Increased Chemotherapeutic Activity of Camptothecin in Cancer Cells by siRNA-Induced Silencing of WRN Helicase

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Werner syndrome helicase (WRN) participates in a wide range of DNA activities, including replication, double-strand DNA break repair, telomere and retrovirus long terminal repeat maintenance. Mutations of the *WRN* gene cause Werner syndrome (WS), an autosomal recessive premature ageing disorder associated with various symptoms related to ageing. In this study, we investigated the siRNA that specifically down-regulates WRN expression. WRN silencing increased markedly the chemotherapeutic activity of camptothecin (CPT) on cancer cells in terms of the extent of efficacy and lowering effective drug dosage, accompanied by suppressing recovery from DNA damage caused by CPT. Here, we propose a potential combination therapy of *WRN*-siRNA and CPT, looking forward to minimizing the inevitable adverse effects associated with cancer chemotherapy.

Key words Werner helicase; camptothecin; siRNA; anti-cancer drug

Werner syndrome (WS) is an autosomal recessive disorder causing symptoms of premature ageing.[3] In 1996, the Werner syndrome gene (*WRN*) responsible for WS was identified.[4] The gene product, WRN, acts as a DNA helicase (WRN helicase) with exonuclease activity.[5] WRN greatly participates in DNA metabolism by facilitating cellular processes, including DNA replication, recombination, repair, and transcription in cooperation with other cellular proteins. WRN may participate in the retrovirus replication.[6] WS patients lack functional WRN mainly because of mutations that cause premature termination in WRN synthesis, produce incomplete WRN molecules lacking C-terminal nuclear localization signals and so result in impaired nuclear transportation of WRN.[7]

The genotoxic clastogen 4-nitroquinoline-1-oxide (4NQO) induces a more distinct increase in both break and interchange aberrations in WS patient cells than in control cells from normal individuals or from patients with other diseases.[8] WS patient lymphoblastoid cell lines (LCLs) transformed by Epstein-Barr virus are hypersensitive to 4NQO than LCLs from normal individuals without mutation.[9] Previously we studied the effect of camptothecin (CPT), a DNA topoisomerase I-trapping agent, on several LCLs from non-WS individuals and WS patients, and showed that CPT had a stronger cytotoxic effect on LCLs from WS patients than LCLs from non-WS individuals.[10] Poot *et al.* confirmed and extended our results by showing that CPT more strongly impairs S-phase transit in WS LCLs than in normal LCLs.[11] Lebel and Leder made homozygous WS embryonic stem cells (ES) of mice that lacked normal *WRN* genes at both alleles.[12] Such cells were more markedly sensitive to CPT than were wild-type ES cells.

From these results, we investigated if silencing *WRN* gene expression by siRNA targeting the *WRN* gene (*WRN*-siRNA) increases the sensitivity of cells against CPT. We show here that specific down-regulation of WRN expression increases the cytotoxic effects of CPT in cancer cells, and we discuss the potential of using these findings for cancer therapy.

MATERIALS AND METHODS

Cell Culture and Transfection HeLa cells were cultured in Dulbecco's modified Eagle's medium (Sigma, St. Louis, MO, U.S.A.) supplemented with 10% fetal calf serum and 25 μg/ml gentamicin (Sigma, St. Louis, MO, U.S.A.). Cells in 100 mm dishes were cultured at 37 °C in a humidified atmosphere containing 5% CO_2.

siRNA We preliminarily examined *WRN*-gene-silencing activity with several siRNA sequences by using down-regulations of *WRN* mRNA expression as a marker. The design of siRNAs was done according to the method of Elbashir *et al.*[13] The siRNAs used for silencing the target gene expression consisted of 21 bp duplexes complementary to a unique sequence of the target gene. All these siRNAs had an extra dTdT overhang at their 5′ ends. Among them, *WRN*-siRNA with the following sequence was the most effective in silencing WRN expression that suppressed the expression of *WRN* mRNA by more than 80% in HeLa cells: GUUCUUGUCACGUCCUCUG targeted against 3373—3391 bases of NM000553. Our intensive homology search by using Smith and Waterman method showed that no other human sequence was identical to this sequence except for the *WRN* gene. We used this *WRN*-siRNA in this study. As a negative control, a non-silencing siRNA duplex (*NS*-siRNA) having sequence 5′-UUCUCCGACGUGUCACGUdTdT-3′ was used. Both of these siRNAs were synthesized by using Qiagen-Xeragon (Germantown, MD, U.S.A.). Transfection of cells with these siRNAs (10 nM) was done by using Oligofectamine (Invitrogen, CA, U.S.A.).

Immunoblotting Cells were washed with ice-cold PBS, were pelleted and then were lysed in a sodium dodecyl sulfate (SDS) buffer containing 1% SDS, 2% beta-mercaptoethanol, 20% glycerol, 30 mM Tris–HCl (pH 6.8), and 0.2 M dithiothreitol. The cell lysate was boiled for 5 min and was electrophoresed on 7.5% SDS-polyacrylamide gels. Proteins fractionated on the gels were electrophoretically transferred to polyvinylidene difluoride membranes (Immobilon, Millipore, MA, U.S.A.) and were blocked overnight with 5% skimmed milk in PBS. Then the membranes were incubated

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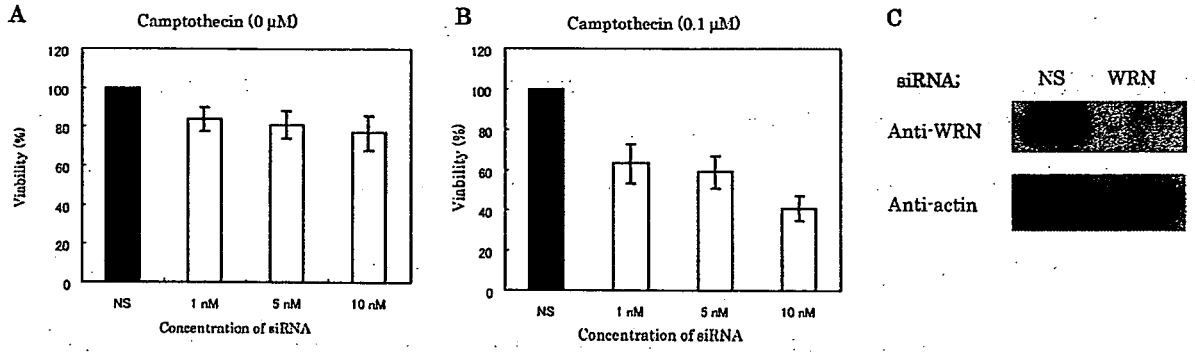

Fig. 1. Dose Effect of *WRN*-siRNAs to Increase CPT-Induced Cytotoxicity in HeLa Cells

The percentage of viability is expressed by viability with *WRN*-siRNA/viability with *NS*-siRNA×100. (A) Percentage of viability determined at 72 h after transfection with *WRN*-siRNAs and *NS*-siRNAs at concentrations 1, 5 and 10 nM, without CPT. (B) Percentage of viability determined at 72 h after transfection with *WRN*-siRNA and *NS*-siRNA at concentrations 1, 5 and 10 nM, in the presence of 0.1 μM CPT added 24 h after transfection. (C) Effect of *WRN*-siRNA (10 nM) on WRN expression levels in HeLa cells assessed by immunoblot analysis. Cell lysates (10 μg) were applied to each lane. Vertical bars indicate standard errors.

with either anti-WRN (4H12)[14] or anti-β-actin monoclonal antibodies (ICN Biomedicals, Aurora, Ohio, U.S.A.) for 60 min at room temperature, were washed with 0.05% Tween-20 in PBS, were incubated with anti-mouse IgG conjugated with horse radish peroxidase (DakoCytomation, Carpinteria, CA, U.S.A.) and then were washed and developed using an enhanced chemiluminescence reagent (ECL) plus (Amersham Biosciences, U.K.).

Confocal Microscopy HeLa cells cultured at 37 °C on microscope cover slips were transfected with 10 nM of *NS*- or *WRN*-siRNA, and were cultured further for 30 h. Then the culture medium was replaced with fresh medium containing 10 μM CPT (Sigma, OR, U.S.A.) and the cells were cultured for 6 h. Then, some CPT-treated cells were cultured in the absence of CPT for an additional 3 h to recover from the CPT effect, were fixed with a 3.7% formaldehyde PBS solution at room temperature for 10 min, and then were permeabilized with a 0.1% Triton X-100 PBS solution for 5 min. To detect WRN helicase and replication protein A (RPA), the cells were blocked by incubating in a 3% skimmed milk PBS solution for 60 min and were incubated overnight with primary antibodies (anti-RPA34; Oncogene, Boston, MA, U.S.A.) at 4 °C. The slides were washed with 0.05% Tween-20 in PBS three times and were incubated with Alexa Fluor 488-conjugated antibody mouse IgG (Molecular Probes, Eugene, OR, U.S.A.) as a secondary antibody for 60 min at room temperature. Fluorescent images were visualized by using a confocal laser-scanning microscope (Fluoview II; Olympus, Tokyo, Japan).

