学位論文

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香川大学大学院医学系研究科

機能構築医学専攻


徳永義昌

# Potent effect of adenoviral vector expressing short hairpin RNA targeting ribonucleotide reductase large subunit M1 on cell viability and chemotherapeutic sensitivity to gemcitabine in non-small cell lung cancer cells

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Yoshimasa Tokunaga [a], Dage Liu [a,*], Jun Nakano [a], Xia Zhang [b], Kazuhito Nii [a], Tetsuhiko Go [a], Cheng-long Huang [c], Hiroyasu Yokomise [a]

[a] *Department of General Thoracic Surgery, Faculty of Medicine, Kagawa University, 1750-1, Miki-cho, Kita-gun, Kagawa 761-0793, Japan*
[b] *Department of Urology, Faculty of Medicine, Kagawa University, 1750-1, Miki-cho, Kita-gun, Kagawa 761-0793, Japan*
[c] *Kitano Hospital, Medical Research Institute, 2-4-20 Ohgimachi, Kita-ku, Osaka 530-8480, Japan*

**Abstract** *Background:* Ribonucleotide reductase large subunit (RRM1) is the main enzyme responsible for synthesis of the deoxyribonucleotides used during DNA synthesis. It is also a cellular target for gemcitabine (GEM). Overexpression of RRM1 is reportedly associated with resistance to GEM and the poor prognosis for many types of malignant tumours. Aim of the present study is to establish gene therapy against RRM1-overexpressing tumours.
*Method:* An adenoviral vector that encoded a short hairpin siRNA targeting the RRM1 gene (Ad-shRRM1) was constructed. Two RRM1-overexpressing non-small cell lung cancer (NSCLC) lines, MAC10 and RERF-LC-MA, were used. Finally, a human tumour xenograft model in nude mice was prepared by subcutaneously implanting tumours derived from RERF-LC-MA cells.
*Results:* Ad-shRRM1 effectively downregulated RRM1 mRNA and protein in both types of NSCLC cells and significantly reduced the percentage of viable cells as detected by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay ($p < 0.005$). Caspase 3/7 analysis revealed that transfection with Ad-RRM1 increased the percentage of apoptotic cells in culture containing either type of RRM1-overexpressing cell ($p < 0.001$). Treatment with Ad-shRRM1 exerted a potent antitumour effect against the RRM1-overexpressing RERF-LC-MA xenografts ($p < 0.05$). Furthermore, Ad-shRRM1-mediated inhibition of RRM1 specifically increased sensitivity to gemcitabine of each type of RRM1-overexpressing tumour

* *Corresponding author:* Tel.: +81 87 891 2191; fax: +81 87 891 2192.
  *E-mail address:* dgliu@kms.ac.jp (D. Liu).

cell. Combination treatment with Ad-shRRM1 and GEM exerted significantly greater inhibition on cell proliferation than Ad-shRRM1 or GEM treatment alone.

*Conclusion:* RRM1 appeared to be a promising target for gene therapy, and Ad-shRRM1 had strong antitumour effects, specifically anti-proliferative and pro-apoptotic effects, against NSCLC cells that overexpressed RRM1. Combination therapy with Ad-shRRM1 and GEM may become a new treatment option for patients with NSCLC.

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## 1. Introduction

Patients with lung cancer generally have poor prognosis, and lung cancer remains the leading cause of cancer death worldwide [1]. Approximately 85% of newly diagnosed lung cancers are non-small cell lung cancers (NSCLCs), which can comprise any of three cell types (adenocarcinoma, squamous cell carcinoma or large cell carcinoma). Although significant advances have been achieved in treatment of NSCLC with molecular-targeted therapies such as gefitinib [2], erlotinib [3] and afatinib [4] (which are each inhibitors that specifically target the epidermal growth factor receptor (EGFR) tyrosine kinase), the median overall survival for patients with advanced NSCLC who are treated with EGFR-targeting agents averages only 24 months [5]. Therefore, it is necessary to develop newer therapies that more effectively combat this disease.

Gemcitabine (GEM) is a deoxycytidine analogue with antitumour activity against a wide variety of cancers and approved as treatment for advanced pancreatic cancer and for combination therapy against NSCLC, breast cancer, biliary tract cancer and ovarian cancers [6]. However, drug resistance often limits its efficacy, and it is important to understand the mechanisms of GEM resistance and to then develop novel strategies to overcome such resistance.

Ribonucleotide reductase (RNR) is an enzyme that catalyses the formation of deoxyribonucleotides needed for DNA synthesis [7,8]. GEM binds to the RNR large subunit M1 (RRM1) and inhibits RNR, thereby depleting cellular pools of deoxynucleotides (dNTPs) [9]. RRM1 is reportedly the key molecule in determining the efficacy of GEM. High levels of RRM1 expression are associated with resistance to GEM in various cancers [10–13]. Additionally, RRM1 overexpression is reportedly related to the proliferative and invasive ability of cancer cells [14,15]. High levels of RRM1 expression are common in NSCLC and correlated to short progression-free and short disease-free overall survival [16–19]. Recently, our clinical studies also revealed that high RRM1 expression is one of the poor prognosis factors for cN2 or cN3 NSCLCs [20]. Overall survival was significantly lower among patients with RRM1-positive tumours than among those with RRM1-negative tumours. Thus, RRM1 may become a potential target for cancer therapies, either alone or in combination with GEM.

