RECQL1 and WRN proteins are potential therapeutic targets in head and neck squamous cell carcinoma

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Abstract

RECQL1 and WRN protein are RecQ DNA helicases that participate in suppression of DNA hyper-recombination and repair. In this study, we report evidence supporting their candidacy as cancer therapeutic targets. In hypopharyngeal carcinomas, which have the worst prognosis among head and neck squamous cell carcinomas (HNSCC) that are rapidly rising in incidence, we found that RECQL1 and WRN proteins are highly expressed and that siRNA-mediated silencing of either gene suppressed carcinoma cell growth in vitro. Similarly, siRNA administration in a murine xenograft model of hypopharyngeal carcinoma markedly inhibited tumor growth. Moreover, combining either siRNA with cis-platinum (II) diammine dichloride (CDDP) significantly augmented the in vivo anticancer effects of this drug that is used commonly in HNSCC treatment. Notably, we observed no recurrence of some tumors following siRNA treatment in this model. Our findings offer a preclinical proof-of-concept for RECQL1 and WRN proteins as novel therapeutic targets to treat aggressive HNSCC and perhaps other cancers.
Introduction

Two-thirds of head and neck squamous cell carcinoma (HNSCC) cases have locally advanced lesions with or without regional lymph node involvement (1). Despite recent advances in therapeutic procedures, the 5-year survival rate for patients with HNSCC has remained ≤50% for the last few decades (2). Hypopharyngeal carcinoma has one of the worst prognoses among HNSCC, and the 5-year survival rate is approximately 30% to 35% (3). Hypopharyngeal carcinoma is prominently invasive, and total pharyngolaryngectomy, which is the curative surgical treatment, causes serious functional problems that include vocal, swallowing and chewing disorders (4). In addition, hypopharyngeal carcinoma often metastasizes to lymph nodes or distant organs and therefore, innovations to improve disease-survival and larynx preservation are needed.

The RecQ family helicases participate in prevention of hyper-recombination and repair of DNA, and are highly up-regulated in transformed cells and tumor cells (5, 6). Partial functional redundancy among 5 RecQ helicases is possible, but they cannot
completely substitute each other. Each helicase plays a distinct role for DNA repair (7-9), and furthermore causes different mutational diseases: dysfunction of WRN, BLM and RTS helicases causes Werner, Bloom, and Rothmund-Thomson syndromes, respectively. Notably, only WRN helicase possesses an exonuclease activity (10).

RECQL1 and WRN proteins are members of the RecQ helicase family and play an important role in maintenance of genomic stability (5, 11-14). RECQL1 functions as a Holliday junction-resolving enzyme and is involved in a mismatch-repair pathway; and the defect is possibly linked to tumorigenesis of colorectal cancers (15).

RECQL1-deficient mice are sensitive to ionizing radiation (14), but can grow without any apparent abnormalities. The *RECQL1* silencing by small interfering RNA (siRNA) induces cancer cell-specific cytotoxicity (5, 16), and is effective in reduction of tumor growth in carcinoma cells originating from lung, hepatic, pancreatic, and colon carcinomas (16). The effect of *RECQL1*-siRNA is suggested to be affected by: (i) the speed of cell cycle progression, (ii) the checkpoint status, such as p53 mutations, and (iii) levels of expressed contents of RECQL1 (16). WRN also participates in DNA
dynamics such as non-homologous end joining, homologous recombination, and base-excision repair (6, 11, 17). WRN functions at telomeres during the S-phase and replication of G-rich telomeric DNA (18). WRN functional loss causes telomeric loss without lagging-strand replication (19). WRN-deficient cells are hypersensitive to camptothecin and 4NQO (20), but can grow without any apparent abnormalities (20, 21). The modification of the expression of the WRN gene is known to affect growth in colorectal tumors and the effect of anticancer drugs (11, 22). Recently, it was reported that inhibition of WRN helicase activity impaired cell growth and proliferation, induced apoptosis in a WRN-dependent manner, and resulted in elevated γ-H2A.X and proliferating cell nuclear antigen (PCNA) foci (23).

