Lentivirus-mediated shRNA targeting of cyclin D1 enhances the chemosensitivity of human gastric cancer to 5-fluorouracil

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Abstract. Gastric cancer is one of the major public health problems. Despite new chemotherapeutic treatments, the prognosis of gastric cancer remains poor. 5-Fluorouracil (5-FU) is used as a standard chemotherapy drug in gastric cancer. However, 5-FU resistance develops frequently and is a main cause of chemotherapy failure in human gastric cancer. Overexpression of cyclin D1 is related to rapid cell growth, a poor prognosis and increased chemoresistance in several types of cancers. In this study, we investigated whether treatment of gastric cancer cells with shRNA targeting cyclin D1 (ShCCND1) or 5-FU, alone or in combination, influences the activation of phosphorylated AKT (pAKT) and pNFkB, which are markers that are increased in 5-FU chemoresistance. We also investigated the effect of combined treatment with ShCCND1 and 5-FU on cell growth and chemosensitivity to 5-FU in the gastric cancer cell line AGS. The data showed that ShCCND1-mediated cyclin D1 downregulation in AGS cells significantly inhibited cell proliferation, cell mobility and clonogenicity. In addition, combined treatment with ShCCND1 and 5-FU significantly decreased the survival rate of AGS cells, compared to single-treatment with either agent. These results demonstrated that ShCCND1 increases 5-FU chemosensitivity, a conclusion that is also supported by the concomitant reduction in expression of pAKT and pNFkB, increase of G1 arrest and induction of apoptosis. Taken together, these data provide further evidence that therapeutic strategies targeting cyclin D1 may have the dual advantage of suppressing the growth of cancer cells, while enhancing their chemosensitivity.

Introduction

Gastric cancer is one of the major public health problems and the main factor of cancer-related deaths in Eastern Europe and East Asia (1). Chemotherapy, including 5-FU, cisplatin and adriamycin, is a commonly used treatment method in gastric cancer (2). A significant survival advantage of 5-FU-based chemotherapy has been reported in patients with metastatic cancer as well as in those who have undergone surgery (3,4). Although such treatments have increased the survival rate of gastric cancer patients, many patients treated with 5-FU-based chemotherapy have recurrence. Although resistance to 5-FU-based treatments is a major cause for recurrence, the mechanisms that drive the development of 5-FU resistance in cancer patients are poorly understood.

The cyclin D1 gene encodes for the proteins in the cyclin-dependent kinase (CDK)4/CDK6 complex. Cyclin D1 and CDK4/6 form a complex that phosphorylates and inactivates retinoblastoma (RB) protein, a tumor suppressor (5). The phosphorylation of RB results in the release of E2Fs, which then continues to activate genes that are necessary for advancing into the G1/S phases of the cell cycle (6). In accordance with its growth-promoting role, cyclin D1 can behave as an oncogene. Indeed, overexpression and/or rearrangement of the cyclin D1 gene is seen in several types of human cancers, including gastric cancer (7,8). Therefore, suppression of cyclin D1 expression with a specific targeting method may serve as a powerful treatment for human gastric cancers and can be achieved via RNA interference (RNAi), one of the most effective methodologies for gene targeting.

Previous studies have demonstrated that overexpression of cyclin D1 increases resistance to radiotherapy or chemotherapeutic drugs in various cancer cells (9-13). Moreover, downregulation of cyclin D1 is related to induction of apoptosis and chemosensitivity in T7n cells (14). However, downregulation of cyclin D1 induces chemosensitivity to cis-diamminedichloroplatinum in human oral squamous cell carcinoma (15). The disparate effects of cyclin D1 downregulation may arise from the differential responses to the different drugs and cell type-specific effects, among other reasons. Therefore, cyclin D1 appears to act not only as a pro-survival factor but also as a pro-apoptotic factor depending on specifics.
of the experiment, such as the cell type and chemotherapy drug (16). AKT is a crucial molecule in protecting against cellular apoptosis and it plays a pivotal function in the regulation of normal cell growth and proliferation (17,18). Previous studies have reported that the expression of AKT is changed in several human cancers and that this dysregulated expression may contribute to chemoresistance (19-21). Therefore, AKT is an attractive target of strategies aimed at overcoming chemoresistance in cancers.