Although RNA interference (RNAi) is a powerful method for gene-specific silencing, successful application of RNAi in cancer gene therapy depends on efficient delivery of small interfering RNA (siRNA) into the cells [21,22]. Stable effective siRNA molecules can be produced via short hairpin RNAs (shRNAs) that are expressed under the control of an RNA polymerase III-dependent promoter [23,24]. Furthermore, adenoviral vectors have been widely used to express transgenes not only in experimental conditions [25,26], but also in clinical settings [10]. Therefore, in order to establish an effective treatment for GEM-resistant tumours, an adenoviral vector that encodes a shRNA targeting RRM1 (Ad-shRRM1) was constructed. We found that Ad-shRRM1 effectively downregulated RRM1 expression both at the mRNA and protein level. We also used Ad-shRRM1 to examine the potential of RRM1 as a target for gene therapy *in vitro* and *in vivo*. Finally, we found that a combined treatment involving Ad-shRRM1 and GEM had effective antitumour activity in NSCLC cells.

## 2. Materials and methods

### 2.1. Cell lines

Six human NSCLC tumour cells, including lung carcinoma A549 cells, MAC10, RERF-LC-MA, NCI-H358, EBC-1 and LUDLU-1 cells, were investigated. Two RRM1-overexpressing cells, adenocarcinoma MAC10 cell and squamous cell carcinoma RERF-LC-MA cell were subjected for silencing experiment. These cell lines were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium (SIGMA–ALDRICH, St. Louis, MO, United States of America (USA)) supplemented with 10% foetal bovine serum at 37 °C in a 5% $CO_2$ atmosphere.

### 2.2. Design and transfection of siRNA

Three siRNA oligonucleotides targeting RRM1 were designed using the siRNA Design Support System (Takara Biomedicals, Japan). The sense strand

sequences were: RRM1-siRNA-A, 5'-GGAUAUGGU CCUAUAGGUUTT-3'; RRM1-siRNA-B, 5'-GGAA UGUUACUCCUACAGATT-3' and RRM1-siRNA-C, 5'-GGACUAAUGGCAAUUCCAATT-3'. The siRNA transfection was performed in a total volume of 3 ml containing each siRNA (final concentration of 25 nM) and 25 µl of TransIT-TKO Transfection Reagents (Mirus, Madison, WI).

## 2.3. Construction of adenoviral vectors

The shRNA template [forward strand: 5'-RRM1-siRNA1 sense strand (GGACTAATGGCA ATTCCAA) + loop (TAGTGCTCCTGGTTG) + RRM1-siRNA1 antisense strand (TTGGAATTGCCA TTAG TCC) + polymerase III terminator (TTTTT TA)] was synthesised. To produce a plasmid vector expressing shRNA (plasmid-RRM1), this shRNA template was cloned into a pBAsi-hU6 plasmid vector (Takara). Thereafter, an adenoviral vector was constructed using an Adenovirus Expression Vector Kit (Takara). The insert with the human RNA polymerase III-dependent U6 promoter and shRNA template was produced from plasmid-shRRM1 using restriction enzyme digestion by EcoRV and it was then ligated into a pAxcwit cosmid vector. A replication-deficient recombinant adenoviral vector expressing shRNA targeting RRM1 under the control of the human U6 promoter (Ad-shRRM1) was constructed using the COS-TPC method [23,27]. A control adenoviral vector expressing shRNA against the scrambled sequence of RRM1-siRNA-A was also constructed (Ad-Scramble). Constructed adenoviral vectors were amplified in 293 HEK cells and purified by CsCl ultracentrifugation.

## 2.4. Quantitative real-time polymerase chain reaction (RT-PCR)

Total cellular RNA was extracted using the acid guanidinium thiocyanate procedure. First-strand cDNA synthesis was performed using TaqMan reverse transcriptase (Applied Biosystems, Branchburg, NJ, USA). TaqMan real-time quantitative PCR was performed with the ABI Step One Plus (Applied Biosystems, Foster City, CA). The primers and probes were from the Assays-on Demand Gene Expression Assay (RRM1 assay ID Hs00168784_mL, Applied Biosystems). The sample was run in triplicate. The comparative threshold cycle method was used to calculate the gene expression in the sample relative to the value in the MAC10, RERF-LC-MA cells, using glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (ID 4326317E, Applied Biosystems) as a control for normalisation among samples.

## 2.5. Western blot analysis

Cells were harvested and re-suspended in lysis buffer (62.5 mM Tris–HCl, 2% sodium dodecyl sulphate (SDS), 10% glycerol, 4 M urea). Protein samples (40 µg) were each diluted into a 30 µl solution of lysis buffer and 5% 2-mercaptoethanol and heated at 95 °C for 5 min. The protein extracts were separated by 10% SDS–polyacrylamide gel electrophoresis (PAGE). The separated proteins were transferred to nitrocellulose membrane. Using antigen–antibody complex reaction kit (SNAP i.d. 2.0, Merck Millipore), the membranes were blocked (0.5% dry milk and 0.2% Tween 20) in phosphate buffered saline (PBS) and incubated with the primary specific antibodies detecting RRM1 (10526-1-AP; Cosmobio, 1:250) for 10 min. The membranes were then incubated with horseradish peroxidase (HRP)-labelled secondary antibodies for 10 min. The proteins were visualised on enhanced chemiluminescence film. Finally, the blots were reprobed using a rabbit anti-human GAPDH (sc25778; Santa Cruz Biotechnology, Santa Cruz, CA, 1:10,000) to ensure equal loading and transfer of proteins. Each experiment was repeated three times with consistent results.

