Tissue distribution and cancer growth inhibition of magnetic lipoplex-delivered type 1 insulin-like growth factor receptor shRNA in nude mice

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The targeted delivery of therapeutic genes into specific tissues, as well as the determination of the biological fate and potential toxicity of nanoparticles, remains a highly relevant challenge for gene-based therapies. Type 1 insulin-like growth factor receptor (IGF-1R), an important oncogene, is frequently over-expressed in lung cancer and mediates cancer cell proliferation as well as tumor growth. In our previous studies, we have successfully applied gene delivery mediated by commercially available nanoparticles (CombiMAG) under a magnetic field, which suppresses IGF-1R expression in a non-small cell lung cancer cell line (A549) in vitro. In the present study, we aimed to investigate the biological distribution and target tumor suppression of magnetofection, as well as its potential toxicity via CombiMAG-carrying plasmids expressing green fluorescent protein (GFP) and short hairpin RNAs (shRNAs) targeting IGF-1R (pGFPShIGF-1Rs) in tumor-bearing mice. The peak expression in various organs appeared 48 h after transfection. Transgene expression via magnetofection was 3-fold improvement than via lipofection. On the 30th day after injection, the tumor size and weight of the CombiMAG-treated group (789.32 ± 39.43 mm³, 164.5 ± 9.1 mg) were significantly decreased compared with those of the lipofection group (893.83 ± 31.23 mm³, 105.5 ± 6.1 mg) were significantly decreased compared with those of the lipofection group (893.83 ± 31.23 mm³, 105.5 ± 6.1 mg; *P < 0.05), and the suppression rate was ~36%. After a 30-day observation, the injection of CombiMAG did not cause any apparent toxicity. Therefore, IGF-1R shRNA nanoparticles can be valuable and safe delivery agents for RNA interference therapy to tumors in vivo.

Keywords lung cancer; insulin-like growth factor 1 receptor; nanoparticle magnetofection; small interference RNA; in vivo

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Introduction

Lung cancer is one of the most common cancers and a major killer worldwide. Non-small cell lung cancer (NSCLC) is the main form [1]. Given their propensity to early metastasis and developing resistance to a wide range of anti-cancer drugs, the prognosis of lung cancer patients is usually poor. Therefore, innovative strategies for effectively treating lung cancer are urgently needed.

The knockdown of over-expressed oncogenes that are essential for NSCLC proliferation, such as insulin-like growth factor 1 receptor (IGF-1R), by RNA interference can inhibit cancer growth [2]. In our previous studies, we constructed IGF-1R-specific short hairpin RNA (shRNA)-expressing plasmids and transfected them into A549 cells. The proliferation, adhesion, invasion, and chemoresistance of A549 cells were inhibited [3–6]. However, currently available non-viral vectors carry shRNA or plasmid DNA into lung cancer cells with low transfection efficiency in vitro, and even lower efficiency in vivo, which hinder the targeted delivery of therapeutic genes to tumor sites [7,8].

Magnetofection exploits the magnetic force exerted on gene vectors associated with magnetic particles to draw the vectors toward, possibly even into, target cells and tissues [9,10]. In addition to enhancing gene transfection into cultured cells in vitro, the approach can be used to achieve in vivo gene delivery into targeted tissues and organs in the body under the control of a magnetic field [11,12]. Liposomal magnetofection is a process wherein nucleic acids are associated with magnetic nanoparticles in combination with cationic lipids to form magnetic lipoplexes. These complexes are then concentrated on the surface of targeted cells under the influence of a magnetic field. However, the feasibility of liposomal magnetofection for gene transfer to lung cancer tissue in vivo has never been systematically analyzed.

In our previous study, we successfully delivered commercially available nanoparticles (CombiMAG) with Lipofectamine 2000 (Lp2000) to an NSCLC cell line (A549) in vitro under an external static magnetic field, which suppressed IGF-1R expression [13]. However, the application of targeted gene delivery via the vein system for the gene therapy of lung cancer, as well as its...
biological effects and toxicity, has not yet been elucidated. In the current study, we injected plasmids expressing green fluorescent protein (GFP) and shRNAs targeting IGF-1R (pGFPShIGF-1R) combined with CombiMAG and Lp2000 complex into the mice via the tail vein. We then evaluated the biological distribution in various organs, lung cancer xenograft growth inhibition, and potential toxicity under the influence of a magnetic field.

Materials and Methods

Cell line and culture
A549 cells belonging to NSCLC cell lines were purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). The A549 cells were grown in RPMI-1640 (Invitrogen, Carlsbad, USA) supplemented with 10% fetal bovine serum (Invitrogen), glutamate (10 ml/l, Invitrogen), 100 U/ml streptomycin, and 100 U/ml penicillin (Genom, Shanghai, China) at 37°C in a humidified atmosphere containing 5% CO2.

Animals
Male BALB/cAnNCj-nu mice (4 weeks old) were obtained from Shanghai Slac Laboratory Animal Co. Ltd (Shanghai, China). The mice were maintained in a specific pathogen free environment and handled according to the institutional guidelines of Zhejiang University established for animal care and use. On the 30th day after injection, all animals were euthanized and the tumors were collected and weighed.

Plasmid expressing GFP and shRNA targeting IGF-1R
pGFPShIGF-1R was purchased from Invitrogen, and the complementary sequences from 30 to 50 ds-oligos encoding shRNAs were as follows: TGCTGAAAGGTAGCCGTCCTGAGGCTGTTTTGGCCACTGACTGA CAGCCTCAGCGGCTACCTTT.

