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Improved Antitumor Effect of Survivin Responsive Conditional Replication Adenovirus in Combination with Cisplatin in Lung Cancer

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Abstract

Object: A chemotherapy drug such as cisplatin or diamminedichloroplatinum (DDP) is an alkylating agent that is widely used to treat many cancers despite its associated severe side effects. Biotherapy involving oncolytic adenoviruses also has proven anti-tumor efficacy. Survivin, an inhibitor of apoptosis is highly expressed in tumor cells. The aim of this study was to examine the synergistic effects of combined therapy including Survivin-responsive adenovirus and cisplatin in the treatment of lung cancer and to further reveal the mechanism involved.

Methods: Two lung cancer cell lines, NCI-H292 and NCI-H66, were obtained from the cell collections center at CAS-China. A Survivin-responsive conditionally replicating adenovirus (CRAd) with a deleted E1B region was developed in our laboratory. The anti-tumor efficacy of combined *in vitro* and *in vivo* treatment (DDP plus CRAd) was assessed with MTS assays and a subcutaneous mouse model, respectively. The expression of Coxsackie adenoviral receptor (CAR) on cancer cell surfaces was determined through RT-PCR analysis.

Results: The MTS assays revealed maximal tumor inhibition rates of 70% and 60% obtained when DDP and CRAd were used at doses of 64 μg/ml and 800 MOI, respectively. Nearly identical inhibition was observed with a combined treatment approach (DDP+CRAd) with lower doses of DDP (4 μg/ml) and CRAd (100 MOI). In vivo studies also revealed that tumor suppression was significantly higher in the combined treatment group. RT-PCR analysis showed that CAR expression was much higher in the combined treatment groups.

Conclusion: Our study indicates that the combined treatment approach, including survivin promoter-regulated CRAd and DDP, is therapeutically more effective against lung cancer not only because of the synergistic tumor-inhibition of the two treatments but also because of the additional tumor-specificity of CRAd resulting from Survivin promoter insertion.

Keywords: Lung cancer; Adenovirus; Cisplatin; Survivin; Conditional replication; Chemotherapy; CAR; Gene therapy

Introduction

Lung cancer is the second most common type of malignant tumor and the most common cause of cancer mortality [1]. Tobacco utilization is by far the strongest lung cancer risk factor, and leading to nearly 70% of deaths worldwide (1.59 million deaths in 2012) [2]. Among the two prominent subtypes of lung cancer, small-cell lung cancer (SCLC) is the most severe [3]. The distinguishing features of SCLC include a peculiar compact-cell structure with scarce cytoplasm and rampant growth.

Chemotherapy, radiation, and immunotherapy, as well as surgery, are globally well-recognized strategies for treating cancers. Cisplatin or DDP is a chemotherapeutic drug often used alone to treat lung cancer or in combination with other chemo-agents; however, severe side effects associated with chemo-toxicity and drug resistance issues have substantial effects on its clinical application [4]. Irrespective of higher initial therapeutic achievements, nearly all lung cancer patients have an early recurrence because of chemo-resistance and metastasis, thus keeping the five-year survival rate below 5% to 10% [4,5]. Combined treatments, including adenoviruses and chemotherapeutic agents such as cisplatin, are being researched to overcome the disadvantages of standard treatments to obtain improved results [6,7].

In recent years, extensive clinical verifications of the use of oncolytic adenoviruses as potent anticancer agents have been reported. Adenoviruses, owing to their broad cell targets, mild side effects, greater gene carrying capacities [8,9], high recombinant adenoviral particles per ml (10^{12} to 10^{13}), and comparatively lower potential for insertional mutation in host genes, are preferred not only as oncolytic agents but also as vectors for gene delivery in gene therapy [10]. The very first reported adenoviral agent, ONYX-015, has proven tumor-specific potency [11] but lacks a potency threshold that can support its use as a monotherapy to achieve long-term therapeutic goals in cancer treatment [12]. The role of coxsackievirus/adenovirus receptor (CAR) in adenoviral cell entry and the requirement of its high expression on tumor cells for successful gene therapy have been elucidated in many recent investigations [13]. After initial recognition and attachment to CAR on the cell surface, adenoviral internalization into coated endosomes is facilitated by viral pentone base motif interaction with integrin, αvβ3, and $\alpha v\beta 5$ [14]. The virus is then translocated into the nucleoplasm via a nuclear pore complex and is replicated there [15].