## 2.6. In vitro cell viability assay

Tumour cells were seeded in 96-well plates at concentration of 4000 cells/well. The cell viability was determined by a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenylte-trazolium bromide (MTT) assay using a Cell Proliferation Kit I (Roche, Mannheim, Germany). Tumour cells were incubated with 10 µl of MTT labelling reagent for 4 h, and then were incubated with 100 µl of solubilisation solution overnight. Finally, the cell viability in each well was measured in terms of optical density at a wavelength of 570 nm, with 750 nm for reference. Each cell viability assay was performed in triplicate and repeated three times.

## 2.7. Human tumour xenograft model in nude mice

Tumour xenografts were prepared by implanting approximately 8 mm³ fragments of tumours derived from RERF-LC-MA cells subcutaneously into the back of 6-week-old male nude mice. When the tumour volume reached approximately 200 mm³, the mice were randomly divided into three groups (five mice/group): groups treated with Ad-shRRM1, Ad-Scramble and control group treated with PBS. Intra-tumoural injection with adenoviral vectors (at $2 \times 10^9$ PFU, respectively) or 0.5 ml of PBS was performed every 4 days

for 28 days. The tumour volume was calculated by the following formula: tumour volume = (length) × (width)$^2$ × 0.5. Animal experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals from Kagawa University.

## 2.8. Apoptosis assay by detecting the activity of caspase 3/7

The activity of both caspase 3 and 7 was evaluated simultaneously using the ApoTox-Glo™ Triplex Assay (Caspase-Glo substrate, G6320, Promega) together with cell viability (GF-AFC Substrate) and cell cytotoxicity (bis-AAF-R110 Substrate) according to the manufacturer's instructions. Caspase-3/7 cleavage of the substrate releases luciferin, indicating the activity of caspase-3/7. The assay was performed in the same cell-based assay well (4000 cells/well, 96 wells plate; 20 MOI for 1 h). RRM1-overexpressing tumour cells were incubated for 24 h at 37 °C. The substrate GF-AFC and bis-AAF-R110 were added and fluorescence was measured after 30 min incubation at 37 °C. Luminescence was detected after 30 min incubation with the Caspas-Glo3/7 substrate.

## 2.9. Cell sensitivity to chemotherapeutic agents

Regarding the cell sensitivity to GEM, we carried out MTT assay for investigating the *in vitro* drug concentration that inhibited cell growth by 50% (IC$_{50}$). MAC10 and RERF-LC-MA, RRM1-overexpressing NSCLC cells, were seeded in 96-well plates at concentration of 4000 cells/well and allowed to attach overnight. Then cells were infected with control virus or Ad-shRRM1 at three different concentrations. Twenty-four hours later, cells were treated with GEM or cisplatin at concentration varying from 0 to 100 μmol/l. Three days after exposure to the agents, the cell viability was evaluated with MTT assay.

## 2.10. Statistical analysis

Data are expressed as the mean ± SD. Differences among the treatment groups were assessed using Mann–Whitney $U$-test. Differences were considered to be significant when $p < 0.05$.

## 3. Results

### 3.1. Selection of RRM1-overexpressing tumour cells

The normalised RRM1 expression ratio was evaluated in six human NSCLC cells by real-time PCR. Most of assessed NSCLC cells showed relative higher expression of RRM1 (Fig. 1A). As a result, two tumour cells, MAC10 cells, adenocarcinoma of the lung, and RERF-LC-MA cells, squamous cell carcinoma of the lung, were selected as RRM1-overexpressing tumour cells for subsequent experiment.
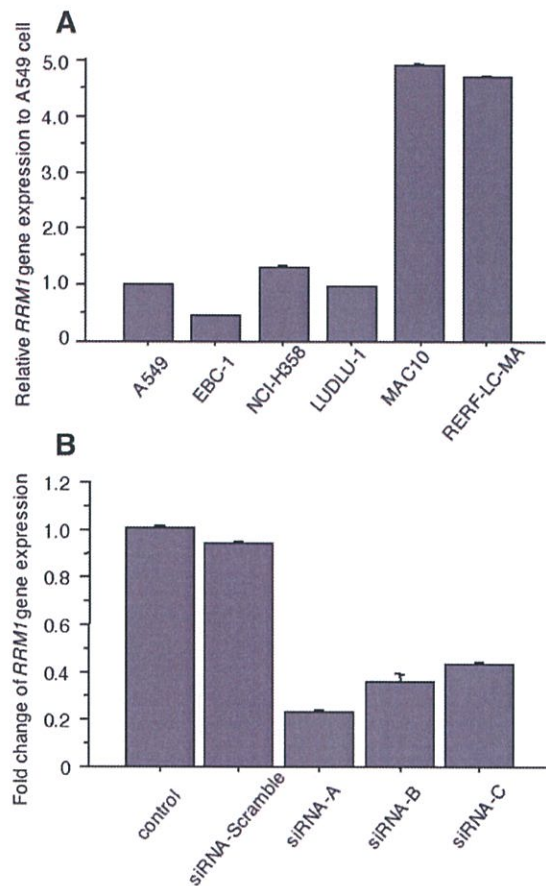


