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Original Article

EphA2 targeted intratumoral therapy for non-small cell lung cancer using albumin mesospheres

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Abstract: Lung cancer, primarily non-small cell lung cancer (NSCLC), is the leading cause of cancer mortality and the prognosis of patients with advanced or metastatic NSCLC is poor. Despite significant advances in diagnosis and treatment, little improvement has been seen in NSCLC mortality. Recently, Intratumoral Chemotherapy, a direct local delivery of chemotherapeutic drugs, has shown promise in clinical studies. However, toxicity and high dosage of chemotherapeutic agents used for treatment are a limitation. Moreover, these drugs damage indiscriminately, cancerous as well as normal tissues. Thus, a novel therapeutic strategy that targets only malignant tissue sparing normal tissue becomes an urgent issue. Ephrin receptor-A2 (EphA2), a new biomarker, is over-expressed in NSCLC, but not on normal epithelial cells. Receptor EphA2 is a cell surface protein, which upon binding to its ligand EphrinA1 undergo phosphorylation and degradation which attenuates NSCLC growth. Targeting the tumor, sparing the normal tissue and enhancing the therapeutic effects of ligand proteins are the goal of this project. Thus a novel method, intratumoral EphA2 targeted therapy, has been developed to target the oncogenic receptors on tumor tissue by using albumin mesosphere (AMS) conjugated ephrinA1 in mice bearing NSCLC tumors.

Keywords: Mesospheres, non-small cell lung cancer, gene therapy, protein therapy, ephrinA1, intratumoral therapy

Introduction

Lung cancer has been the leading cause of death in both male and female among all cancers worldwide, and the number of new cases is the second highest only behind prostate and breast cancers [1]. Lung cancer is majorly classified as small cell (13%) and non-small cell (87%) for their different histology and purposes of treatment. The 5-year survival rate in all stages of non-small cell lung cancer (NSCLC) patients is about 18% and this high rate of mortality has not been significantly improved over years due to the lack of effective therapeutic methods. To improve the treatment for NSCLC patients, endobronchial intratumoral chemotherapy, a new procedure involving direct injection of cancer drug using bronchoscopic needle-catheters, has been explored clinically and showed enhanced effectiveness on reducing tumor burden with relief of endobronchial obstruction and symptoms of dyspnea [2-4]. To further improve the effectiveness of NSCLC treatment, an efficient controlled release system for the therapeutic drugs is in need to provide a prolonged high drug concentration in the micro-environment on the tumor sites and minimized potential systemic toxicity.

Since the transmembrane protein receptors are believed to be involved in the prognosis of most cancers, the overexpressed transmembrane receptors are becoming more and more important in cancer therapy and the gene therapy using ligand proteins for the treatment of cancers has widely attracted attentions [5, 6]. In most aggressive cancer cells including NSCLC, EphA2 receptor, one of the Ephtransmembrane tyrosine kinases, has been found to be overexpressed on the cell membrane; however, the expression of EphA2 is not found in normal cells and tissue [7-10]. More than 90% of NSCLC