The NFкB pathway is one of the main anti-apoptotic signaling pathways and is aberrantly activated in many cancer cells. Previous studies have described that some chemotherapy drugs, including 5-FU, can activate NFкB and, consequently, markedly suppress apoptosis (22,23). Therefore, NFкB is closely linked to 5-FU chemoresistance in many cancer cells (24).

The aim of this study was to develop a lentivirus-mediated shRNA expression system targeting cyclin D1 to generate a stable silencing effect of sufficient efficiency for delivery of cyclin D1-specific shRNA (ShCCND1). We also sought to examine whether treatment with ShCCND1 or 5-FU, alone or in combination, influences the activation of phosphorylated AKT (pAKT) and pNFкB and to determine the effect of the combined treatment with ShCCND1 and 5-FU on cell growth and chemosensitivity to 5-FU in AGS cells.

Materials and methods

Cell line and chemicals. AGS gastric carcinoma cells were purchased from the Korean Cell Line Bank (KCLB, Seoul, Korea). Cells were maintained in RPMI-1640 medium with 10% fetal bovine serum, glutamax and 1% penicillin/streptomycin at 37°C in an atmosphere of 5% CO₂ in a humidified chamber. The 293TN human embryonic kidney cells were purchased from System Biosciences (SBI, Mountain View, CA, USA) and maintained in high-glucose Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, glutamax and 1% penicillin/streptomycin at 37°C in an atmosphere of 5% CO₂ within a humidified chamber. The anticancer agent 5-FU was purchased from Sigma-Aldrich (St. Louis, MO, USA).

Western blot analysis. AGS cells were lysed in radioimmunoprecipitation assay buffer (Sigma-Aldrich) containing a protease inhibitor cocktail (Sigma-Aldrich). AGS cells (ShScramble and ShCCND1) were centrifuged at 13,000 rpm for 10 min. The protein concentration of the cell extract was determined using the BCA™ Protein Assay kit (Thermo Scientific, Rockford, IL, USA). The protein samples were boiled in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer and then electrophoresed on 10% SDS-polyacrylamide gels. The proteins were transferred onto nitrocellulose membranes and the membranes were blocked with 5% non-fat dry milk and incubated overnight with antibodies against cyclin D1 (Santa-Cruz Biotechnology, Santa Cruz, CA, USA), pAkt (Cell Signaling Technology, Beverly, MA, USA), pNFкB (Cell Signaling Technology) and β-actin (Santa-Cruz Biotechnology) at 4°C. Subsequently, the membranes were incubated with either anti-rabbit or anti-mouse secondary antibodies (Santa-Cruz Biotechnology). Specific antibody-protein complexes were detected with the ECL Test kit (KPL, Gaithersburg, MD, USA). Densitometric analysis was performed using ImageJ software.

Cell proliferation and colony formation assay. Cell proliferation was analyzed using the Cell Counting kit-8 (CCK-8; Dojindo Laboratories, Kumamoto, Japan). ShScramble and ShCCND1 cells were plated on 96-well plates at a density of 10⁴ cells/well and cultured for 24 h. Next, 10 µl of CCK-8 solution was added to each well. After a 2-h incubation, an enzyme-linked immunosorbent assay (Tecan Sunrise, Sunnyvale, CA, USA) was performed and the absorbance of the samples was measured at 450 nm.

To assess the colony formation ability, AGS cells containing shRNA were seeded (500 cells/well) in 6-well plates. After 24 h, the culture medium was replaced by RPMI-1640 containing hexadimethrine bromide (5 µg/ml; Sigma-Aldrich). Then, AGS cells were infected with the pseudovirus stock. From the next day onward, AGS cells were maintained in RPMI-1640 medium containing puromycin (1 µg/ml). The stable puromycin-resistant cell lines containing pScramble or pCCND1 obtained by subcloning on the selective medium were named as ShScramble and ShCCND1, respectively. The transduction efficiency of the stable AGS cells expressing shRNA was measured by flow cytometry.
plates. Cell culture medium was replaced fresh RPMI-1640 every 2 days. After 3 weeks, cells were stained with 1% crystal violet for 30 min. To identify colonies, the medium was removed and 3.7% formaldehyde was added. Subsequently, the number of colonies in each well was determined by counting the stained colonies under a light microscope. Image analysis was conducted using Metamorph version 7.5.6.0 software (Molecular Devices, CA, USA).

