Targeting of CDC20 *via* Small Interfering RNA Causes Enhancement of the Cytotoxicity of Chemoradiation

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Abstract. *Background: Cell division cycle 20 homologue (CDC20), which encodes a protein that promotes chromosomal separation, is highly expressed in several carcinomas, including pancreatic cancer. Materials and Methods: To ascertain whether this gene could be a potential therapeutic target, the RNA interference technique was applied using small interfering RNA (siRNA) to knockdown CDC20 expression. Results: The CDC20 siRNA showed more than 90% inhibition of CDC20 expression at both the transcriptional and translational levels and the specific knockdown of CDC20 expression inhibited the cell growth of human pancreatic carcinoma cells in vitro. Suppression of CDC20 induced accumulation of the cells in the G_2/M-phase of the cell cycle. In addition, the knockdown of CDC20 caused enhancement of the cytotoxicity of paclitaxel and increased the effect of γ-irradiation against pancreatic carcinoma cells. Conclusion: CDC20 is a promising target for gene specific therapy in human pancreatic cancer.*

Cell division cycle 20 homologue (CDC20) is thought to play a key role in cell division during the mitotic phase of the cell cycle. It has been reported to directly bind to the anaphase-promoting complex/cyclosome (APC/C) with hCDH1 (fizzy/cell division cycle 20 related 1 (Drosophila)) and activate APC/C by which anaphase is initiated and mitosis is progresses (1). CDC20 interacts directly with Pds1 (securin) and the destruction box of Pds1 is necessary for this interaction. It provides a link between the substrate and the core APC/C and the destruction box is essential for efficient CDC20-substrate interaction. Thus, CDC20 promotes the degradation of Pds1 by APC/C (2, 3).

Overexpression of CDC20 in human pancreatic cancer as compared with that in the normal tissue has been reported from both *in vitro* and *in vivo* studies (4). High levels of expression of the gene have also been reported by cDNA microarray analyses in human pancreatic cancer and lung cancer as compared with that in the normal epithelium in the pancreatic ducts and lung (5), and in gastric cancer (6).

Based on these findings, CDC20 might be a promising candidate for gene-targeted therapy. The use of siRNA against cancer-specific gene targets, such as EphA2 or CEACAM6, in pancreatic cancer cells both *in vitro* and *in vivo* has been reported (7, 8). In this study, the effect of the specific knockdown of CDC20 by small interfering RNA (siRNA) on the growth of human pancreatic cancer cells, changes in the DNA histogram and cancer cell response to paclitaxel and irradiation was investigated.

Materials and Methods

Pancreatic cancer cell lines and cell culture. The human pancreatic cancer cell line, Panc-1,was purchased from American Type Culture Collection (Manassas, VA, USA). The Panc-1 cells were maintained in Dulbecco's modified Eagle medium (DMEM) /F-12 containing 10% fetal bovine serum.

Small interfering RNA (siRNA) and transfection. Double-stranded RNAs, 21 nucleotides in length, were synthesized and purified by Dharmacon Research (Lafayette, CO, USA) using the following primers; sense strand, GGGAAUAUAUAUCCUCUGUUU, and antisense strand, 5'-PACAGAGGAUAUAUAUUCCCUU, corresponding to the coding region of human CDC20 (accession number NM001255, GenBank). Control small siRNAs were also purchased from Dharmacon Research. These siRNAs were dissolved in a buffer (100 mM KCl, 30 mM HEPES, 1 mM $MgCl_2$, pH7.5) to a final concentration of 20 μM prior to being used. Then, 1×10^4 cells/ml were plated on 24- or 96-well plates and incubated for 48 hours before the siRNA transfection. In most of the following

Abbreviations: CDC20: cell division cycle 20 homologue; siRNA: small interfering RNA; APC/C: anaphase-promoting complex/cyclosome.

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experiments, 100 nM of siRNAs with the RNAiFect Transfection Reagent (Qiagen, Hilden, Germany) were used for transfection.