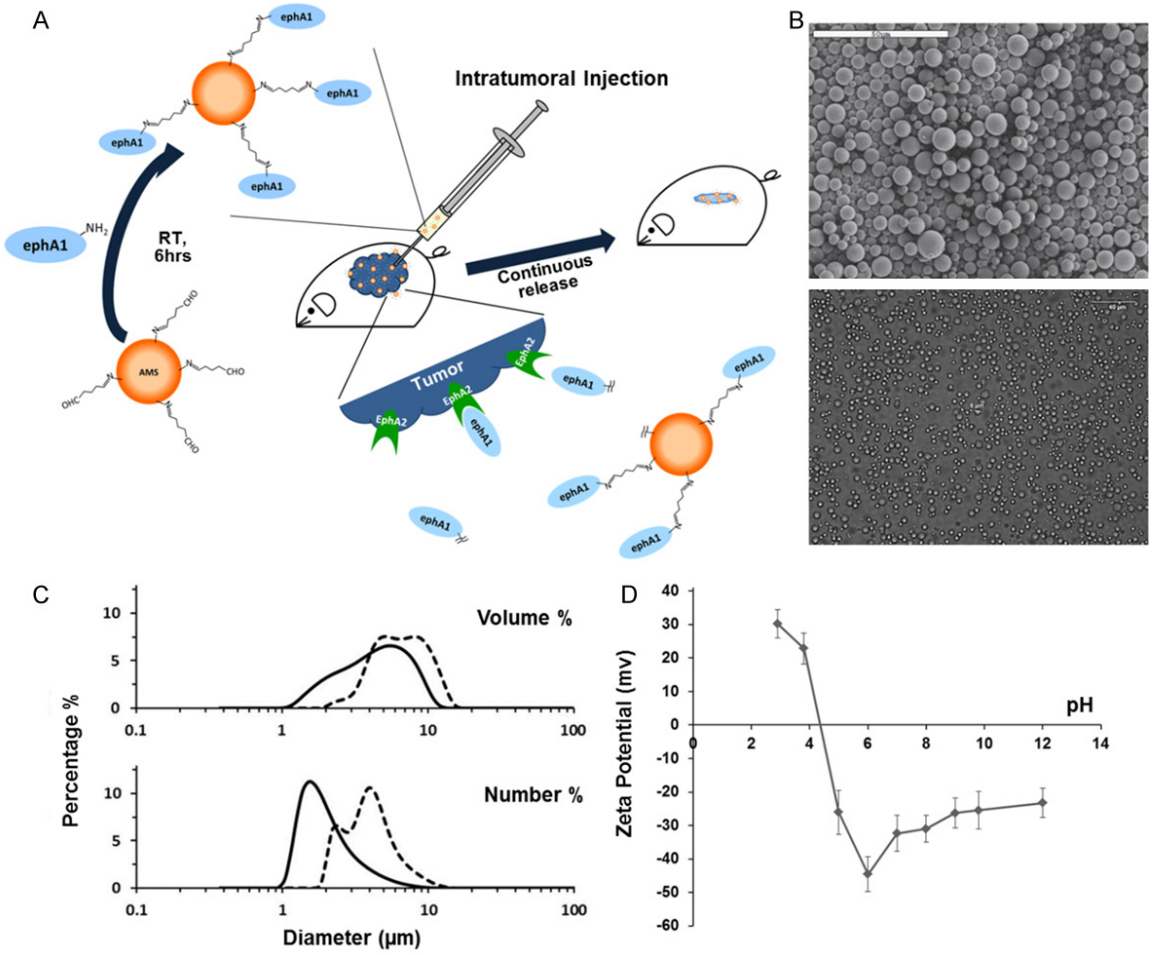

Figure 1. Protein delivery strategy and characterizations. A. The ligand protein ephrinA1 (ephA1) of EphA2 receptors were conjugated with albumin mesospheres (AMS) and intra-tumorally delivered to the mouse model bearing NSCLC. B. Microscopic images of AMS-ephrinA1. Spherical shape of particles was observed in images of optical microscopy (bottom) and scanning electronic microscopy (top). The AMS-ephrinA1 showed a particle size range of 1 to 10 μm under the microscopies. The AMS-ephrinA1 was dispersed in PBS to be observed under optical microscope. C. Volume and number size distribution of AMS-ephrinA1. The particle size distribution of dry AMS-ephrinA1 (in acetone) are shown in solid line and the dash lines present the distribution of AMS-ephirnA1 dispersed in 1 × PBS. D. The variation of zeta potential of AMS with pH of the dispersion solution. The zeta potential of AMS ranged from -30 to -40 mV at pH 7, and the lowest zeta potential value is around -45 mV at pH 6. The isoelectric point is between pH 4 and 5.

patient samples have been reported to overexpress receptor EphA2 and the expression of EphA2 was detected in all the NSCLC cell lines tested [11]. More importantly, ephrinA1, the ligand of EphA2 receptor, has been reported to inhibit proliferation and migration in lung cancer cells via the downregulation of EphA2 receptor expression, by binding to the EphA2 receptors on the cell membrane with a glycosylphosphatidylinositol anchor [12, 13]. The recombinant ephrinA1Fc has also been shown to target EphA2 in NSCLC cells and suppress tumor growth and invasion for treatment and prevention. The specific targeting of EphA2 receptors by ephrinA1 can minimize systemic toxicity and conserve the maximal adjacent normal tissue.

Controlled release and stabilization of proteins at high concentration have attracted strong interests in drug delivery for treatment of various diseases [14]. Protein delivery using nano- and micro-sized microspheres based on various biodegradable polymers, including poly (lactic-co-glycolic acid), lecithin and biocompatible hydrogels, has been widely developed and

studied in recent years [15-18]. Proteins have been loaded in the microsphere matrix, physically adsorbed on the surface and covalently conjugated with reactive functional groups on the particle surface [19-22]. However, protein denaturation during preparation has always been a consideration for protein delivery using particles, especially for some ionic polymers [23]. In this regard, for controlled release of therapeutic proteins, the albumin-based particles may prove more effective as a stabilizing matrix for adsorbed or conjugated proteins.

In our previous study, we have prepared the albumin mesospheres (AMS) and shown the high in-vitro effectiveness of ligand protein delivery [24]. The richness of active functional groups in the hydrophilic protein molecules enables the AMS to be easily modified and conjugated with other biomolecules [25]. EphrinA1 conjugated with albumin mesospheres (AMS-ephrinA1) were characterized and the antitumor activity had been examined in-vitro using A549 human NSCLC cells. The high stability, low cytotoxicity and high phagocytic rate of the AMS-ephrinA1 have been shown and its improved efficiency on inhibiting cell growth and migration in NSCLC cells had been demonstrated. Thus, based on the promising foundation of our previous in-vitro studies, the in-vivo activity of the mesosphere conjugated ephrinA1 were investigated in this report using the subcutaneous xenograftmice model. The strategy is schematically shown in **Figure 1A**.

Material and methods

Preparation of AMS-ephrinA1

The preparation and characterizations of AMS-ephrinA1 were performed as previously described [24, 26]. Briefly, the albumin mesospheres were prepared by using the water/organic solvent dispersion system. The prepared AMS-ephrinA1 were washed by acetone and air-dried at room temperature for storage.