Cytotoxicity Test To determine the sensitivity to CPT, HeLa cells cultured in 48-well plates were transfected with various doses of siRNA using Oligofectamine. After 24 h, the cells were incubated in Dulbecco's modified Eagle's medium containing various concentrations of CPT. After 48 h (*i.e.*, after 72 h starting from siRNA transfection), the cell viability was measured by using a WST-8 assay (Nacalai Tesque, Kyoto), a modified MTT assay.[15]

RESULTS AND DISCUSSION

siRNA-Mediated Suppression of WRN Expression Immunoblot analysis showed that treatment of HeLa cells with *WRN*-siRNA for 30 h almost completely suppressed WRN expression, but *NS*-siRNA added as a negative control

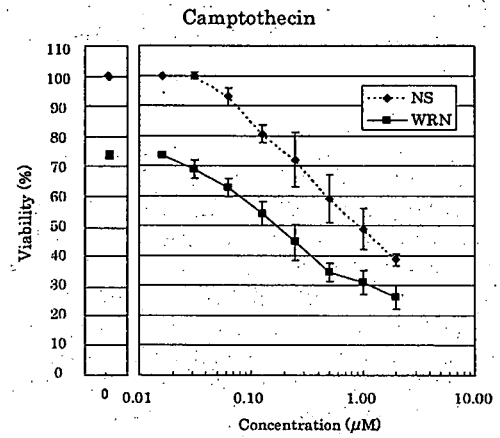

Fig. 2. Dose Effect of CPT to Induce Cytotoxicity in HeLa Cells in the Presence of *WRN*-siRNA

HeLa cells were transfected with *WRN*- or *NS*-siRNA at concentration 10 nM, and were treated with a serial dilutions of CPT added 24 h after transfection. After 48 h of culture, cell viability was monitored by WST-8 assay. The percentage of cell viability was expressed by cell viability in the presence of 10 nM *NS*-siRNA with various concentrations of CPT/cell viability in the presence of 10 nM *NS*-siRNA without CPT×100 (NS), and by cell viability in the presence of 10 nM *WRN*-siRNA with various concentrations of CPT/cell viability in the presence of 10 nM *NS*-siRNA without CPT×100 (WRN). Each point represents a mean of 9 samples and vertical bars indicate standard deviation.

did not affect the initial level of WRN (Fig. 1C). When the effect of 1, 5, 10 nM *WRN*-siRNAs on the growth of HeLa cells was examined by culturing the transfected cells for 96 h, *WRN*-siRNA alone weakly suppressed cell viability at these very low concentrations: cell viability was down-regulated by about 20% compared with cells treated with *NS*-siRNA (Fig. 1A). This reduction of the viable cell population might be mostly due to a delay in cell cycling as measured by flow cytometry analysis (unpublished observation). However, when *WRN*-siRNA-treated HeLa cells were incubated with CPT, cell viability was markedly reduced dependent on the dose of *WRN*-siRNA, and cells treated with *NS*-siRNA were totally unaffected (Fig. 1B).

We compared the cytotoxic dose of CPT with HeLa cells treated with 10 nM *NS*-siRNA and *WRN*-siRNA by changing the concentrations of CPT (Fig. 2). The IC_{50} of CPT was markedly decreased by 5.3-fold from 0.95 to 0.18 μM for *WRN*-siRNA-treated HeLa cells compared with the IC_{50} of CPT with *NS*-siRNA-treated HeLa cells. These results indicate that *WRN*-silenced HeLa cells were more susceptible to

CPT.

Failure of WRN-Silenced Cells to Recover from DNA Damage Response WRN in the nuclei is mainly in the nucleolus,[14,16] but it relocates to the nucleoplasm and colocalizes with RPA34 protein to form nuclear foci at the DNA double-strand break points that are generated by genotoxic reagents such as CPT.[17] The RPA34 protein does not bind to duplex DNA, but it binds to the single-strand regions of DNA unwound by DNA helicase for repair. Thus, nuclear foci consisting of WRN or RPA34 or both are assumed to be excellent markers representing damaged DNA loci generated by CPT treatment. Therefore, experiments were done to investigate how down-regulation of WRN affects nuclear RPA34 foci formation and DNA repair during the recovery process of DNA damage (double-strand breaks) caused by CPT. In the absence of CPT, most of WRN was found in the nucleolus while RPA34 was dispersed in the nucleoplasm as reported before[17] (Fig. 3A). Down-regulation of WRN expression by siRNA treatment, however, made some fraction of RPA34 form nuclear foci in the nucleoplasm (Fig. 3B). This nuclear relocation of RPA may be interpreted that RPA34 assemble in the legions of endogenous DNA damages which are formed during DNA replication and remain unrepaired due to the absence of WRN in the slightly delayed cell cycle progression as shown in Fig. 1A.

When HeLa cells were treated with CPT for 6 h, many nu-

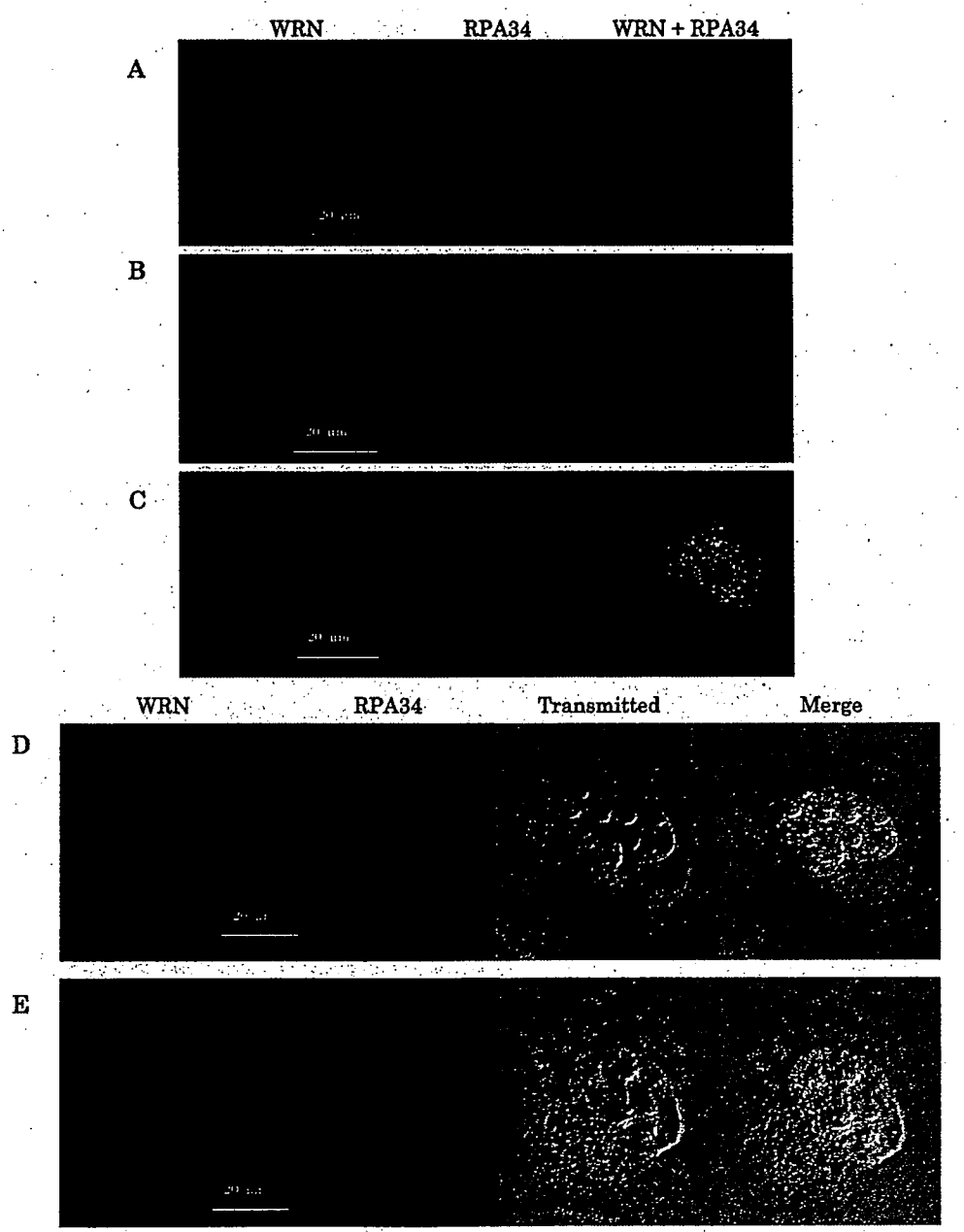

Fig. 3. Induction and Recovery of DNA Damage by CPT in HeLa Cells Assessed by Immunofluorescence

(A) Most of WRN was found in the nucleolus while RPA34 was dispersed in the nucleoplasm in the absence of CPT. (B) Down-regulation of WRN made some fraction of RPA34 form nuclear foci in the nucleoplasm. (C) DNA damage was induced by adding 10 nM of CPT during 6 h in HeLa cells. The nuclear foci at DNA-damaged sites stained with anti-WRN and anti-RPA34 antibodies were obvious and were co-localized. (D, E) At 30 h after transfection with *WRN*-siRNA or *NS*-siRNA, cells were cultured in the presence of 10 nM of CPT for 6 h. Then, the cells were further cultured for 3 h in the absence of CPT to recover DNA damage.

clear foci stained by both anti-WRN and RPA34 antibodies appeared in the nucleoplasm, as we previously reported.[17] The same staining profiles were also obtained with HeLa cells transfected with *NS*-siRNA used as negative control (Fig. 3C). Further culture of these cells in the CPT-free medium for 6 h causes most WRN proteins in the nuclear foci to return to the nucleolus leaving fewer numbers and smaller sizes of RPA34 foci on the nucleoplasmic DNA, suggesting that DNA repair was accomplished and that most nuclear foci consisting of WRN and RPA34 disappeared (Fig. 3D).

Under the same conditions, except the *NS*-siRNA was replaced by *WRN*-siRNA, CPT treated HeLa cells maintained many and significantly large RPA34 foci in the nucleoplasm even after removal of CPT at more than 6 h, suggesting that DNA repair was not completed and that the single-strand region was left unrepaired in the RPA34-bound form (Fig. 3E). It should be noted that most nuclear foci were not stained with WRN antibody as expected from the WRN siRNA treatment, nor was WRN helicase found in the nucleolus because of the silencing of WRN helicase. The data clearly showed that silencing WRN prevented repair of CPT-mediated DNA damages in the CPT-treated HeLa cells, and permitted the accumulation of DNA damage that explained the increased cytotoxicity of CPT in the combined treatment of CPT and *WRN*-siRNA. Similar effects were observed with cancer cell lines other than HeLa cells (data not shown). The data also explain why WS cells previously showed increased sensitivity to genotoxic reagents, including CPT.

