Cell division cycle-associated protein 1 overexpression is essential for the malignant potential of colorectal cancers

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Received September 4, 2013; Accepted October 21, 2013

Abstract. To identify new cancer biomarkers and therapeutic targets for colorectal cancers (CRCs), we performed immunohistochemical analysis using tissue microarrays covering archival tumor tissue samples from 434 CRC patients and antibodies to cell division cycle-associated protein 1 (CDCA1) that was originally identified as an oncoantigen by our gene expression profile database, and compared its expression with several clinicopathological factors. Strong CDCA1 positivity was associated with poorer prognosis for patients with CRC (P=0.019) and multivariate analysis confirmed its independent prognostic value. In addition, transfection of siRNAs against CDCA1 suppressed its expression and induced apoptosis of CRC cells. These results suggest that CDCA1 could be a prognostic biomarker and a potential therapeutic target for CRCs.

Introduction

Colorectal cancer (CRC) is the third most commonly diagnosed cancer in males and the second in females worldwide, and is the fourth leading cause of cancer death in males and the third in females worldwide (1). Although several molecular target therapies have been developed for CRCs, the prognosis of this disease in advanced stages is still poor. Therefore, personalized therapies using biomarkers and new molecular therapies are expected to improve current therapies. For example, cetuximab, a therapeutic antibody to an epidermal growth factor receptor (EGFR), was reported to be effective in CRC treatment. The randomized phase III study (CRYSTAL: Cetuximab Combined (EGFR), was reported to be effective in CRC treatment. The randomized phase III study (CRYSTAL: Cetuximab Combined KRAS mutant, but with fewer adverse events.

Oncoantigens are proteins with oncogenic functions that are overexpressed in malignant cells of various origins and in normal reproductive tissues such as the testis, ovary, and placenta (4,5). Oncoantigens are considered a candidate biomarker and therapeutic target for cancer therapy. We performed genome-wide gene expression analyses and subsequent tissue microarray analyses of solid tumor tissues using a cDNA microarray containing 25,000 genes or expressed sequence tags (ESTs) (6-10). To date, we have isolated several oncoantigens involved in development and/or progression of cancer (11-42). These data revealed that cell division cycle-associated protein 1 (CDCA1) was overexpressed in cancer tissues including CRC and lung cancer tissues. In our previous reports, CDCA1 proteins were detected in many lung cancers with varying histologic types and were associated with a poorer prognosis for patients with non-small cell lung carcinomas (NSCLC). Knockdown of CDCA1 expression with siRNA significantly suppressed growth of NSCLC cell lines (16). In addition, the HLA-A0201-restricted peptides derived from CDCA1 induced peptide-specific cytotoxic T lymphocytes (CTLs), suggesting that CDCA1 is a likely target for molecular therapy and/or immunotherapy (39). CDCA1 mRNA expression was also observed in CRCs and tissues of gastric cancers, and was correlated with cancer growth (43). Moreover, CDCA1 was associated with a decrease in progression-free survival of multiple myeloma patients, and a decrease in probability of biochemical-free survival in localized prostate cancer (44).

CDCA1 plays a role in regulating mitosis. CDCA1, and its binding partner KNTC2, are members of the Ndc80 complex, which comprises the two Ndc80 sub-complexes (KNTC2)-Nuf2 (CDCA1) and Spc24-Spc25 (46). The CDCA1-KNTC2

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Key words: cell division cycle-associated protein 1, oncoantigen, molecular target, biomarker, colorectal cancer

DOI: 10.3892/ijo.2013.2177
complex is highly conserved in prokaryotic and eukaryotic cells, and plays an important role in kinetochore functions and the spindle checkpoint (46). Although some reports describe CDCA1 expression in human cancers, no report has revealed the function of CDCA1 in colon cancer growth/survival in detail, or the clinical or prognostic value of CDCA1 protein as a tissue biomarker for various colon cancers.