Particle size distribution and zeta potential

The particle size distribution of AMS conjugated with BSA (AMS-BSA) was determined by a Coulter LS 13320 laser diffraction particle size analyzer (Beckman Coulter, Inc., Brea, CA, USA). To determine the particle size distribution of AMS, acetone and PBS were used as the suspension fluid and AMS were dispersed in acetone and PBS in advance. The laser obscuration range used was between 8 and 12% during measurement. The zeta potential of the AMS-eprhinA1 was determined using Smoluchowski mobility relations with Brookhaven ZetaPlus (Brookhaven Instruments Corporation, Holtsville, NY, USA).

Loading efficiency and in-vitro protein release rate

The fluorescence-labeled BSA was used as the model protein to study the loading efficiency and in-vitro protein release rate of conjugated proteins from the AMS. The BSA proteins were labeled with IRDye 800CW NHS ester (LI-COR, Lincoln, NE, USA) and purified by using PD MiniTrap G-25 (GE Healthcare Bio-Sciences, Pittsburgh, PA, USA). To determine the loading efficiency of model proteins in AMS, the AMS were dispersed in PBS containing model proteins and mixed by vortex. After hours of incubation, the supernatants were collected and the absorbance was measured at 780 nm.

The in-vitro release rate of model protein from the conjugated AMS was determined in PBS solution containing 0.5 μg/ml trypsin or higher, to simulate the actual concentration of trypsin in body, which is mostly in the range from 100 to 400 ng/ml [29, 30]. One milligram of model protein conjugated AMS were dispersed in 1 ml trypsin/PBS solution and placed on a rotator at 37°C. After intervals ranging from 1 hour to 7 days, the dispersions were centrifuged at 5,000 rpm for 10 min and 100 μl supernatants were collected for measurement. After each sample collection, 100 μl fresh trypsin solution was added into each tube. The absorbance spectra were collected by the SpectraMax M5 plate reader (Molecular Devices, Sunnyvale, CA, USA). The protein release percentage was calculated by using the formula (protein release/total loading amount of proteins in AMS) × 100.

Mouse model development

A NSCLC lung carcinoma line, A549, was maintained in male *C57BL/6* donor mice and passaged approximately every 14 days. All the animal procedures were approved by the Institutional Animal Care and Use Committee (IACUC), University of Florida. The experimental

animals, male nude mice, were implanted subcutaneously with 1 mm^3 fragments of fresh tumor tissue using a 13 gauge cancer implant needle. At 14 days post-implant (approximately 1 cm tumor diameter), tumors were sized with Vernier calipers and the mice assigned to treatment groups (n = 6). The volume of the tumor was calculated as for an oblate spheroid, i.e. volume = π/6 $(width)^2$ (length).

On day 0, intratumoral injections were performed with a 25 gauge needle. Each animal received three 0.15 ml injections, perfusing the center and periphery of the tumor. On day 1, each animal received an additional four 0.15 ml injections. Tumor sizes and animal weights were monitored for 2 to 4 weeks (n = 3). Representative animals from various groups were euthanized and the tumors removed and fixed for histological analysis. Tumor weight and tumor volume were determined. Treatment groups consisted of (1) 1-5 μm AMS-ephrinA1 suspended in sterile phosphate buffered saline (1 × PBS) or (2) free ephrinA1 in PBS. Control groups consisted of PBS alone and unloaded AMS capped with BSA in PBS. AMS conjugated proteins and AMS were completely dispersed in PBS by ultra-sonication with a micro-tip attachment prior to injection.

Western blot analysis

The collected tumor tissues were lysed in lysis buffer, as reported previously [13]. Equal amounts of protein (20 μg per lane) were loaded. Proteins in the samples were separated onto denaturing sodium dodecyl sulfate (SDS)-7.5% polyacrylamide gels (Bio-Rad Laboratories, Hercules, CA, USA) and were transferred electrophoretically onto polyvinylidenedifluoride (Immobilon-P) membranes (Millipore, Billerica, MA, USA). The blots were blocked overnight at 4°C with BSA and were incubated with mouse anti-focal adhesion kinase (FAK) and anti-EphA2 antibody at 1:1000 dilution for 1 hour at room temperature (Zymed Laboratories, San Francisco, CA, USA). After washing, blots were incubated with the second antibody (horseradish peroxidase-conjugated antimouse immunoglobulin G antibody) at a dilution of 1:2000 for 1 hour. EphA2 receptors were detected by using enhanced chemiluminescence (Amersham Pharmacia Biotech, Piscataway, NJ, USA). Prestained protein markers were included for molecular mass determination (Bio-Rad).

Immuno-histochemical analysis

Biopsy sections of tumor tissues from untreated mice and mice treated with AMS and AMS-ephrinA1 were analyzed. Immunohistochemical staining was performed by the standard adivin-biotinylated enzyme complex (ABC) method (Vectastain ABC kit, Vector Laboratories, Burlingame, CA, USA). Briefly, tissue sections were de-paraffinized, rehydrated through xylenes and graded ethanol/water series. Sections were then incubated in peroxide solution for 30 minutes to quench endogenous peroxidase activity. After buffer wash, the sections were incubated in order with diluted normal blocking serum, primary antibody (mouse anti-EphA2 and anti-Caspase 3) and biotinylated secondary antibody solutions, and buffer wash were applied after each incubation. The sections were treated with the Vectastain ABC reagent and then incubated with peroxidase substrate solution until stain intensity developed. The sections were lightly counterstained with hematoxylin and mounted. The negative controls were prepared by replacing primary antibody with normal mouse serum. The images were obtained using Nikon DIAPHOT 300 at 40 × magnification.