The enzymatic activity of WRN to unwind long duplex DNA substrates is stimulated by RPA,[18] and WRN directly interacts with RPA as verified by co-immunoprecipitation of purified proteins.[19] WRN forms nuclear foci in response to genotoxins, including CPT, etoposide, 4NQO and bleomycin.[17] These WRN foci overlap with RPA foci almost entirely and with the foci of Rad51 partially, suggesting cooperative functions of these proteins in response to DNA damage.

The results of this study obtained by using *WRN*-siRNA at cellular levels were consistent with the following clinical results to treat colorectal cancer patients by using the CPT analogue Irinotecan, commonly used in the clinical setting to treat this tumor type.[20] The WRN function was eliminated in some of the human cancer cells by transcriptional silencing associated with CpG island-promoter hypermethylation. Importantly, *WRN* gene hypermethylation in colorectal tumors is a predictor of good clinical response to Irinotecan, due to the reduced WRN expression and increased CPT cytotoxicity. These findings highlighted the importance of *WRN* epigenetic inactivation in human cancer, leading to hypersensitivity to chemotherapeutic drugs.

WRN expression is augmented in rapidly growing cells, such as Epstein-Barr virus-infected and immortalized B limphoblastoid cells, much higher than in virus-infected and mortal B limphoblastoid cells.[21,22] Also, a wide variety of cancer cells show very high expressions of WRN in the same way as WRN in human fibroblast cells transfected with SV40 T antigen.[21] All these findings imply that many of cancer cells are perhaps "addicted to" (that is, physiologically dependent on) the specifically overexpressed WRN participating in DNA repair to maintain their rapid proliferation, analogously to the theory of cancer cell addiction to certain oncogenes proposed by Weinstein[23]; these kind of genes and gene products have been pursued as an ideal chemotherapeutic target for anti-cancer agents with few adverse effects.[24] In addition, administration of WRN-siRNA to cancer cells by an appropriate drug delivery system, such as positively charged liposome that are incorporated more efficiently in rapidly growing cancer cells than non-cancerous resting cells, should lower the cytotoxic dose of CPT preferentially in cancer cells, benefiting patients by fewer adverse effects in their normal tissues and cells.

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Induction of mitotic cell death in cancer cells by small interference RNA suppressing the expression of RecQL1 helicase

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RecQL1 DNA helicase of the human RecQ helicase family participates in DNA repair and recombination pathways during cell-cycle replication. When we examined the effect of RecQL1 suppression on cell growth, we found that *RecQL1* silencing by small interference RNA efficiently prevented proliferation of a wide range of cancer cells by inducing mitotic catastrophe and mitotic cell death. In contrast, such mitotic cell death was not seen in the growing normal fibroblasts used as controls, even if RecQL1 expression was fully downregulated. Our results support the hypothesis that endogenous DNA damage that occurs during DNA replication and remains unrepaired in cancer cells due to *RecQL1* silencing induces cancer cell-specific mitotic catastrophe through a less-strict checkpoint in cancer cells than in normal cells. We speculate that normal cells are exempt from such mitotic cell death, despite slow growth, because cell-cycle progression is controlled strictly by a strong checkpoint system that detects DNA damage and arrests progression of the cell cycle until DNA damage is repaired completely. These results suggest that RecQL1 helicase is an excellent molecular target for cancer chemotherapy. (*Cancer Sci* 2008; 99: 71–80)

The RecQ DNA helicase family is conserved in all organisms and participates in the maintenance of genomic integrity of cells. Microorganisms such as *Escherichia coli*, *Saccharomyces cerevisiae*, and *Schizosaccharomyces pombe* contain only one RecQ helicase, but higher eukaryotes have more than one RecQ helicase. In human cells, the RecQ helicase family comprises RecQL1 (also known as RecQL or RecQ1), BLM, WRN, RTS (also known as RecQL4), and RecQ5.[1–6] We showed that these RecQ helicases are expressed in the cell nucleus, are upregulated with DNA replication, and participate in DNA repair during cell proliferation.[7] Bloom, Werner, and Rothmund–Thomson syndromes are recessive genetic disorders of humans caused by the absence of the corresponding helicase, resulting in genomic instability in patients' cells and causing susceptibility to cancer and accelerated phenotypes of aging.[8–10] Patients with Werner and Rothmund–Thomson syndromes show premature aging phenotypes and have an increased risk of sarcomas, whereas patients with Bloom syndrome have an increased risk of many kinds of tumors.[11,12] Diseases caused by mutation of RecQL1 and RecQ5 are unknown. WRN functions in non-homologous end joining, homologous recombination repair, base-excision repair, and telomere maintenance, whereas BLM functions in homologous recombination repair, mismatch repair, and telomere maintenance.[9,10] Although RecQL1 helicase was the first human RecQ helicase family member to be characterized,[1] its biological functions have not been well studied because no human disease has been associated with mutations in RecQL1. RecQL1 protein is highly upregulated in rapidly growing cells, including various cancer cells, and it is expressed in human peripheral B cells in association with blast formation from the resting state induced by treatment with phorbol 12-myristate 13-acetate.[7] Human RecQL1 unwinds specific DNA *in vitro* in an ATP-dependent manner,[13,14] and can increase base matching independently of ATP.[15] RecQL1 is assumed to participate in the mismatch-repair pathway *in vivo* because it binds to the incision activity of human EXO1 and the mismatch repair recognition complex MSH2/6.[16] Also, RecQL1 was reported to function as a Holliday junction-resolving enzyme during cell proliferation. Consequently, downregulation of RecQL1 expression in HeLa cells by RNA interference (RNAi) increases sister chromatid exchange (SCE) resulting from unprocessed Holliday junction structures.[17] Although RecQL1-deficient mice, produced by gene targeting of RecQL1[18] (Sakamoto *et al.*, unpublished data, 1999), show no apparent phenotypic differences when compared with wild-type mice, embryonic fibroblasts from RecQL1-deficient mice are sensitive to ionizing radiation.[18] Collectively, these findings showed that RecQL1 suppresses chromosomal instability by participating in DNA repair associated with cell proliferation, but its function seems to be non-essential and complemented by other cellular repair systems.

Cells in the process of replication have various kinds of endogenous DNA damage in the chromosomes resulting from mismatch or replication errors caused by stalled replication folks and other anomalous DNA structures. In proliferating cells, however, cellular DNA damage checkpoints coordinate an arrest at G_1 and G_2 phase of the cell-cycle to allow for the DNA repair process to eventually avoid mitotic catastrophe or mitosis of cells with damaged DNA. Mitotic catastrophe or mitotic cell death is a cellular event in which replicating cells having DNA damage are unable to maintain G_2 arrest and die as they enter mitosis with terminal dUTP nick end labeling (TUNEL)-positive apoptotic phenotypes.[19–22] In cancer chemotherapy, mitotic catastrophe is an important mechanism required to induce cell death by anticancer agents that damage DNA. This process of mitotic catastrophe is facilitated by defects in the G_1 or G_2 checkpoints of the cell cycle that are common in cancer cells. The G_2 checkpoint is especially important, and defective G_2 arrest causes an inability of the cells to repair DNA damage, permitting cells to enter mitosis with DNA damage.[20–23] Cells that enter mitosis with DNA damage are arrested in M phase for long periods without segregation of chromosomes, and they undergo mitotic death.[24]

In the present study, we investigated the effect of *RecQL1* silencing on the proliferation of cancer cells using RNAi technologies.[25] We introduced RecQL1 small interference RNA (siRNA) into several different human cancer and normal cell lines to downregulate the expression of RecQL1 and studied its

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inhibitory effect on cell proliferation. The results show that RecQL1 siRNA at low concentrations (10 nM or less) kills a wide range of cancer cells by inducing mitotic catastrophe, but it does not kill normal cells treated similarly. Detailed analysis using time-lapse fluorescence video microscopy showed that mitotic catastrophe is induced specifically in cancer cells, occasionally accompanying unequal cell division resulting in non-proliferating and seemingly senesced cells. These results clearly indicate that RecQL1 DNA helicase, which participates in the DNA repair of cancer cells, is an excellent molecular target, and RecQL1 siRNA itself is a promising anticancer drug candidate having limited adverse effects on normal cells.

Materials and Methods

Cells and cell culture conditions. The cancer cell lines HeLa (endocervical carcinoma), A549 (lung carcinoma), PC-3 (prostate carcinoma), KP4, MIA PaCa-2 (pancreatic carcinoma), Hep3B (liver carcinoma), HCT116 (colon carcinoma), PA-1 (ovarian carcinoma), MKN45 (stomach cancer), MCF7 (breast cancer), and U2OS (osteosarcoma), the normal cell lines W138 and HUVEC, and ARPE19 (a normal spontaneously arising retinal pigmented epithelium cell line) were obtained from American Type Culture Collection (ATCC; Manassas, VA, USA). T24 (bladder carcinoma) and TIG3 (normal lung diploid fibroblasts from human fetus) were obtained from Riken Cell Bank (Tsukuba, Japan). These cell lines were incubated at 37°C in a humidified chamber supplemented with 5% CO_2. The culture medium and method were as described in the supplier instructions. The protein extracts from 5637 and SNK86 (bladder carcinoma), MDA-MB-435 (breast cancer), SW620 and Colo205 (colon carcinoma), siHA (endocervical carcinoma), U251 and SF295 (glioma), Huh7 and HepG2 (liver carcinoma), Caki-1, OS-RC-2 and RCC10RGB (kidney cancer), K562, U937 MOLT4, Jarkat and Namalwa (leukemia), NCI-H23 and H522 (lung carcinoma), C32, G361 and A375 (melanoma), HDF (normal fibroblast), OVCAR3 and OVCAR5 (ovarian cancer), MKN74 and HGC-27 (stomach cancer), LNCaP (prostate carcinoma), Saos2, HTB82 and HT1080 (sarcoma), were used for immunoblotting analysis.

