Overexpression of CDC25B Phosphatase as a Novel Marker of Poor Prognosis of Human Colorectal Carcinoma

Ichiro Takemasa, Hirofumi Yamamoto, Mitsugu Sekimoto, Masayuki Ohue, Shingo Noura, Yasuhiro Miyake, Takashi Matsumoto, Tomohiko Aihara, Naohiro Tomita, Yasuhiro Tamaki, Isao Sakita, Nobuteru Kikkawa, Nariaki Matsuura, Hitoshi Shiozaki, and Morito Monden

Department of Surgery II, Osaka University Medical School, Osaka 565-0871, Japan [H. T., H. Y., M. S., M. O., S. N., Y. M., T. M., Y. T., I. S., H. S., M. M.]; Department of Surgery, Osaka National Hospital, Osaka 540-0006, Japan [N. K.]; Department of Pathology, School of Allied Health Science, Faculty of Medicine, Osaka University, Osaka 565-0871, Japan [N. M.]; and Department of Surgery, Kansai Rosai Hospital, Hyogo 660-0864, Japan [T. A., N. T.]

ABSTRACT

There is evidence to suggest that CDC25B phosphatase is an oncogenic protein. To elucidate the role of CDC25B in colorectal carcinoma, we examined the expression of CDC25B at the mRNA and protein levels. Reverse transcription-PCR assay indicated that CDC25B was overexpressed in tumor tissues relative to normal mucosa in 6 of 10 cases. Using immunohistochemistry, we identified high expression of CDC25B in 77 of 181 colorectal cases (43%). Univariate analysis showed that high expression was a significant predictor for poor prognosis compared with low expression (5-year survival rate: 59% versus 82%, respectively; P < 0.0001). Multivariate analysis indicated that CDC25B was an independent prognostic marker (risk ratio for death, 3.7; P < 0.0001) even after controlling for various factors such as lymph node metastasis, tumor size, degree of differentiation, and depth of invasion. Furthermore, the level of CDC25B expression clearly predicted the outcome of patients with Dukes’ B and Dukes’ C tumors. On the other hand, CDC25A mRNA was overexpressed in 9 of 10 colorectal cancer cases, and immunohistochemistry for CDC25A showed high expression in 52 of 111 cases (47%), but no significant correlation with prognosis. Our findings suggest that CDC25B is a novel independent prognostic marker of colorectal carcinoma and that it may be clinically useful for selecting patients who could benefit from adjuvant therapy.

INTRODUCTION

Colorectal carcinoma is one of the most common malignancies in the world. The prognosis of patients with this disease has not changed during the last 30 years; treatment is based mainly on surgical removal of the tumor, and approximately 50% of patients die from this malignancy. Dukes’ classification of tumor stage, which is based on the extent of invasion of carcinoma cells into the colonic wall and the presence of metastasis in regional lymph nodes or distant organs, is often used to determine the prognosis of colorectal cancer (1). However, prognosis varies greatly in patients at intermediate stages Dukes’ B and C (2). Therefore, it is important to identify a biological marker that is independent of the above-mentioned clinicopathological factors to guide clinicians in selecting appropriate treatment.

The cell cycle is a complex process in which many molecules are involved. Central to this process are the CDKs and their catalytic partners, cyclins. They are negatively regulated by CDK inhibitors (e.g., p16, p21, and p27) and positively activated by CDK-activating kinase (3, 4). CDC25 phosphatase is a novel class of CDK activator. In mammalian cells, CDC25A, CDC25B, and CDC25C are three CDK-activating phosphatases that remove the inhibitory phosphates of threonine and tyrosine residues in ATP-binding sites of CDKs at different points of the cell cycle (5–7). In the CDC25 family, CDC25A and CDC25B types appear to be potential oncogenes because they have been found to transform primary murine fibroblasts in cooperation with either mutated Ha-ras or loss of Rbl (8). In fact, overexpression of CDC25A and CDC25B has been demonstrated in non-Hodgkin’s lymphoma, human carcinomas of the breast and lung, and head and neck tumors (8–11). Dysregulation of cell cycle progression is one evident alteration in human malignancies (12, 13). Colorectal carcinomatous tissues overexpress CDC1 and CDC2, possibly overexpress CDC4, and overexpress cyclins D1 and E (14–17). The CDK inhibitor p21 was previously identified, methylation of the p16INK4A gene occurs in the promoter region, and p27Kip1 appears to be decreased in a subset of colorectal carcinomas (18–21).