Quantitative PCR

The collected tissues were cut in small pieces and homogenized in buffer RLT containing 10 μl β-mercaptoethanol per milliliter. RNA extraction and purification were performed by using the RNeasy Mini Kit (Qiagen, Valencia, CA, USA) and the complementary DNA was generated using Enhanced Avian Reverse Transcriptase (Sigma). The primers and SYBR Green JumpStart Taq Ready Mix used to perform qPCR were purchased from Sigma-Aldrich. The primers used in the qPCR reaction: EphA2, sense: 5'-TTCAGCCACCACAACATCAT-3', antisense: 5'-TCAGACACCTTGCAGACCAG-3'. h18S, sense: 5'-AAACGGCTACCACATCCAAG-3', antisense: 5'-TAACGAGGATCCATTGGAGG-3'.

Statistical analysis

SigmaStat 3.5 (SYSTAT Software, Inc. San Jose, CA) was used for the statistical analysis. Results were expressed as mean ± SD. Experiments with more than one treatment were assessed using the Kruskal-Wallis and the Mann-Whitney '*U*' tests. If the *p*-values were

Table 1. Particle size statistics of AMS-ephrinA1 in acetone and PBS

Dispersing solvent		Mean diameter (μm)	Mode	SD	90%<	50%<
Acetone	Volume	5.01	5.88	2.45	8.54	4.66
	Number	2.29	1.59	1.17	3.71	1.92
PBS 1 ×	Volume	7.24	8.54	2.86	11.40	6.75
	Number	4.40	4.05	1.85	6.76	4.06

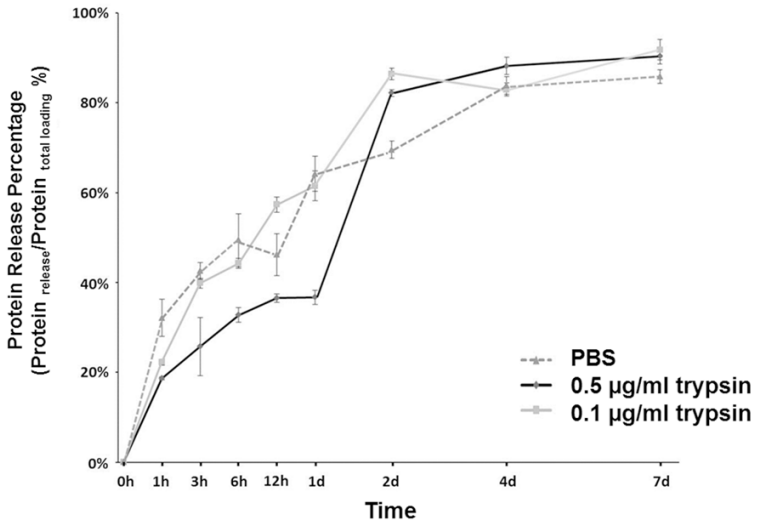

Figure 2. Protein release rate from AMS system. The release rate of model protein was determined in PBS and trypsin solutions. Above 80% of loaded proteins were released out within 2 days. The protein release percentage were determined using the following equation; Protein release rate = (protein release/total loading amount of proteins in AMS) × 100%.

<0.05 the differences were considered statistically significant.

Results

Characterizations of AMS-protein system

The prepared AMS-ephrinA1 was observed under microscope before the in-vivo studies. The AMS-ephrinA1 showed 1 to 10 μm size range and excellent dispersity in PBS under the microscope in **Figure 1B**. To further confirm the particle size of the AMS-protein system, the particle size distribution of AMS-BSA in acetone and PBS were determined. The data is shown in **Table 1** and **Figure 1C**. The swelling degree of the AMS-BSA dispersed in PBS compared to acetone is 3.0 in volume size and 7.1 in number size (the swelling degree = volume of particles in PBS/volume of particles in acetone). The AMS-BSA shows a high stability in acetone and PBS. The zeta potential of the AMS-BSA was determined in aqueous solution with pH ranging from 3 to 12. In the result shown in **Figure 1D**, the zeta potential of AMS-BSA ranged from -30 to -40 mV at pH 7, and the lowest zeta potential value is lower than -45 mV at pH 6. The loading efficiency of model protein, BSA, in AMS was measured to be 30 ± 9.0%, and the release rates in PBS and trypsin solutions are shown in **Figure 2**. The protein release rate in PBS was higher than the release in trypsin solution at the first hours of incubation, however, the protein release in high concentration of trypsin showed higher protein release after 4 days of incubation. Above 80% of loaded proteins were released out within 2 days while incubated with 0.5 μg and 0.1 mg per milliliter trypsin.