Conditional replication adenoviruses (CRAds) proliferate in and specifically lyse tumor cells [16]. CRAds, which acquire tumor selectivity either through gene deletions, as in Onyx-015 [17], or by induction of tumor-specific promoters, as survivin, hTERT, and prostate specific

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antigen [18], rely on CAR for proliferation; the expression of CRAds on cancer cell surfaces is scarce [19,20]. Modifications in the adenoviral tropism [21] and polylysine and heparin sulfate insertions in the fiber knob have sublimated the issue of limited CAR expression [17].

Among different tumor suppressor genes, the p53 gene is defective in many human cancers [22] and has been extensively used in cancer gene therapy. Normal cells exhibit very low p53 gene expression, and their activation and up-regulation require prior oncogenic activation [23]. Gendicin, the first gene therapy-based product treating head and neck carcinoma, was launched in China in 2003. It incorporates wild-type p53 genes into a replication incompetent adenovirus 5 [24]. Tumor-specific replication of oncolytic viruses based on the p53 gene has been criticized by many studies showing the presence of the functional p53 gene in tumors [25] and p53-independency of viral replication [26-31].

By virtue of its very high tumor-specific expression, non-detectability in normal cells, and broad anti-tumor spectrum, an inhibitor of apoptosis protein (IAP), Survivin, is being explored for use in gene therapy with a higher cancer targeting specificity [32,33]. Reports of transcriptional regulation of Survivin expression in cancerous cells [34,35] and genome-wide study (GWAS)-based evidence have placed survivin among the top five "transcriptomes" in cancer tissues with weak or undetectable expression in normal tissues of same organ [36] and have highlighted the potential of this protein in cancer treatment.

Many clinical investigations have positively evaluated the synergistic effects of combination therapies (gene therapy and chemo-therapy) [37,38]. Adenovirus in combination with chemotherapy has become an effective tool in cancer treatment [8]. We constructed CRAd incorporated with Survivin promoter (Sur-P) and sought to explore the therapeutic effect and mechanism of CRAd (Sur-P controlled) combined with cisplatin in the treatment of lung cancer both *in vitro* and *in vivo*.

Materials and Methods

Cell culture

The lung cancer cell lines NCI-H292 and NCI-H661 were procured from the Cell Collection Center, Shanghai (CAS-China); the adenoviral E1A-region containing HEK-293 cell line, used for multiplication of CRAd, was acquired from Microbix Biosystems Inc. The lung cancer cell lines H292 and H661 and the human embryonic kidney 293 cell line were cultured and propagated in RPMI-1640 medium containing 10% FBS (Gibco-BRL, HyClone); DMEM plus fetal bovine serum (10%) was used to culture other cells at recommended conditions for incubation (37°C, 5% CO_2).

Adenovirus preparation

Conditional replication adenoviruses (CRAds) that replicate majorly in cancer cells have been proven to be efficient anticancer agents. We developed a Survivin-responsive CRAd in which adenoviral E1A was regulated by the promoter of Survivin; additionally, the E1B region was deleted. The virus exhibited strong cancer-selective phenotypes without reduced anticancer effects. Ad-Luc expressing firefly luciferase gene was used as a control virus.

In vitro cell inhibition assay

Cells were seeded in 24-well plates at a density of 1×10^5 cells in each well. After 24 hours, each well was treated with different concentrations of DDP and CRAd viruses. Two treatment groups were prepared by adding both CRAd and cisplatin sequentially. Differently treated cells were transferred to a 96-well plate in three wells with 5×10^3 cells per well; cells were observed for 5 days with MTS/PMS reagents added at standard incubating conditions (37°C, 5% CO_2 for 180 minutes). Next, the absorbance at 490 nm was detected using a microplate reader.

Semi-quantitative reverse transcription-PCR

Total RNA was extracted from cells according to the manufacturer's procedure, by using TRIzol reagent (Ambion, life technologies); each sample was then converted to cDNA using an RT-reagent kit (TakaRa). The primers designed for CAR and GAPDH (control) were as listed below: CAR Forward: 5'-CCACCTCCAAAGAGCCGTAC-3', CAR Reverse: 5'-ATCACAGGAATCGCACCC-3'; GAPDH Forward: 5'-GATTGTTGCCATCAACGACC-3', and GAPDH Reverse: 5'-GTGCAGGATGCATTGCTGAC-3'. The expected PCR-product sizes of CAR and GAPDH were 218 bp and 371 bp, respectively. The amplified products were observed under UV-light via gel electrophoresis using 1 % agarose gel stained with ethidium bromide.