We present evidence that CDCA1 plays a significant role in the malignant potential of CRC, and is a promising diagnostic and prognostic biomarker, as well as a therapeutic target for treating CRC.

Materials and methods

Colorectal cancer cell lines and tissue samples. The human CRC cell lines, Caco-2, CCD-841, COLO205, LoVo, HCT116, HT-29, SW48, SW480, SW620 and SW948 were purchased from American Type Culture Collection (ATCC; Rockville, MD, USA). All cells were grown in monolayers in the appropriate medium (Table I), supplemented with 10% fetal bovine serum (FBS) (Nichirei Biosciences, Tokyo, Japan), and were maintained at 37°C in atmospheres of humidified air. Eight CRC tissue samples and adjacent normal colorectal tissue samples were obtained from patients undergoing surgery at Shiga University of Medical Science Hospital. In addition, we obtained 434 CRC and adjacent normal colorectal tissue samples for immunostaining on tissue microarrays from CRC patients without distant metastases who underwent surgery at Kanagawa Cancer Center Hospital. Individual institutional ethics committees approved this study and the use of all clinical materials.

Semi-quantitative reverse transcription-PCR. Total RNAs were extracted from cultured cells and clinical tissues using Maxwell 16 LEV simplyRNA Purification kit (Promega, Madison, WI, USA) according to the manufacturer's protocol. The RNAs from cultured cells and clinical tissues, as well as commercially available mRNAs from normal human colon and rectum samples (Agilent Technologies, Santa Clara, CA, USA) were reversely transcribed using ReverTra Ace® qPCR RT kit (Toyobo, Osaka, Japan). Semi-quantitative reverse transcription-PCR (RT-PCR) experiments were carried out with the following synthesized CDCA1-specific primers or with β-actin (ACTB)-specific primers as an internal control: CDCA1, 5'-GAGAAACTGAACTCCAGGAAT-3' and 5'-CTGATACCTTTTGCCCTCACC-3'; ACTB, 5'-GCC ACCCCACCTCCTCCTAA-3' and 5'-CAGCAGGCTCAT CATTCAA-3'. RT-PCR reactions were optimized for the number of cycles to ensure product intensity within the logarithmic phase of amplification.

Western blot analysis. Cells were lysed in Pierce RIPA buffer (Thermo Scientific, Waltham, MA, USA) that included 1% protease inhibitor cocktail (Thermo Scientific). After homogenization, the cell lysates were incubated on ice for 30 min and centrifuged at 14,000 rpm for 15 min to separate the supernatant from cellular debris. The amount of total protein was estimated using a Quick Start Bradford Protein Assay kit (Bio-Rad, Hercules, CA, USA), and the proteins were then mixed with SDS sample buffer and incubated at 37°C for 15 min before loading them into a 12% SDS-PAGE gel. After electrophoresis, the proteins were transferred onto an Amersham Hybond-P PVDF Membrane (GE Healthcare, Buckinghamshire, UK). Membranes were blocked using 4% Block Ace (Dainippon Pharmaceutical, Osaka, Japan), and incubated with anti-Nuf2 (alias CDCA1) antibody (catalog no. ab96147; Abcam, Cambridge, MA, USA) and mouse anti-β-actin antibody (catalog no. 8H10D10; Cell Signaling Technology, Danvers, MA, USA). In the final step, the membranes were incubated with enhanced chemiluminescence (ECL) anti-rabbit IgG, horseradish peroxidase (HRP)-linked whole antibody, ECL anti-mouse IgG and HRP-linked whole antibody (GE Healthcare). Protein bands were visualized using ECL detection reagents (GE Healthcare).