Recent studies have demonstrated overexpression of CDC25A phosphatase in azoxymethane-induced murine colon cancer (22), but the expression and biological significance of CDC25A and CDC25B in human colorectal carcinoma have not yet been elucidated. Of considerable interest is that CDC25 phosphatase, especially the CDC25B type, is associated with the malignant properties of some human carcinomas. For example, expression of CDC25B is a poor prognostic factor for breast cancer when assessed by in situ hybridization and CDC25B overexpression was associated with aggressive non-Hodgkin’s lymphoma (8, 9). Furthermore, transgenic mice that overexpress the CDC25B gene display enhanced sensitivity to the carcinogen 9,10-dimethyl-1,2-benzanthracene (23) or develop mammary gland hyperplasia (24).

To investigate the role of CDC25B phosphatase in the progression of colorectal carcinoma, we examined its expression using immunohistochemistry in 181 primary human colorectal carcinomas and analyzed the correlation between prognosis and the level of CDC25B protein. Western blot analysis and RT-PCR were used to quantify the expression levels of CDC25B protein and mRNA in 10 paired samples of colonic normal mucosa and carcinomas. In addition, we compared the expression of CDC25A and proliferation marker Ki-67 with that of CDC25B in a subset of specimens. The present findings indicate that CDC25B is a novel, independent prognostic marker for colorectal carcinoma.
ingful statistical analysis of the population sample. None of the patients had been treated preoperatively with chemotherapy or radiotherapy. Only one patient with rectal carcinoma had radiation after surgery. Chemotherapy was applied after surgery in 45% of patients with Dukes’ B stage tumor, 81% of patients with Dukes’ C stage tumor, and 57% of patients with Dukes’ stage D tumor using 5-fluorouracil or its derivatives, occasionally combined with mitomycin C. The mean postoperative follow-up period was 65.9 ± 35.5 months. The resected surgical specimens were fixed in formalin, processed through graded ethanol, and embedded in paraffin. A portion of each tissue sample was frozen immediately in liquid nitrogen and stored at −80°C until use for RT-PCR and immunoblotting.

Clinical Features. The selected patients included 79 (44%) males and 102 (56%) females, with a mean age at surgery of 60 ± 10 years (range, 40–86 years). The primary tumors were evenly distributed in the colon and rectum and ranged in size from 0.7–13.0 cm (mean size, 4.8 ± 1.9 cm). The majority of tumors were well-differentiated carcinomas (56%), followed by moderately differentiated carcinomas (40%) and poorly differentiated carcinomas (4%). Dukes’ staging included 35 (19.3%) Dukes’ A patients, 61 (33.8%) Dukes’ stage B patients, 69 (38.2%) Dukes’ stage C patients, and 16 (8.7%) Dukes’ stage D patients.

Antibodies. Mouse antihuman CDC25B mAb and its blocking peptide, which was used as an immunogen (NH2-terminal of human CDC25B; amino acids 109–122), were obtained from Transduction Laboratories (Lexington, KY). The positive control lysate from HeLa cells was also obtained from Transduction Laboratories. Rabbit polyclonal antibodies for CDC25B and CDC25A and their blocking peptides were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The CDC25B polyclonal antibody was against the COOH-terminal of murine CDC25B. The mouse antihuman Ki-67 mAb was purchased from DAKO (Carpinteria, CA; Ref. 21).

Specificity of Antibodies in Immunohistochemistry. Specificity of staining obtained with CDC25B antibodies and CDC25A antibody was assessed first by an absorption test in which immunogens were used to generate the antibodies. This test resulted in the disappearance of staining. For negative control, nonimmunized mouse or rabbit IgG (Vector Laboratories, Burlingame, CA) or PBS alone was used as a substitute for the primary antibody to exclude possible false positive responses from secondary antibody or from nonspecific binding of IgG. These control samples showed no cell staining. Staining of Ki-67 was performed as described previously (21), using tonsil samples as a positive control.

H&E Staining and Immunohistochemistry. Tissue sections (4-μm thick) were deparaffinized in xylene, rehydrated, and stained with H&E. The specimens were histologically diagnosed by two pathologists from the Department of Pathology, Osaka University Medical School. For immunostaining, sections were mounted on charged glass slides, boiled for antigen retrieval (21), and then processed for immunohistochemistry on the TecxMate Horizon automated staining system (DAKO, Glostrup, Denmark) using the Vectastain ABC peroxidase kit (Vector Laboratories) as described previously (25). In the primary antibody reaction, the slides were incubated with appropriate antibodies for 1 h at room temperature. The dilution of each antibody was as follows: (a) CDC25A polyclonal antibody, 1:50; (b) CDC25B mAb, 1:200; (c) CDC25A polyclonal antibody, 1:50; and (d) Ki-67 mAb, 1:50.