Fig. 1. (A) Relative ribonucleotide reductase large subunit M1 (RRM1) gene expressions in human non-small cell lung cancer cell lines (in referencing to A549 cell line). (B) RRM1 gene expressions in lung adenocarcinoma MAC10 cells after transfection of small interfering RNAs (siRNAs) targeting RRM1. One of three experiments with similar results is shown. Scramble, scrambled oligonucleotides as a negative control against RRM1-siRNA1.

### 3.2. Identification of specific sequence of siRNA targeting RRM1

Three chemically-synthesised siRNAs designed from different sequences of the RRM1 were investigated. Seventy-two hours after the siRNA transfection into MAC10 cells, RRM1-siRNA-A caused the strongest down-regulation of the RRM1 gene expression (0.233 ± 0.01; Fig. 1B). Therefore, RRM1-siRNA-A was selected as the specific target site to construct RRM1-inhibiting adenoviral vector (Ad-shRRM1).

### 3.3. Ad-shRRM1 efficiently down-regulates the RRM1 expression

Two RRM1-overexpressing tumour cells, MAC10 cells and RERF-LC-MA cells, were transfected with Ad-shRRM1 at a multiplicity of infection (MOI, PFU/cell) of 10 and 20 (Fig. 2A and B). Ad-shRRM1 effectively knocked down the RRM1 gene expression in

both of these RRM1-overexpressing tumour cells in a dose-dependent manner ($p < 0.005$ versus Ad-Scramble, respectively). The down-regulation of RRM1 protein expression was also detected after transfection with Ad-shRRM1 in all the RRM1-overexpressing tumour cells (Fig. 2C and D).

### 3.4. Ad-shRRM1 inhibits the growth of RRM1-overexpressing tumour cells

We investigated the inhibitory effect of Ad-shRRM1 against RRM1-overexpressing tumour cells (Fig. 3A and B). The percentages of viable cells significantly decreased in both of these RRM1-overexpressing tumour cells transduced with Ad-shRRM1 ($p < 0.005$ versus Ad-Scramble, respectively). Transduction with Ad-shRRM1 strongly reduced the percentage of viable cells in these RRM1-overexpressing tumour cell lines in a time-dependent manner. These results indicate the essential role of RRM1 in regulating cell proliferation of NSCLC cells.

### 3.5. Antitumour activity of Ad-shRRM1 against RRM1-overexpressing tumour xenograft

A RRM1-overexpressing RERF-LC-MA tumour xenograft model was prepared in nude mice. The

tumour volumes at day 28 were $1254 \pm 536$ mm$^3$ in the PBS-treated groups, $1355 \pm 527$ mm$^3$ in the Ad-Scramble-treated groups and $509 \pm 208$ mm$^3$ in the Ad-shRRM1-treated group (Fig. 4A and B). The Ad-shRRM1 treatment significantly inhibited RERF-LC-MA xenografts in comparison with the PBS treatment and Ad-Scramble treatment ($p < 0.05$, respectively). Based upon these results, Ad-shRRM1 was proved to have anti-tumour effects in vivo.

### 3.6. Ad-shRRM1 increases the apoptosis in RRM1-overexpressing tumour cells

Using the AproTox-Glo triplex assay, the cell viability, cell toxicity and apoptosis were tested in the same cell-based well. The percentages of viable cells significantly decreased in the RRM1-overexpressing tumour cells transfected with Ad-shRRM1 ($p < 0.05$ versus Ad-Scramble, respectively, Fig. 5A). Correspondingly, the cell toxicity increased gradually after Ad-shRRM1 infection (Fig. 5B). Moreover, cell apoptosis, indicated by activity of caspase 3/7, rapidly reached a high level and remain stable after Ad-shRRM1 transfection (Fig. 5C and D). The infection with Ad-shRRM1 strongly induced apoptosis in the RRM1-overexpression NSCLC cells. Consequently, the downregulation of RRM1 can inhibit cell growth and
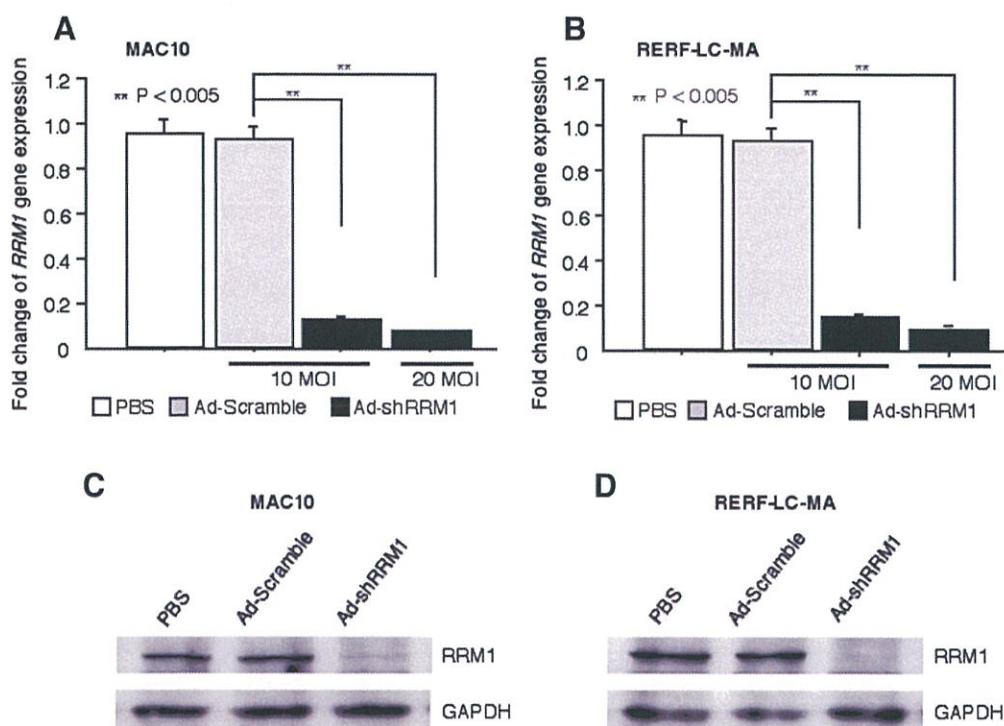


