁴ Current studies have shown that anaphase-promoting complex (APC) is the critical E3 ligase that targets Skp2 for ubiquitylation and degradation, therefore averting abnormal entry into S phase.^{5,6} However, the correlation between Cdh1 and tumorigenesis through regulation of Skp2 has not yet been validated although Skp2 has served as a clinical marker for several types of cancer.^{7–9}

The APC is a multifunctional E3 ligase, regulating several critical cellular events, including mitotic progression, DNA replication, cellular differentiation, genomic integrity, and signal transduction.^{10–17} Recent evidence has drawn our attention to connect APC function with human diseases. Pathological and epigenetic studies have demonstrated that dysfunction in several components of the APC pathway, including APC6, Cdc16, Cdc23, and Cdh1 or Cdc20, is correlated with different types of cancer such as colon cancer, B-lymphoma, gastric, and lung cancer.^{18–21} Activation of APC is controlled by two WD40 family proteins, Cdh1 and Cdc20. Cdh1 in association with APC results in determining APC function in G₁ and postmitotic processes, whereas interaction of Cdc20 with APC leads to controlling chromatid separation during

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mitosis.^{11,22–25} Recognition of substrate by the substrate-specific activator, Cdh1/Cdc20, is facilitated by several well characterized degrons/recognition domains, including destruction box (RXXL), KEN box, and A box present in the substrate.²⁴ Although results from current studies suggest the role of APC is involved in cancer formation, the underlying mechanism by which APC is implicated in the above carcinogenesis remains primarily unknown. Dissection of the APC pathway in human cancer will facilitate our understanding of APC in tumorigenesis.

Recent studies have revealed that APC targets Skp2 for degradation and therefore prevents abnormal entry into S phase.^{5,6} Activation of APC by Cdh1 in G₁ inhibits premature Skp2/SCF-mediated destruction of p27 and as a result prevents premature entry into S phase. These findings suggest that the Cdh1-dependent APC activity not only targets mitotic cyclins for destruction from the end of mitosis to the G₁ phase but is indispensable for controlling the appropriate G₁/S transition, suggesting that deregulation of Cdh1/APC-dependent proteolysis of these substrates is thereby important and likely to be involved in initiation of tumor. The above previous notions have suggested that Cdh1/APC could be an important molecule as a tumor suppressor regulating the oncoprotein Skp2, whereas the significance of Cdh1/APC and its correlation with Skp2 has not yet been shown in tumorigenesis.

To validate the connection of Cdh1 and Skp2 with tumor formation, we have analyzed the expression profile of these molecules examining human tissue array and determining the clinicopathological relevance with respect to components of Cdh1/APC-Skp2 cascade. In addition, using RNA interference, we have further dissected the function of Cdh1 and Skp2 in suppressing or enhancing cell growth in both nonmalignant or cancer colon cell lines. Our study demonstrates that Cdh1 expression inversely correlates well with the expression of Skp2 in the cancer or normal condition. Suppression of Skp2 protein levels by enhancing Cdh1 function results in decrease of tumor cell growth. The present results suggest that Cdh1 may function as a critical component in the suppression of colon tumor.

Materials and Methods

Cell Lines and Culture Medium

Establishment of primary mouse embryonic fibroblasts and bone marrow stromal cells from c57BL/6J mouse were reported previously.²⁶ Bone marrow stromal cells were cultured in minimal essential medium- α (Invitrogen, Carlsbad, CA) with 10% fetal calf serum, 1 mmol/L L-glutamine (Invitrogen), 100 U/ml penicillin, 100 μ g/ml streptomycin at 32°C in 7% CO₂. Primary mouse embryonic fibroblast cells were grown in a mixture of 50% Dulbecco's modified Eagle's medium (Invitrogen) and 50% Dulbecco's modified Eagle's medium F12 (Invitrogen) supplemented with 10% fetal calf serum, 1 mmol/L L-glutamine, 100 U/ml penicillin, 100 μ g/ml streptomycin at 32°C filled with appropriate humidified gas mixture (5% O₂, 5% CO₂, 90% N₂). Primary cancer cell lines UM-22A and UM-22B

were provided by Dr. Thomas Cary (University of Michigan, Ann Arbor, MI) and grown in Dulbecco's modified Eagle's medium (Invitrogen), 10% fetal calf serum, 100 U/ml penicillin, 100 μ g/ml streptomycin at 37°C in 5% CO₂.