Small interference RNA and RNAi. RecQL1 gene silencing activity was examined with several siRNA sequences by using downregulation of RecQL1 mRNA expression as a marker. The siRNA were designed according to the method of Elbashir *et al.*[25] They consisted of 21-bp duplexes complementary to a unique sequence of the target gene and were used to silence target gene expression. The siRNA (21 bp) targeting RecQL1 mRNA were synthesized chemically (NIPPON EGT, Toyama, Japan) and all siRNA sequences (21-mer) had overhanging 3′-dTdT at the 3′ terminus: 5′-GUUCAGACCACUUCAGCUUdTdT-3′ (position 273–291). A sequence homology search using the Smith and Waterman method showed that no other human siRNA sequence was identical to this sequence except for siRNA targeting the *RecQL1* gene. Sequence-specific gene silencing was confirmed using cDNA microarray analysis with the Affimetrix GeneChip system (Human Genome U133 Plus 2.0 Array). A total of 47 000 open reading frames were examined to determine whether the expression of other genes was silenced in HeLa and A549 cells at 48 h after treatment with RecQL1 siRNA.

Non-silencing (NS) siRNA contains a duplex of strands: 5′-CUUACGCUGAGUACUUCGAdTdT-3′ and 5′-UCGAAGUACUCAGCGUAAGdTdT-3′. The sequence of NS siRNA represents part of the firefly luciferase gene (*GL3*; Promega) and was used as a negative control in the transfection experiments. For transfection, at 24 h after plating, the cells were incubated with 2.5–160 pmol siRNA duplex using Oligofectamine or Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. In view of the different efficiencies of transfection among various cell lines, prior investigations were made individually to find the optimal conditions by changing the transfection reagent or slightly modifying the concentrations or ratio of siRNA and transfection reagents.

Reverse transcription–polymerase chain reaction. At 30 h after transfection, total RNA was extracted from cultured cells using an RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. Reverse transcription–polymerase chain reaction (RT-PCR) analyses were done using the ABI Prism 7000 Sequence Detection System and Taqman probes and primers (ABI, Foster, CA, USA). The *β-actin* gene was used as the internal standard.

Cell proliferation assays. Cell proliferation was measured by colorimetric assays of cell viability based on cleavage of the tetrazolium salt 4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate (WST)-8 (Nacalai Tesque, Kyoto, Japan) by mitochondrial dehydrogenase at 72 or 96 h after transfection. The absorbance of formazan dye formed was measured at 450 nm at 3 h after adding the reagent.

Flow cytometric analysis. Trypsin-treated cells were washed with phosphate-buffered saline (PBS) and were fixed in ice-cold methanol for 2 h. The cells were treated with pancreatic RNaseA (Nippon Gene, Toyama, Japan), stained with propidium iodide (Sigma, St Louis, MO, USA) for 30 min, and then analyzed by flow cytometry. Fluorescence was measured using EPICS XL (Beckman Coulter, Tokyo, Japan). For each sample, 7000 events were analyzed.

Immunoblotting. The cells were washed with ice-cold PBS, pelleted, and then lysed in a sodium dodecylsulfate (SDS) buffer containing 1% SDS, 2% β-mercaptoethanol, 20% glycerol, 30 mM Tris-HCl (pH 6.8), and 0.2 M dithiothreitol. The cell lysate was boiled for 5 min and electrophoresed on 7.5% SDS-polyacrylamide gels. Proteins fractionated on the gels were transferred electrophoretically to polyvinylidene difluoride membranes (Immobilon; Millipore, MA, USA) and were blocked overnight with 5% skim milk in PBS. The membranes were then incubated with either anti-RecQL1 polyclonal antibody,[26] or anti-β-actin monoclonal antibody (ICN Biomedicals, Aurora, OH, USA) for 1 h at room temperature, washed with 0.05% Tween-20 in PBS, incubated with antimouse IgG conjugated with horseradish peroxidase (DakoCytomation, Carpinteria, CA, USA) and then washed and developed using an enhanced chemiluminescence reagent (ECL plus; Amersham Biosciences, UK).

Immunocytostaining. To examine DNA damage-repair loci, HeLa cells were cultured at 7×10^3 cells in eight-well chamber slides and transfected with 40 nM siRNA. At 60 h after transfection the cells were fixed with 3.7% formaldehyde in PBS. Antibodies against phosphohistone H2AX (phosphorylation of histone H2AX on Ser^{139}) (Upstate, Lake Placid, NY, USA), phospho-ATM (phosphorylation of ATM on Ser^{1981}) (Rockland, Gilbertsville, PA, USA), and 53BP1 (p53-binding protein 1) (Oncogene, San Diego, CA, USA) were used. Alexa Fluor488-conjugated antirabbit, antigoat, and antimouse immunoglobulins (Molecular Probes Invitrogen, Carlsbad, CA, USA) were used as secondary antibodies. To examine the activation of spindle checkpoint and mitotic arrest in the RecQL1 siRNA-treated HeLa cells, cells in M phase were stained with antibodies specific for human tubulin (Oxford Biotechnology, UK) and Mad2 proteins. The anti-Mad2 antibody was kindly provided by Dr H. Saya of the Keio University Medical School. Fluorescence images were visualized using a confocal laser scanning microscope (Fluoview; Olympus, Tokyo, Japan).

Sister chromatid exchange assay. The SCE assay was carried out as described by Morimoto and Wolff with slight modifications.[27] Briefly, at 8 h after siRNA transfection, A549 cells grown to 40% confluency were grown for an additional 48 h in the presence of 20 μM BrdU. The cells were incubated with 0.2 μM colcemid (Nacalai Tesque, Kyoto, Japan) and

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harvested with trypsin. The cells were made to swell for 20 min in 75 mM KCl and were fixed in methanol : acetic acid (3:1). They were then dropped onto moist –20°C prechilled glass slides and dried on a 90°C hot plate. The cells were allowed to age overnight and were then stained with 50 μg/mL Hoechst 33528 in phosphate buffer (pH 6.8) for 20 min at room temperature. The cells in phosphate buffer (pH 8.0) were covered with glass coverslips and bleached under an ultraviolet lamp at a distance of 2–3 cm for 20 min on a 60°C prewarmed plate. They were then stained with 5% Giemsa stain for 5 min, air-dried, mounted in Mountquick (Daido Sangyo, Saitama, Japan) under glass coverslips, and analyzed using AxioVision 4.1. (Carl Zeiss, Germany). Pictures were taken using an AxioCam (Carl Zeiss).

Results

Inhibition of HeLa cell proliferation by downregulation of *RecQL1* expression. RNA interference using RecQL1 siRNA for *RecQL1* silencing decreased the amount of polyA-containing RecQL1 mRNA in HeLa and TIG3 cells by more than 85% within 24 h. Immunoblot analyses clearly showed that RecQL1 helicase protein disappeared from the cells at 48 h of culture after RecQL1 siRNA transfection (Fig. 1a). At 96 h after transfection, the number of viable HeLa cells transfected with RecQL1 siRNA decreased markedly to less than 10% of those treated with NS siRNA (Fig. 1b; HeLa). In contrast, the viability of TIG3 cells was unaffected, even though *RecQL1* silencing was similar to HeLa cells (Fig. 1b; TIG3). These results were striking because the proliferation of HeLa cells representing cancer cell lines was preferentially inhibited by *RecQL1* silencing, but both NS siRNA-treated HeLa and TIG3 cells proliferated equally well. Because the inhibitory effect of *RecQL1* silencing on the proliferation of HeLa cells was distinct from TIG3 cells, we investigated *RecQL1* silencing to assess the potential use of RecQL1 siRNA as an anticancer drug free of adverse effects. To confirm whether the RecQL1 siRNA used in this experiment downregulated only the expression of *RecQL1*, cDNA microarray analysis was done with HeLa and A549 cell mRNA. The results indicate that *RecQL1* was the only gene silenced to ~1/20 of the original expression level among the 47 000 genes investigated. Similar levels of sequence-specific RecQL1 mRNA downregulation were also observed with other siRNA molecules designed from other parts of the RecQL1 mRNA (data not shown).

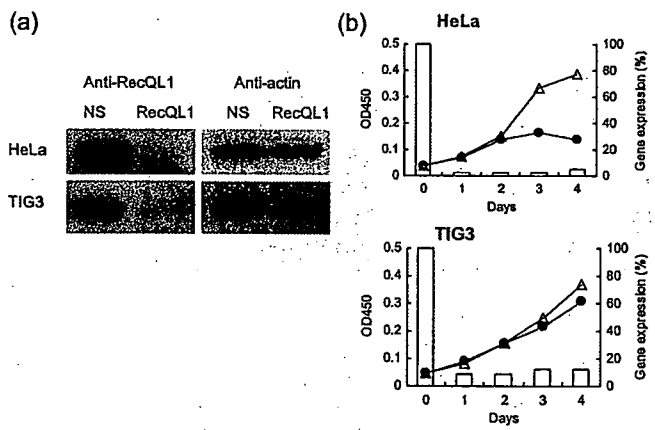

Fig. 1. Effects of RecQL1 small interference RNA (siRNA) on the expression of human RecQL1 helicase and cell viability. (a) After transfection with either RecQL1 siRNA or non-silencing (NS) siRNA, HeLa and TIG3 cells were cultured in siRNA-free medium. After 48 h, the amount of RecQL1 protein in the lysates of *RecQL1*-silenced HeLa and TIG3 cells was measured using immunoblotting with the anti-RecQL1 antibody described by Tada *et al.*[26] RecQL1 helicase protein was greatly reduced in the RecQL1 siRNA-treated cells, but it was unaffected in NS siRNA-treated cells (two left panels). The amounts of actin protein in HeLa and TIG3 cells were unaffected before and after siRNA treatment (data not shown) and between NS siRNA and RecQL1 siRNA treatments (two right panels). (b) Growth of HeLa and TIG3 cells for 96 h after transfection with RecQL1 siRNA or NS siRNA. Solid circles show cells treated with 40 nM RecQL1 siRNA. Open triangles show cells treated with 40 nM NS siRNA. The viable cells were counted by MTT assays measuring the optical density of the formazan dye produced by active mitochondrial dehydrogenase at 450 nm. The histogram shows the proportion (%) of RecQL1 mRNA in cells treated with NS siRNA and RecQL1 siRNA. The RecQL1 mRNA levels were measured using semiquantitative reverse transcription–polymerase chain reaction. The levels were downregulated to less than 10 and 15% of the original levels in HeLa and TIG3 cells, respectively, by *RecQL1* silencing. The RecQL1 mRNA level of NS siRNA-transfected HeLa cells was calculated as 100%.