Fig. 2. Ribonucleotide reductase large subunit M1 (RRM1) gene expressions and protein expressions in RRM1-overexpressing human lung cancer cells after transfection with adenoviral vectors. (A) RRM1 gene expressions in lung carcinoma MAC10 cells, and (B) RERF-LC-MA cells. RRM1 protein expression in (C) MAC10, and (D) RERF-LC-MA RRM1-overexpressing cells. Western blot analyses were performed at 72 h after transduction with adenoviral vectors at a multiplicity of infection MOI of 20 and one of three experiments with similar results is shown. MOI, multiplicity of infection; *$p < 0.005$ versus Ad-Scramble treatment.
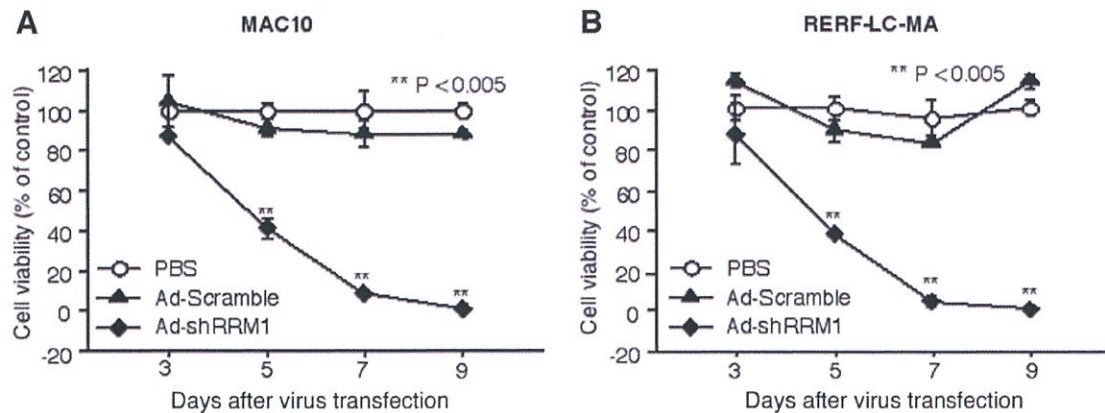
Fig. 3. The effect of adenoviral vector that encoded a short hairpin siRNA targeting the ribonucleotide reductase large subunit M1 gene (Ad-shRRM1) on growth of cell lines. Cell viability evaluated by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay after transduction with adenoviral vectors, in comparison with phosphate buffered saline (PBS)-treated control cells. (A) MAC10 cells, adenocarcinoma of the lung (B) RERF-LC-MA cells, squamous cell carcinoma of the lung. MOI, multiplicity of infection; $^*p < 0.005$ versus Ad-Scramble treatment.
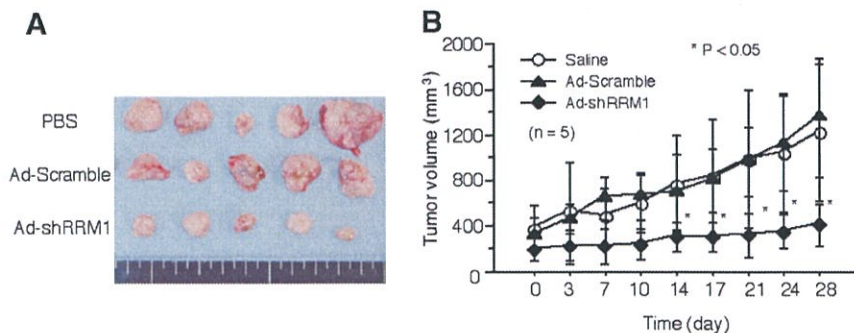


Fig. 4. In vivo anti-tumour effect of adenoviral vector that encoded a short hairpin siRNA targeting the ribonucleotide reductase large subunit M1 gene (Ad-shRRM1) on ribonucleotide reductase large subunit M1 (RRM1)-overexpressing RERF-LC-MA xenografts. RERF-LC-MA tumours, treated with control phosphate buffered saline (PBS), Ad-Scramble and Ad-shRRM1, were enucleated 28 days after the initiation of treatment. (A) Appearance and (B) tumour volumes of RRM1-overexpressing RERF-LC-MA xenografts in nude mice. $^*p < 0.05$ versus Ad-Scramble and PBS treatment.

induce cell apoptosis in the RRM1-overexpression NSCLC cells.