Immunohistochemical Assessment. All immunostained tissue sections were evaluated in a coded manner without knowledge of the clinical and pathological parameters. For assessment of CDC25B, both cytoplasmic and nuclear staining were evaluated. For each section, five high-power fields were selected at random, and at least 700 cells were evaluated. The results were expressed as a percentage of positively stained cells. In addition, the cytoplasmic staining intensity for CDC25B was evaluated as follows: (a) weak, 1; (b) moderate, 2; or (c) strong, 3. Carcinoma samples containing >75% immunoreactive cells with strong staining intensity (intensity ≥3) were classified as high expressors of CDC25B, and the remaining samples were classified as low expressors of CDC25B. Agreement in the above-mentioned tissue evaluation between the two investigators (H. Y. and I. T.) was 98%. In cases of disagreement, the two investigators reached the final evaluation by consensus after reexamining the tissue using a multihread microscope. Staining was repeated in 50% of cases to check for possible technical errors, but similar results were obtained. For assessment of CDC25A and Ki-67, cells with positive nuclear staining were counted as described above.

Western Blot Analysis for CDC25B. Approximately 100 mg of each sample were homogenized in 1 ml of lysis buffer [50 mM Tris (pH 8.0), 150 mM NaCl, and 0.5% NP40] with protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, and 10 μg/ml leupeptin). The homogenate was centrifuged at 14,000 rpm for 20 min at 4°C. The resulting supernatant was collected, and total protein concentration was determined using the Bradford protein assay (Bio-Rad, Hercules, CA). Western blotting was performed as described previously (26). Briefly, 100 μg of the total protein were subjected to 10% PAGE, followed by electroblotting onto a polyvinylidine difluoride membrane. After blocking in 5% skim milk, the membrane was incubated with 1 μg/ml CDC25B antibody, followed by incubation with the secondary antibody at a dilution of 1:3,000. For detection of the immunocomplex, the enhanced chemiluminescence Western blot detection system (Amersham, Aylesbury, United Kingdom) was used.

RNA Extraction and RT-PCR Analysis. Total RNA was extracted with a single-step method using Trizol reagent (Life Technologies, Inc., Gaithersburg, MD), and cDNA was generated using avian myeloblastosis virus reverse transcriptase (Promega, Madison, WI). Briefly, 1 μg of RNA was incubated at 70°C for 5 min and then placed on ice before the addition of reverse transcription reaction reagents with Oligo(dT)15 Primer. Reverse transcription was performed at 42°C for 90 min, followed by heating at 95°C for 5 min.

Semi-quantitative analysis for expression of CDC25B or CDC25A mRNA was performed with the multiplex RT-PCR technique, using PBGD (27, 28) as the internal standard. To minimize the inter-PCR difference, PCR was performed with PBGD and CDC25A or CDC25B primers in identical tubes under unsaturated conditions, as described previously (25). PCRs were performed in a total volume of 25 μl of reaction mixture containing 1 μl of cDNA template, 1× Perkin-Elmer PCR buffer, 1.5 mM MgCl2, 0.8 mM deoxynucleotide triphosphates, 20 pmol of each primer for CDC25A or CDC25B, 4 pmol of each primer for PBGD, and 1 unit of Taq DNA Polymerase (AmpliTaq Gold; Roche Molecular Systems, Inc., Belleville, NJ). The primer sets of CDC25A and CDC25B were designed to flank at least one intron and tested to ensure amplification of only cDNAs so that amplification of possibly contaminated genomic DNA could be avoided. The sequences of these PCR primers were as follows: (a) CDC25A sense primer, 5′-GAGGAGTCTCCACCTTGGAAG-TACA-3′ (nucleotides 1297–1569); (b) CDC25A antisense primer, 5′-GCCCATCCAAAACCCAGATGCCATAA-3′; (c) CDC25A sense primer, 5′-CACGCCCCTGGCAGAATAAGC-3′ (nucleotides 1059–1475); and (d) CDC25B antisense primer, 5′-ATGACTCTCTTTGTCAGAGCGGTTACGG-3′. The primers for PBGD were synthesized as described previously (28). The sizes of the amplicons for CDC25A, CDC25B, and PBGD were 272, 416, and 127 bp, respectively. The PCR conditions were as follows: (a) initial denaturation at 95°C for 12 min; (b) 35–40 cycles of 95°C for 1 min, 62°C for 1 min, and 72°C for 1 min; and (c) a final extension at 72°C for 10 min. In the next step, 10 μl of each PCR product were electrophoresed on 2% agarose gels and stained with ethidium bromide. The PCR products were scanned by densitometry.