Induction of mitotic death in HeLa but not normal cells by *RecQL1* silencing. To confirm the killing effect caused by *RecQL1* silencing in HeLa cells and to understand the different effects on the proliferation of HeLa and TIG3 cells, we investigated the cell cycle of these cell lines after the cells were treated with RecQL1 siRNA (40 nM). As another control representing normal cells, ARPE-19 cells were used to analyze the effect of *RecQL1* silencing (Fig. 2). Comparative flow cytometric analysis at 48, 72, and 96 h after transfection showed that: (i) *RecQL1* silencing affected the progression of HeLa cells most severely, producing $subG_1$ cells that represented dead cells at 48 h after RecQL1 siRNA treatment; (ii) the $subG_1$ HeLa cells increased as the culture continued, resulting in simultaneous reduction of cells arrested in G_2 phase; (iii) both TIG3 and ARPE-19 cells showed no apparent $subG_1$ cells throughout the culture after RecQL1 siRNA treatment, but TIG3 cells showed slightly increased numbers of cells in G_1 phase, suggesting that the cell cycle of these cells entered S phase and quickly stopped, whereas ARPE-19 cells were slowed by arrest at both the G_1 and G_2 phases; and (iv) NS siRNA treatment under the same conditions did not affect the cell-cycle progression of HeLa, TIG3, or ARPE-19 cells.

These data suggest that *RecQL1* silencing induced reproductive death in HeLa cells, often referred to as mitotic cell death or mitotic catastrophe, which occurs characteristically in checkpoint system-mutated cancer cells when they have DNA damage during replication. This conclusion was consistent with findings made by analysis with time-lapse fluorescence video microscopy that recorded the replication of cells in the presence or absence of RecQL1 siRNA. Many *RecQL1*-silenced HeLa cells have a round shape representing the M phase-arrested state with condensed chromosomes (Fig. 3a). The duration of the M phase-arrested state of *RecQL1*-silenced HeLa cells was strikingly increased to an average of 6 h, more than 10-fold greater than the 0.5 h of non-silenced cells, showing the characteristics of mitotic catastrophe resulting from DNA damage in G_2 checkpoint-mutated cancer cells. To confirm that this M-phase arrest by *RecQL1* silencing resulted from activation of the spindle checkpoint, we analyzed the subcellular distribution of Mad2 proteins. Treatment with RecQL1 siRNA induced hypercondensed chromosomes that were not aligned regularly, differing from control chromosomes aligned on the mitotic plate (Fig. 3b; tubulin-stained chromosomes; NS siRNA vs RecQL1 siRNA). Multiple foci of Mad2 immunostained with Mad2-specific antibody were seen readily on the kinetochores of NS siRNA-treated cells, but Mad2 foci in cells arrested by *RecQL1* silencing were not detectable (Fig. 3b; Mad2). This incomplete assembly of chromosomes and the absence of Mad2 are characteristics of precatastrophic cells and are consistent with the activation of spindle checkpoints described by Nitta *et al.*[24] In the experiments with A549 human lung cancer cells in which *RecQL1* was silenced in the presence or absence of nocodazole and the Cdc2 inhibitor

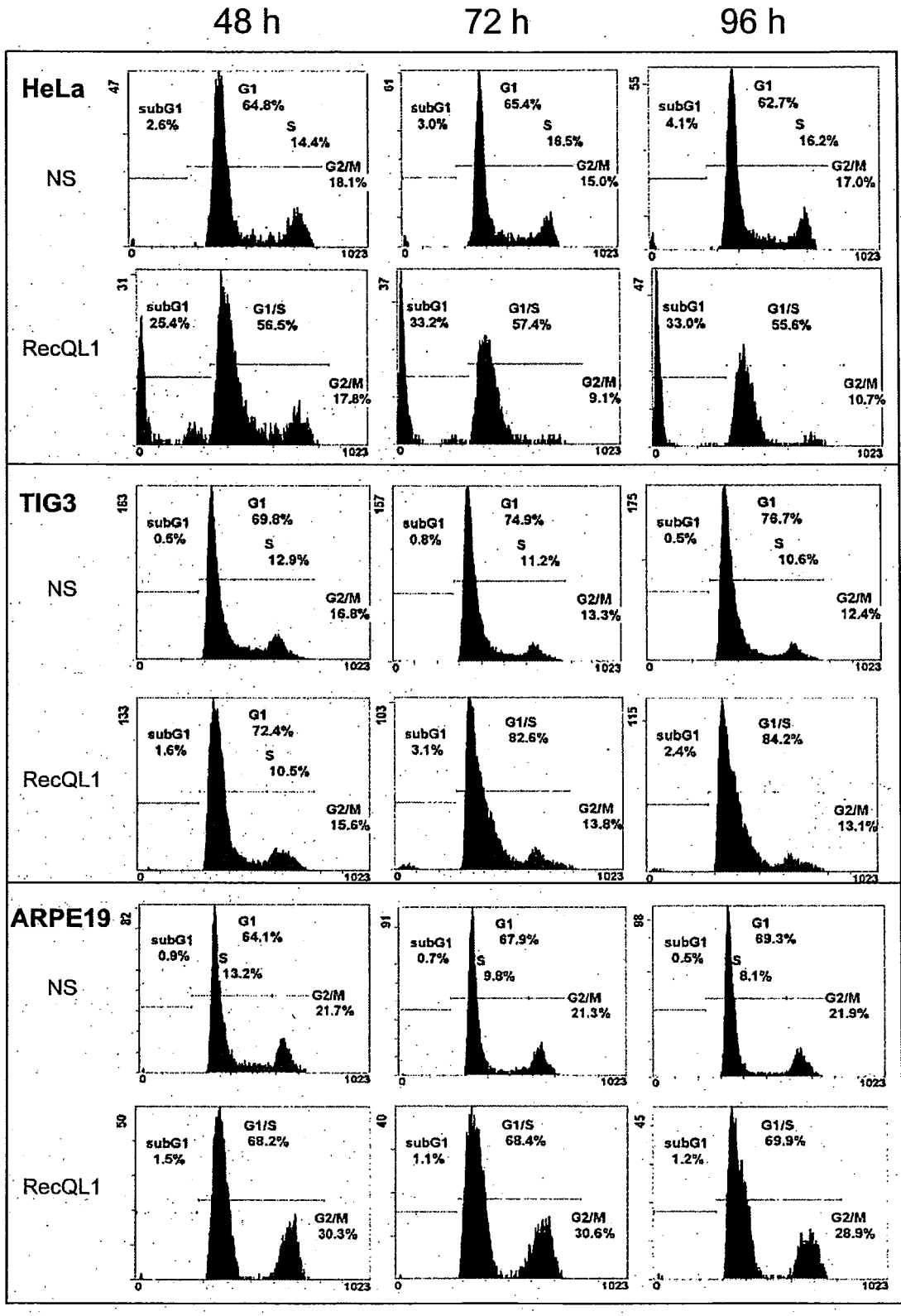

Fig. 2. Induction of mitotic cell death by RecQL1 small interference RNA (siRNA) in HeLa cells but not in TIG3 and ARPE19 cells. HeLa: The effect of *RecQL1* silencing on the cell cycle of HeLa cells was monitored by flow cytometric analysis at 48, 72, and 96 h after HeLa cells were treated with 40 nM RecQL1 siRNA. The percentage of cell populations in the subG$_1$, G$_1$, S, and G$_2$/M phases are shown in each flow cytometric profile. The analysis data for HeLa cells treated with non-silencing siRNA are also shown as the control. TIG3 and ARPE19: The effect of *RecQL1* silencing on the cell cycle of TIG3 and ARPE19 cells that represent growing normal cells. The effect was compared with that in HeLa cells. It is noticeable that no or very few cells were in the subG$_1$ fraction with TIG3 and ARPE19 cells, in contrast to HeLa cells.

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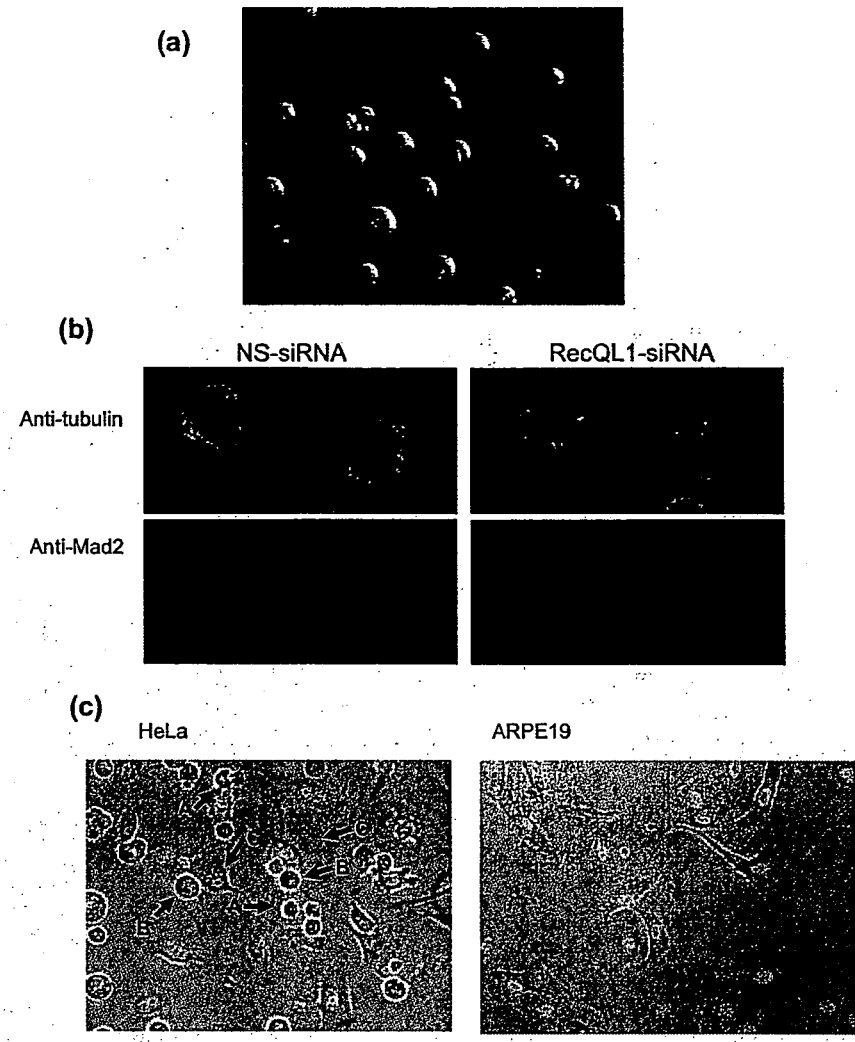