Immunocytochemistry. Cultured cells were washed twice with PBS(-), fixed in 4% formaldehyde solution for 30 min at room temperature and rendered permeable by a 3-min treatment with PBS(-) containing 0.1% Triton X-100. Cells were covered with CAS Block (Invitrogen, Carlsbad, CA, USA) for 7 min to block non-specific binding before the primary antibody reaction. Then the cells were incubated with anti-Nuf2 antibody. The immune complexes were stained with Alexa Fluor 488 goat anti-rabbit IgG (Life Technologies, Grand Island, NY, USA) and mounted with Vectashield mounting medium with DAPI (Vector Laboratories, Burlingame, CA, USA), and viewed using a motorized inverted microscope IX81 (Olympus, Tokyo, Japan).

Immunohistochemistry and tissue microarray analysis. To investigate the significance of CDCA1 overexpression in clinical CRCs, we stained tissue sections using Envision® kit/horseradish peroxidase (HRP; DakoCytomation, Glostrup, Denmark). Anti-Nuf2 antibody was added after blocking of endogenous peroxidase and proteins and each section was incubated with HRP-labeled anti-rabbit IgG as the secondary antibody. Substrate-chromogen was added and the specimens were counterstained with hematoxylin and eosin (HE). Tumor tissue microarrays were constructed according to previously published procedures, using formalin-fixed CRCs that were surgically resected (11-13). Tissue areas selected for sampling
were determined by the visual alignment with the corresponding HE-stained sections on slides. Three, four or five tissue cores (diameter, 0.6 mm; height, 3-4 mm) taken from donor tumor blocks were placed into recipient paraffin blocks using a tissue microarrayer (Beecher Instruments, Sun Prairie, WI, USA). A core of normal tissue was punched from each case. Sections (5-µm thick) of the resulting microarray block were used for immunohistochemical analysis. Positivity and staining intensity were recorded as absent, weak or strongly positive.

**Statistical analysis.** We used contingency tables to correlate clinicopathological variables, such as gender, age, histologic type, and pathologic tumor-node-metastasis (TNM) stage, with CDCA1 protein expression levels determined by tissue microarray analysis. Survival curves were calculated from the surgery date to the CRC-related time of death or to the last follow-up observation. Kaplan-Meier curves were calculated for each relevant variable and for CDCA1 expression; differences in survival times among patient subgroups were analyzed using the log-rank test. Univariate analysis was performed using the Cox proportional-hazard regression model to determine associations between clinicopathological variables and mortality. We first analyzed associations between death and possible prognostic factors including age, gender, pathologic tumor classification and pathologic node classification, taking into consideration one factor at a time. Then, a multivariate Cox analysis was applied on backward (stepwise) procedures that always forced strong CDCA1 expression into the model, along with any or all variables that satisfied an entry level P-value of <0.05. As the model continued to add factors, independent factors did not exceed an exit level of P<0.05.

**Results**

**CDCA1 gene expression in CRC tissues and cell lines.** We previously demonstrated using gene expression profile analysis that CDCA1 was overexpressed in cancer tissues including CRC tissues (data not shown). We also checked Gene Expression Omnibus (GEO; http://www.ncbi.nlm.nih.gov/geo; ID: 88360759) and found that CDCA1 gene expression in colorectal cancer tissues was higher in all of 17 clinical CRC tissues than in their corresponding normal tissues. Because our original gene expression profile database and the publicly available database revealed high CDCA1 expression levels in clinical CRCS, we performed a semi-quantitative RT-PCR analysis of CDCA1 gene expression in both the cancer and corresponding normal colorectal tissues isolated from 8 CRC patients and the 10 CRC cell lines. CDCA1 gene was highly expressed in 4 of the 8 CRC tissues and in 6 of the 10 CRC cell lines, but was not detected in any normal colorectal tissues (Fig. 1).
Figure 2. CDCA1 protein expression in CRC cell lines. (A) CDCA1 protein expression in four CRC cell lines examined using western blot analysis. ACTB expression served as a quantity control. (B) Subcellular localization of CDCA1 protein. The cells were immunocytochemically stained using anti-CDCA1 antibody (green) and DAPI (blue).