Tumor model

Six to eight-week-old female BALB/C nude mice were purchased from the Chinese Academy of Sciences. Ethical guidelines for the use of animals as experimental entities from the NIH were followed; H292 and H661 cells (4×10^6) were subcutaneously (SC) inoculated into the right flanks of the mice. Tumors were visible on the 15th day in H292 cells, but tumor formation in H661 cells was not significant.

In vivo tumor inhibition assay

H292 (8×10^6) and H661 (8×10^6) cells were inoculated subcutaneously into the right flanks of BALB/C nude mice. Nude mice with tumors were separated into three groups, each consisting six animals. The groups were treated as follows: (a) DDP treatment group (b) CRAd treatment group (c) DDP plus CRAd treatment group (cisplatin followed by CRAd); Ad-Luc virus transfected to each treatment group. Effects of treatments were evaluated by measurement of tumor volumes in one month.

Statistical analysis

Student's *t*-test was used to evaluate the statistical significance and data values are presented as with standard deviation. Data value at $p<0.05$ was considered statistically significant.

Results

Cisplatin dose optimization

We conducted an *in vitro* cell inhibition assay to optimize the dose of cisplatin (DDP) in human lung cancer cell lines, H292 and H661, by MTS/PMS assay prior to testing the combined treatment (DDP+CRAd). Both cell lines were treated with different DDP concentrations ranging from 0.25 μg/ml to 64 μg/ml and were observed for five days. The results showed that cisplatin inhibited H292 and H661 cell growth in a dose-dependent manner. Only ten percent inhibition was observed at a 0.25 μg/ml DDP dose, whereas maximum inhibition (76%) was observed at 64 μg/ml, as shown in Figure 1. Although the inhibitory rate in the H292 cell line was comparatively higher, no significant differences were found between the two cell lines. This experiment clearly verified DDP dose-dependent inhibition. However, higher chemo doses are accompanied by profound side effects and chemo resistances [39]. Our study of combined treatment also sought to provide significant therapeutic effects by using chemo-doses as low as possible.

Characterization of survivin responsive CRAd

Similarly, CRAds alone were tested for their cell-specific infection, cancer cell inhibiting potency, and optimum multiplicity of infection (MOI). Cancer cell lines were treated with different concentrations of CRAd such as 50, 100, 200, 400 and 800 MOIs. MTS/PMS assay revealed that CRAd significantly suppressed the growth of H661 cells, but growth inhibition in the H292 cell line was not significant. Figure 2 shows that at 100 MOI, almost 45% growth inhibition was observed in H661 cells, but to achieve the same level of growth inhibition in the H292 cell line, doses 10-fold higher or more were necessary. The major cause of this large difference may be the tissue-based differences in CAR expression on tumor cell surfaces, as reported by a previous study [40]. CRAd growth inhibition efficiency is directly related to its MOI; the higher the viral dose, the more pronounced the growth inhibition. The optimum MOI for CRAd in the lung cancer cell line H661 is 800, showing above 60% inhibition of cancer cells.

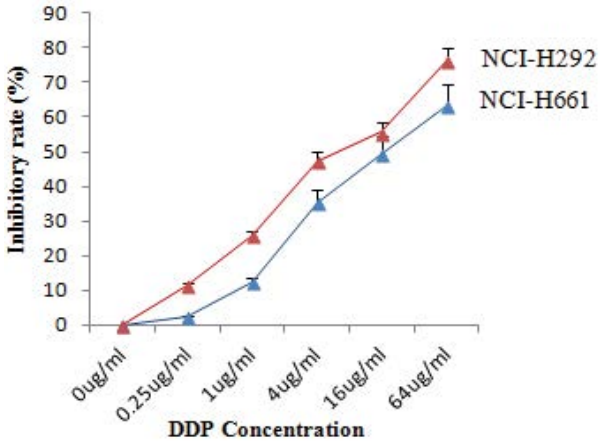

Figure 1: *In vitro* study for DDP-dose optimization. Both lung cancer cells lines, H292 and H661, were treated with different DDP concentrations ranging from 0.25 μg/ml to 64 μg/ml and were observed for five days before evaluation with MTS/PMS assay. Both cell lines exhibited comparable inhibition rates at identical cisplatin doses. The data shown above are the average of triplicate experiments. Error bars represent standard deviation (SD).