Because hypopharyngeal carcinomas show a high growth activity, they are expected to express high levels of RecQ family proteins, suggesting that RECQL1 and WRN could be potential therapeutic targets. Therefore, we examined whether RECQL1 and WRN are highly expressed in hypopharyngeal carcinomas, whether RECQL1- or WRN-siRNA has an inhibitory effect on hypopharyngeal cancer cell growth in vitro
and *in vivo*, and whether the combined therapy with cis-platinum (II) diammine dichloride (CDDP) and either siRNA could enhance the anticancer effect *in vivo*. In addition, the present study discusses the mechanisms of *in vivo* cancer cell death caused by the combined treatments with CDDP and siRNA.
Materials and Methods

Cell cultures. The cancer cell lines, FaDu, D-562 (hypopharyngeal carcinoma), HeLa (endocervical carcinoma), MCF7, T47D, and SKBr3 (breast carcinoma) were purchased from American Type Culture Collection. TIG-108 human normal fibroblast was from the Japanese Collection of Research Bioresources. These cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM) or RPMI 1640 containing 10% fetal bovine serum. All cell culture media were supplemented with penicillin (50 units/ml) and streptomycin (50 mg/ml). These cell lines were incubated at 37°C in a humidified chamber supplemented with 5% CO₂.

WST-8 assay. FaDu, D-562, MCF7, T47D and TIG-108 cells were cultured in 96-well plates, and cell viability assays were carried out using a Cell Counting Kit-8 (DOJINDO, Kumamoto, Japan). WST-8 reagent solution was added to each well, and the absorbance at 450 nm (OD450) was then measured using a microplate reader after the microplate was incubated with the reagent for 3 h at 37°C, in accordance with the manufacturer’s instructions.
Protein extractions and Western blot analysis. The expressed proteins of each cell line were analyzed by immunoblotting. Cells were ruptured in ice-cold lysis buffer (40mM Hepes, 120mM NaCl, 1mM ethylenediaminetetraacetic acid, 10mM pyrophosphate, 10mM glycerophosphate, 50mM NaF, 0.5mM Na3VO4, 1% Triton X-100, and containing the protease inhibitor). Cell extracts were kept on ice for 20 min and centrifuged at 15,000 rpm for 10 min (4°C). The supernatants were boiled with SDS sample buffer. Proteins separated by SDS-PAGE were transferred to polyvinylidene difluoride filters. After blocking of the filters with TBS-T (10 mM Tris-HCl (pH 7.6), 150 mM sodium chloride, 0.1% Tween 20) containing 5% bovine serum albumin (BSA), the filters were incubated for 1 h with the primary antibodies in TBS-T containing 2% BSA at 4°C. The filters were then washed in TBS-T and incubated for 30 min in horseradish peroxidase-conjugated anti-mouse IgG (GE Healthcare, Amersham Place, UK) diluted 1:10,000 in TBS-T containing 2% BSA. After several washes with TBS-T, immunoreactivity was detected using the ECL
system (GE Healthcare, Amersham Place, UK) according to the procedures recommended by the manufacturer.

**Tissue samples and pathologic data.** Fifty-two primary hypopharyngeal and fifty-nine primary breast cancer specimens were obtained by biopsy and surgical resection, respectively, at Kyoto Prefectural University of medicine from 1997 to 2008. Hypopharyngeal carcinomas were all squamous cell carcinomas. The samples were obtained from 5 females and 47 males with a median age of 65 years (range 45-81 years). Four cases were stage I, 4 cases were stage II, 4 cases were stage III, and 40 cases were stage IV. Breast cancers were all invasive ductal carcinoma. All the breast cancer samples were obtained from females with a median age of 76 years (range 27-86 years). Nine cases were stage I, 38 cases were stage II, 23 cases were stage III, and 1 case was stage IV. In this study, breast cancers were used as one of the representatively mild malignancies, which have been well controlled by the presently curative protocols and have better prognosis than hypopharyngeal carcinomas. Data were collected from clinical and pathological records with the written informed
consent of individual patients and after approval by the Ethics Committee of the institute.

**Antibodies and reagents.** Anti-WRN (4H12 or 8H3) and anti-RECQL1 (Q1N3) antibodies were reported previously (6, 17, 24), and provided from GeneCare Institute (Kanagawa, Japan). Anti-α tubulin (DM 1A), anti-cleaved caspase 3 (Ab-4), anti-phospho-histone H2A.X (γ-H2A.X; 20E3), anti-anti-MPM-2 (FOXM1,0.T.181), and anti-Ki67 (MM-1) were purchased from SIGMA (Tokyo, Japan), Oncogene Research Products (La Jolla, CA), Cell Signaling Technology (Beverly, MA), Abcam (Cambridge, UK), and Leica Microsystems(Tokyo, Japan), respectively. CDDP was provided by NIPPON KAYAKU (Tokyo, Japan), dissolved with N, N-dimethylformamide, and diluted 1:200 with purified water. The final concentration of CDDP was 50 µg/ml. Lipofectamine RNAi MAX Reagent was purchased from Invitrogen (Carlsbad, CA). Atelocollagen (AteloGene Local Use) was purchased from KOKEN (Tokyo, Japan).