Statistical Analysis. Statistical analysis was performed using the Statview J-5.0 program (Abacus Concepts, Inc., Berkeley, CA). The postoperative period was measured from the date of surgery to the date of the last follow-up or death. The Kaplan-Meier method was used to estimate death from colorectal cancer, and the log-rank test was used to examine statistical significance. A Cox proportional hazards model was used to assess the risk ratio under simultaneous contributions from several covariates. The associations between the discrete variables were assessed using Fisher’s exact test. Mean values were compared using the Mann-Whitney test. P < 0.05 was accepted as statistically significant.

RESULTS

Western Blot Analysis for CDC25B. Western blot analysis was performed on colorectal carcinoma surgical specimens and the corresponding normal tissues using anti-CDC25B mAb. The lysates from HeLa cells, which have been used as a positive control for CDC25B (29), yielded a M, 63,000 band for the CDC25B protein (Fig. 1, Lane 1). Normal mucosa generally expressed low levels of CDC25B (Lanes 2, 4, 6, and 8), whereas colorectal carcinoma tissues showed wide variability in the expression of CDC25B (strong expression, Lane 3;
moderate expression, Lanes 5 and 7; weak expression, Lane 9). When 10 paired samples were examined, 8 of 10 (80%) carcinomas showed overexpression of the CDC25B protein, which displayed a 2-fold band density, compared with their corresponding normal mucosa. When the same series of carcinoma samples was immunostained with the CDC25B antibody, the level of CDC25B in each sample paralleled the results obtained by Western blot analysis (data not shown).

**Immunohistochemistry for CDC25B.** Immunostaining was performed with anti-CDC25B mAb. In normal colonic mucosa, CDC25B was detected in the cytoplasm of colonic mucosal cells (Fig. 2A). The CDC25B protein was randomly distributed from the bottom to the top of the normal epithelium, and the intensity of staining was generally weak, except in occasional cases. The germinal center of the lymph follicle exclusively expressed CDC25B with weak intensity. In carcinoma tissues, CDC25B protein was localized mainly in the cytoplasm (Fig. 2B). CDC25B expression was noted in 175 of 181 carcinoma cases (97%), with a wide variability in the expression level, which ranged from 15% to 100%. Approximately half of the samples displayed strong staining (Fig. 2B), whereas a subset of carcinomas showed weak staining for the protein (Fig. 2C). All carcinoma sam-

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**Fig. 1. Western blotting using antihuman CDC25B antibody.** Normal mucosa generally expressed low levels of CDC25B (Lanes 2, 4, 6, and 8), whereas colorectal carcinoma tissues showed wide variability in the expression of CDC25B (strong expression, Lane 3; moderate expression, Lanes 5 and 7; weak expression, Lane 9). Lysates from HeLa cells served as a positive control showing a M, 63,000 band for CDC25B.

**Fig. 2. Immunostaining with antihuman CDC25B antibody in normal colonic mucosa (A) and colorectal carcinoma tissues (B and C).** A, in normal colonic mucosa, weak expression of CDC25B was noted from the bottom to the top of the normal epithelium. B, a representative colorectal carcinoma that expressed a high level of CDC25B. The intensity of staining was judged as strong (intensity = 3), and the percentage of CDC25B-positive cells was 100%. C, a representative colorectal carcinoma that expressed a low level of CDC25B. Intensity was weak, and the percentage of CDC25B-positive cells was <5%. D, immunostaining with the antihuman CDC25A antibody. CDC25A protein was localized mainly in the nucleus in both normal tissue (left) and carcinoma tissue (right). In the normal epithelium, relatively intense nuclear expression was noted in the lower parts of the gland. Intense staining was also observed in infiltrating lymphocytes. A and D, ×25; B and C, ×50.
mRNAs was performed using paired normal-carcinoma mRNA expression indicated no significant association with the expressors among 59 low CDC25A expressors. Further analysis of those of CDC25B immunostaining with respect to the level of 75%. When we compared the results of CDC25A immunostaining to the respective indices in high CDC25A- and low CDC25A-expressing colorectal carcinomas. In normal mucosa, such cells were distributed at random. The Ki-67 index between high and low expressors were not statistically significant.