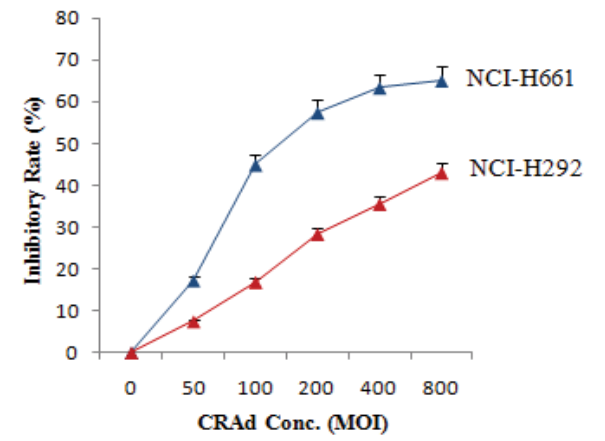

Figure 2: *In vitro* study for CRAd-dose optimization. Both lung cancer cells lines, H292 and H661, were treated with CRAd at different MOIs ranging from 50 to 800 and were then evaluated with MTS/PMS assays. Both cell lines exhibited significantly different inhibition rates at respective MOIs. The data shown above are the average of triplicate experiments. Error bars represent standard deviation (SD).

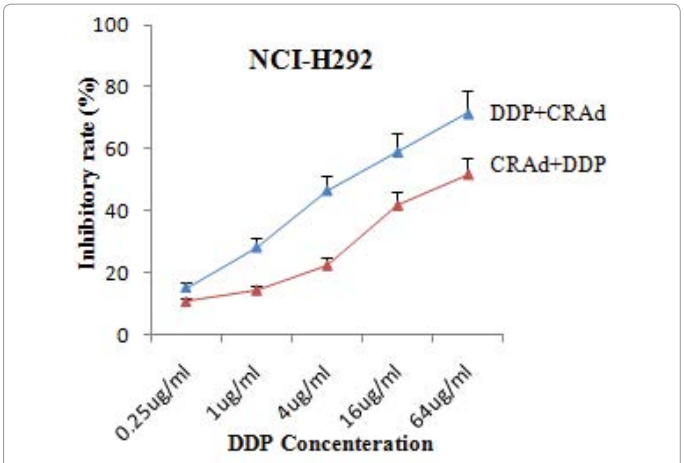

Figure 3: Inhibitory effects of combined treatment with different DDP-concentrations on NCI-H292. MTS/PMS assays reveal that lung cancer cell line H292 exhibit dose-dependent inhibition with DDP. Among two sequence approaches in combined treatment, DDP applied prior to CRAd (DDP+CRAd) showed higher inhibitory rates. At 64 μg/ml DDP-Conc, more than 70% inhibition was observed. The data shown above are the average of triplicate experiments. Error bars represent standard deviation (SD). Data value at $p<0.05$ was considered statistically significant.

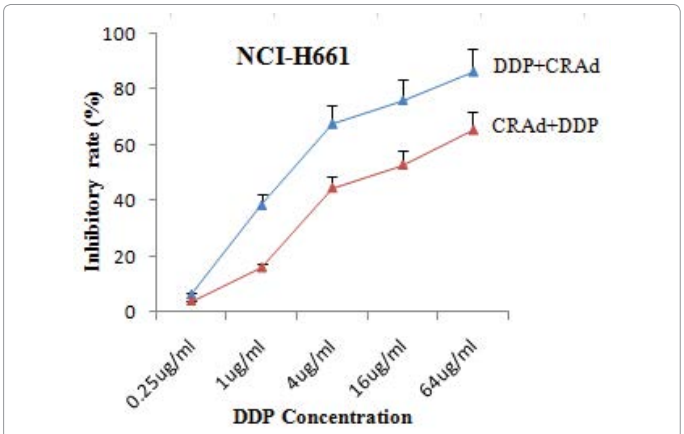

Figure 4: Inhibitory effects of combined treatment with different DDP-concentrations. MTS/PMS assays revealed that the lung cancer cell line H661 exhibited dose-dependent inhibition with DDP. Among two sequence approaches in combined treatment, DDP applied prior to CRAd (DDP+CRAd) showed higher inhibitory rates. At 64 μg/ml DDP-Conc, more than 80% inhibition was observed. The data shown above are the average of triplicate experiments. Error bars represent standard deviation (SD). Data value at $p<0.05$ was considered statistically significant.