Figure 3. CDCA1 protein expression in CRC tissues and its association with poor clinical outcomes for CRC patients. (A) Immunohistochemical evaluation of CDCA1 expression on tumor tissue microarrays. Examples are strong, weak and absent CDCA1 expression in cancer tissues, and no expression in normal tissues. (Original magnification, x100). (B) Kaplan-Meier analysis of survival of patients with CRC (P=0.019 by the log-rank test) according to the CDCA1 expression levels.
CDCA1 protein expression and its subcellular localization in CRC cells. To determine the CDCA1 protein expression and its subcellular localization in CRC cells, we performed western blotting and immunofluorescence analyses using anti-CDCA1 antibody, CDCA1-positive CRC cells (SW480, SW620 and SW948), and CDCA1-negative HT-29 cells. The band was detected using western blotting in CDCA1-positive SW480, SW620 and SW948 cells, whereas no signal was detected in CDCA1-negative HT-29 cells (Fig. 2A). In addition, through an immunofluorescence analysis, we detected CDCA1 protein primarily in the nucleus and cytoplasm of CDCA1-positive SW480, SW620 and SW948 cells, but not in CDCA1-negative HT-29 cells (Fig. 2B).

Association of CDCA1 overexpression with poor clinical outcomes for CRC patients. To verify the biological and clinicopathological significance of CDCA1 in clinical CRCs, we examined CDCA1 protein expression with immunohistochemical analysis using anti-CDCA1 antibody and tissue microarrays for the 434 CRC cases without distant metastases that underwent surgical resection. CDCA1 staining was observed primarily in the cell nucleus and cytoplasm of CDCA1-positive SW480, SW620 and SW948 cells, but not in CDCA1-negative HT-29 cells (Fig. 2A). In addition, through an immunofluorescence analysis, we detected CDCA1 protein primarily in the nucleus and cytoplasm of CDCA1-positive SW480, SW620 and SW948 cells, whereas no signal was detected in CDCA1-negative HT-29 cells (Fig. 2B).

Growth-inhibitory effects of siRNAs against CDCA1. To assess the role of CDCA1 in CRC cell growth and/or survival, we knocked down CDCA1 expression in the CDCA1-positive CRC cell lines SW480 and SW948 using two siRNAs against CDCA1 (si-CDCA1-1 and -2), along with two control siRNAs (siRNAs for EGFP and LUC). Transfection of si-CDCA1s into CRC cells reduced CDCA1 protein levels, and significantly reduced cell viability (Fig. 4). These results indicate that CDCA1 is indispensable for growth and survival of CRC cells.

Table II. Association between CDCA1 positivity in colorectal cancer tissues and patient characteristics (n=434).

<table>
<thead>
<tr>
<th></th>
<th>CDCA1 Strong positive</th>
<th>CDCA1 Weak positive</th>
<th>CDCA1 Absent</th>
<th>P-value (Strong versus weak, absent)</th>
</tr>
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<tr>
<td>Gender</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Male</td>
<td>249</td>
<td>74</td>
<td>73</td>
<td>102</td>
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<tr>
<td>Female</td>
<td>185</td>
<td>58</td>
<td>35</td>
<td>92</td>
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<tr>
<td>Age (years)</td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>&lt;65</td>
<td>215</td>
<td>74</td>
<td>54</td>
<td>87</td>
</tr>
<tr>
<td>≥65</td>
<td>219</td>
<td>58</td>
<td>54</td>
<td>107</td>
</tr>
<tr>
<td>Histologic type</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>tub1, tub2, pap</td>
<td>385</td>
<td>116</td>
<td>99</td>
<td>170</td>
</tr>
<tr>
<td>por1, por2, sig, others</td>
<td>49</td>
<td>16</td>
<td>9</td>
<td>24</td>
</tr>
<tr>
<td>pT factor</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tis+T1+T2</td>
<td>132</td>
<td>43</td>
<td>32</td>
<td>57</td>
</tr>
<tr>
<td>T3+T4</td>
<td>302</td>
<td>89</td>
<td>76</td>
<td>137</td>
</tr>
<tr>
<td>pN factor</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N0+N1</td>
<td>364</td>
<td>108</td>
<td>89</td>
<td>167</td>
</tr>
<tr>
<td>N2</td>
<td>70</td>
<td>24</td>
<td>19</td>
<td>27</td>
</tr>
</tbody>
</table>