Semi-quantitative PCR and quantitative real-time PCR. The total RNA was extracted from the cells using an RNeasy Mini Kit (Qiagen). One μg of RNA per sample was reverse- transcribed into cDNA using TaqMan reverse transcription reagents (Roche Diagnostics, Basel, Switzerland). For the semi-quantitative PCR, the forward primer for CDC20 was 5'-TCCAAGGTTCAGAC CACTCC-3'and the reverse primer was 5'-GATCCAGGCC ACAGAGGATA-3'. The PCR reaction was conducted by PC801 (Astec, Tokyo, Japan). For real-time PCR, the forward primer for CDC20 was 5'-CTACAGCCAAAAGGCCACTC-3' and the reverse primer was 5'-GATCCAGGCCACAGAGGATA-3'. The Cybergreen Dye assay was used with the Light Cycler (Roche Diagnostics) for the real-time PCR reaction. GAPDH was used as the internal control for both PCRs.

Western blotting. Cell extracts were prepared with NP40 cell lysis buffer (50 mM Tris, pH7.4, 250 mM NaCl, 5 mM EDTA, 50 mM NaF, 1 mM Na_3VO_4, 1% Nonidet P40, 1 mM phenylmethylsulfonyl fluoride (PMSF)) (Biosource, Camarillo, CA, USA). The total protein concentration was measured using the BCA (bicinchoninic acid) protein assay kit (Pierce, Rockford, IL, USA). Cell extracts containing 50 μg of total protein were separated on 10% SDS-PAGE gels, and the resolved proteins were transferred to a nitrocellulose membrane (Biorad, Hercules, CA, USA). The membranes were then incubated with rabbit monoclonal anti-p55CDC antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) diluted 1:200. Thereafter, the membranes were incubated with anti-rabbit IgG-horseradish- peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology, Inc.).

Cellular proliferation assay. The cells were seeded at a density of 1×10^4 cells per well into 96-well plates containing 100 μl of the appropriate medium per well. Transfection of the RNA oligonucleotides was conducted after 48 hours' incubation (37˚C, 5% CO_2). Until 5 days after the transfection, every 24 hours, 20 μl of 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTS solution) was added to each well, and after 1 hour's incubation, the absorbance was measured at 490 nm using a microplate reader, ImmunoReader NJ-2001 (Nihon InterMed, Tokyo, Japan).

Flow cytometry. After the ethanol fixation of the cells, they were incubated with 0.5 mg/ml RNase (Qiagen), and stained with 1 ml of 30 μg/ml propidium iodide. Then, the DNA content was analyzed with a dual laser flow cytometer (Becton Dickinson, San Jose, CA, USA). The percentages of the cells in various stages of the cell cycle in the DNA histogram were estimated using the ModFit LT software (Verity Software House, Topsham, ME, USA).

Chemosensitivity to paclitaxel. The cells were seeded at a density of 1×10^4 cells per well into 96-well plates in 100 μl of the appropriate medium. After 48 hours' incubation (37˚C, 5% CO_2), the cells were transfected with 20 nM of CDC20 siRNA or control siRNA. Then, 48 hours after the transfection, the cells were exposed to a series of dilutions of paclitaxel. The concentration of paclitaxel ranged from 7×10^{-7} nM to 7×10^{-1} nM. Finally, after 72 hours' incubation in the presence of paclitaxel, the cell viability was measured by MTS assay. The IC_{50} value of each set was calculated using the GraphPad Prism (GraphPad Software, San Diego, CA, USA).

Sensitivity to radiation. The cells were seeded at a density of 1×10^4 cells per well into 96-well plates in 100 μl of the appropriate medium. After 48 hours' incubation (37˚C, 5% CO_2), the cells were transfected with 20 nM of CDC20 siRNA or control siRNA. Thereafter, 24 hours after the transfection, the exponentially growing cells were exposed to radiation from a Cs-137 Gamma Cell (Atomic Energy, Ontario, Canada). The radiation dose ranged from 2 Gy to 8 Gy. Seventy-two hours after the irradiation, the cell viability was measured by MTS assay.

Statistical analysis. Results were presented as means±SD. Data comparisons were made by Student's *t*-test. Values of $p<0.05$ were considered to be statistically significant.

Results

Suppression of CDC20 expression by siRNA. The semi-quantitative RT-PCR revealed almost complete suppression of CDC20 expression at the transcriptional level at 24 hours after the transfection of CDC20 siRNA (Figure 1A). The real- time PCR revealed a CDC20 mRNA expression level of 1208±278 in the control siRNA-transfected cells as compared to 90±41 in the CDC20-specific siRNA-transfected cells ($p=0.002$) (Figure 1B). The Western blotting revealed 94% reduction in the level of the p55CDC expression, a product of CDC20, at the translational level following the CDC20 siRNA transfection (Figure 2).

