Targeting of angiopoietin 2-small interfering RNA plasmid/chitosan magnetic nanoparticles in a mouse model of malignant melanoma *in vivo*

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Received August 11, 2015; Accepted March 3, 2017

DOI: 10.3892/ol.2017.6443

Abstract. The aim of the present study was to observe the *in vivo* targeting characteristic of angiopoietin 2-small interfering RNA (Ang2-siRNA) plasmid/chitosan magnetic nanoparticles in an established nude mouse model of malignant melanoma (MM) under an external magnetic field. The nude mouse MM model was first established, then divided into 3 groups, including the control group, the non-targeting group and the target group, the control group was given normal saline and the non-targeting and targeting groups were administrated particles through the tail vein; the non-targeting group was not under external magnetic field and the control group and the targeting group were under external magnetic field for 60 min. The mice were then sacrificed and the tumor tissues were stained with hematoxylin and eosin and Prussian blue in order to verify the particle distributions in the tumor tissues. The control group exhibited negative Prussian blue staining in the tumor tissues, the non-targeting group demonstrated weakly positive Prussian blue staining in tumor tissues and the targeting group revealed strongly positive Prussian blue staining in tumor tissues. Ang2-siRNA plasmid vector/chitosan magnetic nanoparticles directly moved towards tumor tissues under the action of external magnetic field, thus it demonstrated good targeting characteristic.

Introduction

Cancer is the greatest threat towards human lives (1). Currently, the focus of anticancer drug development has migrated from traditional chemotherapies to molecularly targeted therapies with high selections and few side effects (2). In the 1970s, Widder *et al* (3) first proposed the concept of a magnetic targeting drug delivery system and performed experiments investigating drug-bearing magnetic particles. Due to investigation into potential novel targeted drug delivery systems, magnetic nanoparticles have been developed rapidly in cancer-targeting therapies and have become the research focus and hotspot of anticancer drugs in China and other countries (4). In recent years, magnetic nanoparticles have become increasingly widely used in biomedical studies, including magnetic resonance imaging (MRI) contrast enhancement, targeting drug delivery, tumor magnetic thermotherapy and concentration tracing towards specific targeting points (5). Among numerous control delivery systems, magnetic nanoparticles exhibited the highest targeting effectiveness (6).

The principle of magnetic transfection technique, which combined magnetic targeting technology and RNA interference (RNAi) technology, was to combine magnetic nanoparticles with targeted genes by chemical covalent bonds or physical adhesion. The formed magnetic nanoparticles would be able to accumulate directly towards the target organs under external magnetic field, thus serving its roles (7,8). Using this technique, our previous *in vitro* experiments confirmed that angiopoietin 2-small interfering RNA (Ang2-siRNA) chitosan magnetic nanoparticles could inhibit the expression of *Ang2* gene in human malignant melanoma (MM) cells, and the inhibition efficiency was 59.56% (9). In the present study, Ang2-siRNA plasmid/chitosan magnetic nanoparticles were injected into the nude mouse MM model to observe the targeting characteristic of these particles under external magnetic field, in order to determine certain foundations for further *in vivo* targeting intervention studies investigating the tumor growth in MM-transplanted nude mice.

Materials and methods

Preparation of chitosan magnetic nanoparticles. A total of 0.15 g magnetic Fe_3O_4 nanoparticles was dispersed into 20 ml of 1.5% chitosan (relative molecular weight: $1.38x10^6$; deacetylation degree: 90%; Zhejiang Hisun Chemical Co., Ltd., Taizhou, China) under ultrasound and agitation.

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Key words: angiopoietin 2, plasmid, RNA interference, chitosan magnetic nanoparticles, malignant melanoma, targeting

Subsequently, this was added to 80 ml mixed phase solvent of liquid paraffin and petroleum ether (volume ratio: 7/5) supplemented with 2 ml Span-80 (emulsifier). The solution was sufficiently emulsified and agitated at 40˚C for 30 min, then 10 ml glutaraldehyde solution (diluted 1 ml 25% glutaraldehyde to 10 ml) was slowly added drop-wise. Following, the solution was incubated at 40˚C in a water bath for 30 min, and then adjusted to pH 9.0 with 1 mol/NaOH solution. The resulting solution was heated to 60˚C. After standing for 1 h, the precipitate was produced. Following thorough washing with anhydrous ether, acetone, anhydrous ethanol and distilled water successively, the chitosan magnetic nanoparticles were obtained.