### 3.7. Inhibition of RRM1 specifically increased lung cancer cell sensitivity to GEM

In these two RRM1-overexpressing tumour cells, the transfection with Ad-shRRM1 reduced the half maximal inhibitory concentration (IC$_{50}$) of GEM. A concentration-dependent manner was observed in the MAC10 cell (Fig. 6A). In the RERF-LC-MA, squamous cell carcinoma of the lung, Ad-shRRM1 showed a very strong effect on inhibiting cell proliferation (Fig. 6B). After inhibiting RRM1, the cell sensitivity to GEM increased nearly by 100 times for MAC10 cell (Fig. 6A) and by 10–1000 times for RERF-LC-MA (Fig. 6B). However, these changes were not observed in both of these cells with the treatment of cisplatin (Fig. 6C and D).

## 4. Discussion

RNR transforms ribonucleoside diphosphates into deoxyribonucleoside diphosphates. Deoxyribonucleoside diphosphates are further phosphorylated into deoxyribonucleoside triphosphates, which are essential to de novo DNA synthesis and DNA repair processes. RNR is a tightly regulated enzyme consisting of homodimers of RRM1 and RRM2 subunits, both of which are required for active site formation [8]. By directly comparing the effect of siRNAs targeted at RRM1 versus RRM2, Reid et al. verified that RRM1 silencing is a more effect by means of inhibiting cell growth than is RRM2 silencing [14].

In the present study, we constructed Ad-shRRM1, an adenoviral vector expressing shRNA targeted at RRM1. Ad-shRRM1 effectively inhibited RRM1 expression both at the mRNA and protein level, and it had a long-lasting inhibitory effect on cell proliferation
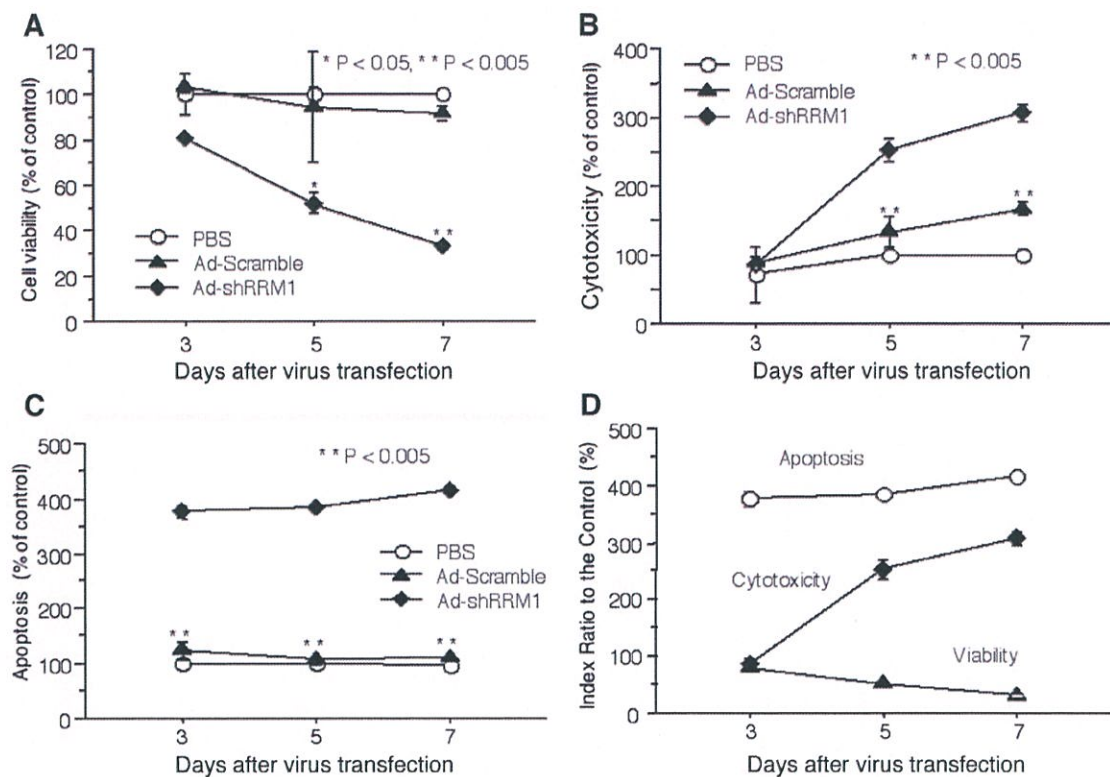
Fig. 5. Comprehensive analysis of cell viability, cytotoxicity and apoptosis using ApoTox-Glo™ Triplex Assay within a single assay well. (A) Cell viability, (B) cytotoxicity and (C) apoptosis in ribonucleotide reductase large subunit M1 (RRM1)-overexpression RERF-LC-MA cell after virus transfection in comparison with phosphate buffered saline (PBS)-treated control cells. (D) Comprehensive result of Ad-shRRM1-treated RERF-LC-MA cells. MOI, multiplicity of infection; $^*p < 0.05$, $^{**}p < 0.005$ versus Ad-Scramble treatment.