Plasmid Preparation and Construction of Small-Interfering RNA Stable Cell Lines

pCS2-Myc-Cdh1 and pCS2-HA-Skp2 plasmids have been engineered and reported previously.¹⁰ Stable knockdown using small interfering RNA (siRNA) for Cdh1 has been reported.¹⁵ Construction of Skp2-siRNA stable cell line Skp2 N-terminal (5'-GAGGAGCCCCGACAGT-GAGA-3') was engineered using pSUPER system (Oligo-Engine, Seattle, WA). Transfection was performed using Lipofectamine2000 (Invitrogen) according to the manufacturer's protocol. Thereafter, positive clones were selected in the presence of puromycin (Promega, Madison, WI) containing medium. Stable Cdh1 knockdown (target sequence, nucleotides 266 to 286) were reported previously using the pSUPER system (OligoEngine).¹⁶ Double knockdown of Cdh1 and Skp2 in HCT116 cells was performed by delivering duplex Skp2 siRNA (Dharmacon, Chicago, IL) (target sequence 5'-ATTCAGCTGGGTGAT-GGTCTC-3') into stable Cdh1 knockdown cells. CyclinB knockdown (target sequence: 5'-GATGGAGCTGATC-CAAAC-3') were reported previously²⁷ using the pSUPER system. As controls, we used the firefly luciferase-targeted oligo nucleotide 5'-CTGACGCGGAAT-ACTTCGA-3'.

Antibodies and Reagent

Antibodies were Cdh1 (Calbiochem, Darmstadt, Germany), Skp2 (Santa Cruz Biotechnology, Santa Cruz, CA), p27 (Santa Cruz), tubulin (Calbiochem), and horseradish peroxidase-conjugated goat anti-mouse and anti-rabbit secondary antibody (Promega). Western blot analysis was performed using an enhanced chemiluminescence detection kit (Amersham, Buckinghamshire, UK). Semiquantification of data were performed using an image densitometer.

Colony Formation by Soft Agar Assay

Twenty-four hours after transfection, viable cells were counted and concentrated at 2.0×10^5 /ml. Cells were seeded into soft agar as described previously²⁸ with slight modification (Dr. E. Flemington, Department of Pathology, Tulane Cancer Center, New Orleans, LA). Briefly, 1% agarose solution was made with sterile water, and 5 ml of agarose were added into a six-well plate until covered completely. Then the agarose was pipetted off, leaving a thin film of agarose on the bottom and side of each well. Cells were then plated at 2.0×10^5 /well. Colony formation was assessed by microscopic inspection ($\times 10$) and counting 7 days after cell seeding. Because the aggregates of the untreated CCD18Co cells did not grow further throughout the experiment period, they have been not considered as colonies.

Cell Cycle Analysis

DNA fragmentation was measured by propidium iodide staining and fluorescence-activated cell sorting analysis. After treatments, cells were harvested and pelleted by centrifugation, and were resuspended and fixed in ethanol. Cells were incubated in the propidium iodide solution (Sigma, St. Louis, MO) with 5 $\mu\text{g}/\text{ml}$ of RNase (Sigma). Flow cytometric analysis of stained cells was performed using a FACScan (Becton Dickinson, Mountain View, CA).

Bromodeoxyuridine (BrdU) Labeling

The proliferative rate of cells grown was measured by assaying 5-bromo-2-deoxyuracil (BrdU) incorporation with commercially available labeling and detection kit (Rosch Diagnosis, Indianapolis, IN). Briefly, 24 hours after transfection, cells were spread and labeled nuclei were detected, according to the manufacturer's instructions. BrdU-labeled indices were determined by visually scoring nuclei stained with 4',6-diamidino-2-phenylindole (Vector Laboratories, Burlingame, CA) in 50 to 100 cells in 10 independent visual fields and thereafter scoring BrdU-positive cells as a percentage of the total cell number.²⁹ Each experiment was repeated at least three times. Values given are the results of mean ($\pm$ SD) value score.

Immunofluorescence

Immunofluorescence analysis was performed using the following first antibodies Cdh1 (rabbit anti-rat, 1:500) and Skp2 (rabbit anti-mouse, 1:100). Second antibodies are Cy2 (anti-mouse, 1:500; Jackson ImmunoResearch, West Grove, PA), Texas-red (anti-rat, 1:100; Jackson ImmunoResearch). Semiquantification of data were performed using Scion Image (Frederick, MD) imaging software.

