Combination therapy with VEGFR2 and EGFR siRNA enhances the antitumor effect of cisplatin in non-small cell lung cancer xenografts

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Abstract. RNA targeting the murine vascular endothelial growth factor receptor 2 (VEGFR2) gene was designed and validated for efficient and robust silencing *in vitro* and was delivered by polyethylenimines (PEI) *in vivo* to investigate the antitumor effect on non-small cell lung cancer (NSCLC) xenografts. The following dosage regimens were tested for their tumor inhibitory effect *in vivo*: VEGFR2 siRNA, epidermal growth factor receptor (EGFR) siRNA, VEGFR2 siRNA+EGFR siRNA, cisplatin alone and VEGFR2 siRNA+ EGFR siRNA+cisplatin. Targeted silencing of both VEGFR2 and EGFR expression by siRNA, combined with low-dose cisplatin, was found to effectively inhibit tumor growth and extend the survival time of mice bearing the NSCLC xenografts. These results suggest that combination therapy using siRNAs and chemotherapy agents might offer a novel strategy for cancer treatment in the future.

Introduction

Without neovascularization tumors are typically restricted in growth to a small mass not more than approximately 0.4 mm in diameter (1). In order to ensure the oxygen and nutrients essential for tumor growth and spread, tumor cells secrete pro-angiogenic factors designed to induce the angiogenesis

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and vasculogenesis by binding their related tyrosine kinase receptor (2). Vascular endothelial growth factor (VEGF) is one of the most well studied pro-angiogenic factors overexpressed in many types of cancer cells (3). Vascular endothelial growth factor receptor 2 (VEGFR2), which is primarily expressed in the endothelial cells (ECs) of the neovasculature (4), is the major tyrosine kinase receptor of VEGF. During angiogenesis, VEGF combines with its cognate receptor tyrosine kinase, VEGFR2 (also known as KDR and FLK1), and activates multiple downstream pathways via signaling intermediates, such as mitogen-activated protein kinases (MAPKs), phosphoinositide 3-kinase (PI3K), AKT, phospholipase Cγ and small GTPases (5).

Epidermal growth factor receptor (EGFR) and its ligand, epidermal growth factor (EGF), also play critical roles in the development of cancer through their effects on cellular proliferation, apoptosis, angiogenesis and metastasis (6). As one type of pro-angiogenic factor, EGFR is overexpressed in epithelial cells of many tumors and provides an essential survival signal to tumor cells by activating the expression of anti-apoptotic Akt, MAPKs, Jak/Stat and protein kinase C (7,8). In addition, knockdown of the EGFR expression in the tumor cell has shown direct or indirect anti-angiogenesis and tumor inhibitory effects (9).

The pivotal roles in the progression of cancer and angiogenesis that EGFR and VEGFR2 play make them attractive targets for anticancer therapy (10). Treatment strategies, by blocking the pathways of either VEGF/VEGFR2 or EGF/EGFR, have produced modest objective responses in clinical trials, and have failed to improve long-term patient survival (11,12). Furthermore, monotherapy approaches, including anti-angiogenesis monoclonal antibodies, or tyrosine kinase inhibitors (TKIs), did not show any survival advantage over chemotherapy alone in patients (13). Markedly, prolonged survival of patients up to five months was only achieved when bevacizumab was combined with traditional chemotherapy (14). This success indicated that the co-administration of cytotoxic drugs (chemotherapy agents) and anti-angiogenesis therapy yields maximal benefits, due to the dual-inhibition of the growth of both tumor and endothelial cells (15). Another presumption of this synergetic antitumor effect was that the normalization of the vascular conducted by

anti-angiogenesis therapy subsequently lowered the pressure inside the tumor, which increased the delivery of cytotoxic drugs to the tumor more effectively (15).

Therefore, siRNA, having the ability to knock down or 'silence' overexpressed or mutated genes involved in the development of cancer, is a powerful antitumor drug candidate. Several RNAi based therapies are under evaluation in the clinical trials (16). The first clinical trial of human cancer therapy based on systemically delivered siRNA by transferrin-tagged, cyclodextrin-based polymeric nanoparticles, has recently been launched (CALAA-01; Calando Pharmaceuticals, Pasadena, CA, USA; phase I) (17). However, the delivery system for siRNA therapy remains the major hurdle in this type of approach (18). Commercially available branched cationic polymers polyethylenimines (PEI) have been utilized to improve the efficiency of *in vivo* siRNA delivery by complexing the negatively charged siRNA to form non-covalent nanoparticles (19). The positively-charged PEI-siRNA complexes protect the siRNA from the degradation, enable effective uptake by cellular endocytosis mechanisms and facilitate subsequent siRNA released from endosomes based on the proton-sponge effect (20).

To explore a potentially novel therapeutic cancer regimen and minimize the side-effects of cytotoxic drugs, we examined the *in vivo* antitumor effects of cisplatin combined with siRNA aimed at silencing the expression of VEGFR2 or EGFR and mediated by PEI complexes. Both receptor tyrosine kinases (VEGFR2 or EGFR) were found to be overexpressed in human non-small cell lung cancer (NSCLC) and closely correlated with the malignant disease progression (21). Herein, siRNA targeting murine VEGFR2 was designed, validated and found to be robust in silencing the expression of VEGFR2 mRNA *in vitro*. Next, the antitumor effects of cisplatin alone, or combined with siRNAs targeting VEGFR2 and EGFR, were investigated and compared in murine A549 NSCLC tumor xenograft models. Finally, siRNA targeting both VEGFR2 and EGFR when combined with cytotoxic chemotherapy resulted in profound antitumor effects and prolonged the survival of mice bearing the tumor xenografts and may, therefore, provide new insight into future clinical anticancer treatments.