AMS-ephrinA1 reduced tumor growth in mouse NSCLC xenograft models

To explore the in-vivo therapeutic efficacy of AMS-ephrinA1 on NSCLC, intratumoral AMS-ephrinA1 treatments were applied on the tumor xenografts in nude mice. As shown in **Figure 3A** and **3B**, tumors in mice treated with AMS-ephrinA1 were significantly reduced at 4 weeks after the treatment. Tumor weight decreased significantly in mice treated with AMS-ephrinA1 when compared to untreated tumor bearing group of animals. The AMS-ephrinA1 showed about 60% and 80% of reduction in tumor mass at 2 and 4 weeks after the treatment. The tumor mass in AMS-BSA injected group of mice did not show any significant changes in the tumor mass. Whereas the intratumoral injection of ephrinA1 alone showed some regression in tumor growth at 2 weeks but at 4 weeks the tumor growth was significantly increased and is comparable to control group. **Figure 3C** demonstrates the tumor volume and tumor

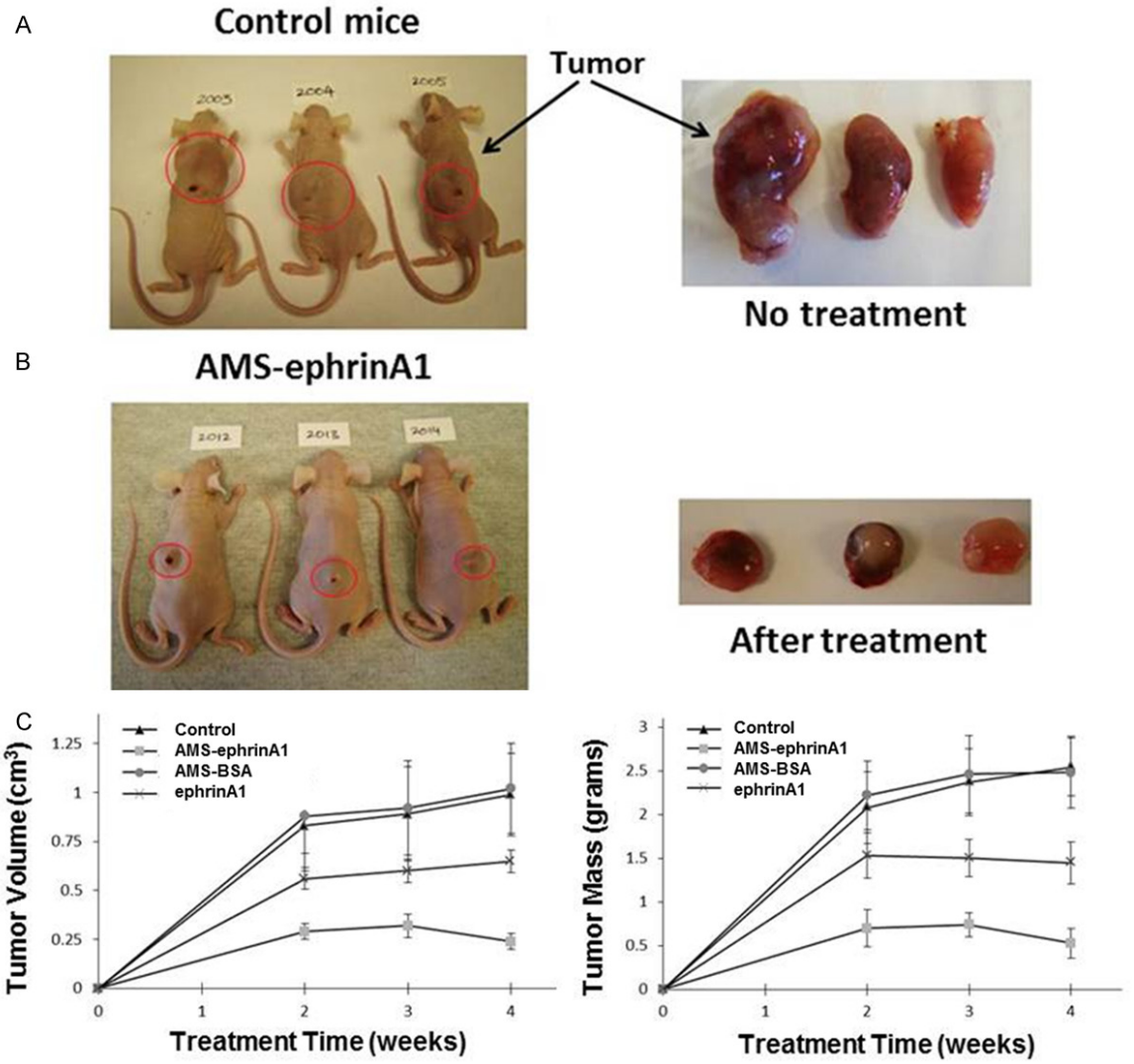

Figure 3. Anti-tumor efficacy of the AMS-ephrinA1 in the nude mice bearing A-549 cells NSCLC adenocarcinoma tumors. Treatment with AMS-ephrinA1 reduced the tumor size in nude mice. Tumors in the nude mice are shown on the left and the resected tumors on the right. (A) Control mice without treatments after 2 weeks and (B) the mice treated with intratumoral AMS-ephrinA1 therapy after 2 weeks. (C) Tumor mass and volume comparison at 2, 3 and 4 weeks after different treatments. (Data as means ± SEM for each treatment group. *$P<0.05$ vs control group).

mass decreases over time in the treatment group, AMS-ephrinA1 versus the other untreated groups. Taken together, this data suggests that AMS-ephrinA1 developed for the treatment of NSCLC tumor in mice is highly effective.