**Immunohistochemistry.** Surgical specimens were transferred to 10% buffered
formalin and fixed overnight. The fixed samples were embedded in paraffin and serially sliced into 4μm sections. After dewaxing, sections were autoclaved at 120°C for 1 min in 10mM sodium citrate buffer (pH 6.0), and immersed in 0.3% H₂O₂. They were then incubated overnight at 4°C with primary antibodies to WRN (diluted 1: 500), RECQL1 (1:500), or γ-H2A.X (1:200). The sections were rinsed with 1×PBS and incubated with the secondary antibody (Simple Stain MAX-PO; Nichirei, Tokyo, Japan) at room temperature for 1 hour. The sections were then colorized with 3,3’-diaminobenzidinetetrahydrochloride and counter-stained with hematoxylin. The stainings were evaluated by two blinded independent observers in all cases.

Small interfering RNA and WST-8 assays.  GL3-, WRN-, and RECQL1-siRNA were provided from GeneCare Institute (Kanagawa, Japan). Firefly luciferase gene sequence GL3 is non-silencing siRNA and was used as a negative control (16). siRNA sequences were as follows: GL3, (sense) 5’-CUUACGCUGAGUACUUCGATT-3’, (antisense) 5’-UCGAAGUACUCAGCGUAAGTT-3’; WRN, (sense) 5’-GUUCUUGUCACGUCUCCUGTT-3’, (antisense)
5'-CAGAGGACGUGACAAGAACTT-3'; \textit{RECQL1}, (sense)

5'-GUUCAGACCACUUCAGCUUTT-3', (antisense)

5'-AAGCUGAAGUGGUCUGAACTT-3'. We examined the silencing effect targeting \textit{RECQL1} and \textit{WRN} by transfecting 40nM of each siRNA with Lipofectamine RNAiMAX reagents. Cells were ruptured in ice-cold lysis buffer and proteins were extracted, as described above. The protein expressions were examined by Western blot analysis. After confirming the silencing effect of each siRNA, we performed WST-8 assays.

**Mice and xenografting of human hypopharyngeal carcinomas.** Eight- to 10-week-old BALB/c nu/nu nude mice (CLEA Japan, Inc., Tokyo, Japan) carrying FaDu or D-562 hypopharyngeal carcinoma were used in the experiments. The use of the animals in the experimental protocols were reviewed and approved by the Committee of Research Center for Animal Life Science in Shiga University of Medical Science. The right dorsal flank of each mouse was injected subcutaneously with $5.0 \times 10^6$ cells of each type of cancer. After the establishment of palpable tumors (about $\geq$
100 mm$^3$), mouse body weight and external tumor volume were determined every two days. Tumor volume was calculated using the formula $A^2 \times \frac{B}{2}$, where $A$ and $B$ represents the smallest diameter and the largest diameter, respectively.

*In vivo evaluation of tumor growth in mice treated with siRNA targeting WRN or RECQL1 or with the combination of CDDP with siRNA.* Forty-Eight mice carrying FaDu hypopharyngeal carcinoma cells were randomized into 8 groups of 6 mice each; CDDP and/or siRNA were administered to the mice on Days 0, 7, and 14. Either CDDP at a dose of 4.0 mg/kg (1,600 μl) or a similar volume of PBS was injected into the peritoneal cavity of each mouse. AteloGene and siRNA were mixed according to the procedures recommended by the manufacturer and administered with siRNA dose 1.0 mg/kg/day around the tumors. Eight experimental groups were as follows: PBS + no siRNA (PBS+NC); PBS + GL3-siRNA (PBS+GL3); PBS + RECQL1-siRNA (PBS+RECQL1); PBS + WRN-siRNA (PBS+WRN); CDDP + no siRNA (CDDP+NC); CDDP + GL3-siRNA (CDDP+GL3); CDDP + RECQL1-siRNA (CDDP+RECQL1); and CDDP + WRN-siRNA (CDDP+WRN). Tumor specimens
were dissected on Day 18 and paraffin sections were prepared as mentioned above.