Materials and methods

Cell lines and culture. A549 cells (from ATCC) were grown in RPMI-1640 (Gibco, China) medium supplemented with 10% fetal calf serum (FCS; Gibco). MS1 cells (MILE SVEN 1 cells; ATCC) were maintained in high glucose DMEM (Gibco) supplemented with 10% FCS. Cell cultures were maintained at 37°C in a humidified atmosphere containing 5% CO_2. Cells were propagated by passage of 1 mM EDTA and 0.25% trypsin-dispersed cells.

siRNA sequence. Based on the design principles of siRNA made by Tuschl *et al* (22), three pairs of VEGFR2/Flk-1 siRNA sequences were designed according to the mouse mRNA sequence (NM_010612). The sequences of VEGFR2 siRNA4 and EGFR siRNA were from Schiffelers *et al* and Weihua *et al*, respectively (23,24). A scrambled siRNA was used as a control. All the sequences were synthesized by RiboBio Co., Ltd., (Guangzhou, China) and are shown in Table I.

Transfection experiments. For *in vitro* tests, siRNAs were delivered to MS1 cells using Lipofectamine™ 2000 (Invitrogen) according to the manufacturer's instructions. Briefly, cells were seeded in a 6-well plate at a density of 5×10^4 cells/well 24 h before transfection. siRNA complexes were added to cells when cultures reached 50% confluence at a final concentration of 50 nM in the absence of serum. Following incubation at 37°C for 4 h, the culture medium was replaced with 2 ml of fresh medium supplemented with 10% fetal bovine serum. Cells were cultured under standard conditions for a further 48 h before being examined by semi-quantitative RT-PCR or quantitative RT-PCR. For western blot analysis, cells were cultured for a further 72 h.

siRNA/PEI complex. For *in vivo* experiments, siRNAs were encapsulated by branched PEI (Mw 25 kDa, Sigma Aldrich) with an N/P ratio =8 (the molar ratio of nitrogens in the PEI to the ratio of phosphates in the siRNA). Theoretically, 1 μg of siRNA (Mw 13,300 g/mol) contained 3 nmol of phosphate, and 1 μg PEI contained 10 nmol of amine nitrogen. Briefly, PEI was diluted in HEPES buffer at pH 7.4 to make stock solution with a concentration of 1.02 mg/ml. Then, 0.5 nmol (6.5 μg) of siRNA was dissolved in 0.05 ml of HEPES buffer and incubated for 10 min, and appropriate amounts of PEI solution (15.65 μg) were pipetted to the siRNA solution resulting in the desired N/P ratio (N/P ratio=8). The mixtures were vortexed for 5 sec and incubated at room temperature for at least 20 min to allow complex formation. The final complex was prepared immediately before intratumoral injection.

Semi-quantitative RT-PCR. Total-RNA was extracted from cells 48 h after transfection by the TRIzol reagent (Invitrogen). Total-RNA (1 μg) was reverse-transcribed into cDNA by ReverTra Ace-α-® cDNA Synthesis kit (Toyobo). The RT products were then amplified using Takara Taq™ DNA polymerase (Takara). For normalization of RNA loading, β-actin was also amplified from each sample. The expression of VEGFR2 mRNA in MS1 cells was analyzed by PCR using the following primers: forward, 5'-AGAACACCAAAAGAGAGGAACG-3'; reverse, 5'-GCACACAGGCAGAAAGTAG-3'. Primers for β-actin mRNA amplification were: forward, 5'-CAACT GGGACGATATGGAGAAG-3', reverse 5'-TCTCCTTCTGC ATCCTGTCAG-3'. PCR was carried out at 95°C for 2 min, followed by 20 cycles of 30 sec at 94°C, 30 sec at 54°C and ended with 1 min at 72°C. The PCR products were analyzed by 1.2% agarose gel (containing 0.05% ethidium bromide), photographed under UV light and quantified by ImageJ software. The data are presented as the ratio of the expression level of interest protein to that of β-actin expression.

Quantitative real-time PCR. The mRNA expression levels of VEGFR2 and EGFR in tumors were determined by two-step quantitative real-time reverse transcription PCR. Total-RNA was isolated from tumor tissue using the TRIzol reagent (Invitrogen) according to the manufacturer's instructions. For cDNA synthesis, 1 μg of extracted RNA was transcribed using the ReverTra Ace-α-® cDNA Synthesis kit (Toyobo). Real-time monitoring of PCR amplification of the cDNA was performed in a Real-Time PCR Detection System (Bio-Rad Laboratories) using Real Master Mix (SYBR-Green) (Tiangen,

Table I. Sequences of siRNAs used in the study.

Name	Sequence (5'-3')
VEGFR2-siRNA1	Sense strand: 5'-CCGGAAAUCUGGAGAAUCAdTdT-3' Antisense strand: 3'-dTdTGGCCUUUAGACCUCUUAGU-5'
VEGFR2-siRNA2	Sense strand: 5'-CGGAGAAGAAUGUGGUUAAdTdT-3' Antisense strand: 3'-dTdTGCCUCUUCUUACACCAAUU-5'
VEGFR2-siRNA3	Sense strand: 5'-CGGAUGAUCAAGAGAAAUAdTdT-3' Antisense strand: 3'-dTdTGCCUACUAGUUCUCUUUAU-5'
VEGFR2-siRNA4	Sense strand: 5'-GCUCAGCACACAGAAAGACdTdT-3' Antisense strand: 3'-dTdTCGAGUCGUGUGUCUUUCUG-5'
EGFR siRNA	Sense strand: 5'-CUGACUCCGUCCAGUAUUGAUdTdT-3' Antisense strand: 3'-dTdTGACUGAGGCAGGUCAUAACUA-5'
Control siRNA	Sense strand: 5'-CGUGAUUGCGAGACUCUGAdTdT-3' Antisense strand: 3'-dTdTGCACUAACGCUCUGAGACU-5'

siRNA, small interfering RNA; VEGFR2, vascular endothelial growth factor receptor 2; EGFR, epidermal growth factor receptor.