Immunohistochemical Staining Using Human Colon Cancer Tissue

Samples were deparaffinized in xylene and rehydrated in a series of graded alcohols, and the antigen was retrieved in 0.01 mol/L sodium citrate buffer, thereafter sections were treated with 0.6% hydrogen.³⁰ Samples were incubated using rat anti-human Cdh1 antibody (1:150), rabbit anti-human Skp2 antibody (1:100), and rabbit anti-human p27 antibody (1:150). Sections were thereafter treated with biotinylated mouse anti-rat immunoglobulin (Jackson ImmunoResearch) and donkey anti-rabbit antibody (Vector Laboratories) followed by incubations with avidin-biotin peroxidase complex solution (DAKO Cytomation, Carpinteria, CA) and 3-amino-9-ethylcarbazole solution (DAKO Cytomation). The counterstaining was performed using Mayer's hematoxylin (Sigma). Tissue arrays were purchased from US Biomax. (Rockville, MD). To verify the specificity and optimal concentration of the antibody, each antibody and its concentration was ver-

ified using the test tissue array slides (CO601t, CO701t, CO811t).

Scoring of Immunohistochemical Staining

Cdh1, Skp2, and p27 immunohistochemical staining were examined under the microscope (Olympus, Melville, NY), and staining intensity and subcellular localization were evaluated twice in a blinded manner based on the preagreed staining scoring standard from specialized pathologists Dr. D. Roodman (Department of Hematology and Oncology, University of Pittsburgh) and Dr. S. Cheng (Department of Pathology, University of Pittsburgh). Staining intensity was scored separately using the following scoring criteria: 0 to 1, negative or low staining intensity in >50% of tumor cells or moderate to high in <50% of the cells (hereafter referred to as low); and 2 to 3, moderate to high staining intensity in >50% of tumor cells (hereafter referred to as high).³¹

Statistical Analysis

Levels of statistical significance were evaluated with data from at least three independent experiments by using the two-tailed Student's *t*-test. The χ^2 test, Fisher's exact test, and correlation test were used for statistical analysis of immunostaining results and analysis of clinicopathological data. *P* < 0.05 was considered statistically significant. All data were analyzed with SPSS14.0 (Chicago, IL) for Windows.

Results

Abrogation of Cdh1/APC Pathway in Cancer

It has been demonstrated that Skp2 and p27 are important proteins with their deregulation correlated with tumorigenesis.^{9,32,33} Skp2 is thought as an oncogenic protein-enhancing carcinogenesis, whereas p27 is defined as a tumor suppressor-inhibiting tumorigenesis.³³⁻³⁵ Previous biochemical studies have shown that both Skp2 and p27 are high-turnover proteins governed by the ubiquitin-proteasome pathway.⁹ Current studies have implicated Cdh1/APC in having a pivotal role in orchestrating the Skp2-p27 axis, thereby preventing precocious entry into S phase.^{5,6,10} To investigate the regulatory axis of Cdh1/APC-Skp2-p27 with malignant tumor status, we analyzed the expression of Cdh1 in various types of human malignant tissues. Using tissue array, we examined 176 tissue samples, which have 15 different types of cancer tissue together with the adjacent nonmalignant tissues and scored staining intensity. In our tumor tissue analyses, both tumor and adjacent normal tissue were examined for Cdh1. Mean values of score are obtained with statistical analyses. As shown in Figure 1A, in several organs, Cdh1 is relatively highly expressed in adjacent nonmalignant tissues compared with the cancer tissues, especially in colon and breast tissues. In addition, statistically significant differences between cancer and nonmalignant adjacent tissues were observed in breast, co-

Figure 1. Abrogation of Cdh1, APC, Skp2, and p27 in cancer. **A:** Expression profile of Cdh1 in 15 different types of malignant tumors with the adjacent nonmalignant tissues as measured by immunohistological analysis (**a**). As indicated, Cdh1 is expressed relatively higher in brain, breast, colon, esophagus, liver, ovary, rectum, skin, and stomach cancer compared with adjacent normal tissues. Moreover, significant difference of Cdh1 expression is detected between cancer and normal tissue of colon and breast, where expression of Cdh1 is significantly lower in cancer tissues than normal tissues. Each value (**b**) shows mean score of the positive Cdh1 staining from at least six different samples. **B:** Cdh1 and p27 are down-regulated, whereas Skp2 is up-regulated in both breast and colon cancer cells (**a**). Quantification of protein expression in normal and cancer cells (**b**). **C:** Immunocytochemical analysis of Cdh1 and Skp2 in breast (**a**) and colon cancer cells (**b**). Both Cdh1 (red) and Skp2 (green) are expressed and co-localized in the nucleus. Estimation of Cdh1 and Skp2 expression is reflected by fluorescent signal in nonmalignant and cancer cells using surface plotting. Each value (**c**) shows relative intensity/pixel from at least five independent views.