Relationship between CDC25B Expression and Clinicopathological Parameters. Further analysis showed that 77 cases (43%) were high expressors of CDC25B, whereas the remaining 104 cases (57%) were classified as low expressors of CDC25B. There was a significant correlation between high expression of CDC25B and tumor size (P = 0.035) or distant metastasis (P < 0.0001; Table 2). There was no correlation between high expression of CDC25B and age, gender, site of tumor, lymph node metastasis, degree of invasion, degree of differentiation, and Dukes’ stage.

**Immunohistochemistry for CDC25A.** Because CDC25A is overexpressed in azoxymethane-induced murine colon carcinoma (22), its expression was compared with that of CDC25B. In the above-mentioned series of colorectal carcinomas used for analysis of CDC25B expression, 111 samples were randomly selected and examined for CDC25A expression. CDC25A protein was localized mainly in the nucleus in both normal and carcinoma tissues (Fig. 2D). In the normal epithelium, CDC25A protein was weakly expressed at the top and upper half of the gland, but a relatively intense nuclear expression was noted in the lower parts of the gland. The germinal center of the lymph follicle exclusively expressed marked CDC25A expression and served as a positive control within the sections. Furthermore, infiltrating lymphocytes frequently showed intense staining. In colorectal carcinoma tissues, the CDC25A protein was detected in 108 of 111 cases (97%), with a wide range of nuclear expression (range, 30–100%; median, 75%). The carcinoma specimens were divided into two groups, 52 high expressors and 59 low expressors, according to the percentage of nuclear CDC25A-positive cells, using a cutoff level of 75%. When we compared the results of CDC25A immunostaining with those of CDC25B immunostaining with respect to the level of expression, there was an appreciable mismatch, with 22 low CDC25B expressors among 52 high CDC25A expressors and 21 high CDC25B expressors among 59 low CDC25A expressors. Further analysis of CDC25A expression indicated no significant association with the clinicopathological parameters listed in Table 2.

**RT-PCR Analysis.** RT-PCR analysis for CDC25B and CDC25A mRNAs was performed using paired normal-carcinoma mRNA extracts. The relative value of the CDC25A or CDC25B band to the PBGD band was calculated for each sample, and the T:N ratio was determined in each case. In five representative cases, the T:N ratio was 7.0, 12.9, 3.0, 3.4, and 2.2 for CDC25A and 6.3, 4.1, 1.3, 0.7, and 1.2 for CDC25B (Fig. 3).

<table>
<thead>
<tr>
<th>Stage</th>
<th>Positive</th>
<th>Negative</th>
<th>Total (%)</th>
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</thead>
<tbody>
<tr>
<td>Dukes’ A</td>
<td>35 (19%)</td>
<td>54 (31%)</td>
<td>89 (50%)</td>
</tr>
<tr>
<td>Dukes’ B</td>
<td>41 (23%)</td>
<td>57 (34%)</td>
<td>98 (55%)</td>
</tr>
<tr>
<td>Dukes’ C</td>
<td>22 (12%)</td>
<td>72 (41%)</td>
<td>94 (53%)</td>
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**Relationship between Ki-67 and CDC25B and CDC25A.** To examine the possible involvement of CDC25B and CDC25A in cellular growth, the expression of Ki-67, a marker for proliferation, was examined by immunohistochemistry in 111 specimens of colorectal carcinoma. In normal mucosa, cells positive for nuclear Ki-67 were localized exclusively at the bottom of the glands, whereas in carcinoma tissue, such cells were distributed at random. The Ki-67 index was determined as described previously (21), and the results showed a wide variability in the percentage of Ki-67-positive cells (range, 18–88%; mean value, 49.6 ± 15.1%). The mean Ki-67 indices in high CDC25B- and low CDC25B-expressing colorectal carcinomas were 52.5 ± 15.1% and 49.8 ± 15.4%, respectively. In comparison, the respective indices in high CDC25A- and low CDC25A-expressing tumors were 51.8 ± 14.2% and 49.8 ± 15.4%. Differences in Ki-67 index between high and low expressors were not statistically significant for either CDC25B subtype. In addition, comparative immunohistochemical analysis using serial sections revealed no significant association between CDC25A- or CDC25B-expressing cells and Ki-67-positive cells (data not shown).