AMS-ephrinA1 suppressed EphA2 expression and promoted apoptosis in tumor tissues of mice

Efficient delivery of ephrinA1 leads to inhibition of tumor growth via suppressing the level of EphA2 expression in cancer cells. In the immunohistochemistry images in **Figure 4**, continuous high intensity of EphA2 staining was observed in the tumor tissue of the mice without treatment and treated with AMS-BSA. Especially on the border area of tumors, higher level of EphA2 expression was presented by stronger staining. In the mice treated with AMS-ephrinA1, EphA2 expression was significantly reduced and the expression of Casp3 was induced in the tumor tissue after the treatment. Significant reduction of EphA2 staining and increased Casp3 expression was observed in whole tumor area in the treated mice. The tumor tissue apoptosis was further confirmed

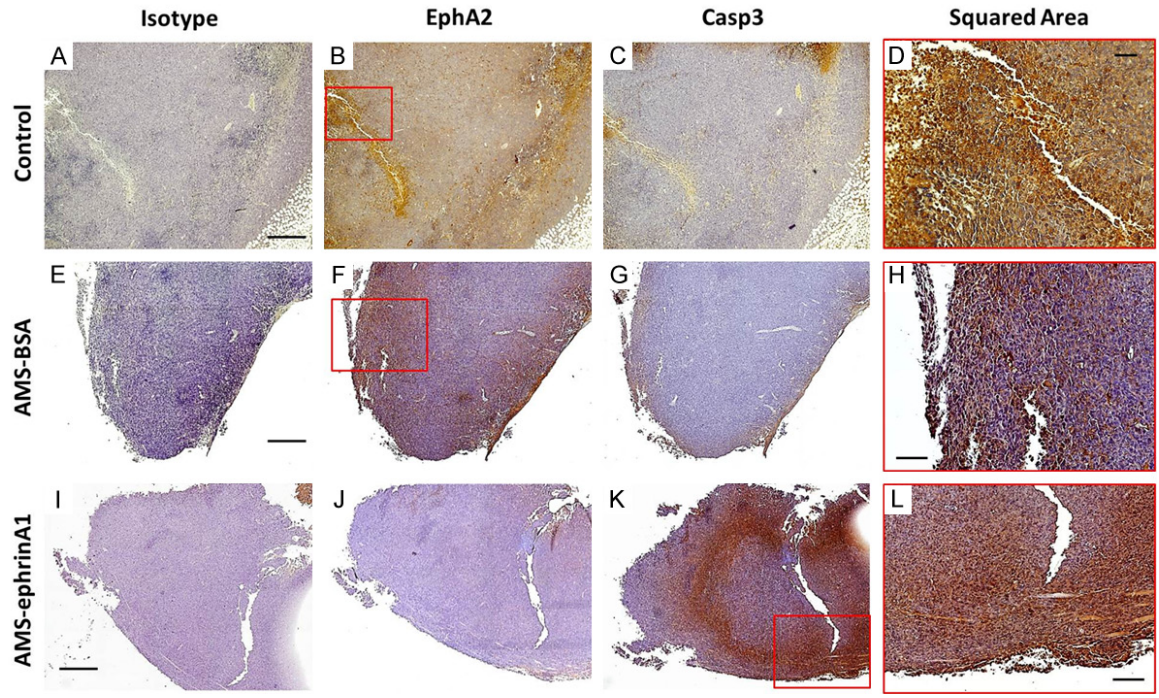

Figure 4. Immuno-histochemical detection of EphA2 in mouse NSCLC tumor tissue sections. Tumor cells showed high intensity of EphA2 staining in the mice without treatment (Control) and treated with AMS-BSA. EphA2 expression was significantly reduced and Casp3 (Caspase-3) was induced in the tumor cells of NSCLC after the treatment of intratumoral AMS-ephrinA1 injection (scale bars on the images in first column are 500 μm and images in first three columns share the same scale bar; scale bars are 100 μm in the images in last column).

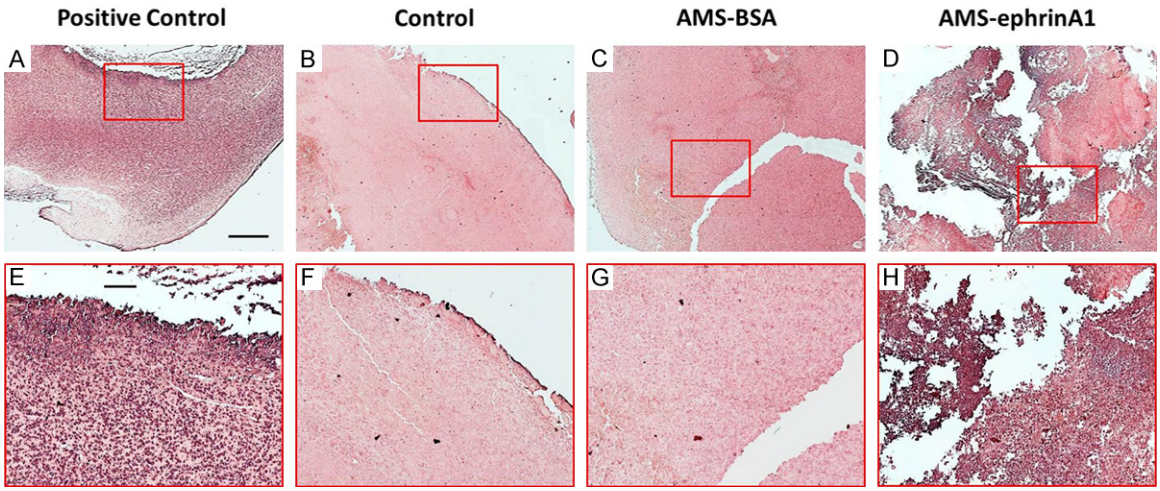

Figure 5. Tumor tissue apoptosis determined by TUNEL assay. AMS-ephrinA1 promoted apoptosis in NSCLC tumor tissues. Tissues without treatment were incubated with DNase in the group of positive control (scale bars are 500 μm on images in the first row and 100 μm on second row).

by using TUNEL assay, as shown in **Figure 5**. The results indicated that the treatment of AMS-ephrinA1 induced high level of apoptosis in the NSCLC tumors compared to the control groups.