Combination of Ang2-siRNA plasmid and chitosan magnetic nanoparticles. A total of 1 mg chitosan magnetic nanoparticles were added to 1 ml PBS buffer (pH 7.4) and ultrasonically agitated (200 W, 3 min). Subsequently, 2 ml polylysine (diluted with PBS buffer to a concentration of 0.1 mg/ml) was added, mixed well and incubated at room temperature for 10 min. The Ang2-siRNA plasmid was then combined with the polylysine-modified chitosan magnetic nanoparticles with ratios of 1:1, 1:10, 1:100 and 1:1,000 (quality ratio), respectively, followed by incubation at room temperature for 1 h. Routinely vaccinated and cultured MM A-375 cells (purchased from Type Culture Collection of the Chinese Academy of Sciences, Shanghai, China) were seeded into the 6-well plate ($1.0x10^5$ cells/well). The Ang2-siRNA plasmid/chitosan magnetic nanoparticles were added to the wells, followed by incubation at 37˚C, 5% CO_2 for 48 h. The expression of red fluorescent protein was observed under DVM6 optical microscope (Leica Science Lab, Leica Camera AG Berlin, Germany).

Establishment of MM-transplanted nude mouse model. Routinely vaccinated and cultured MM A-375 cells were seeded in 10 cm dishes ($1.0x10^6$ cells/dish). When the cells in logarithmic growth phase grew to >90% confluency, 0.25% trypsin was added for a 3 min digestion period, then Dulbecco's modified Eagle's medium (DMEM; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) was added to terminate digestion. The cells were transferred into a 1 ml centrifuge tube for 3 min centrifugation at 256 x g and 4˚C. Subsequently, the supernatant was discarded and cell culture medium was added to pipet the cells into tumor cell suspensions, this was centrifuged (256 x g at 4˚C for 3 min) and the supernatant was discarded. Cells were washed twice with PBS by centrifugation, then serum-free high glucose DMEM was added to prepare the cell suspensions. The cells were counted using a BX61 fluorescent microscope (Olympus Corp., Tokyo, Japan) and the cell concentration was adjusted to $5x10^7$/ml. Subsequently, 100 μl cell suspension was subcutaneously inoculated using a micro-injector into the right armpit of nude mice. A total of 15 nude BALB/c male mice (specific pathogen free, 6 weeks old, 20-25 g) were provided by Shanghai Wu Animals Center, Shanghai, China (license number, SCXK (Min) 2012-0001). They were raised in housing conditions (22-25˚C; 55±5% humidity) in a 12 h dark/light cycle with free access to food and water. The present study was approved by the Animal Ethics Committee of Fujian Medical University (Fuzhou, China).

Table I. Transfection efficiency of angiopoietin 2-small interfering RNA plasmid/chitosan magnetic nanoparticles towards human malignant melanoma cells.

Quality ratio	Total no. of cells[a]	Total no. of cells[b]	Transfection efficiency[c], %
1:1	0	118	0.00
1:10	10	107	9.35
1:100	63	103	61.17
1:1,000	35	84	41.67

[a]Emitting red fluorescence under a mercury air lamp. [b]Under a normal light source. [c]Determined according to the cell counting results.

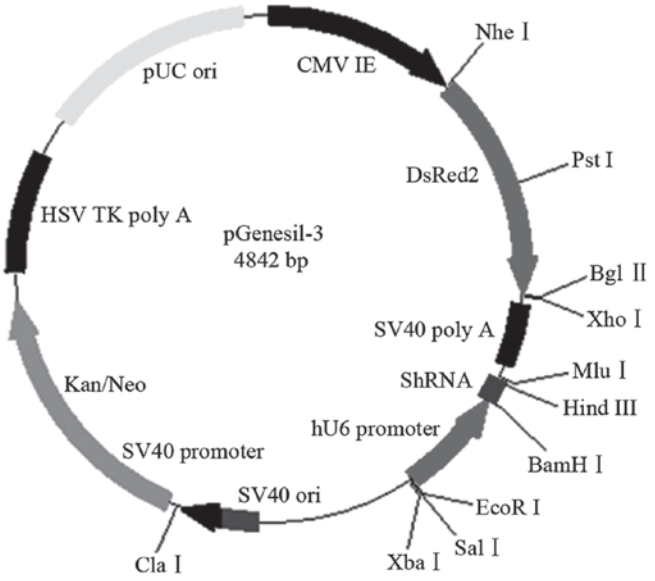

Figure 1. pGenesil-3 recombinant plasmid (Ang2-siRNA plasmid). Ang2, angiopoietin 2; p, plasmid; siRNA, small interfering RNA; shRNA, small hairpin RNA; HSV TK poly A, herpes simplex virus thymidine kinase polyadenylation.