The sections were immunohistochemically evaluated. The primary antibodies were
directed against Ki67 (diluted 1:50), cleaved caspase 3 (1:1000), MPM-2 (1:500), and
γ-H2A.X. The percentage of positively stained tumor cells per ≥ 1,000 tumor cells was
calculated for each antibody. The anti-tumor effect of each siRNA was also examined
in D-562 xenografts. Twenty-four mice were randomized into 4 groups of 6 mice each.

The 4 intervention groups were as follows: PBS+NC; PBS+GL3; PBS+RECQL1; and
PBS+WRN.

**TdT-mediated dUTP nick end-labeling (TUNEL) assays.** Apoptotic cells of the
dissected tumor samples were detected by a TUNEL assay using the *In situ* Apoptosis
Detection Kit (TaKaRa, Shiga, Japan) according to the manufacturer's protocol.

Proteinase K was treated at a concentration of 20 mg/ml for 5 min. Detection was
performed using 3,3’-diaminobenzidinetetrahydrochloride and counter-stained with
methyl green. The apoptotic index (AI) was assessed as the percentage of positively
stained tumor cells per a high-powered field.
**Statistical analyses.** All the data were analyzed statistically using Stat View 5.0 for Windows (Stat View Inc., NC). OD450 of the WST-8 assay, xenografted tumor volume, and the percentage of positive cells for each immunohistochemical or TUNEL assay were statistically analyzed for differences among the therapeutic groups using one-way factorial analysis of variance (ANOVA) and multiple comparison tests accompanied by Fisher’s significance. Statistical significance was defined as a *P*-value of < 0.05.
Results

**RECQL1 and WRN proteins are abundantly expressed in hypopharyngeal carcinoma.** The hypopharyngeal carcinoma cell lines, FaDu and D-562, grew more rapidly than the breast carcinoma cell lines, MCF7 and T47D, and TIG-108 human fibroblast (Fig. 1A). The expression levels of both WRN and RECQL1 proteins were very high in FaDu and D-562, compared with those in HeLa. In contrast, the expression levels in MCF7, T47D, SKBr3, and TIG-108 were lower than those in HeLa (Fig. 1B). FaDu, D-562, T47D, and SKBr3 showed mutant p53 accumulation (data not shown). HeLa has been used as a blotting control; HeLa expresses a high level of RECQL1 compared with normal cell lines (5). Thus, hypopharyngeal cancer cells expressed abundant amounts of RECQL1 and WRN, whereas breast cancer cells expressed them at a similar level to that of normal cells. In addition, we confirmed the expressions of WRN and RECQL1 proteins in clinical specimens. Both proteins were strongly stained in hypopharyngeal cancer cells compared with stromal fibroblasts, while they were less stained in breast cancer cells (Fig. 1C). The rates of
WRN-positive cells were significantly higher (92.7%) in hypopharyngeal carcinoma than those (59.8%) of breast cancers (Mann-Whitney U test, $P < 0.0001$). On the other hand, the mean RECQL1-positive cell rates were not significantly different between them: 87.7% and 78.9%, in hypopharyngeal and breast carcinoma (Mann-Whitney U test, $P = 0.31$), respectively (Fig. 1D). These comparative findings were also confirmed among similar TNM stages of both cancers (Supplementary Fig. S1A-B). Both WRN- and RECQL1-proteins were highly expressed, and WRN was significantly higher than RECQL1 in hypopharyngeal carcinoma (Wilcoxon signed-ranks test, $P = 0.0009$; Supplementary Fig. S1C). In breast carcinoma, however, RECQL1-positive cells were significantly more frequent than WRN-cells, with case-by-case variations (Wilcoxon signed-ranks test, $P = 0.0075$; Supplementary Fig. S1D).

We assessed the extent of DNA damage by staining with anti-phospho-histone H2A.X ($\gamma$-H2A.X) antibody in hypopharyngeal and breast carcinomas. Abundant $\gamma$-H2A.X was expressed in cell lines (FaDu and D-562; Supplementary Fig. S2A) and clinical samples of hypopharyngeal carcinoma, but not in cell lines and clinical
samples of breast carcinoma (the difference between clinical samples of hypopharyngeal carcinoma and breast carcinoma: Student t-test, $P = 0.0061$; Supplementary Fig. S2B-C). In hypopharyngeal carcinoma, RecQ proteins are expressed at high levels probably in order to deal with the DNA damage as assessed by the staining of $\gamma$-H2A.X.