Beijing, China). Primers used for VEGFR2 amplification were the same as previously mentioned. Primers used for EGFR amplification were: forward, 5'-AGGACCAAGCA ACATGGTCA-3'; reverse, 5'-CCTTGCAGCTGTTTTCA CCT-3'. Levels of the GAPDH housekeeping gene mRNA were determined as control with the following primers: forward, 5'-GAACGGGAAGCTCACTGG-3'; reverse, 5'-GC CTGCTTCACCACCTTCT-3'. Each reaction was carried out at 95°C for 2 min, followed by 40 cycles of 95°C for 10 sec and 60°C for 30 sec. The levels of VEGFR2 or EGFR mRNA were normalized to that of GAPDH mRNA after the confirmation that the amplification efficiency of the two genes was comparable.

Western blot analysis. Cell suspensions were homogenized in lysis buffer with protease inhibitors (Nanjing KeyGen Biotech. Co., Ltd., Nanjing, China). The samples were subjected to electrophoresis on 10% SDS-polyacrylamide gels and transferred to a polyvinylidene difluoride membrane (Millipore). After blocking with 5% non-fat milk at 4°C overnight and washing three times with a 0.1% Tween-20 solution in Tris-buffered saline (TBST), the membranes were incubated with a primary monoclonal anti-mouse VEGFR2/Flk-1 antibody (R&D Systems) at 4°C overnight. The membranes were then washed and incubated with HRP-conjugated secondary donkey anti-rat IgG (H+L) antibody (Proteintech Group, Inc.), and detected with Novex® ECL Chemiluminescent Substrate Reagent (Invitrogen). Then, the membranes were stripped and reprobed for detection of β-actin with rabbit anti-β-actin antibody (Abmart, Shanghai, China) and developed as above following incubation with the goat anti-rabbit secondary antibody (Boster, Wuhan, China). For the detection of VEGFR2 protein expression levels, tumor tissues were homogenized then centrifuged and the supernatants were collected. Equal amounts of protein were subjected to western blot analysis as described above. Briefly, membranes were incubated with primary monoclonal anti-mouse VEGFR2/Flk-1 (Abcam)

antibody at 4°C overnight. The membranes were washed three times in TBST and incubated with the corresponding HRP-conjugated secondary antibody, as mentioned above, and then developed for the blots. Next, the membranes were stripped for the detection of β-actin and developed as above. Finally, the density of bands were scanned and the ratio of band intensities for VEGFR2, which were normalized to that of β-actin, were calculated using ImageJ software.

NSCLC A549 tumor xenograft model and growth inhibition. *In vivo* experiments were carried out at the Center of Experimental Animals of Sun Yat-sen University (Guangdong, China), according to standardized animal care guidelines, and the mice were housed in pathogen-free conditions. This study was approved by the Center of Experimental Animals of Sun Yat-sen University (permit no. SYXK2007-0081). A549 cells were harvested by trypsinization, washed twice in PBS, centrifuged and re-suspended in PBS. A total of $5x10^6$ cells in 0.2 ml of buffered saline were injected s.c. into the flank region of athymic nude mice (BALB/C, nu/nu, 4-6 weeks) and tumors were allowed to grow for 10 days. The tumor volume was measured with a caliper every three days and calculated by the formula: Volume = 1/2 x length x width^2, where length represented the longest tumor diameter and width represented the shortest tumor diameter (25). When tumor volume reached 30-100 mm^3, the animals were randomized into different groups for therapy testing. Each treatment group consisted of four to five mice. Mice were injected with: i) saline (intratumorally, twice a week); ii) PEI alone (intratumorally, twice a week); iii) 0.5 nmol VEGFR2 siRNA-PEI complexes (intratumorally, twice a week); iv) 0.5 nmol EGFR siRNA-PEI complexes (intratumorally, twice a week); v) 0.5 nmol siControl-PEI complexes (intratumorally, twice a week); vi) (0.25 nmol VEGFR2 siRNA + 0.25 nmol EGFR siRNA)-PEI complexes (intratumorally, twice/week); vii) 5 mg/kg cisplatin (intraperitoneally, once a week); and viii) (0.25 nmol VEGFR2 siRNA + 0.25 nmol EGFR siRNA)-PEI complexes + 3 mg/kg cisplatin (siRNA,
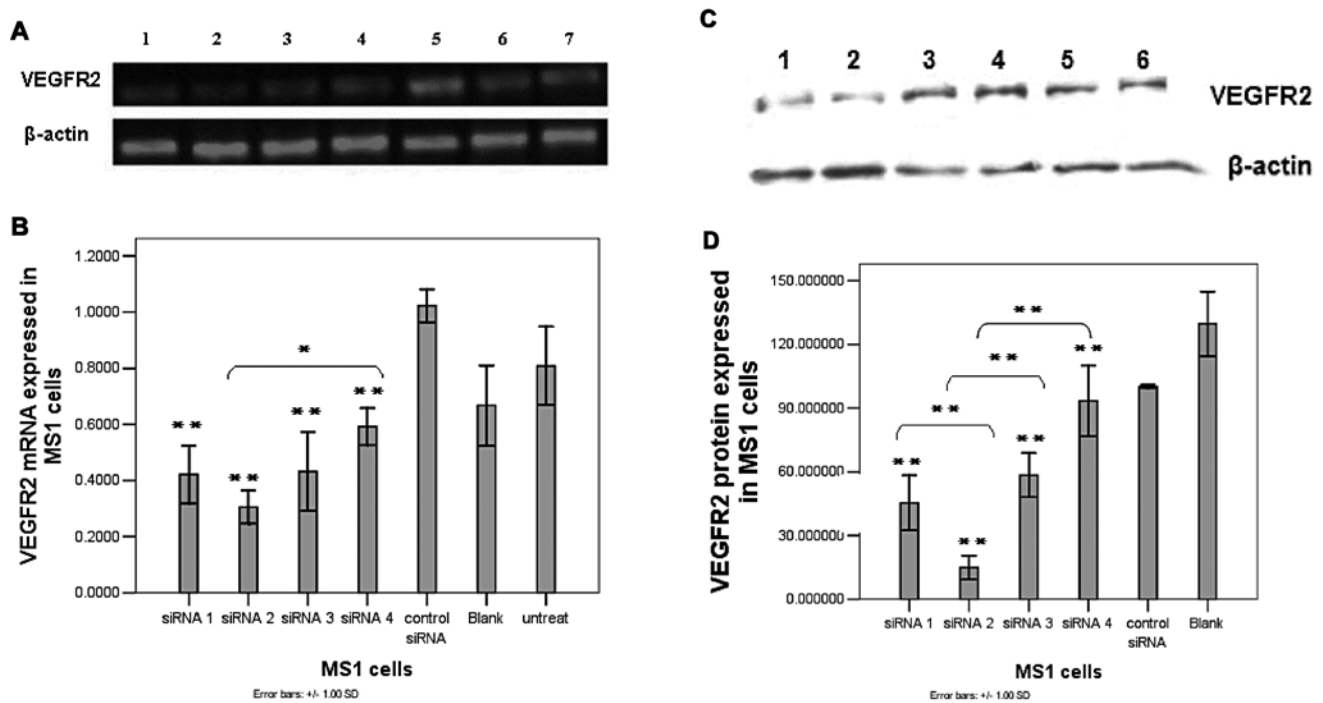
Figure 1. siRNA significantly reduces VEGFR2 expression of both mRNA and protein in MS1 cells. (A) Agarose gel electrophoresis of semi-quantitative RT-PCR. Lane 1, siRNA 1; lane 2, siRNA 2; lane 3, siRNA 3; lane 4, siRNA 4; lane 5, control siRNA; lane 6, blank (Lipofectamine™ 2000 only); lane 7, untreated (normal MS1 cells). Representative bands are shown from 3 experiments. (B) Downregulation of VEGFR2 mRNA in MS1 cells determined by semi-quantitative RT-PCR. (C and D) Western blot analysis of VEGFR2 in MS1 cells transfected with siRNA. β-actin was also amplified as an internal control. Columns, mean; bars, SD. *P<0.05; **P<0.01, vs. the control siRNA-treated group.