Magnetic targeting positioning experiment of Ang2-siRNA plasmid vector/chitosan magnetic nanoparticles in nude mice. Following successful establishment of the nude mouse model and when the subcutaneous tumors grew to ~6x6 mm in size, the mice were randomly divided into 3 groups, with 5 mice in each group. The targeting group was injected with 0.4 ml chitosan magnetic nanoparticle solution through the tail vein, then 4,000 GS magnetic field was added close to the right armpit subcutaneously following anesthesia using 4% chloral hydrate (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany), 60 min later the magnetic field was removed and the mice were sacrificed. The non-targeting group was injected with 0.4 ml of plasmid-coupled particles (35.35 mg/kg) through the tail vein, this was not performed under a magnetic field. Following 60 min, the mice were sacrificed. The control group was injected with 0.4 ml saline through the tail vein, this was performed under a magnetic field; following 60 min, the mice were sacrificed by cervical dislocation. The tumor tissues were stripped to prepare paraffin tissue sections, followed

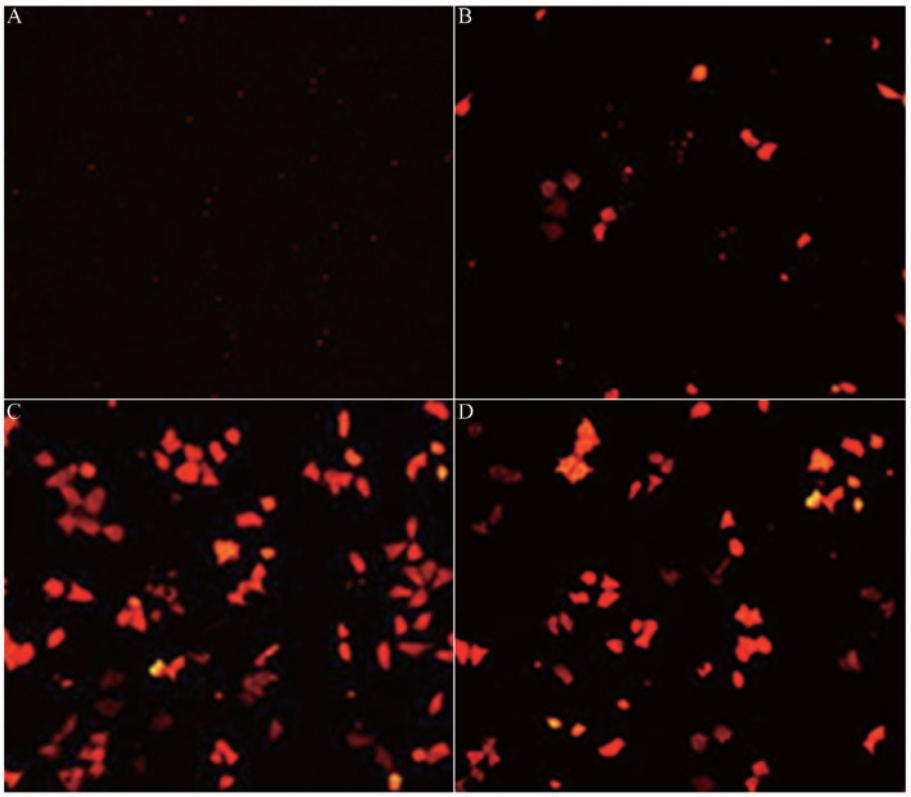

Figure 2. Transplantation conditions of malignant melanoma A-375 cells at various quality ratios (magnification, x100). Auality ratio of Ang2-siRNA plasmid to polylysine-modified chitosan magnetic nanoparticles: (A) 1:1, (B) 1:10, (C) 1:100 and (D) 1:1,000.

by hematoxylin and eosin (H&E) staining and Prussian blue staining in order to verify the particle distributions inside the tissues using a DVM6 optical microscope (Leica Microsystems GmbH, Wetzlar, Germany).