Inhibitory effect of CDC20 siRNA on the cell proliferation. The MTS assay revealed that the cell growth was inhibited significantly by 56% following transfection of 100 nM of the CDC20 siRNA ($p<0.05$), the growth curve of the control siRNA-transfected cells was almost identical to that of the untreated cells (Figure 3).

Effect of suppression of CDC20 expression on the cell cycle. The inhibition of CDC20 expression by the CDC20 siRNA was expected to inhibit cellular proliferation with arrest at metaphase of the cell cycle. To confirm this supposition, flow cytometry was conducted to investigate the DNA contents of the cells. As shown in Figure 4, an increase in the number of cells in the G_2/M-phase was observed in the CDC20 siRNA-transfected cells as compared with that in the control siRNA-transfected cells. The percentage of cells in the G_2/M-phase of the cell cycle was 22.1±5.5% in the CDC20 siRNA-transfected cells, in contrast to 8.0±1.4% in the control siRNA-transfected cells ($p<0.05$).

Suppression of CDC20 expression and the cytotoxic effect of paclitaxel. At the 20 nM dose rate, the CDC20 siRNA

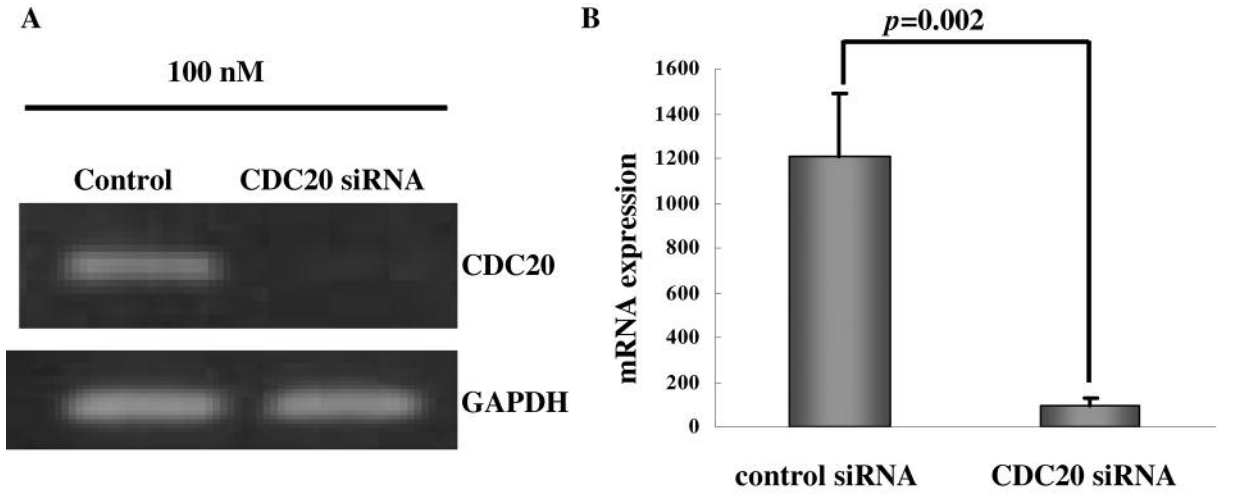

Figure 1. *Suppression of the CDC20 mRNA expression level by siRNA in Panc-1 cells. Analysis was performed by semi-quantitative PCR (A) and real-time PCR (B).*

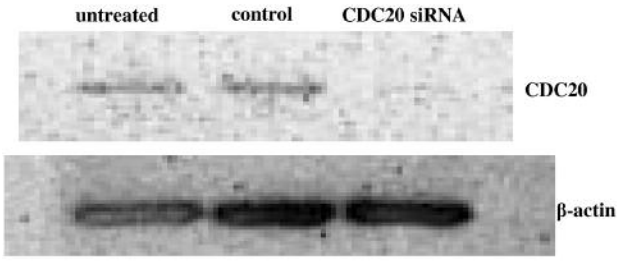

Figure 2. *Suppression of CDC20 protein expression by siRNA in Panc-1 cells. Analysis was performed by Western blotting 72 hours after the siRNA transfection.*