intratumorally, twice a week; cisplatin, intraperitoneally, once a week, one day after the first delivery of siRNA). siRNAs were complexed with PEI prior to injection as previously described. The animals were sacrificed when they became moribund or when the experiment was terminated and the tumors were carefully dissected and snap frozen for RNA, protein and immunohistochemical analysis.

Statistical analysis. All descriptive statistics, including the means ± SD, were performed. For parametric variables in the experiments, ANOVA was used along with Fisher's least-significant difference (LSD) or Dunnett's T3 method, depending on the homogeneity of variance using the software program SPSS 13.0. Kaplan-Meier curves were used for *in vivo* experiments and the log-rank test was used for statistical comparisons. All of the analyses were two-tailed. A P-value of <0.05 was considered to indicate statistically significant difference and a P-value of <0.01 was considered to indicate very significant difference. Each experiment was repeated three times.

Results

Reduction of both mRNA and protein expression of VEGFR2 in MS1 cells mediated by siRNA. Four siRNA sequences targeting VEGFR2 were assessed for silencing efficiency *in vitro*. Three of the siRNA sequences (siRNA1-3) were designed according to the mouse mRNA sequence (NM_010612) and based on the siRNA design made by Tuschl *et al* (22) and one siRNA (sequence 4) was published previously (27). To evaluate gene silencing, VEGFR2 siRNA (100 pmol) and a scrambled siRNA control were transfected into MS1 cells overexpressing

VEGFR2 and the VEGFR2 mRNA expression level was determined 48 h later by semi-quantitative reverse transcription PCR. Agarose gel electrophoresis showed that the expression of VEGFR2 mRNA was markedly dowregulated in response to the siRNA treatment. The analysis of semi-quantitative reverse transcription PCR revealed an approximate 60-75% reduced level of VEGFR2 mRNA mediated by siRNA2, compared to that of the 50-60% reduction by siRNA1 or 3, and ~40% reduction by siRNA4, respectively. However, the cells treated with control siRNA showed a high level of VGFR2 mRNA compared to that in untreated MS1 cells (Fig. 1). In addition, all the VEGFR2 mRNA expression levels were compared to expression levels in control siRNA-treated cells (Fig. 1B) to confirm siRNA specificity.