**Tumor cell growth was significantly inhibited in vitro by RECQL1- or WRN-silencing in hypopharyngeal carcinoma cells.** As the expression levels of both RECQL1 and WRN were very high in the hypopharyngeal carcinoma cell lines, these proteins are expected to play important roles in the cancer cell growth. The *in vitro* hypopharyngeal cancer cell growth was assessed during treatment with RECQL1- and WRN-siRNA. Each siRNA caused significant growth inhibition in FaDu and D-562 (One-way factorial ANOVA and multiple comparison tests accompanied by Fisher’s significance, $P < 0.05$). On the other hand, neither siRNA could effectively inhibit the growth of MCF7, T47D, or TIG-108 cells (Fig. 2). These results suggested that RECQL1 and WRN play important roles in cancer cell growth and therefore, are
potential targets for anticancer therapy in hypopharyngeal carcinoma.

**In vivo therapeutic effect of RECQL1- or WRN-siRNA.** The anti-tumor effect of siRNA targeting *RECQL1* or *WRN* was assessed in nude mice carrying FaDu hypopharyngeal carcinoma (Fig. 3A). Treatment with siRNA (1mg/kg once a week) targeting *RECQL1* or *WRN* (PBS+RECQL1 and PBS+WRN), suppressed tumor growth significantly during Days 7-11 compared with the control treatment, PBS+NC and PBS+GL3 (One-way factorial ANOVA and multiple comparison tests accompanied by Fisher’s significance, *P* < 0.05: Fig. 3B). Moreover, we confirmed similarly the anti-tumor effect of each siRNA in D-562 xenografts (Supplementary Fig. S3).

**Combined effect with CDDP and siRNA targeting RECQL1 or WRN in vivo.** In an attempt to maximize tumor cell death, we investigated in FaDu xenografts whether the combined therapy with CDDP and siRNA targeting *RECQL1* or *WRN* enhanced the anti-tumor effect of the siRNA. This combination therapy was highly effective in inhibiting tumor growth (Fig. 3C) and was significantly different from the
control treatment (PBS+NC and PBS+GL3) and from the monotherapy with CDDP (CDDP+NC and CDDP+GL3). The combination of RECQL1-siRNA with CDDP was more effective than each of the monotherapies. The combination of WRN-siRNA with CDDP was the most effective in tumor growth suppression (One-way factorial ANOVA and multiple comparison tests accompanied by Fisher’s significance, \( P < 0.05 \); Fig. 3C). The tumor growth was completely suppressed in 2 out of 6 mice of the treatment group (CDDP+WRN), and the tumors never recurred during the experimental period. None of the groups experienced side-effects such as body-weight loss.

**Combination treatment with CDDP and siRNA targeting RecQ increased DNA damage and induced apoptosis and/or mitotic catastrophe.** In vivo proliferation and cell death accompanied by mitotic catastrophe and apoptosis were assessed by various immunohistochemistry and TUNEL assays on the treated cells in FaDu xenografts.

Ki67 labeling indices of all groups were 71-79%, and showed no significant difference between them (Supplementary Fig. S4A). None of the treatments in the present study showed any significant effects on the proliferation.
TUNEL was performed for the evaluation of apoptosis. In the control groups (PBS+NC and PBS+GL3) and monotherapeutic groups of CDDP (CDDP+NC and CDDP+GL3), the apoptotic indices were less than 1.0%. Administration of RECQL1- and WRN-siRNA induced apoptosis with indices of 1.56% and 6.23%, respectively. A combination of CDDP and siRNA targeting RECQL1 or WRN showed indices of 5.38% and 5.00%, respectively. Thus, combining CDDP with RECQL1-siRNA enhanced strongly the apoptotic index compared with that of the monotherapeutic RECQL1-siRNA (Fig. 4A). We also performed cleaved caspase 3 staining to evaluate the caspase-pathway, and these data were similar to those of the TUNEL assay (Supplementary Fig. S4B).