**Scratch wound-healing assay.** AGS cells were seeded on 60-mm plates. When cells were >90% confluent, a scratch was made using the tip of a pipette (27). After incubation for 48 h, cells that were protruding from the border of the wound were observed and photographed using a Zeiss Axiovert 200 inverted microscope (Carl Zeiss MicroImaging, Thornwood, NY, USA) equipped with a 10x objective. Image analysis was conducted using Metamorph version 7.5.6.0 software.

**In vitro cellular chemosensitivity to 5-FU.** To evaluate the cytotoxic effect of different concentrations of 5-FU, cells were seeded in 96-well plates at a density of 2x10³ cells/well. After overnight incubation, the culture medium was replaced with fresh medium containing various concentrations of 5-FU (0.1, 1, 5 and 15 µg/ml); a control set was included in which fresh medium alone was added, without any 5-FU. Cell viability was evaluated with the CCK-8 assay 72 h after exposure to 5-FU.

To evaluate the cytotoxic effect of the combined treatment with ShCCND1 and 5-FU, a time-course cell viability assay was performed using the CCK-8 kit. Cells were seeded in 96-well plates at a density of 2x10³ cells/well. After overnight incubation, the cells were treated with 5-FU (15 µg/ml). Cell viability was evaluated with a CCK-8 assay after 24, 48 and 72 h.

**Analysis of cell cycle and apoptosis.** To analyze the cell cycle status, 10⁶ AGS cells were washed and resuspended in phosphate-buffered saline containing 5 µl of RNase A (10 mg/ml; Sigma-Aldrich) and 10 µl of propidium iodide (PI; 1 mg/ml; Sigma-Aldrich). After incubating the samples for 1 h in the dark, the cell cycle distribution was assessed using a fluorescence-associated cell-sorting (FACS) assay with FACSCalibur (Becton-Dickinson, Rutherford, NJ, USA) and the results were analyzed with the CellQuest software (Becton-Dickinson).

To evaluate apoptosis, AGS cells were suspended in binding buffer at a cell density of ~10⁶ cells/ml. Aliquots (100 µl) of this cell suspension were incubated with Annexin-V-allophycocyanin and propidium iodide (BD Biosciences Pharmingen). After incubation, the samples were mixed with binding solution and analyzed on a flow cytometer (FACSCalibur, Becton-Dickinson). The data were analyzed using the CellQuest software (Becton-Dickinson).

**Statistical analysis.** For statistical analysis, all data obtained were analyzed using the Prism 5 software for Windows (GraphPad Software, San Diego, CA, USA). Statistically significant differences between the various groups were evaluated using the unpaired Student's t-test and Fisher's exact test. The level of statistical significance was set at values of P<0.05.

**Results**

**ShCCND1 inhibits the expression of cyclin D1 and cell proliferation in AGS cells.** AGS cells stably transduced with lentivirus were successfully selected by puromycin-containing medium. The transduction efficiency of the lentivirus-transduced AGS cells, as measured by flow cytometry, was 95.1 and 95.8% for the ShScramble and ShCCND1 cells, respectively (data not shown). The protein levels of cyclin D1 in AGS cells were examined by western blotting. Compared to the AGS cells, ShScramble expressed similar levels of cyclin D1 (data not shown). As shown in Fig. 1A, cyclin D1 expression levels were similar in ShCCND1_1- and ShScramble-transduced cells, whereas ShCCND1_2- and ShCCND1_3-transduced cells had decreased cyclin D1 levels compared to AGS cells (Fig. 1A). Densitometric analysis of the western blotting images indicated a dramatic and significant decrease in cyclin D1 expression in ShCCND1_3-transduced cells, compared to the parental AGS cells (P<0.05). The ShCCND1_3-transduced clone was then used for subsequent studies and named as ShCCND1. To evaluate the inhibitory effect of cyclin D1 knockdown on cancer cell proliferation, the cell viability of ShScramble- and ShCCND1-transduced cells was measured using the CCK-8 assay. The viability of the ShCCND1-transduced cells was significantly diminished compared to that of ShScramble cells (Fig. 1B). Proliferation of these cells was considerably suppressed by ShCCND1-transduced cells after day 1 of culture. These data suggested that ShCCND1 could effectively knockdown the endogenous cyclin D1 expression and inhibit cell proliferation in AGS cells.