The results of real-time PCR and Western blot analyses are shown in **Figure 6A** and **6B**. In the real-time PCR analysis, about 60% reduction of EphA2 expression was detected in the tumor tissues treated with AMS-ephrinA1, while the

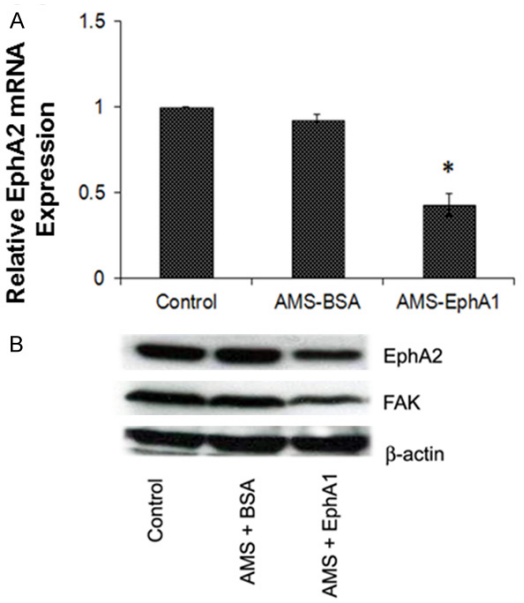

Figure 6. AMS-ephrinA1 down-regulated the expression of EphA2 in mouse tumors. A. In the real-time PCR analysis, mice tumors treated with AMS-ephrinA1 showed about 60% reduction of EphA2 expression. B. The Western blot result shows that AMS-ephrinA1 decreased EphA2 and focal adhesion kinase (FAK) expression in the mouse tumors. The lower band, *β*-actin, was a housekeeping gene to show equal loading of protein.

tumors treated with AMS-BSA showed no significant influence on EphA2 expression. The Western blot result in **Figure 6B** shows that AMS-ephrinA1 decreased EphA2 and focal adhesion kinase (FAK) expression in the mouse tumors. The AMS-BSA showed no effect on the expression of FAK and EphA2. Taken together these results imply that the EphA2 down-regulation induced by AMS-ephrinA1 was specific and significant when compared to AMS-BSA treated or control group.

Discussion

The EphA2 transmembrane receptors have been known to be widely overexpressed in NSCLC cells. In our earlier studies, it has been reported that the binding of ephrinA1 to the EphA2 receptors inhibits the expression of EphA2 receptors, leading to attenuation of cell proliferation and tumor growth in malignant mesothelioma and NSCLC cells [12, 13, 27-29]. To efficiently deliver and create a high localized drug concentration on the tumors, ephrinA1 was conjugated with AMS to improve its stability as well as therapeutic efficacy. The excellent biological properties of AMS such as high biocompatibility, low cytotoxicity and high phagocytic rate allow it to be applied for ligand protein delivery. To improve the therapeutic efficacy of AMS applied on NSCLC patients and to prevent the occurrence of clogging, the AMS conjugated with ephrinA1 was prepared majorly in the size range of 5 to 10 μm which matches the size of blood vessels in human body. In addition, the AMS-protein conjugations shows high stability and great ease of application in their solid powder or aqueous dispersion forms.

Targeted delivery and controlled release of therapeutic proteins have attracted attentions since the therapeutic efficacy of proteins is limited by the conventional drug delivery methods including oral, dermal and intravenous administration [30, 31]. Drug delivery systems applying ligand proteins or peptides as the targeting agents with therapeutic effects have been widely developed [32]. It has been previously reported that the ephrinA1 was conjugated on the surface of nanoliposomal particles encapsulating microRNA let-7 for targeting lung cancer and mesothelioma cells [33]. The combination treatment of ligand protein and small nucleotides showed an improved effectiveness on inhibiting cell proliferation, compared with treatment with each alone. It implies that the AMS-ephrinA1 also has a high potential to be applied in the targeted delivery of combined treatments, such as with chemotherapeutic drugs and nucleotides. In addition, compared to the liquid liposomal dispersions, the solid AMS-ligand protein systems show much higher stability while stored in different circumstances or administrated in human body.

The AMS shows high swelling degree in aqueous environment which enables efficient in-bulk loading of water-soluble proteins in the matrix, in addition to the conjugation on surface. In our study, albumin was used as the model protein in characterizations to understand the in-vitro loading and release behaviors of protein molecules in the AMS system. In the release profile of BSA from AMS-BSA as shown in **Figure 2**, the AMS-BSA showed higher protein release rate in PBS in the first hours of incubation; however, addition of trypsin in solution induced higher protein release rate after days of incubation. This hints that the additional proteins or enzymes in the environment may hinder the protein diffusion from AMS, and the

enzymatic digestion of trypsin may cause degradation on particle surface to release out conjugated protein molecules [34].