tub, tubular adenocarcinoma; tub1, well-differentiated type; tub2, moderately-differentiated type; pap, papillary adenocarcinoma; por, poorly-differentiated adenocarcinoma; por1, solid type; por2, non-solid type; sig, signet ring cell carcinoma.
Table III. Cox's proportional hazards model analysis of prognostic factors in patient with colorectal cancers.

<table>
<thead>
<tr>
<th>Variables</th>
<th>Hazards ratio (95% CI)</th>
<th>Unfavorable/favorable</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Univariate analysis</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CDCA1</td>
<td>1.813 (1.097-2.997)</td>
<td>Strong/weak, absent</td>
<td>0.020^a</td>
</tr>
<tr>
<td>Gender</td>
<td>0.775 (0.464-1.295)</td>
<td>Male/female</td>
<td>0.330</td>
</tr>
<tr>
<td>Age (years)</td>
<td>1.189 (0.725-1.952)</td>
<td>≥65/&lt;65</td>
<td>0.493</td>
</tr>
<tr>
<td>Histologic type</td>
<td>0.643 (0.258-1.604)</td>
<td>tub1, tub2, pap/por1, por2, sig, others</td>
<td>0.344</td>
</tr>
<tr>
<td>pT factor</td>
<td>14.222 (3.477-58.166)</td>
<td>T3+T4/Tis+T1+T2</td>
<td>&lt;0.001^a</td>
</tr>
<tr>
<td>pN factor</td>
<td>1.299 (0.706-2.393)</td>
<td>N2/N0+N1</td>
<td>0.401</td>
</tr>
<tr>
<td><strong>Multivariate analysis</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CDCA1</td>
<td>1.977 (1.195-3.269)</td>
<td>Strong/weak, absent</td>
<td>0.008^a</td>
</tr>
<tr>
<td>pT factor</td>
<td>14.877 (3.636-60.874)</td>
<td>T3+T4/Tis+T1+T2</td>
<td>&lt;0.001^a</td>
</tr>
</tbody>
</table>

^aP<0.05 (Fisher's exact test). tub, tubular adenocarcinoma; tub1, well-differentiated type; tub2, moderately-differentiated type; pap, papillary adenocarcinoma; por, poorly-differentiated adenocarcinoma; por1, solid type; por2, non-solid type; sig, signet ring cell carcinoma.

Figure 4. Growth-inhibitory effects of CDCA1 siRNAs on CRC cells. Western blotting showing suppression of CDCA1 expression mediated by CDCA1-specific siRNAs, si-CDCA1-1 and si-CDCA1-2, in CRC cell lines SW480 and SW948, respectively. ACTB protein expression served as a quantity control for protein levels (A and C). MTT assay demonstrating a decrease in cell numbers by knockdown of CDCA1 in SW480 cells (si-CDCA1-2 and si-EGFP; P=0.0084; unpaired t-test) and SW948 cells (si-CDCA1-2 and si-EGFP; P=0.0136; unpaired t-test) (B and D).

an increased sub-G1 fraction in SW480 and SW948 cells transfected with si-CDCA1-1, compared with those transfected with control siRNAs (Fig. 5A). In addition, caspase 3/7 assay detected an increase in caspase 3/7 activity, whereas TUNEL assay showed an increase in TUNEL staining in SW480 and SW948 cells transfected with siRNAs for CDCA1, compared with those transfected with control siRNAs. These results independently demonstrated activation of caspase cascades and subsequent DNA fragmentation in CRC cells transfected with siRNAs for CDCA1 (Fig. 5B and C).
Discussion

Significant advances in the development of molecular-targeting drugs for cancer therapy have been achieved in the last two decades. However, the number of patients that respond to the presently available treatments is limited, and a subset of the patients suffers from severe adverse reactions without clinical benefits. Therefore, it is critical to develop new anticancer agents that are highly specific to malignant cells and have a minimum risk of adverse side effects.