**ShCCND1 inhibits focus formation and cell motility in AGS cells.** To test whether the knockdown of cyclin D1 affects the clonogenic potential, which is one of the important characteristics required for tumor formation in vivo, a colony formation assay was performed, as described previously (28). The number of foci in ShCCND1-transduced cells (N=12.7) was lower than that in ShScramble-transduced cells (N=63.7, P<0.001; Fig. 2A). Moreover, because decreased clonogenic potential is usually associated with invasive ability in cancer cells, the cell motility of AGS cells was analyzed using a classic wound-healing assay (28). The wound area in the ShCCND1-transduced cells was significantly decreased compared to that in ShScramble-transduced cells (Fig. 2B); the wound area was 31.3 and 14.6% in ShCCND1- and ShScramble-transduced cells, respectively (P<0.001). These results demonstrated that ShCCND1 significantly decreased the focus-formation potential of the cells, which correlates with the formation of cancer in nude mice and their migratory capacity (29).

**Combined treatment with ShCCND1 and 5-FU inhibits cell growth to a greater extent than treatment with either agent alone.** High expression of cyclin D1 protein has been associated with increased resistance of cancer cells to chemotherapeutic agents, such as 5-FU (12,13). Therefore, we investigated whether ShCCND1 could enhance the sensitivity of AGS cells to 5-FU. As shown in Fig. 3A, 5-FU treatment (0.1, 1 and 5 µg/ml) decreased cell survival in a dose-dependent manner in AGS and in ShScramble- and ShCCND1-transduced cells. However, treatment with a high dose of 5-FU (15 µg/ml) decreased cell
survival in ShCCND1-transduced cells (P<0.05), but not in AGS and ShScramble-transduced cells. As shown in Fig. 3B (left panel), cell viability gradually increased in AGS and ShScramble- and ShCCND1-transduced cells during culture without 5-FU. Compared to AGS and ShScramble-transduced cells, ShCCND1 cells exhibited significantly decreased cell
viability on day 3 (P<0.05). These results reconfirmed that ShCCND1 inhibited the proliferation of AGS cells. Under the 5-FU (15 µg/ml) treatment, the viability of ShCCND1-transduced cells was significantly decreased compared to that of AGS cells (P<0.01) on day 2 and compared to AGS (P<0.01) and ShScramble-transduced (P<0.001) cells on day 3 (Fig. 3B right panel). These findings indicate that combined treatments with 5-FU and ShCCND1 more effectively inhibited cell growth than treatment with 5-FU or ShCCND1 alone. These results indicated that ShCCND1 increases the sensitivity of AGS cells to 5-FU.

Combined treatment with ShCCND1 and 5-FU affects apoptosis and cell cycle regulation. To determine the potential effects of ShCCND1 on the cell cycle, ShCCND1-transduced cells were analyzed by flow cytometry. As shown in Fig. 4A, the G1-phase distribution was 76.7, 74.9 and 87.9% in cells treated with 5-FU, ShCCND1 and a combination of both agents, respectively. The percentage of cells arrested in the G1 phase in the combination-treated cells was much higher than that in ShScramble-transduced (P<0.001), ShCCND1-treated (P<0.001) and 5-FU-treated cells (P<0.05). This increase was accompanied with a concomitant reduction in the percentage of cells in the S and G2 phases of the cell cycle. These data indicated that G1 phase arrest is induced in ShCCND1-transduced cells and that the combined treatment with ShCCND1 and 5-FU elicits a synergistic increase in G1 phase arrest, compared to treatment with ShCCND1 or 5-FU as single agents. In addition to cell cycle arrest, apoptotic cell death was measured by flow cytometry to assess the effect of the combined treatment on cell death. As shown in Fig. 4B, cellular apoptotic indices were 13.02% in cells without treat-