(up to 9 days after transfection, Fig. 3). Ad-shRRM1-mediated knockdown of RRM1 expression significantly inhibited *in vitro* cell proliferation (Fig. 3) and *in vivo* growth of tumours comprising lung cancer cells that overexpress RRM1 (Fig. 4A and B). These results provided further evidence that RRM1 is a highly appropriate target for cancer treatment. Previous studies also highlight the benefit of targeting RRM1 overexpression. Reid et al. first reported the effective inhibition of RRM1 using siRNA decreased cell proliferation [14]. And then, Wonganan et al. demonstrated *in vivo* inhibition of tumour growth in mice model with systemic administration of RRM1-specific siRNA [28]. RRM1 almost certainly affects tumourigenesis; indeed, high levels of RRM1 expression are associated with resistance to GEM in various cancers [10–13].

Furthermore, our data also indicated that RRM1 not only inhibited cell proliferation of RRM1-overexpressing cells, but it also significantly increased the apoptosis as indicated by a rapid increase in caspase 3/7 activity (Fig. 5). Thus, RRM1 seemed to be important for maintaining cell viability through promoting proliferation and inhibiting apoptosis. Regarding the role of RRM1 in cell proliferation, it is usually thought that inhibition of RRM1 results in depletion in deoxyribonucleoside diphosphate (dNDP); thereafter RRM1

inhibition affects DNA synthesis and repair. However, recent findings indicated that RRM1 has a critical role as an important regulatory factor. Other recent studies also describe a potential role of RRM1 in cell cycle regulation. Wang et al. reported that siRNA-mediated inhibition of RRM1 not only significantly reduced the dNTP pool, but also decreased Ras/Raf expression and MMP-9 activities and levels of p-MEK, p-ERK and NF-kappaB; together these effects result in growth retardation and reduced invasion in gastric cancer cells [15]. Taricani et al. also demonstrated genetic and physical interactions between RNR and CHK1 in mammalian cells [29]. Although our findings did not demonstrate changes in signalling from the Ras pathway (data not shown), there was no doubt that RRM1 had an important role in maintaining cell viability. Further study is necessary to explore the underlying mechanisms of RRM1 in regulating cell viability.

RRM1 is recognised as a key molecule in determining the efficacy of GEM. GEM is an analogue of deoxycytidine, which is phosphorylated by deoxycytidine kinase and further by a nucleoside monophosphate kinase, generating difluorodeoxycytidine 5′-diphosphate. This product binds to the substrate binding site and inactivates the RRM1 subunit, whereas the triphosphorylated form is incorporated into DNA [9,30,31]. Preclinical studies
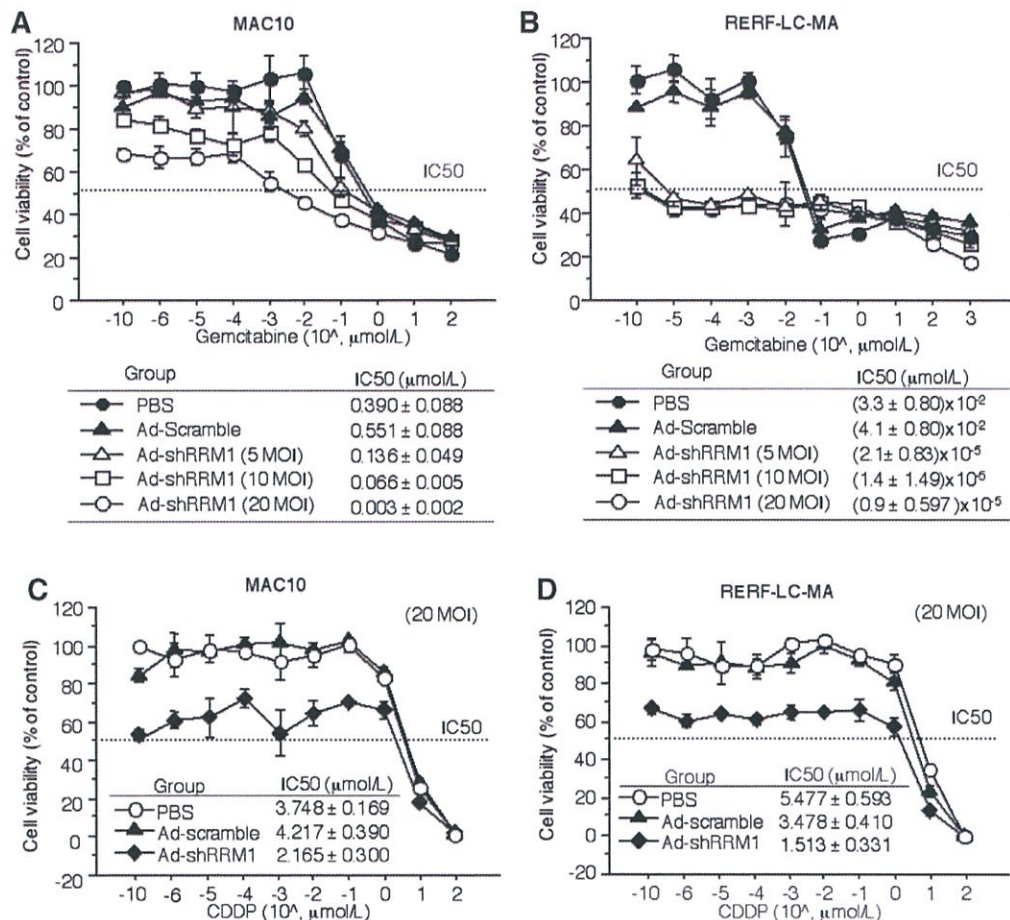
Fig. 6. Adenoviral vector that encoded a short hairpin siRNA targeting the ribonucleotide reductase large subunit M1 gene (Ad-shRRM1) specifically sensitises ribonucleotide reductase large subunit M1 (RRM1)-overexpressing cells to gemcitabine. $IC_{50}$ was evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay after transduction with adenoviral vectors, in comparison with phosphate buffered saline (PBS)-treated control cells. (A) Lung adenocarcinoma MAC10 cells, (B) Lung squamous cell carcinoma RERF-LC-MA cells. $IC_{50}$, drug concentration that inhibited cell growth by 50%; MOI, multiplicity of infection; CDDP, cisplatin.