We selected the 100 MOI dose of CRAd to test the efficiency of the combined treatment, CRAd+DDP.

Increased tumor suppression by combining CRAd with cisplatin

In vitro studies carried out with CRAd as a monotherapy indicated that 100 MOI was the optimal dose for infecting tumor cells (Figure 2). Thus, for *in vitro* combined treatment, we used a 100 MOI CRAd dose and observed its tumor inhibiting efficiency with various concentrations of cisplatin (0.25 μg/ml to 64 μg/ml). To evaluate whether changing the sequence in combined treatment would affect its efficiency, we used two sequential approaches for combined treatment: (a) using 100 MOI CRAd for four hours and then adding different concentrations of cisplatin for three hours; (b) using different concentrations of cisplatin

for three hours and then adding 100 MOI CRAd for four hours. Figures 3 and 4 indicate that both combined gene therapy and chemotherapy and sequential approaches improved the cancer cell killing potency. In both types of cancer cells, H292 and H661, a sequential approach (DDP followed by 100 MOI of CRAd) proved superior in its tumor inhibition ability. The results of *in vitro* experiments strongly favored our combined treatment approach because of its synergistic effects on tumor inhibiting capability (Figures 3 and 4).

Enhanced CAR expression on lung cancer cell surfaces in combined treatment

To explore the molecular mechanism underlying the enhanced anti-tumor efficiency of combined treatment (cisplatin+CRAd), we performed RT-PCR by using CAR-specific primers and GADPH as the internal control. As indicated in Figure 5, lanes 2 and 3 showed very high expression of CAR in the combined treatment in both cancer cell lines H292 and H661. Cisplatin sensitizes both lung cancer cell lines

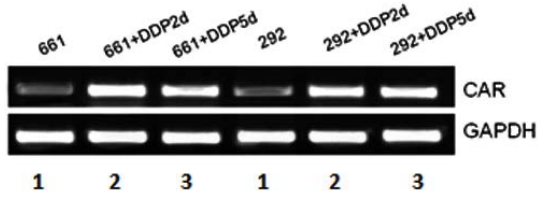

Figure 5: RT-PCR analysis of CAR expression. In both lung cancer cell lines (NCI-H292, NCI-H661), CAR mRNA expression was enhanced in the combined treatment groups (661+DDP, 292+DDP) in comparison to untreated cell lines (H661 and H292). GADPH was used as the internal control.

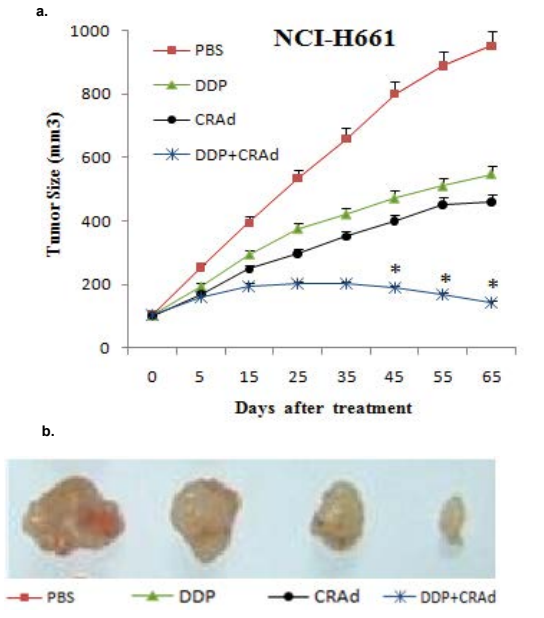

Figure 6: (a) In vivo antitumor effect of DDP+CRAd in female BALB/C nude mice inoculated with H661 cells (8×10^6). Four groups of tumor-bearing mice were injected intratumorally with PBS (4 mg/kg), CRAd (100 MOI) alone, and intraperitoneally with cisplatin (4 mg/kg), and cisplatin+CRAd (cisplatin first and CRAd later). A line graph was plotted to observe the antitumor efficacy using the average of tumor volumes measured with vernier calipers. Error bars represent standard deviation (SD). The combined treatment (DDP+CRAd) group showed the highest tumor suppression among all four groups. Data value at $p<0.05$ was considered statistically significant. **(b)** Solid tumor collected in one month of treatment. Combined treatment group (DDP+CRAd) showed maximum tumor size reduction.