To investigate mitotic catastrophe, MPM-2 immunohistochemistry was performed (Fig. 4B). Administration of RECQL1- and WRN-siRNA weakly increased the fraction of MPM-2 positive cells from 5% to 15-20%. CDDP induced a marked increase in MPM-2 positivity, with all of the indices about 60-70%. In particular, the combined therapy with CDDP and RECQL1-siRNA most strongly increased the fraction of
MPM-2 positive cells, with an index of 82%.

Furthermore, because both CDDP and siRNA targeting the RecQ family genes have been involved in causing DNA damage of tumor cells, we investigated the extent of DNA damage with γ-H2A.X staining (Fig. 4C). The percentages of γ-H2A.X-positive cells were low (≤ 3%) in control groups (PBS+NC and PBS+GL3), but were higher than 5% in all of the therapeutic groups, and maximally reached 16% in the CDDP+RECQL1-siRNA group. There were significant differences between CDDP+RECQL1-siRNA and all other groups (One-way factorial ANOVA and multiple comparison tests accompanied by Fisher’s significance, $P < 0.05$).
Discussion

Normal cells avoid mitosis during the repair process of damaged DNA at the G1 or G2 checkpoint of cell cycle. In contrast, defects in G1 or G2 checkpoint are common in cancer cells continuing cell proliferation in the presence of DNA damage, failing in G2 arrest and leading to mitotic catastrophe (5). Although defects in G2-checkpoint, including p53-mutation, lead to mitotic catastrophe, highly upregulated RecQ-helicases can salvage, the cancer cells from DNA-damage-induced cell death, including mitotic catastrophe (19, 25-27). Therefore, RECQL1 silencing can induce effective cell death in some cancer models (5, 16).

HNSCC, especially hypopharyngeal carcinoma, grows rapidly, suggesting that RecQ family proteins are expressed abundantly. The present study has supported this prediction. The high growth rate and the high-level expressions of both RECQL1 and WRN proteins in hypopharyngeal carcinomas in comparison with breast carcinomas may be consistent with the fact that hypopharyngeal carcinomas are more aggressive and have clinically worse outcomes than breast carcinomas. The expressions of both
RECQL1 and WRN correlated with γ-H2A.X intensities in hypopharyngeal rather than breast carcinomas probably in order to deal with the DNA damage caused by mitotic catastrophe.

A siRNA delivery system using liposome and/or collagen-based transfection method may be promising in HNSCC therapy, because local administration and/or the arterial infusion therapy with siRNA are possible to perform in HNSCC. In contrast, in other internal malignancies, specific and selective drug delivery is still difficult. In addition, siRNA administration at 1.0 mg/kg, once a week, is actually suitable for clinical application, and the administration protocol is better than the protocol reported previously stating that tridaily administration of RECQL1-siRNA (2 mg/kg) was used (16). To obtain the individualized medicine, it is necessary to target the molecules adapted for each carcinoma, case-by-case. As we did in the present study, specific siRNAs targeting individual molecules would be one of the best and simplest options.

The mechanisms of cell death, including apoptosis, autophagy, mitotic catastrophe, and necrosis still remain to be elucidated (28). Silencing of RECQL1 induces mitotic
catastrophe (5), and CDDP is involved in apoptosis, mitotic catastrophe, and necrosis (29-32). The data in the present study were mostly consistent with these reports, as described below.

The investigation of apoptosis with the TUNEL and cleaved caspase 3 assays revealed that CDDP monotherapy did not induce apoptosis in FaDu xenografts. Some reports showed that CDDP is a cytotoxic agent that induces DNA damage resulting in a very high percentage of necrotic cells and affects apoptosis (32, 33). Vakifahmetoglu et al. (30) showed that CDDP treatment led to apoptosis in the presence of a functional wild-type p53, but in the absence of functional p53 mitotic catastrophe occurred. In this study, FaDu hypopharyngeal carcinoma cells harbor a mutant p53, and CDDP induced nonapoptotic cell death, including mitotic catastrophe. \textit{RECQL1}-siRNA did not activate the caspase pathway by itself. However, when it was combined with CDDP, \textit{RECQL1}-siRNA strongly activated the caspase pathway and TUNEL-positive apoptosis. Notably, the present observation is consistent with the previous report suggesting the role of RECQL1 in CDDP resistance (34). On the other hand,
$WRN$-siRNA activated the caspase pathway and induced apoptosis by itself. When it was combined with CDDP, $WRN$-siRNA did not additionally enhance the caspase pathway and apoptosis.