In our previous in-vitro studies, the ephrinA1 conjugated with AMS showed enhanced inhibiting effects on cell proliferation, cell migration and tumorosphere growth, when compared with the treatment of ephrinA1 alone [24]. In the present in-vivo study, we further demonstrated that the AMS-ephrinA1 showed significantly improved inhibiting effects on tumor growth compared to the free ephrinA1 alone in the mouse model. Sustained therapeutic effects of AMS-ephrinA1 on suppressing cancer tumor growth have been shown at 2 and 4 weeks after treatment. The AMS-ephrinA1 treatment showed 80% of reduction on tumor mass after 4 weeks of treatment, whereas the ephrinA1 showed only 20% reduction. The tumors treated with AMS-ephrinA1 showed an observable difference on the tumor appearance. The tumors treated with AMS-ephrinA1 showed a firm and restrained spherical shape in appearance, whereas the untreated tumors appeared soft and irregular in shape. The AMS conjugated with BSA (AMS-BSA) showed no significant inhibiting effects on tumor growth inhibition in mice. The present study demonstrates that the AMS is an excellent low-toxic ligand protein carrier which can provide localized controllable protein release without compromising the therapeutic effects of conjugated proteins.

The cell-cell contacts in cancer cells are highly unstable and the loss of cell-cell interaction results in cancer formation [35]. In lung cancer and malignant mesothelioma cells, the binding of EphA2receptor to the ligands that are anchored to the cell membrane of adjacent cells leads to inhibited cell proliferation, tumor growth and migration [12, 13]. Efficient ligation of EphA2 by ephrinA1 regulates abnormal cell proliferation via activating kinases which are critical signaling intermediates for downstream signaling pathway modulating malignant behavior. Thus the method of protein delivery plays a critical role influencing the treatment efficacy of therapeutic ligand proteins. The goal of protein delivery is to provide sustained protein concentration level on the target sites and stabilize protein structure in human body in order to achieve efficient ligation between the ligand proteins and receptors. In addition, proteins are stabilized against unfolding, denaturation or gelation by the presence of macromolecules or bioconjugates [36, 37]. In our results, the AMS-ephrinA1 showed high bioactivity and efficacy on inhibiting the EphA2 expression via binding on the EphA2 receptors. In the oncogenic signal pathways, the expression of EphA2 has been shown to be associated with the focal adhesion kinase (FAK) which involves in cellular adhesion and spreading, additionally, relates to invasion and metastasis of cancers [24]. In EphA2 signaling pathway in lung cancers, the activation of EphA2 receptor through binding with ephrinA1 leads to phosphorylation of EphA2 from the protein tyrosine phosphatase, and which is followed by the dephosphorylation of FAK and dissociation of FAK-EphA2 complex. Thus the FAK proteins on cancer cell membrane are down-regulated with the suppression of EphA2 receptors and leads to inhibition of tumor growth. In **Figure 6B**, the effective suppression of EphA2 and FAK proteins in the mice tumors by AMS-ephrinA1 reveals that the ephrinA1 conjugated on AMS has high affinity binding onto the EphA2 receptors and to block the oncogenic pathways.

In addition to inhibiting tumor growth, the AMS-ephrinA1 has a potential to suppress cancer cell migration and cancer metastasis. In the previous in-vitro study, the AMS-ephrinA1 showed significant hindering effects on cell spreading and invasion in the wound healing and tumorosphere growth assays [24]. Compared to the treatment with free ephrinA1 or liposome-conjugated ephrinA1, the AMS-ephrinA1 showed the most obvious hindrance on cell spreading and invasion, especially on the fibroblast-like mesothelioma cells, from the edges of cell monolayers or tumors. It implies that the enhanced suppressing effects of the ephrinA1 conjugated AMS on cell migration and invasion might be due to the matrix stiffness and steric hindrance of AMS. Although the suppressing effects of AMS-ephrinA1 on tumor invasion and cancer metastasis is not directly demonstrated in this report, the high potential of AMS-ligand protein on cancer metastasis suppression has been revealed.

Conclusions

The albumin based mesosphere as a drug carrier has shown high biocompatibility, stability, phagocytosis and low cytotoxicity. Through direct intratumoral therapy, the protein delivery

and controlled release system using the solid AMS provides a sustained high concentration of proteins on the tumor local sites. The AMS-ephrinA1 showed significant inhibiting effects, 80% reduction in mass, on NSCLC tumor growth in mice. The ligand protein conjugated on the AMS was shown to bind on the EphA2 membrane receptors and effectively block the oncogenic pathway in lung cancer cells. The treatment with AMS-ephrinA1 has enhanced effectiveness on tumor inhibition compared to the treatments with ephrinA1 alone. It indicates that the protein delivery system using AMS provides not only high protein stabilization but also enhanced the therapeutic effectiveness of ligand proteins. Based on the promising first-stage in-vivo testing in this report, the intratumoral EphA2 targeted therapy using AMS-ligand protein system may have a high potential to enter the next stage clinical trials and be applied in the combined therapy with other drugs and biomolecules. However, this warrants further studies.

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Disclosure of conflict of interest

None.

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