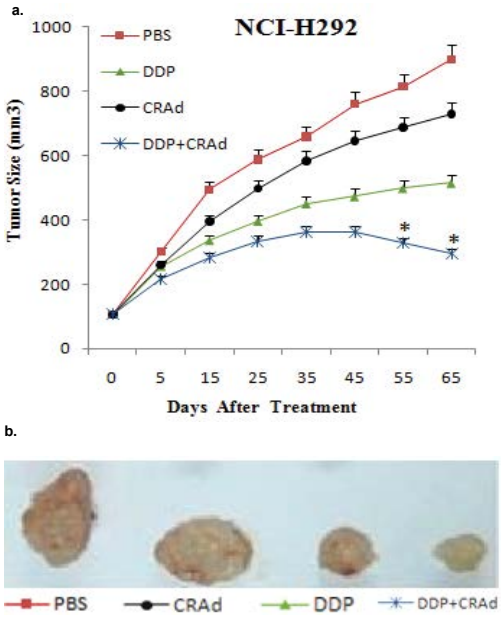

Figure 7: (a) In vivo antitumor effects of DDP+CRAd in female BALB/C nude mice inoculated with H292 cells (8×10^6). Four groups of tumor-bearing mice were injected intratumorally with PBS (4 mg/kg), CRAd (100 MOI) alone, and intraperitoneally with cisplatin (4 mg/kg), and cisplatin+CRAd (cisplatin first and CRAd later). A line graph was plotted to determine the antitumor efficacy using the average of tumor volumes measured with vernier calipers. Error bars represent standard deviation (SD). The combined treatment (DDP+CRAd) group showed the highest tumor suppression among all four groups. Data value at $p<0.05$ was considered statistically significant. (b) Solid tumor collected in one month of treatment. Combined treatment group (DDP+CRAd) showed maximum tumor size reduction.

for adenoviral transduction. Our RT-PCR results showing cisplatin enhanced expression of CAR were concordant with the results of previous studies that have successfully established a direct link between enhanced CAR expression and Adv transduction ability [25,41]

Anti-tumor efficacy of CRAd combined with cisplatin

In our *in vitro* studies, the synergistic effect of combined treatment (DDP+CRAd) on tumor inhibition, and the significant increase in cell killing efficiency of combined treatment with a sequential approach was firmly established. To further verify and strengthen our *in vitro* findings, we performed *in vivo* studies in lung cancer xenografts. Six- to eight-week-old female BALB/C nude mice were recruited and inoculated with 8×10^6 H292 and H661 cells via subcutaneous injection in the right flank. Measurements of tumor size in terms of volume showed that H292 cell lines formed substantial tumors, whereas tumor formation in H661 cells was weak. DDP doses greater than 4 μg/ml resulted in very strong tumor inhibitory effects but were accompanied by continuous weight loss in mice and ultimately in death in *in vivo* studies (data not shown). We used 4 mg of DDP/kg body weight as the optimum dose in our *in vivo* experiments to test the combined treatment efficacy. We divided tumor-bearing mice into four groups and injected them intratumorally with PBS or CRAd (100 MOI) alone and intraperitoneally with cisplatin (4 mg/kg), and cisplatin+CRAd (cisplatin first and CRAd later). A line graph was plotted to determine the *in vivo* antitumor efficacy of different treatments. As shown in Figures 6 and 7, the results of *in vivo* studies highlighted the sharp differences in anti-tumor efficacy between four treatment groups, in both cancer cell lines, with DDP plus CRAd treatment being the most effective tumor cell growth inhibitor in comparison with DDP or CRAd monotherapy.