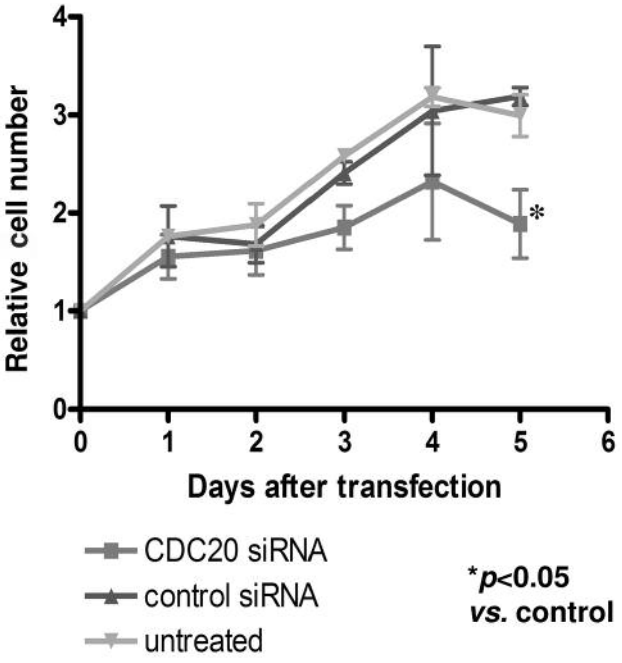

Figure 3. *CDC20 siRNA suppression of proliferation of Panc-1 cells in vitro. The cell proliferation was significantly inhibited 5 days after the transfection of CDC20 siRNA.Values are means±S.D. *p<0.05 vs. that in the control siRNA-treated cells.*

alone had no effect on the cell growth of the Panc-1 cells (data not shown). The cytotoxicity of paclitaxel was thus significantly more pronounced with the addition of the CDC20 siRNA. The calculated IC_{50} value of paclitaxel was 7×10^{-5} nM for the CDC20 siRNA-transfected cells, whereas it was only $7\times10^{-2.6}$ nM for the cells with knockdown of CDC20 (Figure 5).

Suppression of CDC20 expression enhances the sensitivity of pancreatic cancer cells to irradiation. Figure 6 shows the dose-response curves of radiation against the Panc-1 cells. The cell viability decreased in a radiation dose-dependent manner. The viability of the cells transfected with 20 nM of CDC20 siRNA was significantly decreased to 57% of that in the control siRNA-transfected cells following exposure to 8 Gy radiation ($p<0.001$). These observations suggest that the CDC20 siRNA transfection increased the cytotoxic effect of radiation by 1.75-fold.

Discussion

In the present study CDC20 was shown to play a significant role in the progression of the cell cycle and proliferation of pancreatic carcinoma cells. Overexpression of CDC20 destabilized wild-type p21, and the degradation of p21

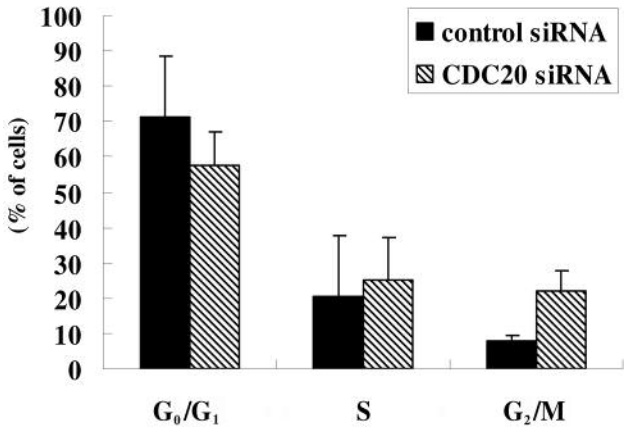

Figure 4. *Effect of CDC20 suppression on the cell cycle of Panc-1 cells. The DNA contents were analyzed by flow cytometry.*