Fig. 3. Dynamics of mitotic catastrophe induced preferentially in HeLa cells by *RecQL1* silencing. (a) Increased population of M phase-arrested HeLa cells induced by *RecQL1* silencing. The process of HeLa cell death induced by *RecQL1* silencing was observed by time-lapse fluorescence video microscopy. HeLa cells cultured in small interference RNA (siRNA)-free medium for 60 h after treatment with RecQL1 siRNA (40 nM) are shown. (b) Immunocytostaining analysis of HeLa cells treated with RecQL1 siRNA. HeLa cells were treated with RecQL1 siRNA or non-silencing siRNA at concentrations of 40 nM and were cultured for 60 h. Cells arrested at M phase had duplicated chromosomes stained red by TO-PRO3, and the proteins involved in chromosome assembly were detected by staining with specific antibodies to human tubulin and Mad2 (green). (c) Differential effects of RecQL1 siRNA on HeLa and ARPE19 cells. The effects of *RecQL1* silencing on HeLa and ARPE19 cells, representing cancer and normal cells, respectively, were compared. The ARPE19 cells treated with RecQL1 siRNA were distinct from HeLa cells in several features. The figures show the morphology of HeLa and ARPE19 cells at 72 h culture after treatment with 40 nM siRNA. In HeLa cell populations, most cells died of mitotic catastrophe (arrowhead A shows the remnants), some cells died after first forming a round shape in M phase (arrowhead B), and the rest of the senesced-like cells remained as giant cells (arrowhead C). As the control, APRE19 cells treated similarly with RecQL1 siRNA and cultured for 72 h are shown. The RecQL1 siRNA-treated ARPE19 cells showed no sign of mitotic cell death, despite an apparent delay in cell proliferation.

purvalanol A, the data showed that cells did indeed die of mitotic catastrophe. Purvalanol A inhibits Cdc2–cyclin B at an IC_{50} of 4 nM.[28] The results showed that: (1) cells treated with RecQL1 siRNA in the presence of nocodazol also showed a long duration of M phase and died like cells treated with RecQL1 siRNA alone, indicating that the cells died of mitotic catastrophe; (2) notably, cell death caused by mitotic arrest mediated by RecQL1 siRNA was prevented by silencing Mad2, an important key factor for spindle checkpoint activation, with co-transfection of Mad2 siRNA, and these results were consistent with previous findings by Nitta *et al.*;[24] and (3) however, as we expected, the Cdc2 inhibitor purvalanol A (at 1 μM) prevented cell death mediated by RecQL1 siRNA by inhibiting entry into M phase. These findings collectively and clearly indicate that A549 cell death induced by *RecQL1* silencing is due to mitotic catastrophe. These events occurring in cancer cells associated with *RecQL1* silencing are interpreted as mitotic catastrophe, described previously by several groups of researchers.[19–24] Most of the *RecQL1*-silenced HeLa cells underwent apoptosis-like death directly after the long M phase-arrested state, and the remaining cells underwent a sequence of aberrant mitoses, generating giant cells, such as cells having large and flat surface areas, having multiple nuclei and many vacuoles, and cells staying in a non-replicating state, sharing several morphological phenotypes with senesced cells (Fig. 3c; arrows, RecQL1 siRNA-treated cells, 72 h culture). In contrast, TIG3 and ARPE-19 cells showed no such cell death or aberrant cell division, even though the cell-cycle progression was markedly delayed (data not shown).

Induction of mitotic cell death in a wide range of cancer cells by *RecQL1* silencing. We studied whether mitotic cell death is induced in other cancer cell lines by *RecQL1* silencing. The cancer cell lines A549, PC3, T24, MIA, PaCa-2, and Hep3B were tested for the effect of RecQL1 siRNA treatment (Fig. 4). All tested cancer cell lines underwent mitotic cell death when treated with 2.5–160 nM RecQL1 siRNA, producing many numbers of $subG_1$ cells characteristic of dead cells and cell debris, but the degree of cell death varied depending on the cancer-cell species (Fig. 4a). Cells that survived were apparently senesced giant cells having large surface areas and were not active in proliferation. In contrast to cancer cells, the normal cell lines WI38 and HUVEC showed no signs of cell death, similar to the other normal cell lines TIG3 and ARPE-19. These results clearly indicate that *RecQL1* silencing induces mitotic cell death preferentially in cancer cells. It was surprising that cell death was not induced in the four normal cell lines even if their RecQL1 expression was completely silenced and cell cycles slowed. Thus, the results suggest that RecQL1 siRNA has excellent potential to be tested as a candidate anticancer drug because it shows no killing effects on normal cells. RecQL1

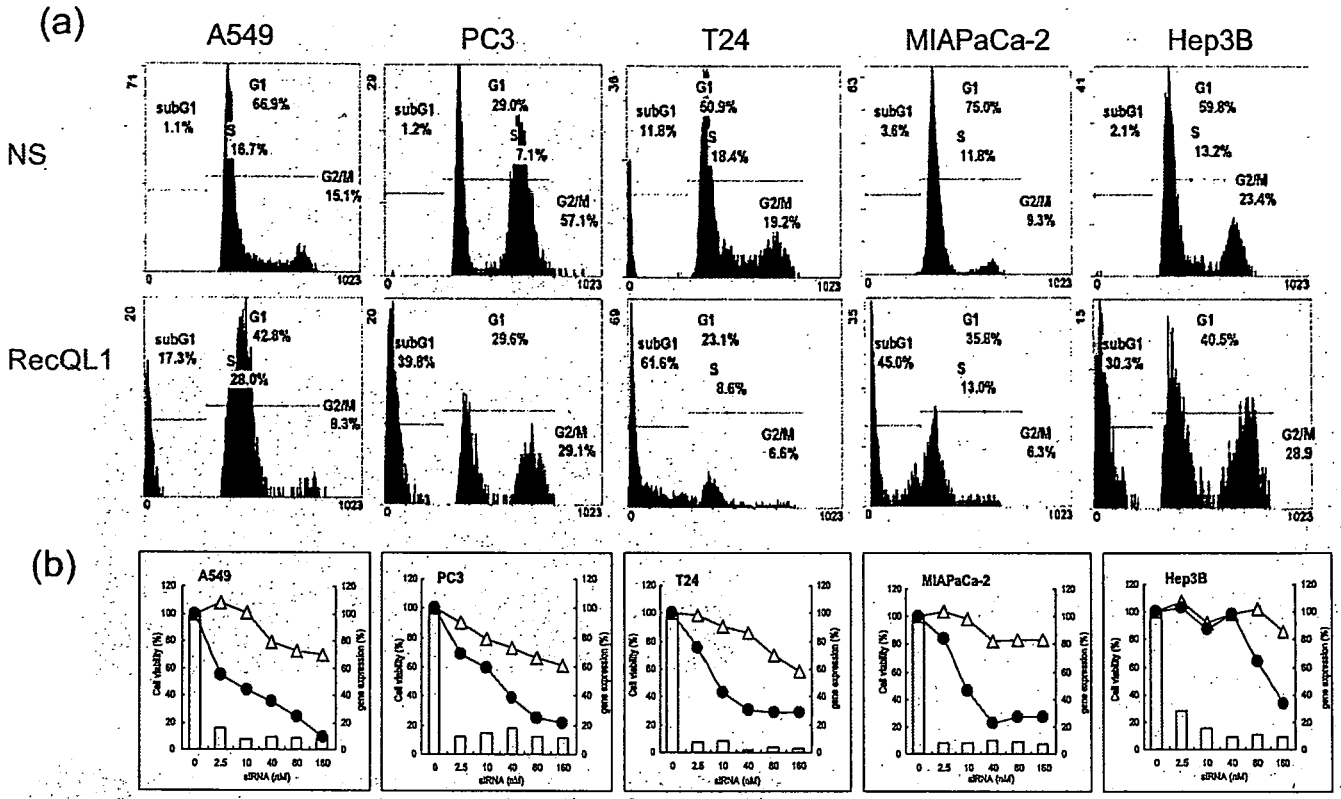

Fig. 4. Cell-cycle distribution of various cancer cells after treatment with RecQL1 small interference RNA (siRNA). (a) The cancer cell lines A549, PC3, T24, HCT116, and Hep3B originally from lung, prostate, bladder, colon, and liver cancers, respectively, were treated for 18 h with 40 nM RecQL1 siRNA in the presence of Oligofectamine or Lipofectamine 2000 and were analyzed using flow cytometry after 72 h of culture. NS, non-silencing siRNA-treated cells; RecQL1, RecQL1 siRNA-treated cells. (b) Dose–response curves of RecQL1 siRNA concentrations that inhibit growth of cancer cells. Cells were transfected with serially diluted concentrations of RecQL1 siRNA or GL3 siRNA, and were cultured for 72 h. The viability of cells was measured by colorimetric assay. Open triangles show NS siRNA-treated cells, and closed circles show RecQL1 siRNA-treated cells. The histograms show the levels of RecQL1 mRNA in the *RecQL1*-silenced cells that are represented as the proportion (%) of NS-treated cells.

helicase may also be a promising molecular target for use in anticancer drug screening. *RecQL1* silencing was tested similarly in various other cancer cell lines, including KP4, HCT116, PA-1, MKN45, MCF7, and U2OS. The growth of most, if not all, of these cells was inhibited at a low concentration of 40 nM RecQL1 siRNA, resulting in similar mitotic catastrophe (Fig. 4).