lon, and rectum cancer. Furthermore, using serial sections of the same specimen, we examined the Skp2 levels as well as p27 levels (Supplemental Figure S1A, available at <http://ajp.amjpathol.org>) and found that Skp2 levels were significantly higher ($P < 0.05$), whereas abundances of p27 levels were significantly lower in breast, colon, and rectum cancers.

To confirm further the correlation between Cdh1 and Skp2-p27 axis in colorectal cancer as well as breast cancer, we have accessed the expression of Cdh1, Skp2, and p27 in different types of cancer as well as nonmalignant cell lines including breast and colon cells, which contain primary established nonmalignant (mouse embryonic fibroblasts and bone marrow stromal cells) and cancer (UM-22A, UM-22B) cells. As shown in Figure 1B, expression levels of Cdh1 and p27 were lower in these cancer cell compared with nonmalignant cell lines ($P < 0.05$), whereas expression levels of Skp2 were higher in cancer cells but lower in nonmalignant cells (Supplemental Figure S1B, available at <http://ajp.amjpathol.org>).

To confirm the results obtained from the above immunoblotting, we have performed immunocytochemical analysis in colon and breast cancer cell lines (HCT116, MCF7) compared with nonmalignant (CCD-18Co, MCF10A) cells. As shown in Figure 1C, both Cdh1 and Skp2 are principally expressed in the nucleus, and they co-localize within the nucleus in both colon and breast cells. Quantification analyses demonstrated that expression of Cdh1 in nonmalignant cells is ~30% higher than in cancer cells whereas Skp2 is ~40% lower in the nonmalignant cells than in cancer cells (Figure 1C). The results based on immunocytochemical analyses are consistent with the results from the initial immunoblotting data. Taken together, the results based on tissue array, immunoblotting, as well as immunocytochemical analyses suggest a role for Cdh1 upstream of Skp2-p27 cascade in tumorigenesis and further implicate the function of Cdh1 in protein degradation in the tumor formation.

Overexpression of Skp2 or Depletion of Cdh1 Enhances Cell Proliferation in Nonmalignant Colon Cells

To analyze the role of Skp2 in promoting cellular growth and function of Cdh1 potentially in suppressing proliferation via degradation of Skp2,^{5,6,9,36} we have performed overexpression of Skp2 and depletion of Cdh1 using RNA interference, respectively, in nonmalignant colon cells. Based on Skp2 overexpression or Cdh1 knockdown, we measured their downstream protein expression and further estimated their effects on cellular oncogenesis and proliferation. We initially engineered a Cdh1 shRNA stable cell line (Figure 2B).^{10,15} As shown in Figure 2A, overexpression of Skp2 in CCD18Co cells results in significant drop of p27 protein levels. Consistent with previous data, depletion of Cdh1 using RNA interference leads to significant elevated levels of Skp2, which in turn causes the attenuation of p27 levels in CCD18Co cells (Figure 2B).

To access the ability for oncogenic colony formation and the capacity for accelerating cell growth in response to alteration of Cdh1 and Skp2, we conducted an anchorage-independent growth assay and further measured cell cycle profile as well as BrdU-positive cells based on CCD18-Co cells. As shown in Figure 2C, either depletion of Cdh1 or overexpression of Skp2 in CCD18Co cells promoted the number as well as the size of colonies on soft agar, whereas only nonappreciable colonies was found for control cells. Results from fluorescence-activated cell sorting analyses showed that either depletion of Cdh1 or overexpression of Skp2 in CCD18Co cells significantly elevated the fraction of cells in S phase (Figure 2Da). Analysis based on BrdU staining demonstrated that either depletion of Cdh1 or overexpression of Skp2 resulted in an increase of BrdU-positive stained cells (Figure 2D, b and c). In summary, the results based on the above analyses suggest that increase of Skp2 protein levels or loss of Cdh1 could lead to the aberrant cell cycle, which in turn would promote oncogenesis in nonmalignant colon cells.