The combined treatment by $WRN$-siRNA with CDDP had no additional effect on mitotic catastrophe, but, nevertheless, this therapy had the most prominent anticancer effect in this study. It is possible that the excess cell death, including necrosis—in addition to that caused by apoptosis and mitotic catastrophe—was caused by the combination of $WRN$-siRNA with CDDP. WRN-null cells from Werner syndrome patients show hypersensitivity to several types of DNA-damaging agents like cross-linking agents (CDDP, mitomycin C) (35), a DNA topoisomerase I-trapping agent (camptothecin) (20, 36) and hydroxyurea (37). Silencing of $WRN$ in cancer cells increases the chemotherapeutic activity of camptothecin (17) and CDDP (7, 38). The epigenetic inactivation of WRN leads to repress WRN proteins, and increases chromosomal instability and sensitivity to chemotherapeutic drugs in cultured cells; and enhanced the clinical sensitivity to camptothecin in human colorectal tumors (22).
WRN interacting with DNA-PKcs and/or KU70-80 may participate in DNA repair of double-strand breaks through the non-homologous end-joining pathway (39-42). WRN also participates in telomere maintenance (11, 18). Loss of normal WRN function causes telomere shortening, and substantial shortening of telomeres mediates cellular senescence and/or apoptosis to inhibit tumorigenesis (42-44). Thus, WRN-siRNA could exert its effect through inhibition of DNA repair and alternative lengthening of telomeres (ALT), which is non-telomerase mechanism for telomere maintenance (18, 45). Although further studies on these interacting pathways are needed for clarifying the mechanism of the combined anticancer therapy with WRN-siRNA and CDDP, WRN-siRNA could enhance the anti-cancer effect of CDDP leading to cell-growth suppression and cell death.

We investigated mitotic catastrophe in tumor cells in vivo. Mitotic catastrophe is defined as a type of cell death caused by aberrant mitosis (25, 28, 46). The MPM-2 monoclonal antibody binds to a phosphorylated amino acid-containing epitope present on more than 40 proteins of G2-M-phase eukaryotic cells (47). MPM-2 stainings
reflect cell populations in the G2-M-phase, and the aberrant increase is associated with mitotic catastrophe (48). CDDP monotherapy increased the MPM-2 positive rate, and the additional administration of *RECQL1*-siRNA enhanced the rate much more. However, additional administration of *WRN*-siRNA had no additional effect on the MPM-2 positive rate. The cell death caused by combining *WRN*-siRNA with CDDP could not be adequately explained through mitotic catastrophe.

The DNA damage caused by siRNA or CDDP was assessed by γ-H2A.X stain reflecting an early DNA double-strand breaks (49). Our data showed that the combined therapy with CDDP and *RECQL1*-siRNA enhanced the DNA damage maximally. When the cancer cells were treated with both *RECQL1*-siRNA and CDDP, they couldn’t repair the DNA damage, and fell into G2-M-phase arrest and apoptosis. Our findings were similar to hypersensitivity of *RECQL1*-deficient cells against genotoxic agents such as camptothecin and ionizing radiation (14, 50). On the other hand, the combined therapy with CDDP and *WRN*-siRNA reduced the number of cancer cells maximally, but the DNA-damage evaluation could not explain the effect adequately.
WRN-deficient cells are known to be hypersensitive to CDDP and fall into cell death (35), and our study could indicate the similar phenomena.

Taken together, this study provides preclinical proof-of-concept for two RecQ DNA helicases as novel therapeutic targets to treat aggressive head and neck cancers. The combination of RECQL1-siRNA with CDDP resulted in maximal apoptosis and mitotic catastrophe. On the other hand, WRN-siRNA down-regulated WRN expression leading to the failure of DNA repair and induced caspase-dependent apoptosis. This study has first demonstrated the difference in the mechanisms of anticancer effect between RECQL1 and WRN silencing. Further elucidation of the mechanism behind the apoptosis and mitotic catastrophe caused by the combination of WRN-siRNA with CDDP should be necessary. CDDP combined with WRN-siRNA might induce cell death such as necrosis in addition to the caspase-dependent apoptosis.
Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References


45. Hagelstrom RT, Blagoev KB, Niedernhofer LJ, Goodwin EH, Bailey SM. Hyper


**Figure Legends**

Figure 1.