***RecQL1* silencing elicits DNA damage response in HeLa cells.** To investigate whether mitotic cell death induced by *RecQL1* silencing is caused by unrepaired DNA damage due to the absence of RecQL1 helicase, we monitored DNA damage in HeLa cells treated with RecQL1 siRNA using the DNA damage response marker antibodies γH2AX, ATM (pS1981), and 53BP1. The proteins of these markers were recruited to sites of DNA damage or replication blockade or both.[29,30] In HeLa cells, when *RecQL1* expression was suppressed by transfection of RecQL1 siRNA, the number of nuclear foci containing the four markers increased within 60 h of transfection and was not seen in the NS siRNA-transfected control cells (Fig. 5a,b). In addition, the number of SCE increased significantly in RecQL1 siRNA-treated A549 cells (Fig. 5c, arrows). Thus, the present study confirmed the previous findings of LeRoy *et al.* that *RecQL1* silencing increases SCE in several cancer cell lines, including HeLa.[17] In normal cells, by contrast, no noticeable chromosomal abnormality was detected, but the number of nuclear foci consisting of DNA damage-response markers was increased by *RecQL1* silencing (data not shown). These results indicate that *RecQL1* silencing permits accumulation of endogenous DNA damage derived from the DNA replication process of growing cancer cells. Such DNA damage is carried over to M phase by bypassing G_2 arrest in cancer cells that mostly lack a G_2 checkpoint system and are therefore unable to arrest at G_2 phase. However, cell-cycle progression in normal cells is arrested before M phase by the intact G_2 checkpoint, permitting ample time for DNA repair and resulting in the prevention of mitotic catastrophe. Consistent with this speculation, the differential effect of *RecQL1* silencing is dependent on growth because the incidence of mitotic cell death was decreased in HeLa cells under conditions where progression of the cell cycle was delayed by culturing cells at confluent, high cell density (data not shown).

Cellular features required for susceptibility to mitotic cell death induced by impaired repair function due to *RecQL1* silencing. Induction of mitotic catastrophe has so far been investigated only from the aspect of extraneous DNA damage caused by the attack of genotoxic agents or incorporation of modified nucleotides, such as 5-fluorouracil derivatives, both of which are used broadly in cancer chemotherapy. To our knowledge, this is the first study in which the induction of mitotic catastrophe of cancer cells was investigated from the aspect of managing repair systems by silencing a specific gene with RNAi and increasing endogenous DNA damage. To gain further insight into such mitotic catastrophe resulting from impaired repair, we investigated the genetic and cell biological background of some of the cancer cell lines used in this study to elucidate the cellular features that facilitate mitotic death by *RecQL1* silencing.

First, *RecQL1* expression was measured using western blotting (Fig. 6, left panel). The levels of *RecQL1* expression in cancer cells were higher than or equal to those in the normal cell lines

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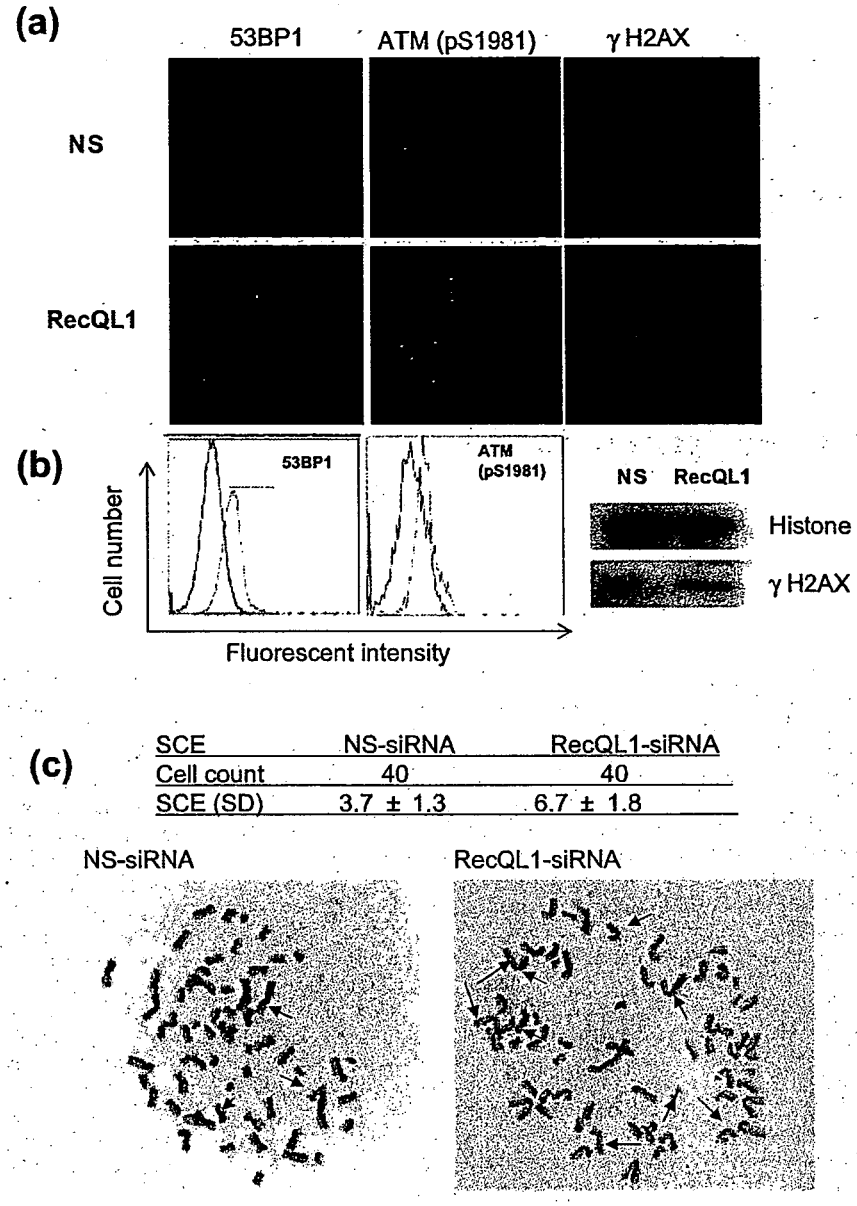


Fig. 5. Accumulation of DNA damage in HeLa cells by inhibition of RecQL1 expression with RecQL1 small interference RNA (siRNA). (a) HeLa cells were transfected with 40 nM RecQL1 siRNA. After culturing for 60 h in siRNA-free medium, cells were immunostained for the DNA damage-marker proteins H2AX, 53BP1, ATM-pS1981, and Chk2-pT68 to examine the presence of DNA damage in cells. NS, cells transfected with non-silencing siRNA (upper panels). RecQL1, cells transfected with RecQL1 siRNA (lower panels). Immunostaining was done with specific antibodies. (b) The upregulation of foci formation with 53BP1 and ATM in the *RecQL1*-silenced cells was monitored by flow cytometric analysis using the same antibodies used in the immunocytostaining experiments (Fig. 5a). The peak with blue color represents NS siRNA-treated cells, the peak with red color represents RecQL1 siRNA-treated cells. We also show the increased level of phosphorylated γH2AX after RecQL1 silencing by specific antibody. (c) Increased frequency of sister chromatid exchange (SCE) in A549 cells. A549 cells were treated with 40 nM RecQL1 siRNA for 8 h and suppression of RecQL1 expression below 10%, as examined using semiquantitative reverse transcription–polymerase chain reaction, was confirmed. After culturing for 48 h in siRNA-free medium, cells were analyzed for the incidence of SCE. NS, non-silencing siRNA-treated A549 cells; RecQL1, RecQL1 siRNA-treated A549 cells. Forty each of NS siRNA-treated and RecQL1 siRNA-treated cells were analyzed for the presence of SCE in the chromosomes. The average incidence of SCE per cell was summarized. SCE (SD) denotes the number of SCE with a standard deviation. Arrowheads show SCE.

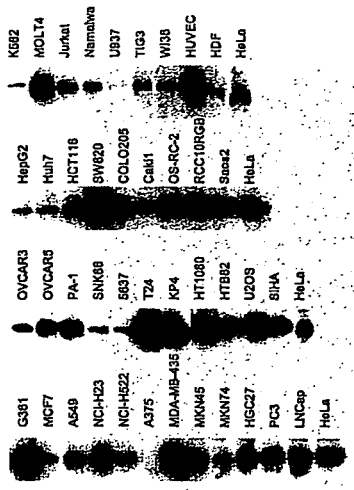
TIG3, WI38, HDF, and HUVEC. K562 and U937 cells that were derived from leukemia showed lower *RecQL1* expression than normal cells. Notably, the expression levels were very high in the SW620, Colo205, MDA-MB-435, MKN45, KP4, and T24 cell lines. and in the U2OS, HTB82, and HT1080 sarcoma cell lines. The expressions were evaluated in relation to susceptibility to mitotic cell death by *RecQL1* silencing. The present study showed that cells having a large copy number of RecQL1 tended to be more sensitive to *RecQL1* silencing.

Second, the influence of the *p53* gene, which is mutated in many cancer cells,[31,32] was assessed because the p53 protein participates in a variety of important cellular functions, including genomic repair, and the cell cycle and checkpoint systems. Although the number of cancer cell lines tested in our study was limited, cells that had mutated p53 were all susceptible to mitotic catastrophe by *RecQL1* silencing, and cancer cells containing wild-type p53 appeared to be more or less resistant to *RecQL1* silencing, similar to normal cells that have wild-type p53 and are freed from mitotic cell death with *RecQL1* silencing.