Figure 3. Effect of ShCCND1 and/or 5-FU on AGS cell viability, as measured by the CCK-8 assay. (A) ShCCND1-transduced cells alone exhibited a dose-dependent decrease in cell survival after 5-FU treatment. (B) Time-course of the effect of ShCCND1 and/or 5-FU on AGS cell viability. Data are presented as mean ± SEM. *P<0.05 and **P<0.01, versus untreated parental AGS cells. *P<0.05 and ***P<0.001, versus ShScramble-transduced cells.

Figure 4. Effect of ShCCND1 and/or 5-FU on cell cycle distribution and apoptosis in AGS cells, as determined by flow cytometry. (A) Combined treatment with 5-FU and ShCCND1 significantly increased the proportion of cells in the G1 phase of the cell cycle, compared to the ShScramble-, ShCCND1- or 5-FU-treated cells. (B) Apoptosis significantly increased in cells treated with the combination of ShCCND1 and 5-FU, compared to the ShScramble-, ShCCND1-, or 5-FU-treated cells. Data are presented as mean ± SEM. *P<0.05, **P<0.01 and ***P<0.001.
ment, 17.03% in cells treated with ShCCND1 alone, 21.62% in cells treated with 5-FU alone and 32.85% in cells treated with the combination. These results show that ShCCND1 in combination with 5-FU leads to significantly increased apoptosis, compared to that in untreated (P<0.05), ShCCND1- (P<0.05) and 5-FU-treated cells (P<0.05). Thus, these data indicate that ShCCND1 increases the apoptotic cell population and combined treatment with ShCCND1 and 5-FU synergistically increases apoptosis. Collectively, these results clearly demonstrate that the anti-proliferative effect of ShCCND1 is mediated via induction of cell cycle arrest and apoptosis.

**ShCCND1 or 5-FU singly or in combination influences the expression of pAKT, pNFκB and downstream signaling molecules.** The AKT/NFκB survival signaling pathway plays a significant role in the progression of various cancers (30,31). Chemoresistance to 5-FU has been reported to be caused by the induction of pAKT and pNFκB (32). Moreover, high expression of cyclin D1 protein is also associated with increased resistance of cancer cells to chemotherapeutic agents such as 5-FU (12,13). Therefore, to test whether the anti-proliferative effect of the combination of 5-FU and ShCCND1 is attributable to the inhibition of AKT/NFκB expression, we investigated the activation status of downstream components of AKT/NFκB by western blotting. As shown in Fig. 5A, the expression of pAKT and pNFκB after 5-FU treatment was reduced, compared to that in untreated cells. Combined treatment reduced pAKT expression to a greater degree than did treatment with ShScramble (P<0.01), ShCCND1 (P<0.05), or 5-FU (P<0.05) alone. In addition, combined treatment with 5-FU and ShCCND1 significantly reduced pNFκB expression compared to that in cells treated with ShScramble (P<0.001), ShCCND1 (P<0.05), or 5-FU (P<0.01) alone. These data suggest that the reduced expression of pAKT and pNFκB by combined treatment with ShCCND1 and 5-FU increases chemosensitivity and overcomes 5-FU resistance in AGS cells.

The expression of cyclin D1 and pRB, as measured by western blotting, was consistent with the cell cycle arrest at G1 phase, as determined by flow cytometry (Fig. 5B). The expression of cyclin D1 in cells treated with 5-FU (P<0.001) or ShCCND1 was reduced compared to that in untreated cells. Combined treatment with 5-FU and ShCCND1 reduced cyclin D1 expression to a greater extent compared to that in untreated cells (P<0.05) and in cells treated with 5-FU or ShCCND1 alone. Analysis of pRB, a downstream molecule of cyclin D1, revealed a similar pattern. The expression level of pRB in cells treated with 5-FU or ShCCND1 was decreased compared to that in untreated cells. Combined treatment with 5-FU and ShCCND1 reduced pRB levels to a greater extent compared to that in untreated cells (P<0.01) and in cells treated with ShCCND1 (P<0.05) or 5-FU alone.