Overexpression of Cdh1 or Knockdown of Skp2 Reduces Cellular Growth in Colon Cancer Cells

To confirm the above results based on the normal colon cells, we further performed depletion of Skp2 or overexpression of Cdh1 in colon cancer cells and subsequently examined the effects on colony formation as well as proliferation. We initially overexpressed Cdh1 in HCT116 cells. As shown in Figure 3A, overexpression of Cdh1 in HCT116 cells primarily reduced Skp2 protein levels leading to elevation of p27 abundance. We next established a Skp2 shRNA stable cell line (Figure 3B).^{10,15} As predicted, depletion of Skp2 resulted in significant increase in p27 levels (Figure 3B; and Supplemental Figure S2A, available at <http://ajp.amjpathol.org>). Additionally, no significant effect of p27 levels was observed by overexpressing Cdh1 in Skp2-depleted cells suggesting that Cdh1-mediated p27 regulation is depend on Skp2 (Supplemental Figure S2A, available at <http://ajp.amjpathol.org>).

Furthermore, we analyzed the oncogenic colony formation and the capacity of accelerating cell growth using HCT116 having either Cdh1 overexpression or Skp2 depletion. As shown in Figure 3C, HCT116 cells have great capacity for colony formation in soft agar while both number and size of colony significantly dropped when either Cdh1 was overexpressed or Skp2 was depleted in HCT116 cells, confirming that the major effects of Cdh1 in p27 regulation is mediated via Skp2 proteolysis (Supplemental Figure S2, A–C, available at <http://ajp.amjpathol.org>).

Consistent with the results based on the anchorage-independent growth assay. Fluorescence-activated cell sorting analysis demonstrated the fraction of cells in S phase was significantly reduced in either Cdh1-overexpressed or Skp2-depleted HCT116 cells (Figure 3Da). In addition, the results from immunocytochemical analysis based on BrdU staining showed that either overexpres-

Figure 2. Effect of overexpression of Skp2 or depletion of Cdh1 in nonmalignant colon cells. **A:** Overexpression of Skp2 results in down-regulation of p27 in nonmalignant colon cells (**a**). Quantification of expression of Skp2 and p27 from three replicate experiments is shown (**b**). **B:** Depletion of Cdh1 by shRNA (**a**). Knockdown of Cdh1 leads to up-regulation of Skp2 and down-regulation of p27 (**b**). Quantification of Cdh1 knockdown experiment from three replicate experiments (**c**). **C:** Depletion of Cdh1 or overexpression of Skp2 promotes anchorage-independent growth in nonmalignant colon cells (**a**). Quantification of anchorage-independent growth analysis from three replicate experiments (**b**). **D:** Effect of Cdh1 knockdown and overexpression of Skp2 on the cell cycle profile of nonmalignant colon cells (**a**). Effect of Cdh1 knockdown or overexpression of Skp2 on cellular proliferation of nonmalignant cells measured by BrdU analysis (**b**). Quantification of BrdU analysis from three replicate experiments (**c**).

Figure 3. Effect of overexpression of Cdh1 or depletion of Skp2 in colon cancer cells. **A:** Overexpression of Cdh1 results in down-regulation of Skp2 in colon cancer cells (**a**). Quantification of expression of Cdh1, Skp2, and p27 from three replicate experiments (**b**). **B:** Engineer of Skp2 shRNA clones (**a**). Knockdown of Skp2 results in elevation of p27 (**b**). Quantification of Skp2 knockdown experiment (**c**). **C:** Depletion of Skp2 or overexpression of Cdh1 suppresses anchorage-independent growth in breast cancer cells (**a**). Quantification of anchorage-independent growth analysis from three replicate experiments (**b**). **D:** Effect of Skp2 knockdown or overexpression of Cdh1 on the cell cycle profile of colon cells (**a**). Effect of Skp2 knockdown or overexpression of Cdh1 on cellular proliferation of colon cancer cells measured by BrdU analysis (**b**). Quantification of BrdU analysis from three replicate experiments (**c**).

sion of Cdh1 or depletion of Skp2 led to a significant reduction of BrdU-positive stained cells in HCT116 cells (Figure 3D, b and c). Taken together, the above results further suggest that Skp2 promotes oncogenic proliferation while Cdh1 potentially could orchestrate Skp2, thereby suppressing the acceleration of tumor growth.

Correlation of Cdh1/APC to Skp2-p27 Cascade by Tissue Array Analysis of Human Colon Tissue

Our results based on the loss of function analyses of Cdh1/APC-Skp2-p27 using RNA interference in combination with the overexpression analyses in both normal and colon cancer cells demonstrated that Cdh1 plays a pivotal role in dictating Skp2-p27 function in cellular proliferation for colon cancer cells. This result suggests that deregulation of Cdh1 could contribute to aberration of Skp2/p27 in colon cancer.