Third, we investigated whether the presence or absence of telomerase activity in cancer cells affected the susceptibility to mitotic catastrophe. Of the 10 cancer-cell lines, only the U2OS cells were telomerase negative, similar to normal cells, and all other cancer cells were telomerase positive. Telomerase is known to be one of the important elements for maintaining genomic integrity.[33] However, the presence of telomerase activity did not seem to protect U2OS cells from mitotic catastrophe by *RecQL1* silencing. These results indicate that cancer cells that contain a large copy number of RecQL1 helicase, as well as mutated (or inactive) p53, are most susceptible to mitotic catastrophe induced by *RecQL1* silencing, irrespective of telomerase activity.

Discussion

Of the five human RecQ family helicases, RecQL1 was the first to be characterized by purifying the protein from HeLa cells and subsequently cloning the encoding gene.[1] Because of high amino acid homology to the RecQ helicase of *E. coli* that participates



Cell lines	TP53 status	RecQL1 expression	Cell death
Tumor			
MCF7	WT	+	–
A549	WT	++	++
HCT-116	WT	++	++
MKN45	WT	+++	–
PC-3	mut	++	++
KP4	WT	+++	++
T24	mut	+++	+++
PA-1	mut	++	+++
HeLa	WT	++	+++
U2OS	WT	+++	+++
Normal			
TIG3	WT	+	–
WI38	WT	+	–
HUVEC	WT	++	–
ARPE-19	WT	ND	–

Fig. 6. RecQL1 expression in various cancer cells and degrees of susceptibility of cells to mitotic catastrophe by *RecQL1* silencing. Immunoblot analysis (left panel): The amount of RecQL1 helicase in the growing cells was measured by immunoblotting using 10 μg cell protein. A protein extract of HeLa cells (same extract) was used as a common standard for different groups of western blotting experiments to further normalize the immunoblotting reaction. Right panel: The right panel illustrates the relative expression levels: +++, ++, and + representing high, medium, and low expression, respectively. The degrees of susceptibility to mitotic cell death were compared by measuring the viability of RecQL1 small interference RNA (siRNA)-transfected cells after 72 h of culture. The cell viability was compared also by flow cytometric analysis as shown in Fig. 4. The combined result was taken into consideration in the assessment of susceptibility of cells to mitotic cell death caused by RecQL1 siRNA: +++, ++, and + represents more than 80, 60, and 40% dead cells, respectively, and – shows no mitotic cell death. The status of *p53* gene mutation is summarized in the table, which shows the status of the *p53* gene by WT (wild type) or mut (mutated).

in the resumption of DNA synthesis after DNA damage[34,35] and prevents illegitimate recombination of *E. coli*,[35,36] it was named RecQL1 (RecQ like human helicase-1). RecQL1 helicase is highly upregulated in rapidly proliferating cancer cells and other transformed cells, such as Epstein–Barr virus-infected B lymphocytes and Simian virus (SV)-40-infected fibroblasts or SV-40 T antigen-transfected fibroblasts, suggesting that RecQL1 helicase may be needed to repair DNA during the cell cycle.[7] LeRoy *et al.* reported that RecQL1 helicase acts on Holliday junctions in proliferating human cell lines,[17] to resolve stalled DNA structures and to facilitate the continuation of DNA replication. RecQL1 helicase is reported to be in the Piwi complex, but its biological function in this complex is unclear.[37]

In the present study, we showed that the *RecQL1* silencing by RNAi kills various cell lines derived from human cancers by inducing mitotic catastrophe. In contrast, all four normal cell lines of different origins tested (fibroblasts [TIG3 and HDF], endothelial cells [HUVEC], and epithelial cells [APRE19]) showed tolerance to mitotic cell death by *RecQL1* silencing, although these normal cell lines have slightly different cell-cycle profiles (e.g. Fig. 2), perhaps because of different backgrounds in their checkpoint abilities. Although less frequent, this mitotic catastrophe in cancer cells generated cells with senesced phenotypes as a result of a long duration in M phase and aberrant mitosis. Notably, the same *RecQL1* silencing induced no mitotic catastrophe in normal diploid cells. What then is the basis underlying selective induction of cell death in cancer-derived cell lines? A plausible explanation is that cancer cells fail to correct DNA damage, including stalled DNA structures such as Holliday junctions caused by the absence of RecQL1, and the accumulation of such DNA damages increases in the M phase-arrested cancer cells because of incomplete checkpoint systems. Our results with DNA damage-response markers (Fig. 5a,b) clearly indicate an increased accumulation of DNA damage in cancer cells caused by *RecQL1* silencing. Most cancer cells are deficient in G_1 and, more importantly, G_2 checkpoint function and thus fail to arrest the cell cycle at G_1 and G_2 phases to permit cells to engage in DNA repair. Instead, the cells proceed in the cell cycle to M phase where chromatin is condensed and DNA repair is not permitted. Thus, cells eventually undergo cell death as they enter mitosis.[19,20,24] In cancer cells, mitotic cell death due to DNA damage is most probably avoided during the S and G_2 phases by highly upregulated repair enzymes, such as RecQL1, which coordinates well with DNA replication to remove DNA damage. Erenpreisa and Cragg characterized the main features of mitotic cancer-cell death.[21] Some of the features relevant to this study are: (1) the absence or delay of the G_1/S checkpoints; and hence (2) the absence of interphase apoptosis coupled with the G_1/S checkpoint; (3) delay or inability of the G_2 function due to a defective G_2 checkpoint; and hence (4) a sequence of aberrant mitoses that end in mitotic death; and (5) formation of (multinuclear) giant cells. As other features of mitotic catastrophe, Nitta *et al.* added a prolonged M-phase arrest without segregation of chromosomes and a prerequisite activation of the spindle checkpoint.[24]

Our data obtained from time-lapse microscopic analysis were consistent with the features described for cancer cells entering into mitotic catastrophe with DNA damage. Most of the *RecQL1*-silenced cancer cells showed an M phase longer than 6 h and death from mitosis. Occasional generation of abnormal aneuploid HeLa cells with senescent phenotypes or A549 pre-catastrophic cells having multiple SCE (Fig. 5c) may also be produced under these conditions of genomic instability by the absence of spindle checkpoint activation. In this context, Nitta *et al.* reported that suppression of a spindle checkpoint in DNA-damaged cells leads to escape from catastrophic death and to subsequent abnormal mitosis,[24] suggesting that the spindle checkpoint is the key element to decide cell death or aberrant mitosis causing multinuclear giant cells.

In contrast, the checkpoint system in normal cells remains intact, controls cell-cycle progression stringently, and cooperates more with a cellular-repair system than in cancer cells, resulting in transient cell-cycle arrest until the DNA problems are resolved by recruiting an appropriate repair system. In the case of mutations in p53 (the product of the tumor suppressor gene), which occur in many if not most cancer cells, among the 10 cancer-derived cell lines tested, MCF7 and MKN45, which have wild-type p53, were resistant to mitotic cell death by *RecQL1* silencing, whereas A549, HCT-116, KP4, and U2OS cells, all of which are sensitive to *RecQL1* silencing, have wild-type p53, suggesting that these cells may contain defects other than p53 in the checkpoint (Fig. 6). However, there was no cell that had mutant p53 and was resistant to *RecQL1* silencing. Thus, although mitotic cell death by *RecQL1* silencing results from

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checkpoint deficiency of cancer cells, it does not necessarily depend on mutations in *p53*. Further studies are needed to understand which checkpoint system most affects RecQL1-mediated mitotic cell death, but the defective G_1 checkpoint function in many cancer cells does not seem to be important for mitotic cell death by *RecQL1* silencing.

Expression levels of RecQL1 vary depending on the cell species. The expression level of *RecQL1* in cancer cells appears to be correlated with mitotic catastrophe induced by *RecQL1* silencing based on the immunoblotting data shown in Fig. 6. In this context, it is intriguing to refer the notion raised by Weinstein, that cancer cells are often 'addicted to' (that is, physiologically dependent on) the continued activity of specifically activated or overexpressed oncogenes for maintenance of their malignant phenotype;[38] these kinds of genes and gene products have been pursued as ideal chemotherapeutic targets for anticancer agents with few adverse effects.[39] RecQL1 has never been assumed to be an oncogene-related product per se, but it is possible to postulate that cancer cells may also be addicted to greater copy numbers of DNA repair enzyme such as RecQL1 in addition to specified oncogenes, so that DNA damage is resolved in a short time even if the ample time of cell cycle arrest needed for repair at the S or G_2 phase is unavailable due to defects in the checkpoint activity.

Our study suggests that mitotic catastrophe caused by *RecQL1* silencing occurs most efficiently in those cancer cells that have mutated p53 and highly increased expression of RecQL1 helicase. Accordingly, the *RecQL1* silencing-mediated mitotic cell-death preferential in cancer cells is speculated to take place depending on: (1) rapid cell proliferation; (2) addiction to *RecQL1* overexpression; and (3) incomplete checkpoints of cancer cells. The p53-related G_2 checkpoint systems of cancer cells are often inactivated by mutations, and thus cancer cells die directly from M phase when DNA is damaged by treatment with genotoxic chemotherapeutic agents. Our study showed that cancer-cell killing mediated by such DNA damage can also be achieved by endogenous DNA damage occurring naturally during cell proliferation and remaining unrepaired due to *RecQL1* silencing, most importantly evading the use of genotoxic agents that inevitably cause DNA damage to the neighboring cells, irrespective of normal or cancer cells, and growing or resting stage. We believe that: (1) the present study provides a new idea that inhibition of DNA repair enables cancer cells to retain endogenous DNA damage generated during DNA replication and leads them to mitotic cell death due to cancer cell-specific dysfunction of checkpoint systems; and (2) RNAi therapy, which permits highly specific inhibition of the selected molecular target, is an additional way to reduce the adverse events very often associated with cancer chemotherapy.

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