Rapid cell growth and high expression of WRN and RECQL1 in hypopharyngeal carcinomas. A, the growth of hypopharyngeal and breast cancer cells was assessed. Hypopharyngeal carcinoma cell lines, FaDu and D-562, grew more rapidly than breast carcinoma cell lines, MCF7 and T47D, and TIG-108 human fibroblast, as shown by WST-8 assay. B, Western blot analyses of WRN, RECQL1, and α-tubulin in hypopharyngeal and breast cancer cells. The expression levels of WRN and RECQL1 were higher in hypopharyngeal carcinoma cells than the other cells. The similar amount of each cell protein was loaded in each lane of the SDS-PAGE. HeLa cells were used as a blotting control. C, immunohistochemistry of WRN and RECQL1 in representative cases of hypopharyngeal and breast cancers. Cancer cells of hypopharyngeal carcinoma were stained stronger for WRN and RECQL1 than stromal fibroblasts or breast cancer cells. Scale bar; 100μm. D, the rates of cancer cells stained positively for WRN and RECQL1 were compared between hypopharyngeal and breast
cancers. WRN-labeling indices (mean ± standard error) were 92.7 ± 0.7% and 59.8 ± 4.9% in hypopharyngeal and breast carcinomas, respectively. RECQL1-labeling indices were 87.7 ± 1.5% and 78.9 ± 3.1%, respectively. There was significant difference between hypopharyngeal and breast carcinoma in WRN-positive cell rates (*; P < 0.05; Mann-Whitney U test).

Figure 2.

In vitro growth assays in hypopharyngeal and breast cancer cells. GL3 was used as non-silencing siRNA. RECQL1 and WRN silencing were confirmed by Western blots and led to significant growth inhibition in FaDu and D-562 hypopharyngeal carcinoma cells but they could not effectively inhibit the growth of MCF7, T47D or TIG-108 cells. Mean absorbance values (OD450) ± standard errors are shown vertically in the WST-8 assays, and the day numbers after the siRNA treatment are indicated horizontally. The values are derived from quadruplicate experiments (*; P < 0.05; One-way factorial ANOVA and multiple comparison tests accompanied by Fisher’s
significance).

Figure 3.

Therapeutic effects of *RECQL1* - or *WRN*-siRNA, and the combined effect with CDDP and siRNA *in vivo*. A, *in vivo* knockdown effects of *RECQL1* - or *WRN*-siRNA. *RECQL1* - or *WRN*-siRNA reduced effectively each protein expression in FaDu xenografts. Immunohistochemical evaluations. Scale bar = 50 μm. B, *in vivo* therapeutic effects of *RECQL1* - or *WRN*-siRNA. *RECQL1* - and *WRN*-siRNA induced significantly effective tumor growth inhibition in FaDu xenografts (*; P < 0.05). C, *in vivo* combined effect with CDDP and siRNA for *RECQL1* or *WRN*. The combined therapy with CDDP and siRNAs (CDDP+*RECQL1* and CDDP+*WRN*) provided a significantly stronger reduction of tumor growth than CDDP monotherapy (#; P < 0.05). We defined the start of the administration as Day 0. The days on which the treatments were administered are indicated by arrows. Mean tumor volumes ± standard errors are shown. One-way factorial ANOVA and multiple comparison tests
accompanied by Fisher’s significance were used to evaluate results statistically.

Figure 4.

*In vivo* evaluation of apoptosis, mitotic catastrophe, and DNA-damage.

Immunohistochemical labeling indices (left halves) and representative findings (right halves) of each therapeutic group are shown. TUNEL (A), MPM-2 (B), and γ-H2A.X (C) were applied in histological evaluations of apoptosis, mitotic catastrophe, and DNA-damage of cancer cells, respectively. Statistically significant differences between non-therapeutic groups (PBS+NC and PBS+GL3) and the other groups are indicated (*; $P < 0.05$). Significant differences between CDDP monotherapeutic groups (CDDP+NC and CDDP+GL3) and the combined therapeutic groups (CDDP+RECQL1 and CDDP+WRN) are also indicated (#; $P < 0.05$). One-way factorial ANOVA and multiple comparison tests accompanied by Fisher’s significance were used to evaluate them statistically. Scale bar = 50 μm.
RECQL1 and WRN proteins are potential therapeutic targets in head and neck squamous cell carcinoma

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