To correlate the importance of the molecular pathway of Cdh1/APC-Skp2-p27 in controlling G₁/S transition with pathological relevance in colon cancer, we first performed human tissue array analyses using 80 colon tumors as well as adjacent nonmalignant colon tissues (US Biomax). For the semiquantitative evaluation of the tested proteins, we scored the staining intensity in four categories (Figure 4A). As shown in Figure 4B, significantly higher frequency of positive Cdh1 expression was detected in nonmalignant adjacent colon tissue with prominent nuclei localization compared with colon cancer tissue (Figure 4B). Meanwhile, higher frequency of positive Skp2 expression was observed in colon cancer tissue, whereas lower frequency of p27 expression was measured in the cancer area (Figure 4B). Further, significant differences are observed in Cdh1 ($P < 0.01$), Skp2 ($P < 0.01$), and p27 ($P = 0.016$) between colon cancer and adjacent normal colon tissue (Table 1). Moreover, there is a significant correlation between the expression levels of Cdh1 and p27 ($P = 0.00026$), and an inverse correlation is also recognized between Cdh1 and Skp2 ($P = 0.025$), where colon cancer cells have lower expression of Cdh1 and p27 but higher expression of Skp2 (Figure 4C). Overall, our results based on the human colon tissue array are consistent with the results obtained from the culture cell studies.^{5,6}

Clinicopathological Significance of Cdh1 in Patients with Colon Cancer

The results from the tissue array suggest a potential function for Cdh1 in suppressing colon tumor progression. To correlate such results based on the molecular dissection to clinical or pathological relevance, we have performed a clinicopathological analysis for Cdh1/APC-Skp2-p27 axis in patients with colon cancer. In this study, using a different and independent set of 133 colon cancer samples, we analyzed the patients age, tumor size (T1 to T4), lymph node status (N0 to N2), distant metastatic status (M0 to M1), and histological grade (G1 to

G3) then evaluated the significance of Cdh1 in these patients.

Among the 133 patients, 30 patients (22.5%) were positive for Cdh1, which is consistent with the previous observation as shown in Table 1. There are no significant differences between Cdh1-positive and -negative cancers in patient's age ($P = 0.892$), tumor size ($P = 0.236$), lymph node status ($P = 0.624$), and distant metastasis status ($P = 0.252$) (Table 2). However, statistically significant difference are observed in histological grade between Cdh1-positive and -negative colon cancers ($P = 0.009$), where Cdh1-positive cancer is more frequently categorized in lower histological grade tumor (grade 1: Cdh1⁻: 58.2%, Cdh1⁺: 41.8%), while less frequently categorized into higher histological grade tumor (grades 2 and 3: Cdh1⁻: 85.9%, Cdh1⁺: 14.1%) (Figure 5). Results of the clinicopathological analysis indicate that abundant Cdh1 is correlated with low histological grade tumor and therefore suggest that loss of Cdh1 could be associated with aggressive cellular behavior and potential poor prognosis in colon cancer patients. Taken together, these results implicate that Cdh1 has an appreciable function in suppressing colon tumorigenesis and could be a potential prognostic marker in patients with colon cancer.

Discussion

The balance of cell cycle-positive regulators and tumor suppressor is critical to ensuring normal cell cycle progression. Uncontrolled expression of oncogenic proteins such as Skp2 and pivotal tumor suppressor p27 has been implicated in various types of cancer.^{32,34,37} Previous studies have demonstrated that the ubiquitin-proteasomal system plays a vital role in regulating both Skp2 as well as p27. Recent studies have demonstrated that Cdh1 is a critical factor of E3 ligase in orchestrating Skp2-p27 axis, which sheds a light on its potential clinical relevance. The present study confirms the role of Cdh1 in the regulation of cancer formation, particularly in colorectal tumorigenesis through modulating Skp2-p27-cyclin E/CDK2 axis. As illustrated in Figure 6, our results implicate an important insight that Cdh1 in association with APC may contribute to the deregulation of Skp2-p27 cascade in colorectal cancer.