The downregulation of mRNA expression was translated into reduced VEGFR2 protein levels, as determined by western blot analysis 72 h after transfection of siRNA (Fig. 1C and D). Compared to control siRNA, siRNA2 could significantly silence VEGFR2 protein expression by 85% *in vitro*. The reduced expression of the VEGFR2 protein in MS1 cells mediated by siRNA1 and 3 was 55 and 42%, respectively. However, no significant decline of VEGFR2 protein expression was observed in siRNA 4-transfected cells compared to that in control siRNA-transfected cells. Furthermore, the reduced VEGFR2 protein expression results directly reflected the mRNA knockdown data in a siRNA sequence-dependent manner, with siRNA2 standing out as most effective. Therefore, siRNA2 was chosen for the subsequent tumor inhibitory experiments *in vivo* based on its robust capacity to decrease both the mRNA and protein expression levels of VEGFR2 in MS1 cells.
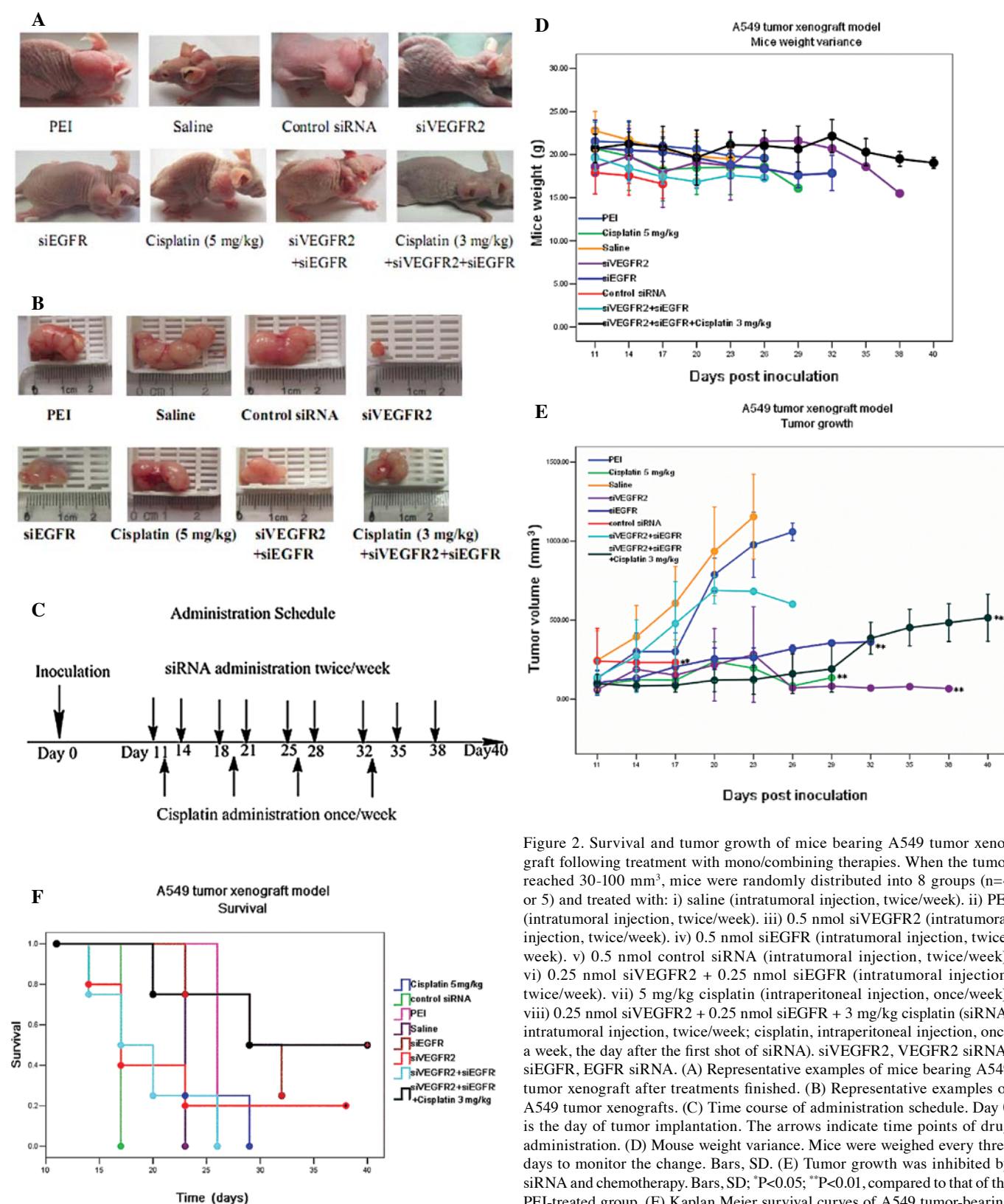
Figure 2. Survival and tumor growth of mice bearing A549 tumor xenograft following treatment with mono/combining therapies. When the tumor reached 30-100 mm³, mice were randomly distributed into 8 groups (n=4 or 5) and treated with: i) saline (intratumoral injection, twice/week). ii) PEI (intratumoral injection, twice/week). iii) 0.5 nmol siVEGFR2 (intratumoral injection, twice/week). iv) 0.5 nmol siEGFR (intratumoral injection, twice/week). v) 0.5 nmol control siRNA (intratumoral injection, twice/week). vi) 0.25 nmol siVEGFR2 + 0.25 nmol siEGFR (intratumoral injection, twice/week). vii) 5 mg/kg cisplatin (intraperitoneal injection, once/week). viii) 0.25 nmol siVEGFR2 + 0.25 nmol siEGFR + 3 mg/kg cisplatin (siRNA, intratumoral injection, twice/week; cisplatin, intraperitoneal injection, once a week, the day after the first shot of siRNA). siVEGFR2, VEGFR2 siRNA; siEGFR, EGFR siRNA. (A) Representative examples of mice bearing A549 tumor xenograft after treatments finished. (B) Representative examples of A549 tumor xenografts. (C) Time course of administration schedule. Day 0 is the day of tumor implantation. The arrows indicate time points of drug administration. (D) Mouse weight variance. Mice were weighed every three days to monitor the change. Bars, SD. (E) Tumor growth was inhibited by siRNA and chemotherapy. Bars, SD; *P<0.05; **P<0.01, compared to that of the PEI-treated group. (F) Kaplan Meier survival curves of A549 tumor-bearing mice. *P<0.05; **P<0.01, compared to that of the PEI-treated group.