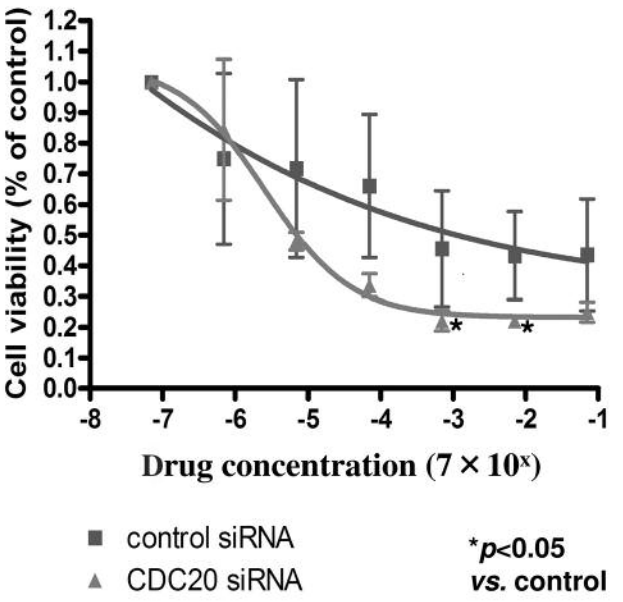

Figure 5. *Effect of CDC20 siRNA on the chemosensitivity of Panc-1 cells to paclitaxel. The cytotoxicity of paclitaxel was significantly enhanced by transfection of the cells with CDC20siRNA. Values are means±S.D.*

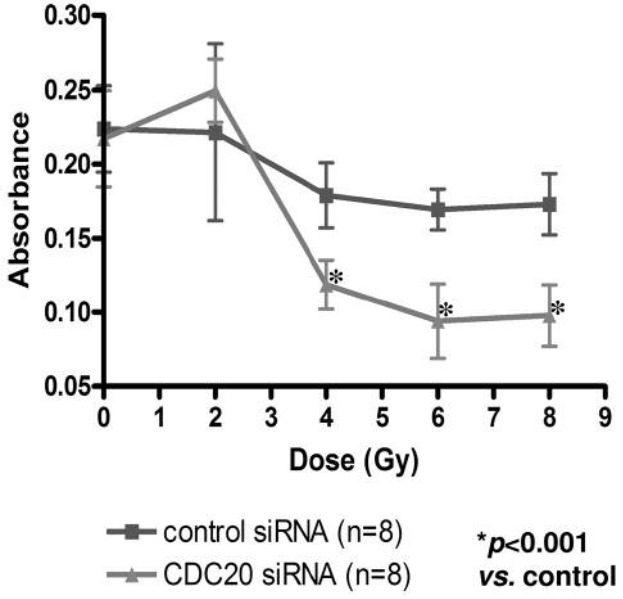

Figure 6. *Effect of CDC20 siRNA on the radiosensitivity of Panc-1 cells. Cells were exposed to various doses of radiation. The cytotoxicity of radiation was significantly enhanced in the cells transfected with the CDC20 siRNA. Values are means±S.D.*

contributes to the full activation of Cdk1 necessary for mitotic events and prevents mitotic slippage during spindle checkpoint activation (9). The overexpression of CDC20 might be expected to lead to accelerated proliferation of cells and the specific knockdown of CDC20 by siRNA did in fact show an inhibitory effect against cell growth *in vitro*. The human pancreatic carcinoma cells with suppressed CDC20 expression accumulated in the G_2/M-phase.

Taxanes, such as paclitaxel, are anticancer drugs that promote polymerization of tubulin and formation of abnormal microtubules (10). With this mode of action, these drugs have been reported to mainly exert their effects during the mitotic phase of the cell cycle. Therefore, cells treated with taxanes also become arrested at the G_2/M-phase of the cell cycle (11), and the cytotoxic effect of these drugs is considered to be mainly exerted at the G_2/M-phase of the cell cycle. The enhanced cytotoxicity of paclitaxel observed in our study might might have been due to the increase in the number of cells in the G_2/M-phase caused by transfection of the CDC20 siRNA.

It is well-known that cells at the G_2/M-phase are exquisitely radiosensitive (12). Several radiosensitizers have been identified, including L-canavanine. Bence *et al.*, reported that L-canavanine, which redistributes cells into the G_2/M-phase of the cell cycle, acts synergistically with radiation against the pancreatic cancer cell line Panc-1, and may have clinical potential in the treatment of pancreatic cancer (13). As shown by the present study, specific knockdown of CDC20 increased the proportion of cells in the G_2/M-phase of the cell cycle and enhanced the cytotoxic effect of γ-irradiation. Thus, CDC20 siRNA may have the potential as a potent radiosensitizer.

CDC20 siRNA has an inhibitory effect on the growth of pancreatic carcinoma cells and enhances the cytotoxic activity of both paclitaxel and irradiation and may be a new promising target for gene-targeted therapy of human pancreatic cancer.

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