show that RRM1 is important to GEM sensitivity in NSCLC [10,12]. Consistently, clinical studies suggest that overexpression of RRM1 is correlated with resistance to GEM-based therapies and, in part, responsible for poor survival of patients with NSCLC [16,32]. Moreover, in our recent clinical study, high expression of RRM1, which was investigated via immunohistochemistry, was a poor prognostic factor for cN2 or cN3 NSCLC among patients regardless of GEM treatment [20]. Overall survival was significantly lower among patients with RRM1-positive tumours than among those with RRM1-negative tumours. Although little controversy exists regarding the role of RRM1 in predicting GEM efficacy, a recent meta-analysis of 18 studies, which each evaluated a GEM-based regimen for patients with advanced NSCLC, verified that low/negative RRM1 expression in advanced NSCLC ($n = 1243$) was associated with a higher response rate to a GEM-containing regimen and with better prognosis [33].

In the present study, we demonstrated that Ad-shRRM1 sensitised RRM1-overexpressing lung cancer cells to GEM as indicated by the 10- to 100-fold decreases in the GEM $IC_{50}$ (Fig. 6). Inhibition of RRM1 with Ad-shRRM1 specifically increased the sensitivity to GEM, but not to cisplatin, in both types of lung cancer cells (Fig. 6C and D). This result was consistent with previous findings reported by Gerold et al. [34]. Especially for the squamous cell carcinoma cells, Ad-shRRM1 had very strong effects with regard to inhibiting cell proliferation and increasing sensitivity to GEM (Fig. 6B).

For most published studies on GEM resistance and RRM1, the cell lines used were artificially generated. Davidson et al. exposed each of two NSCLC cell lines (H358 and H460) to increasing concentrations of GEM *in vitro* to generate GEM-resistant cells, and a primary outcome in each case was increased RRM1 expression [10]. These results were confirmed in a subcutaneous mouse model of colon tumours (Colon

26) in which GEM resistance was generated via prolonged GEM exposure and serial transplantation [12]. However, induction of drug resistance through continuous exposure results in alterations involving multiple genes as demonstrated by these authors. In the present study, we used two lung cancer cell lines that each overexpressed RRM1 before exposure to GEM to avoid the influence mentioned above. Our results further confirmed the role of RRM1 as a major cellular determinant of the cytotoxic effects of GEM in NSCLC cells.

Many chemotherapeutic drugs of the cytotoxic or molecular-targeted type are used clinically to treat NSCLC adenocarcinomas; however, only few effective chemotherapeutic drugs are available for treatment of NSCLC squamous cell carcinomas worldwide. In the present study, Ad-shRRM1 provided similar efficiency in the adenocarcinoma and in the squamous cell carcinoma cell lines. Therefore, inhibition of RRM1 with Ad-shRRM1 may be a useful tool in treatments for patients with NSCLC, especially for those patients with squamous cell carcinoma; notably, Ad-shRRM1 could be used alone or together with GEM.

In summary, RRM1 not only has an important role in regulating cell viability, but it is also a critical target of the anticancer drug GEM. Inhibiting RRM1 with Ad-shRRM1 can kill two birds with one stone. Therefore, RRM1 may be a very good candidate target when developing new treatment strategies for patients with tumours exhibiting RRM1 overexpression. A prospective study is under consideration in our institute using the combined treatment of Ad-shRRM1 and GEM for patients who have GEM-resistant and RRM1 overexpression tumour. Ad-shRRM1 was demonstrated to have strong antitumour effects for RRM1-overexpressing NSCLC cells, and these effects included anti-proliferation and pro-apoptotic effects. Combination therapy with Ad-shRRM1 and GEM may become a new treatment for GEM-resistant NSCLC and especially for squamous cell carcinomas.

## Conflict of interest statement

None declared.

## Acknowledgement

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