Low dose of cisplatin combined with siRNA markedly reduce tumor growth on the well-established human NSCLC xenografts. To test *in vivo* tumor growth inhibition mediated by siRNA combined with low dose chemotherapy, A549 NSCLC xenograft mouse models were established. VEGFR2 siRNA2 (siVEGFR2) (0.5 nmol) (6.5 µg) and PEI (15.65 µg) was freshly complexed as described above with an N/P ratio of 8, and as one dose for each injection. Mice (n=5) were treated twice a week via intratumoral injection with siVEGFR2/PEI complex and received a total of nine administrations (Fig. 2C).

Table II. Adverse effects observed in the experiment *in vivo*.

Groups	Scurf	Purple skin	Itch	Wound	Diarrhea	Weakness
PEI-treated n=4	75%	None	None	25%	None	None
Saline-treated n=4	75%	None	None	None	None	None
siControl-treated n=4	None	75%	None	None	100%	100%
siVEGFR2-treated n=5	100%	60%	40%	20%	100%	60%
siEGFR-treated n=5	None	None	None	20%	None	None
Cisplatin-treated n=5	80%	80%	None	None	100%	100%
siVEGFR2+ siEGFR-treated n=4	100%	None	25%	50%	75%	None
siVEGFR2+ siEGFR+ cisplatin(3 mg/kg)-treated n=4	100%	25%	25%	50%	50%	None

siControl, control siRNA; siVEGFR2, VEGFR2 siRNA; siEGFR, EGFR siRNA. siRNA, small interfering RNA; VEGFR2, vascular endothelial growth factor receptor 2; EGFR, epidermal growth factor receptor; PEI, polyethylenimines.

As shown in Fig. 2A and B, siVEGFR2 could significantly inhibit the growth of s.c. tumor xenografts with the reduction of tumor volume by 77% compared to that in the mice treated by PEI alone (P<0.01). The mean survival time of the siVEGFR2-treated animals was 17 days and only one in five mice survived the experiment (40 days), which may result from the high incidence of side-effects caused by the siRNA (Fig. 2F). In addition, marked weight loss, up to 20%, was another factor contributing to the higher mortality of tumor-bearing mice.

To test whether or not siEGFR could inhibit the tumor growth, freshly prepared siEGFR/PEI complex, containing 0.5 nmol (6.5 µg) siEGFR and PEI (15.65 µg) per injection dose was intratumorally injected twice a week in A549 tumor bearing mice. Similar tumor growth inhibition to that mediated by siVEGFR2 was observed in the siEGFR-treated animals and was significantly different to results found in the PEI-treated animals (P<0.01) (Fig. 2A, B and E). However, the tumor growth inhibitory effect was not found to be significantly different between the siEGFR and siVEGFR2-treated mice. Of note, siEGFR treatment alone resulted in increased survival with a median survival of 29 days. In addition, siEGFR treatment was well tolerated and was associated with fewer adverse events, such as decreased weight loss, in the tumor bearing mice (Fig. 2D and F).

We also examined the *in vivo* antitumor effect of combined therapy with both siVEGFR2 and siEGFR. Due to the drug toxicity, a half dose of 0.5 nmol siRNA was adopted. For the combined siRNA therapy, 0.25 nmol siVEGFR2 and 0.25 nmol siEGFR were mixed with 15.65 µg PEI (N/P=8) for each injection. The siRNA/PEI complex was prepared and intratumorally injected twice a week. At this dosage no adverse effects or notable weight loss was observed in the siVEGFR2+siEGFR treated mice (Fig. 2D), although no signification reduction in the tumor volume or improvement in median survival was found either (Fig. 2E and F). In fact, the median survival time in this group was 17 days which was shorter than that of the siEGFR treated mice (Fig. 2F).

In another cohort of mice bearing A549 tumors, standard cisplatin chemotherapy (5 mg/kg, i.p) (26) was administered once a week (Fig. 2C) and tumor growth was well inhibited by this dosage regimen when compared to PEI-or saline-treated

mice (P<0.01) (Fig. 2E). In spite of the positive effect on tumor inhibition, severe side-effects of the drug were evident in the mice, leading to marked body weight loss and a median survival time of 23 days (Fig. 2D and F). Furthermore, no significant difference in tumor growth inhibition or survival time was observed between the cisplatin-treated mice and the siVEGFR2-treated mice, or between the cisplatin- and the siEGFR-treated mice.

To investigate whether dual-blockade of EGFR and VEGFR2 expression could enhance the antitumor effect of chemotherapy, cisplatin was administrated in combination with siEGFR and siVEGFR2. For this group of mice (siVEGFR2+siEGFR+cisplatin), 0.25 nmol of siVEGFR2 plus 0.25 nmol of siEGFR were complexed with 15.65 µg PEI (N/P=8) as one injection dose followed the next day by an i.p. dose of 3 mg/kg cisplatin (Fig. 2C). Treatment was discontinued two days before mice were sacrificed and resulted in no obvious body weight changes (Fig. 2D) or adverse events compared to cisplatin alone (Table II). In addition, tumor growth was significantly inhibited at the end of the experiment compared to that in the PEI- or saline-treated mice (P<0.01) but no significant difference between this group and siVEGFR2-treated, siEGFR-treated, siVEGFR2+siEGFR-treated, or 5 mg/kg cisplatin alone-treated groups was observed (Fig. 2E). Furthermore, a median survival time of 29 days was observed in this group, which was longer than that of the siVEGFR2+siEGFR-treated animals (P<0.05) (Fig. 2F).