**Analysis of Survival Rates.** In the next step, we analyzed the survival rates according to CDC25B expression in colorectal carcinoma. Univariate analysis showed that high expression of CDC25B, lymph node metastasis, depth of invasion, and degree of differentiation were significant predictors of a poor prognosis (P < 0.0001, 0.0004, 0.026, and 0.027, respectively). Other parameters, such as gender, age, tumor size, tumor site, expression of CDC25A, and Ki-67 index, were not significant predictors of a poor prognosis (Fig. 4A).

<table>
<thead>
<tr>
<th>Gender</th>
<th>High</th>
<th>Low</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male</td>
<td>45 (25%)</td>
<td>57 (31%)</td>
<td>NSa</td>
</tr>
<tr>
<td>Female</td>
<td>32 (18%)</td>
<td>47 (26%)</td>
<td></td>
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</tbody>
</table>

a NS, non-significant.
Dukes’ stage C \((n = 69)\), 55% versus 77% \((P < 0.01)\). In contrast, there was no significant difference in survival rates of patients with Dukes’ stage A and Dukes’ stage D disease stratified by CDC25B level (data not shown).

Multivariate analysis using data from the whole group showed that CDC25B expression and nodal status were significant covariates [relative risk, 3.7-fold \((P < 0.0001)\) and 2.4-fold \((P < 0.01)\), respectively]. However, age, gender, site, size of tumor, depth of invasion, and tumor differentiation were not significant covariates (Table 3). The relative risk of death in patients with carcinomas expressing high levels of CDC25B was 3.7 that of patients with tumors expressing low levels of CDC25B \((P < 0.0001)\). The effect of CDC25B was also clearly observed when patients with Dukes’ B and C stage tumors were analyzed by multivariate analysis in these two groups, a high expression of CDC25B alone was associated with poor prognosis and a relative risk of death due to colorectal cancer of 5.2 \((P < 0.05)\) and 3.1 \((P < 0.05)\), respectively; none of the other factors were associated with prognosis and relative risk of death due to colorectal cancer (data not shown).

**DISCUSSION**

In the present study, we examined the levels of CDC25B gene products using several techniques such as immunohistochemistry, immunoblotting, and RT-PCR. The results of these assays were essentially similar. RT-PCR analysis showed that the proportion of CDC25B overexpression in colorectal carcinoma was 60% at mRNA levels. This rate is compatible or even higher compared to the level reported in other types of carcinomas. CDC25B mRNA or protein was reported to be overexpressed in 32% of breast cancers, 50% of head and neck tumors, 56% of non-Hodgkin’s lymphomas, and 44% of non-small cell lung cancers (8–11). In these studies, overexpression in tumor tissue was defined as a level of CDC25B more than 2–3 times higher than that seen in normal tissues. We followed this criterion (cutoff, CDC25B expression exceeding twice that of normal tissues). In preliminary studies, we found that CDC25B expression was relatively low in hepatocellular carcinoma and in esophageal squamous cell carcinoma, although these carcinomas frequently expressed CDC25A at high levels (data not shown). Therefore, it appears that a high level of CDC25B may be characteristic of colorectal carcinoma and may enhance the growth and survival of these tumors.

Our comparative study revealed several differences in the expression level and cellular localization of CDC25B and CDC25A. RT-PCR and immunohistochemical assays showed that the expression levels of CDC25A and CDC25B were not always similar in individual colorectal carcinoma specimens. Such a difference has also been described in head and neck tumors, non-Hodgkin’s lymphoma, and non-small lung cancer (9–11). These findings suggest that although the two molecules share a high homology in the COOH-terminal domain, their expressions may be regulated separately. Immunohistochemical analysis showed that the CDC25B protein was localized mainly in the cytoplasm, whereas the CDC25A protein was found in the nucleus. These results are in agreement with those of previous studies showing abundant CDC25B protein in the cytoplasm and CDC25A in the nuclear fraction (22–29).