Abrogated Ubiquitin-Dependent Regulation of Skp2-p27 Cascade in Cancer

Uncontrolled regulation of Skp2 and p27 has been demonstrated to be an important molecular basis for the initiation of several types of malignancies because abrogated Skp2 and p27 often lead to abnormal cell cycle progression. Identification of Cdh1/APC as the upstream ubiquitin protein ligase governing the turnover of Skp2 during G₁/S transition unveils the molecular mechanism for orchestrating Skp2 function via a proteolytic regulation. This leads to the question that if Cdh1 could be a potential suppressing component coordinating Skp2-p27 for the control of cyclin E/CDK2 in cancer. To assess the

Figure 4. Human tissue array analysis of proteins in the Cdh1/APC-Skp2-p27 cascade. **A:** Representative picture of negative or positive staining of array sample. **B:** Significant reduction of Cdh1 protein levels was measured in colon cancer tissue, while abundant Cdh1 protein was detected in adjacent nonmalignant tissue. Accumulation of Skp2 was observed in colon cancer tissue, whereas moderate Skp2 protein levels were shown in adjacent tissue. Attenuation of p27 protein levels was examined in cancer tissue, whereas abundant p27 protein was tested in normal tissue. **C:** Significant inverse correlation ($r = -0.297$, $P = 0.0255$) is observed between the level of Cdh1 and Skp2 (**a**). Levels of Cdh1 and Skp2 are plotted according to its IHC score using 80 different tissues. Statistically significant correlation ($r = 0.4584$, $P = 0.00026$) is found between the levels of Cdh1 and p27 (**b**). r , Correlation coefficient; $P < 0.05$ correlations are statistically significant.

potential involvement of Cdh1 in modulating Skp2-p27 in cancer, we first tested the expression of Cdh1 in 15 different types of tumors as well as the adjacent nonmalignant tissues. Thereafter, the expression profile of Cdh1/APC-Skp2-p27 cascade is examined using cell lines. Furthermore, we analyzed the significance of loss of function of Cdh1 as well as Skp2 in either nonmalignant

or cancer cells accompanied with overexpression of Cdh1 and Skp2 in such cells. Results of the present study strongly suggest that the presence of Cdh1/APC-Skp2-p27 cascade with alteration of Cdh1 leading to opposing protein expression pattern for both Skp2 and p27. Overexpression or knockdown of Cdh1 induces a significant change in the S phase population as well as the property

Table 1. Summary of Immunohistochemical Analysis of Cdh1, Skp2, and p27 in Normal Colon Epithelia and Colon Cancer Tissues

	Normal colon tissue		Colon cancer		P
	–	+	–	+	
Cdh1	27.2%	72.8%	76.7%	23.3%	<0.01
Skp2	90.0%	10.0%	33.3%	66.6%	<0.01
p27	37.9%	62.1%	72.4%	27.6%	0.016

+, Positive staining; –, negative staining.

of oncogenic growth, which is consistent with its identified effect in the regulation of Skp2.

Altered Cdh1/APC Function in Cancer

Faithful separation of duplicated genome during mitosis ensures genomic stability. The role of APC has been initially characterized in the control of chromatid separation with dysfunction in APC often resulting in aneuploidy—a hallmark of cancer. Indeed, previous reports from epigenetic studies have further implicated the correlation of APC with tumorigenesis, in which deregulation in components of APC pathway including APC6, Cdc16, Cdc23, and Cdh1 are found in different types of cancer such as colon cancer, B-lymphoma, gastric cancer, and lung cancer. Current pathological analysis has shown that aberrant APC expression is present in multiple types of malignant tumors. Recent demonstration that Emi1, an inhibitor of Cdh1/APC, is involved in the progression of cancer supports this notion.³⁸ Furthermore, the finding that APC mediates transforming growth factor- β signaling targeting SnoN as well as Skp2 also unveiled the poten-

Table 2. Clinicopathological Features of Cdh1-Positive Colon Cancer

	Cdh1 (–), n = 103	Cdh1 (+), n = 30	P
Patients age			N.S (0.892)
>50	31	8	
≤50	72	22	
Tumor size			N.S (0.236)
T1, 2	14	5	
T3	32	16	
T4	52	8	
Unknown	5	1	
Lymph node metastasis			N.S (0.624)
N0	47	17	
N1	30	10	
N2	12	1	
Unknown	14	2	
Distant metastasis			N.S (0.252)
Meta (–)	87	29	
Meta (+)	12	0	
Unknown	4	1	
Histological grade			<0.01 (0.009)
Grade 1	25	18	
Grade 2	35	7	
Grade 3	32	4	
Unknown	11	1	

NS, not significant.