Downregulation of the VEGFR2 expression of both mRNA and protein on the treated tumors. Quantitative RT-PCR was used to analyze the expression level of VEGFR2 mRNA in the tumors from A549 xenografts and showed obvious downregulation of VEGFR2 mRNA in siVEGFR2-, and siVEGFR2+siEGFR+cisplatin-treated tumors compared to that of saline-treated tumors (Fig. 3A). In addition, monotherapy with 0.50 nmol siVEGFR2 showed significantly more downregulated VEGFR2 mRNA in the tumors than tumors that were treated with 0.25 nmol siVEGFR2 + 0.25 nmol siEGFR + 3 mg/kg cisplatin, which indicated a dose-dependent silencing. Notably, the VEGFR2 mRNA level in 5 mg/kg cisplatin-treated tumors was also clearly reduced compared to that in saline-treated tumors (Fig. 3A), which may have resulted
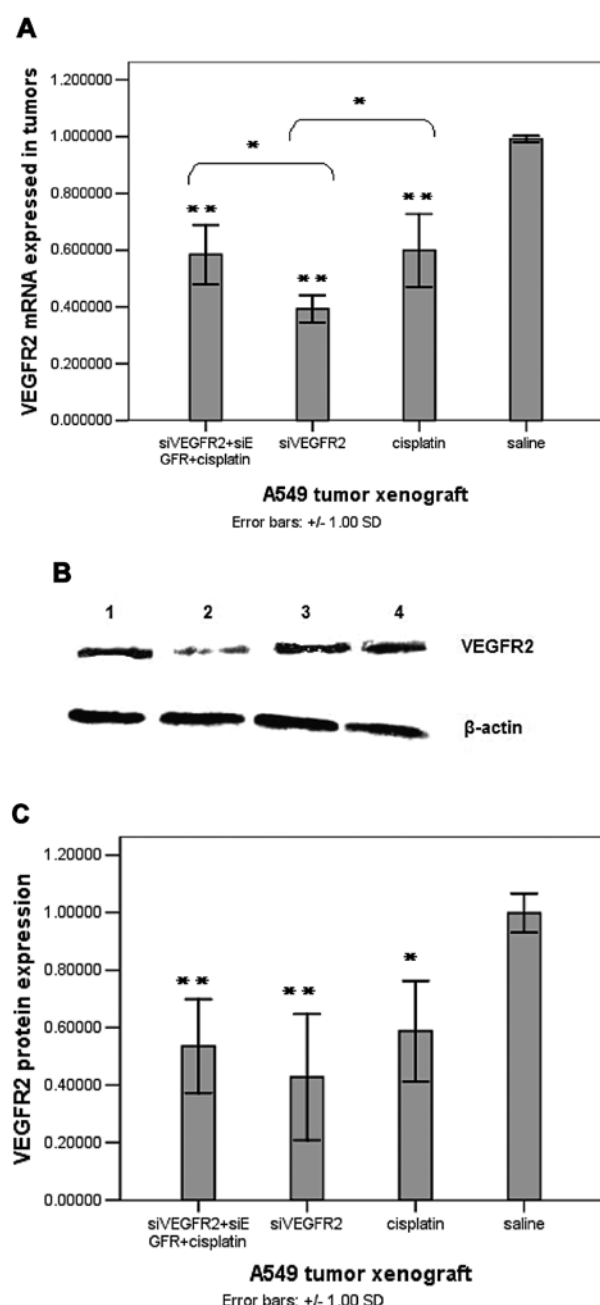
Figure 3. VEGFR2 expression in the tumor tissue. (A) VEGFR2 mRNA expression in the tumors determined by quantitative real-time PCR. (B) Western blot analysis of VEGFR2 protein expression in tumors implanted in nude mice. Representative blots are shown from three experiments. β-actin served as a loading control. Lane 1, 0.25 nmol siVEGFR2 + 0.25 nmol siEGFR+3 mg/kg cisplatin (siRNA, intratumoral injection, twice/week; cisplatin, intraperitoneal injection, once a week the day after the first shot of siRNA). Lane 2, 0.5 nmol siVEGFR2 (intratumoral injection, twice/week). Lane 3, 5 mg/kg cisplatin (intraperitoneal injection, once/week). Lane 4, saline (intratumoral injection, twice/week). (C) The expression level of VEGFR2 protein in the tumors following treatments. All the experiments were performed in triplicates. Columns, means; bars, SD. *P<0.05; **P<0.01, vs. the saline-treated group.

from the sharp reduction of tumor size. There was no significant difference in the VEGFR2 mRNA level between cisplatin-, and siVEGFR2+siEGFR+cisplatin-treated tumors, although there was a significant difference in the VEGFR2 mRNA level found between cisplatin-, and siVEGFR2-treated tumors (Fig. 3A).

Western blot analysis further confirmed the down-regulation of VEGFR2 protein expression in siVEGFR2-, siVEGFR2+siEGFR+cisplatin- (P<0.01), and cisplatin-treated tumors (P<0.05) compared to that in saline-treated tumors (Fig. 3B and C) which was parallel with the reduced level of VEGFR2 mRNA. Furthermore, there was no significant difference in the VEGFR2 protein levels among the siVEGFR2-, siVEGFR2+siEGFR+cisplatin-, and cisplatin-treated tumors (Fig. 3B and C).