**Discussion**

In spite of the availability of new chemotherapeutic treatments, the prognosis of gastric cancer is still poor (33,34).
The survival time of patients with recurrence or metastasis is <2 years, despite application of traditional chemotherapy (35). Moreover, the side effects of cytotoxic chemotherapeutic drugs often result in deterioration of the quality of life in patients. Accordingly, to improve the clinical outcome, more effective and innovative treatments are needed.

5-FU is the standard therapy against gastric cancer and is a well-known apoptosis-inducing drug that has been in use for several decades. However, resistance to 5-FU, a main cause for failure of chemotherapy, frequently develops in human gastric cancer. In previous studies, chemoresistance to 5-FU has been associated with AKT and NFκB activation and was shown to increase with increased expression of pAKT and pNFκB (32,36). Therefore, among the numerous reported mechanisms of 5-FU resistance, in this study, we focused on the cell signaling proteins AKT and NFκB.

Cyclin D1, a cell cycle regulator, is involved in the regulation of the G1-S phase transition of the cell cycle (37). Overexpression of cyclin D1 is associated with rapid cell growth, poor prognosis and increased chemoresistance in various cancers (25,38). Previous studies have distinctly shown that treatment of cancer cells with cyclin D1 antisense results in considerable inhibition of cell growth (38). Recently, silencing cyclin D1 has also been shown to induce apoptosis in some cancer cell lines (25,39). To analyze the effects of silencing cyclin D1 expression on gastric cancer cell function, we first established a stable cell line expressing ShCCND1 that effectively inhibits expression of the cyclin D1 protein. As expected, the results of this study showed that cells stably transduced with ShCCND1 exhibited decreases in cyclin D1 protein levels, cell proliferation rate, cell motility and the ability to form foci. These results are in agreement with those reported in previous studies (9,10,25).

If ShCCND1 can decrease the resistance of gastric cancer to 5-FU, it might be an encouraging potential anticancer therapy that can be administered along with 5-FU for treatment of gastric cancer. To review the role of ShCCND1 in regulating 5-FU sensitivity in gastric cancer, a stable cell line expressing ShScramble or ShCCND1 was used. In this study, AGS gastric cells were insensitive to 5-FU concentrations >5 µg/ml, whereas AGS cells treated with ShCCND1 were sensitive to lower concentrations of 5-FU. Combined treatment with ShCCND1 and 5-FU was more effective than treatment with either agent alone. As expected, cell proliferation was inhibited, G1 arrest was enhanced and apoptosis was induced in AGS cells exposed to ShCCND1 or 5-FU alone or in combination. These results demonstrate that the expression level of cyclin D1 is related to the chemosensitivity to 5-FU and that chemosensitivity might be generated or restored by decreasing the expression of cyclin D1.

The AKT and NFκB proteins play important roles in cancer progression, including cell proliferation, cell invasion, apoptosis and metastasis (30). Furthermore, their activation is also associated with chemosensitivity to 5-FU (40). In a previous report, chemoresistance to 5-FU was shown to be caused by an increase in pAKT and pNFκB expression (32). Moreover, overexpression of cyclin D1 protein also increases resistance of cancer cells to chemotherapeutic agents such as 5-FU (12,13). In this study, combined treatment with ShCCND1 and 5-FU suppressed the expression of pAKT, pNFκB and cyclin D1 proteins to a greater extent than that in cells treated with ShCCND1 or 5-FU alone. In addition, the expression of pRB, which is directly regulated by cyclin D1, was significantly downregulated in cells that received the combined treatment than in those that received the single treatment. These results support the notion that the 5-FU and ShCCND1 combination exerts a synergistic killing effect in human gastric cancer cells and that this effect is attributable to the suppression of pAKT and pNFκB expression. Taken together, the results of this study provide further evidence that therapeutic strategies targeting cyclin D1 may have the dual advantage of suppressing the growth of cancer cells, while concomitantly enhancing their chemosensitivity.

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References