Results

Determination of suitable quality ratio of Ang2-siRNA plasmids and chitosan magnetic nanoparticles. Ang2-siRNA plasmid (Fig. 1) and chitosan magnetic nanoparticles were combined with the quality ratios 1:1, 1:10, 1:100 and 1:1,000, respectively, then transfected into human MM cells. Fluorescence microscopy of A-375 cells (Fig. 2A-D) demonstrated that when the quality ratio was 1:100, the red fluorescence emitted was the strongest (Fig. 2C). The cells in each group were digested into single cell suspensions for cell counting (Table I) and the quality ratio 1:100 was determined to be the appropriate ratio for subsequent experiments as a result.

Construction of MM-transplanted nude mouse model. Fig. 3 revealed that subsequent to subcutaneous inoculation for 5-7 days, subcutaneous tiny nodules (~1 mm) were observed and obvious subcutaneous nodules were observed following 14 days. When the tumor grew to ~6 mm, the tumor-bearing mice were grouped and the success rate of tumor formation by subcutaneous injection was 100%.

H&E staining and Prussian blue staining. Fig. 4A and B demonstrated that there were no particles inside the tumor

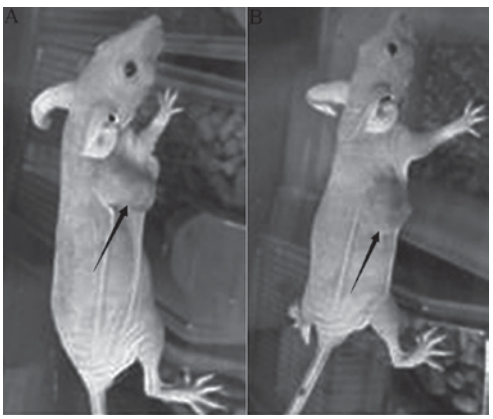

Figure 3. Malignant melanoma-transplanted BALB/c nude mouse model. (A and B) 9-week-old nude mice (in tumor-bearing state) were injected with 0.4 ml small interfering-RNA chitosan-magnetic nanoparticles via the tail vein, and one external magnetic field was applied under mice's right armpit for 60 min. Images were captured 14 days following the seeding of A-375 cells.

tissues of the control group and Prussian blue staining was negative. The non-targeting group exhibited rare particles inside tumor tissues occasionally and Prussian blue staining was weakly positive. The targeting group exhibited aggregation of numerous particles on the capsule of tumor tissues and inside blood vessels and Prussian blue staining was strongly positive.

Discussion

Cancer is one of the three diseases that pose a serious threat to human health (10). MM is a superficial tumor with high

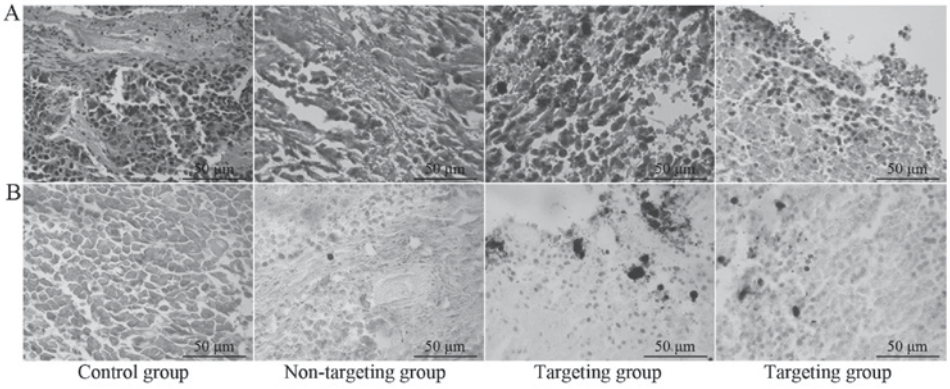

Figure 4. Staining results of malignant melanoma A-375 cell nude mouse transplantation tumor. (A) Hematoxylin and eosin staining. (B) Prussian blue staining.

malignancy and difficult treatment (11). The current conventional clinical treatments have shortcomings including, low specificity and numerous side effects (12). Therefore, increasing the specificity of anticancer drugs is necessary to reduce drug side effects and improve efficacies (13). The study of antitumoral drugs has progressed greatly and targeted drug delivery systems have become a focus in China and other countries (14). A magnetic targeting drug delivery system (MTDDS) is a stable system composed of magnetic substances and drugs with a suitable carrier; the drugs move, re-position, concentrate and accumulate around lesion tissues when exposed to external magnetic field with a specific intensity (15). The high specific characteristic of targeting drug therapy may significantly reduce the side effects of treatment (16).