Discussion

Although chemotherapy is the most commonly practiced strategy among different available anti-cancer treatments worldwide, it still faces challenges of severe side effects and resistance. Efforts to reduce and overcome these drawbacks have led researchers and clinicians toward gene therapy and other tools. Different tumor suppressor genes such as p53 have been manipulated. Adenoviral vectors, despite some usage-limiting factors including cell internalization, tumor cell specificity, and host immune response, have successfully proven their importance in gene therapy. Gendicine, the world's first gene therapy product to treat head and neck cancer, is commercially available in China and uses an adenoviral vector to deliver wild-type p53 genes. Despite the availability of p53-based cancer therapy, this treatment has faced criticism because many studies have discouraged cancer therapies depending on transcriptional activation of the wild-type p53 gene because it is less potent [27-29] and tumor-protective [30] because senescence is an equally important tumor suppression mechanism of p53 [31].

The tumor specificity of ONYX-015 based on a mutated or non-functional p53 gene is still under debate. Some studies have demonstrated that ONYX-015 replication is independent of p53 status [42], but its anti-tumor potential is clear. In our study, we constructed an adenovirus that is similar to ONYX-015 but was modified by incorporating the Survivin promoter (Sur-P) to make it more tumor specific via transcriptional targeting. The Survivin promoter has advantages over other promoters, such as alpha-fetoprotein (AFP) and cytomegalovirus (CMV) promoters; it is expressed in a wide variety of tumors and exhibits very high tumor specific adenovirus-mediated transgenic expression. It is over-expressed in tumor cells and repressed in normal adult tissues [43,44]. Our results demonstrated Sur-P-directed cancer-specific CRAd internalization, replication and subsequent tumor cell lysis with the minimal toxicity of normal cells. It is concordant with findings from many previous studies encouraging the use of Sur-P in cancer gene therapy [34,45-47].

Our experimental results showed that a combined therapeutic approach including cisplatin (DDP) and the oncolytic virus was more effective in the inhibition of tumor cells than the separate application of cisplatin or oncolytic virus; these results are in agreement with the results from many previous studies [48-50]. Adenoviral transduction is dependent on its interaction with the cell CAR [51]. CAR is a 46 kDa transmembrane protein that belongs to an immunoglobulin superfamily [40]. Many studies have reported the dependency of adenoviral transfection on CAR expression [39,52] and the scarcity of CAR expression on tumor cell surfaces [13,53,54]. RT-PCR analysis in our experimental study revealed that the cell surface receptor CAR expression was enhanced by cisplatin, similarly to findings from many previous studies [37,41,43,55].

In the current study, we combined biotherapy with chemotherapy. We engineered an oncolytic virus, CRAd, incorporating the Survivin promoter, whose possible use in gene therapy has been encouraged in many previous studies [43,47,56]. The aim of this study was to enhance the therapeutic index of chemo-gene therapy via a strong tumor-specific viral vector to minimize the toxicity toward normal cells and to further study the molecular mechanisms involved. Our treatment strategy included combining the chemo-agent cisplatin and Sur-P-regulated CRAd such that cisplatin was injected before CRAd; this strategy proved very effective in treating lung cancer. A synergistic trend in tumor growth inhibition was observed after combining two different anti-cancer treatments; this result is concordant with the results of many recent studies [49,50]. The survival rate of mice was increased, and more pronounced tumor inhibitory effects were achieved, probably as a result of lower doses of cisplatin and tumor-specific CRAd replication and subsequent lysis attributed to Survivin promoter.

To our knowledge, this is the first investigation using a Survivin promoter controlled oncolytic virus (CRAd) in gene therapy for lung cancer. This study provides ample evidence for a promising agent, the Survivin promoter, to be incorporated into gene therapy for treatment of not only lung cancer but also other cancers, owing to its high expression in many tumors.

In conclusion, this study revealed that a combined treatment approach, compared with monotherapy, including conditional replication adenovirus and cisplatin has a higher potential of inhibiting lung cancer growth and metastasis *in vitro* and *in vivo*. Furthermore, this treatment strategy has an advantage of being safe because of the additional tumor-specificity of oncolytic virus resulting from the incorporated Survivin promoter and the lower doses of cisplatin used. The results of this study encourage further investigation of conditional replication adenovirus inserted with Survivin promoter because it has the potential to alleviate some if not all of the problems encountered with gene therapy using adenoviral vectors.

Acknowledgements

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