We previously reported that CDCA1 overexpression plays a key role in the proliferation of lung cancer. CDCA1 is one of the highly conserved components of nuclear division cycle complex and is categorized as an oncoantigen that can induce peptide-specific CTLs against solid tumors (16,39). In the present study, we demonstrated that CDCA1 expression is also elevated in many CRC tissues. Similar to previous findings in lung cancer cells, knocking down CDCA1 with siRNA inhibited CRC cell growth, which suggests increased CDCA1 expression is necessary for CRC cell proliferation and/or survival. CDCA1 protein functions at kinetochores for stable microtubule attachment and stable kinetochore localization of centromere-associated protein E (CENP-E) in the cervical cancer cell line HeLa (45-47). When CDCA1 expression was reduced in these cells, kinetochores failed to form attachments with spindle microtubules, which were followed by aberrant chromosome segregation, a prolonged mitotic blockade, and cell death (45-47). This aberrant exit from mitosis has characteristics of both apoptosis and catastrophe (45). Recent investigations also demonstrated that knocking down centrosomal proteins such as aurora A and ninein in HeLa cells led to aberrant spindle formation and subsequent cell death,
which are accompanied by several features of apoptosis (48). Therefore, we presume that apoptosis of CRC cells induced by CDCA1 expression inhibition by siRNA could result from a similar mechanism. However, further studies are needed to clarify the relationship between CDCA1 suppression and mitotic catastrophe or apoptosis. Many proteins that regulate mitosis are aberrantly expressed in human tumor cells when compared with their normal counterparts, and some of these function as oncogenes, such as aurora kinase and polo-like kinase (49,50). Some of these proteins are potential targets for anticancer agents. For example, highly conserved aurora kinases are critical mitotic regulators (49). Several aurora kinase inhibitors, including ZM447439, Hesperadin and VX-680 have been described as anticancer drugs (49). CDCA1 could serve as a valuable target for molecular-targeted therapies, as well as peptide vaccine immunotherapy for CRC.

In addition, we demonstrated that CDCA1 was highly expressed in 55.3% of surgically resected samples obtained from CRC patients and this overexpression was associated with a poorer prognosis. A publicly available microarray database (http://www.prognoscancer.org/) revealed a significant correlation between high CDCA1 expression and a reduced OS period for CRC patients (dataset no. GSE17536; P=0.028054), which independently supports our data that CDCA1 expression has prognostic value for CRC patients. CDCA1 positivity in CRC tissues could provide a clinical prognostic indicator that warrants intensive follow-up in patients and/or addition of adjuvant chemotherapy after surgical treatments.

To examine the mechanisms of CDCA1 activation and overexpression in CRCs, we searched previous publications and databases for CDCA1 including the CGH and genome sequencing (http://cancer.sanger.ac.uk/cosmic/gene/) databases. Missense mutation was indicated in 6 of the 652 CRCs (0.92%), but no CDCA1 gene amplification or translocation was reported in CRCs. Therefore, we speculate that overexpression of CDCA1 may be primarily caused by epigenetic mechanism. Further analysis of CDCA1, including screening using functional assays for an activating mutation or epigenetic regulating mechanisms of CDCA1 may further clarify the oncogenic function of CDCA1.

In conclusion, our data suggest that CDCA1 contributes to the viability and malignant potential of CRC cells, and is a clinically promising prognostic biomarker in addition to a potential molecular target for treating CRC.

References