The targeting of MTDDS included active and passive targeting (17). The active targeting exhibited higher specificity compared with the passive targeting, which mainly involved coupling with the ligand or antibody of targeted cells (18). Otherwise, the particles with magnetic properties migrated directly to the targeted tissues and achieved targeting under an external magnetic field (19). Hsieh *et al* (20) established a mouse model with colon cancer, and injected specific antibodies-containing Fe_3O_4 particles into mice. This revealed that the particles accumulated in the lesions and little was observed in other tissues (20). Zhou *et al* (21) used liposome-encapsulated adriamycin to prepare adriamycin magnetic microspheres, which could specifically accumulate inside tumor tissues under the action of an external magnetic field.

A previous study demonstrated that angiogenesis within tumors was in a chaotic state and generated a large number of immature blood vessels, performing as vascular network distribution disorder, vascular smooth muscle insufficiency, incomplete basement membrane structures and large gaps among endothelial cells (100-600 nm) (22). This type of immature blood vessel is the important cause of continuous aggravation and metastasis of tumors (23). These features increased vascular permeability inside tumors in order to facilitate the penetration of large macromolecules and nanoparticles through the gaps in vascular endothelial cells (24). However, tumor tissues were found to lack a lymphatic system, thus the venous return was slow, resulting in the accumulation of molecules and nanoparticles; this phenomenon demonstrates the high permeability and retention effect of tumors (24). By utilizing this effect, nanoparticles were able to pass through highly permeable tumor blood vessels, resulting in accumulation inside tumor tissues (1).

The sizes of nanoparticles are closely associated with their *in vivo* distribution: When the size of nanoparticles are <400 nm, they are able to penetrate tumor vascular endothelial cells; when the size is >100 nm, they are absorbed by the hepatolienal endothelial reticular system; and when the size is <10 nm, they are mainly excreted by the kidneys (25). Therefore, the nanoparticle sizes should be within 10-100 nm for use as a tumor targeting drug delivery system. The particles resist the permeability reduction of nanoparticles induced by the increased tumor interstitial pressure when particle size is small, thereby increasing the targeting of nanoparticles (26). The average particle size of Ang2-siRNA plasmid vector/chitosan magnetic nanoparticles prepared by the present study was 67 nm (9). Therefore, they were used as a suitable targeting drug delivery system. A previous study revealed that magnetic nanoparticles could generate heat in an alternating magnetic field, thus inducing the apoptosis of tumor cells (27), and achieving the purpose of inhibiting tumor growth with a mechanism alternative to conventional cancer therapy.

In the present study, the histopathological analysis demonstrated that the non-targeting group exhibited occasional particle distribution inside the vessels of tumor tissues and Prussian blue staining was weakly positive. The control group exhibited negative Prussian blue staining inside tumor tissues. Whilst, the targeting group exhibited the aggregation of a large number of particles under the capsule and blood vessels in tumor tissues, and Prussian blue staining was strongly positive, suggesting that the non-targeting group may have blood rich vessels inside tumor tissues. The Ang2-siRNA plasmid vector/chitosan magnetic nanoparticles enter the systemic blood circulation through the tail vein and partial particles may enter tumor tissues due to blood flow, whereas the distribution in the targeting group was due to the external magnetic field. Ang2-siRNA plasmid vector/chitosan magnetic nanoparticles may specifically migrate towards tumor tissues due to the magnetic field.

In conclusion, the present study demonstrated that Ang2-siRNA plasmid vector/chitosan magnetic nanoparticles exhibited a good targeting characteristic and may be considered as a vector for gene therapies. The present study also

provided foundations for further *in vivo* targeting intervention studies investigating the angiogenesis and tumor growth of MM in nude mice.

Acknowledgements

The present study was supported by the Foundation of National Key Clinical Specialty Discipline Construction Program (China; grant no. 2013-GJLCZD), the National Health Planning Scientific Research Foundation-Joint Research Projects of Fujian Provincial Health and Education (Fuzhou, China; grant no. WKJ-FJ-03) and the Projects of Fujian Provincial Natural Science Foundation (Fuzhou, China; grant no. 2016J01527).

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