The major finding of this study is the striking correlation between high CDC25B expression and poor prognosis in patients with colorectal carcinoma (Fig. 4A; \(P < 0.0001\)). When the Cox proportional hazards model was constructed for the entire series, high CDC25B expression remained an independent predictive factor of death; surprisingly, it displayed a higher relative risk for death than lymph node metastasis (relative risk, 3.7 versus 2.4), which is one of the strongest predictors of poor prognosis in colorectal carcinoma, and was used for this purpose in Dukes’ staging system. The mechanism of the negative effect of CDC25B expression on the progression of colorectal carcinoma is not yet clear. Although CDC25B is a positive regulator of the cell cycle, it is unlikely that the prognostic value of CDC25B expression would be due to the rapid growth of carcinoma cells because of a lack of association between CDC25B expression and cell proliferation as assessed by the Ki-67 index. One clue is that high CDC25B expression was frequently noted in patients with distant metastasis, i.e., those with Dukes’ stage D disease. Although the latter by itself is a high risk factor for death, we postulate that CDC25B itself may enhance the malignant nature, apart from distant metastasis, because notable differences in survival rates were also identified in the presence of different levels of CDC25B expression in Dukes’ B and C stage tumors that had escaped from distant metastasis (Fig. 4B). In support of this hypothesis, mechanistic studies of *in vitro* transformation of fibroblasts and CDC25B transgenic mice have shown that CDC25B displays oncogenic properties under certain conditions (8, 23, 24).

Recent studies indicated that CDC25B is involved in G2-M-phase transition through the activation of CDC2 kinase (29–32). Cyclin B is synthesized during S phase and G2 phase and immediately forms complexes with CDC2 in the cytoplasm. The complex is inactivated by phosphorylation of the threonine 14 and tyrosine 15 residues of CDC2 by Wee-1 or Mik1 until G2-M-phase transition (33, 34) but is activated on dephosphorylation by CDC25B. Ectopic expression of the CDC25B gene shows that prophase microtubule nucleation on the centrosomes is a consequence of cytoplasmic CDC25B activity (29). Because the activity of CDC2 kinase is increased in a subset of colon
carcinoma (35), overexpression of CDC25B might contribute to the constitutively active status of CDC2 kinase and accelerate the transition from G2 to M phase. Consequently, alteration of G2-M-phase transition may lead to inappropriate distribution of the chromosome and result in aneuploidy. Indeed, there is evidence that overexpression of Cdc25B causes S phase and G2 phase cells to rapidly enter mitosis, irrespective of the completion of DNA replication (31). Moreover, it has been demonstrated that introduction of CDC25B cDNA into normal mouse embryo fibroblasts leads to aneuploidy (8). Interestingly, aneuploidy is known to be associated with poor prognosis in colorectal carcinoma (36, 37). Introduction of CDC25B cDNA into colon carcinoma cell lines may offer some insight into the underlying mechanisms of ploidy status and other aspects of the malignant properties of colorectal cancers including invasiveness, neovascularization, and metastatic ability.

The present study clearly showed that CDC25B, but not CDC25A and Ki-67, was a significant poor prognostic factor in colorectal carcinoma (Fig. 4A). Previous studies indicated that CDC25A plays a crucial role in G1-S-phase transition (6, 38, 39), whereas CDC25B is essential for the G2-M-phase transition (29–32). Among the components engaged in G2-M-phase transition in the mammalian cell cycle, CDC2 and cyclin B are well-known downstream molecules. CDC25B

![Fig. 4. Survival curves using the Kaplan-Meier method. A, survival curves were drawn for the entire series (n = 181) based on Dukes’ stage and expression of CDC25B, CDC25A, and Ki-67. Note the progressively lower survival rate as the disease advanced from Dukes’ stage A to Dukes’ stage D (P < 0.0001). The 5-year survival rate for Dukes’ stage A, B, C, and D was 96.4%, 87.4%, 69.8%, and 12.5%, respectively. The 5-year survival rate of high expressors of CDC25B was significantly lower than that of low expressors of CDC25B (59% versus 82%; P < 0.0001). B, survival curves for Dukes’ stages B (left) and C (right) based on CDC25B expression. In both groups, the 5-year survival rate was significantly lower in patients with high expression of CDC25B than in those with low levels of CDC25B [Dukes’ stage B (n = 61), 77.1% versus 89.0% (P < 0.05); Dukes’ stage C (n = 69), 55.1% versus 77.0% (P < 0.01)].

Table 3  Multivariate analysis (Cox proportional hazards model)