Toxicity resulting from combination therapy of cisplatin and siRNA in vivo. As an important parameter of the *in vivo* studies, we assessed the toxicity of combination therapy of cisplatin with siVEGFR2, siEGFR alone, or siVEGFR2+siEGFR (Table II). The dosage of 6.5 µg of siRNA per tumor injection itself did not show any acute side-effects, weight loss, or morbidity in the mice, although later in the study some signs of toxicity, such as scurf, itching, wounds, purple skin, diarrhea and weakness, became apparent. Among these, scurf was a common but mild symptom, while the others were more severe and may have contributed to animal death. Symptoms of diarrhea and weakness were mainly found in the cisplatin (5 mg/kg)-, siVEGR2-, and control siRNA-treated groups. Purple skin, which might be credited to damage of the hematopoietic system, were common in the groups of siVEGFR2-, cisplatin (5 mg/kg)-, siVEGFR2+siEGFR+cisplatin (3 mg/kg)-, and control siRNA-treated groups. Itching occurred in mice receiving siVEGFR2. There were few side-effects found in the siEGFR-treated group, except for wounds or tissue lesions manifested at the lateral skin of the tumor. In addition, some mice developed wound lesions on the edge of the ears. The adverse effects seen in our model most likely led to discomfort or indirect impairment of the circulatory system of the mice, and eventually may have contributed to morbidity of the animals. The side-effects resulting from siRNA therapy in animal models in our study, however, might provide a beneficial reference for the future application of siRNA in the clinic.

Discussion

Cancer is a complex disease; and a leading cause of human mortality, it has been described a 'wound that does not heal' (28). After many years of research into cancer therapy, however, little progress has been made. Extensive studies of the subtle network of tumorigenesis and the relationship with vascularization have been carried out (29,30). The formation of a new functional microvasculature derived from pre-existing blood vessels, or angiogenesis, can be induced by the tumor itself as a means to provide all the nutrients and oxygen the tumor needs to grow and metastasize (29). Both endothelial cells and tumor cells are responsible for this pathological process, in which pro-angiogenic factors and the endogenous angiogenesis inhibitors are secreted to play important roles in angiogenesis (31,32). In normal physiological conditions, pro-angiogenic factors and the endogenous angiogenesis inhibitors are in balance (32), however, during tumorigenesis the 'angiogenic switch' is turned on (30). Different pro-angiogenic factors become overexpressed in different types of tumors. Non-small cell lung cancer (NSCLC), one of the most malignant types of cancer, has been found to upregulate

the expression of EGFR and VEGF/VEGFR, both of which are pro-angiogenic factors (33,34).

In the present study, we knocked down the expression of EGFR and VEGFR2 to inhibit the growth of human NSCLC xenografts with siVEGFR2 (siRNA2) and a previously published siEGFR. siRNA was delivered by polyethylenimines (PEI), a type of cationic polymer, which could encapsulate siRNA as nanoparticle complex. Our data indicated that these two receptor tyrosine kinases acted synergistically and both siRNA therapies, particularly the siEGFR, could lead to the inhibition of tumor growth with few adverse effects.

We also observed that a double dosage of siVEGFR2 or siEGFR did not exert a dose-dependent effect, while the siVEGFR2+siEGFR-treated animals receiving a 0.25 nmol (half dose) of each siRNA did indeed display tumor inhibition, while the adverse effects were reduced due to the lower dose of siVEGFR2. The biphasic dose-efficacy curve offered an explanation that a higher dose could not increase the silencing efficacy over a lower dose. Of note, it has been reported that some angiogenesis inhibitors follow a biphasic, U-shaped dose-efficacy curve, unlike dose-dependent chemotherapy agents (35).

Cisplatin has been reported to inhibit tumor growth by cytotoxicity and was found to hinder the DNA replication of tumor cells by activating the transduction of DNA-damage signals (36). Adverse effects caused by cytotoxicity and subsequent drug resistance have restrained the utilization of certain chemotherapy agents in the clinic. The chemotherapy agent we applied in this study was cisplatin with one injection of 5 mg/kg body weight per week. Western blot analysis showed a significant reduction in expression of VEGFR2 in the cisplatin-treated mice. The suppression of the bone marrow and hematopoietic system by cisplatin might contribute to this reduction of VEGFR2 protein expression, whereas killing both the tumor cells and the tumor-associated endothelial cells by destroying the DNA might also lead to the reduced level of VEGFR2 protein observed.

The combination of a chemotherapy agent with the anti-angiogenesis therapy has been termed 'metronomic therapy'. This type of approach can exert an enhanced antitumor effect by combining lower dose anti-angiogenesis inhibitors with standard chemotherapy and has been shown to produce fewer side-effects than seen in conventional dosing of chemotherapy agents (37). It has been hypothesized that anti-angiogenesis inhibitors might lower intratumoral pressure by decreasing vascular leakage to induce the 'normalization' of leaky tumor vessels and thus increase the delivery of the cytotoxicity drugs (38).

With longer survival, fewer adverse effects and, most of all, significant tumor inhibition, the combination of cisplatin with siRNA therapy showed some advantages over monotherapy. Previously it was confirmed that VEGFR2 is expressed not only in endothelial cells, but also in tumor cells, which indicates that targeted inhibition of VEGFR2 expression can inhibit tumor growth both directly and indirectly when mediated by VEGFR2 siRNA (39). Taking the therapeutic effects and the side-effects observed in our study, an optimized clinical dosage regimen should consider a lower dose of siVEGFR2 due to the current lack of an effective delivery system. Intratumoral injection has been shown to be inefficient

with only 0.1% of the given siRNA remaining in the tumor mass, while the remainder might suffer to degradation or induce side-effects (40). Moreover, since siRNA activity is dose-independent, a lower dose of siVEGFR2 might be more beneficial in practical application. Most importantly, the therapeutic regimen with the lower dose of siRNA plus lower dose of cisplatin exerted more benefits on the therapy of tumor than that cisplatin alone (Fig. 2E) and resulted in reduced side-effects (Table II).

In conclusion, targeted knockdown by siVEGFR2 and siEGFR inhibited tumor growth effectively, and a combination of this approach with low dose chemotherapy might offer a novel strategy for the treatment of cancer.

Acknowledgements

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