***, vs **: $p < 0.05$; ***, vs *: $p < 0.05$ (χ^2 -square-test)

Figure 5. Pathological relevance of Cdh1 in colon cancer. Correlation between percentage of Cdh1-positive colon cancer and histological grade. Analytical result indicates that higher numbers of Cdh1-positive cells correlates with lower tumor histological grade in colon cancer.

tial role of Cdh1/APC in tumor inhibition.¹⁶ Previous evidence from several lines has sketched a framework for the involvement of APC in tumor progression.^{18–21} The present results based on tissue array and clinicopathological analysis of Cdh1/APC-Skp2-p27 cascade have confirmed the results of cultured cell-based studies, which further support the idea that the APC pathway is critical in preventing cancer.

Clinical Relevance of Cdh1 in Colon Cancer

The results of tissue array analysis demonstrated that there is a statistically significant inverse correlation between Cdh1 and Skp2 as well as a significant correlation between Cdh1 and p27, which is consistent with the results from cultured cells. The notion provided here is that Cdh1 could have a role as tumor suppressor via down-regulation of Skp2, thereby leading to up-regulation of p27 in colon cancer. In this study, we also tested the significance of Cdh1 using clinical parameters such as patient age, tumor size, status of lymph node metastasis, status of distant metastasis, and histological grade and unveils that patients with positive Cdh1 acquired

Figure 6. Regulation of G₁/S transition by the Cdh1/APC-Skp2-p27 cascade in colon cancer Cdh1/APC is critical in regulating Skp2-p27-cyclin E/CDK2 axis, resulting in averting abnormal entry of S phase.

significantly lower grade of histological grade tumors. Given that lower histological grade tumor is associated with less aggressive biological behavior and subsequently resulted in favorable outcome, significance of Cdh1 is critical in preventing immature entry of S phase and coordinating appropriate cell cycle progression thereby could be a potential prognostic indicator in patients with colon cancer. Current implication is that Cdh1/APC could stabilize p27 through its degradation of Skp2 in response to transforming growth factor- β signaling, which explains the prognostic value of Cdh1 protein levels in patients with colon cancer.^{10,16,39}

Integration of the Present Finding with Current Paradigm

Previous studies have demonstrated that malfunction of the ubiquitin-proteasome pathway can result in tumorigenesis by disrupting the balance between oncoproteins and tumor suppressor proteins.^{40–42} Mitotic regulation and G₁/S transition are key regulatory sites during the cell cycle, where their aberration usually leads to genomic instability and uncontrolled growth. APC and SCF complexes are critical E3 ligase dictating chromatid separation during the mitosis and orchestrating cyclinE/CDK2 during G₁/S transition. Loss of control for these two major ubiquitin-mediated pathways has been correlated to a variety of malignancies.⁴³ This work demonstrates the importance of an ubiquitylation regulatory cascade in tumorigenesis with one E3 ligase (APC) regulating another E3 ligase (SCF) complex resulting in scheduled G₁/S progression. Analyses based on human specimens confirmed the paradigm of Cdh1/APC-Skp2-p27 in cancer formation. The expression patterns of Cdh1/APC-Skp2-p27 in these samples support the notion that this regulatory axis controls cellular proliferation with its dysfunction-promoting carcinogenesis (Figure 6). Cdh1 in association with APC could also target additional proteins other than Skp2, which potentially involves tumorigenesis. Among these targets, cyclinB is one of the major Cdh1/APC substrates, which has been implicated in enhancing certain types of malignant tumor.⁴⁴ Given that proteolysis of cyclinB catalyzed by Cdh1/APC, the potential role of Skp2-p27 axis modulated by the Cdh1/APC in tumorigenesis needs to be addressed. Indeed, cyclin B levels is respond to the status of Cdh1 (Supplemental Figure S3A, available at <http://ajp.amjpathol.org>), whereas cellular proliferation as well as colony formation were more prominent in Cdh1-overexpressing cells compared with cyclin B knockdown cells (Supplemental Figure S3, B and C, available at <http://ajp.amjpathol.org>). These results suggest that Cdh1/APC could play a tumor-suppressing role, which may mediate through several different molecular cascades that supports the previous notion.¹⁶ Further dissection of the role for Cdh1 in governing Skp2-p27 in the tumorigenesis using xenograft human cancer model is necessary to validate its biological function *in vivo*. Combinatorial studies using biochemistry, mouse dissection, and pathological analysis will advance our understanding of Cdh1/APC in cancer.

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