<table>
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<tr>
<th>Categories</th>
<th>P</th>
<th>Risk ratio</th>
<th>95% confidence interval</th>
</tr>
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<tbody>
<tr>
<td>CDC25B (H:L)</td>
<td>&lt;0.0001</td>
<td>3.651</td>
<td>2.058–6.475</td>
</tr>
<tr>
<td>n [m+]:[m–]</td>
<td>0.0032</td>
<td>2.400</td>
<td>1.342–4.294</td>
</tr>
<tr>
<td>Depth (mp:ss)</td>
<td>0.968</td>
<td>1.887</td>
<td>0.892–3.995</td>
</tr>
<tr>
<td>Differentiation (well/mod)</td>
<td>0.2671</td>
<td>1.407</td>
<td>0.770–2.570</td>
</tr>
<tr>
<td>Gender (male:female)</td>
<td>0.6026</td>
<td>0.864</td>
<td>0.497–1.500</td>
</tr>
<tr>
<td>Age (old:young)</td>
<td>0.7825</td>
<td>0.923</td>
<td>0.524–1.628</td>
</tr>
<tr>
<td>Site (colon:rectum)</td>
<td>0.8833</td>
<td>0.958</td>
<td>0.544–1.690</td>
</tr>
<tr>
<td>Size (large:small)</td>
<td>0.9409</td>
<td>0.978</td>
<td>0.536–1.783</td>
</tr>
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</table>

H:L, high:low; n+[m+]:[m–], lymph node metastasis positive; lymph node metastasis negative; mp:ss, muscularis propria:subserosa; well:mod, well differentiated; moderately differentiated.

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phosphatase acts as an upstream effector of the CDC2/cyclin B complex. In contrast, various components are currently known as gatekeepers at G1-S-phase transition, including pRb, cyclin D1, cyclin E, CDK2, CDK4, p21Waf1/Cip1, p27kip1, and p16INK4A, and colorectal carcinoma displays altered expression of these molecules, as described above (14–21). Because CDC25A phosphatase is involved in the complex process of G1-S-phase transition, CDC25A expression alone may not be a sensitive marker. Ki-67 is a good indicator of poor prognosis in certain types of tumors including carcinomas of the liver, breast, and lung (40–42). In contrast, the impact of Ki-67 on prognosis in colorectal carcinoma is controversial, and many investigators have not found a positive correlation in the past (43–45). These findings suggest that features other than proliferation may play an important role in determining the prognosis of patients with colorectal carcinoma.

Dukes’ staging system provides the most reliable information on prognosis and is certainly useful for discriminating patients with early-stage disease from those with very advanced stage disease. However, its prediction of prognosis of patients with intermediate levels of tumor invasion is less accurate. Several investigators have reported that certain biological markers such as urokinase-type plasminogen activator, erbB-2, vascular endothelial growth factor, and E-cadherin (46–49) are useful for identifying those patients with Dukes’ B tumors who are likely to show unfavorable prognosis. We also found that CDC25B was an independent marker for poor prognosis (Fig. 4B). Dukes’ B stage tumors are defined as those without lymph node metastasis. We are not certain at present why these localized tumors showed a difference in prognosis. One possible explanation is that although pathological metastasis cannot be detected, minimal cancer cells might invade blood and lymph vessels because tumors of this stage spread beyond the propria muscularis layer. CDC25B might enhance the probability of such occult metastasis, i.e., micrometastasis. The results of the study reported by Liebers et al. (50) may support this hypothesis because they showed that micrometastasis to regional lymph nodes was indicative of poor prognosis only in stage II tumors. From a clinical point of view, classification of patients with Dukes’ C stage tumor is also important because clinical trials suggest that adjuvant chemotherapy and treatment with mAbs could improve their survival rates (51, 52). Thus, CDC25B may serve as a good marker for treating patients with appropriate adjuvant therapies because we found that high expression of CDC25B alone was an independent indicator of unfavorable prognosis in Dukes’ C stage disease (Fig. 4B). The present study also suggests that CDC25B might be a therapeutic target for advanced colorectal carcinoma. Because high expression of CDC25B is frequently noted in large tumors and in those with distant metastasis, antisense CDC25B constructs or some specific inhibitors might be of clinical use. Although we did not find a significant correlation between CDC25B level and the efficacy of 5-fluorouracil chemotherapy in Dukes’ B and C stage tumors (data not shown), it is of interest that treatment with several reagents that interfere with G2-M-phase transition, including the DNA topoisomerase inhibitors camptothecin and etoposide or the Vinca alkaloid vincristine, has already been found to be clinically feasible. These reagents may suppress those colorectal carcinomas exhibiting a high CDC25B expression.

In conclusion, we have shown in the present study that CDC25B is a novel prognostic marker in patients with colorectal carcinoma. The prognostic value of this protein is equivalent to that of lymph node metastasis and is independent of conventional clinicopathological parameters.

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Overexpression of CDC25B Phosphatase as a Novel Marker of Poor Prognosis of Human Colorectal Carcinoma

Ichiro Takemasa, Hirofumi Yamamoto, Mitsugu Sekimoto, et al.

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