CombiMAG and Lp2000 delivery of pGFPShIGF-1R in vivo
The mice were inoculated subcutaneously with 5 × 10⁶ A549 cells in 100 μl of serum-free RPMI-1640 medium into the right flanks. After 2 weeks, tumor volumes were measured every 3 days using calipers, and calculated using the formula V (mm³) = ab²/2 (where a is the largest dimension and b is the perpendicular diameter). When the subcutaneous tumors grew to ~100 mm³, the mice were randomly divided into three groups and treated as follows: control group (n = 4), mice treated with phosphate-buffered saline (PBS) (200 μl/mouse) via tail vein injection; Lipo group (n = 7), mice treated with pGFPShIGF-1R (50 μg/mouse):Lp2000 (125 μl/mouse) complex via tail vein injection; CombiMAG group (n = 7), mice treated with pGFPshIGF-1R (50 μg/mouse):CombiMAG (50 μg/mouse: polyethyleneimines; Chemicell):Lp2000 (125 μl/mouse) complex via tail vein injection and under a magnetic field within 400 mT. All animals were treated once daily by vein injection (i.v.) for 7 days.

Fluorescence image analysis of plasmid distribution
Three mice from the Lipo and CombiMAG groups were killed at 24, 48, and 72 h after injection. The heart, kidney, liver, lung, spleen, and tumor were quickly removed and analyzed as snap-frozen sections (5 μm) using a freezing microtome (Leica, Solms, Germany). The GFP expression in the tissue sections were visualized by a fluorescence microscope at ×100 magnification.

Western blot analysis
Western blot analysis was performed as previously described [3]. Briefly, 200 μg of the cellular proteins (100 μg for β-actin) from 50 mg of tumor tissue were separated on 10% sodium dodecyl sulfate polyacrylamide gels, and then transferred to Hybond-P polyvinylidene difluoride membranes (Amersham, Piscataway, USA). After the non-specific binding sites were blocked with 5% non-fat milk in PBS-0.1% Tween-20 (PBST) (for detecting IGF-1R), or with 5% non-fat milk in Tris-buffered saline-0.1% Tween-20 (TBST) (for detecting β-actin) for 0.5 h at room temperature (RT), the membranes were probed with anti-IGF-1R antibody (1:200, Lab Vision, New York, USA) for 2 h, or anti-β-actin antibody (1:1000, Santa Cruz, Santa Cruz, USA) for 3 h at RT, and then washed three times with PBST (for IGF-1R) or TBST (for β-actin). The membranes were then incubated with horse-radish peroxidise (HRP)-conjugated goat anti-mouse (or goat anti-rabbit) IgG (1:2500, Santa Cruz) for 1 h at RT, and washed three times with PBST or TBST. The blots were developed by chemiluminescence kit (Roche, Indianapolis, USA), and analyzed using Scion Image software (Scion Corporation, Frederick, USA).

Immunohistochemistry assay
The tumors were fixed for 2 h in 4% paraformaldehyde and sectioned (10 μm). The tissues were stained by the avidin–biotin complex method according to standard protocol. The sections were probed with anti-IGF-1R antibody (1:200, Lab Vision) for 16 h at 4°C, then incubated with HRP-conjugated goat anti-rabbit IgG (1:400, Santa Cruz) for 1 h at RT. The reaction substrate was 3,3-diaminobenzidine (DAB, Sangon, Shanghai, China). Counterstaining was carried out with hematoxylin, and the images were obtained using light microscope (LeicaDM2000, Leica, Solms, Germany).
Serum biochemical analysis
Blood samples were collected after treatment. Serum biochemical analysis was carried out using a biochemical autoanalyzer (VITALAB, Merck, Netherlands) to determine the serum levels of total protein (Pro), albumin, total bilirubin (Bil), aspartate transaminase (AST), alanine transaminase (ALT), glucose, cholesterol, triglyceride, blood urea nitrogen (Bun), creatinine (Cr), calcium, phosphorus, and lactic acid (Lac) dehydrogenase.

Histopathological analysis
The kidney, liver, and lung were removed and fixed in 4% paraformaldehyde for 24 h at RT. Tissue sections (5 μm) were prepared after dehydration and embedded in paraffin. The sections were stained with hematoxylin and eosin (H&E), and subsequently processed for histopathological examination under a light microscope (LeicaDM2000).

Statistical analysis
All quantitative data were presented as means ± SD. The statistical significance of the differences was determined by Student’s two-tailed t-test in two groups and one-way analysis of variance in multiple groups. P < 0.05 was considered statistically significant. All data were analyzed using the SPSS 17.0 software.

Results
pGFPshIGF-1R distribution in organs and tumors
To detect the efficiency of magnetofection, we observed the distribution of pGFPshIGF-1R in various organs at 24, 48, and 72 h after transfection. GFP was detected in all of the organs, such as the heart, kidney, liver, lung, and spleen [Fig. 1(A)], and the peak expression was detected at 48 h after transfection. However, the distribution in the Lipo groups was markedly less than that in the

Figure 1 Tissue distribution of GFP in various organs and tumors The mice were treated with transfection via tail vein injection and sacrificed at indicated time points (24, 48, and 72 h). Tumors and organs were removed from the mice and analyzed as a snap-frozen section (5 μm) using a freezing microtome. (A) The GFP-expressing cells in the tissue sections (heart, kidney, liver, lung, and spleen) were visualized using a fluorescence microscope (×400); (B) The GFP-expression in the tumors were visualized using a fluorescence microscope (×400), and the number of GFP-expressing cells in the tumor sections were counted under five different fluorescence microscope fields at ×100 magnification. *P < 0.05, compared with the Lipo group.
CombiMAG groups in all organs, especially at 48 h. After that, we observed the expression of pGFPshIGF-1R in the subcutaneous tumors. As expected, GFP was highly expressed in CombiMAG groups at three different time points, but only scattered GFP expression was found in Lipo groups [Fig. 1(B)], which suggests that magnetofection can deliver the plasmid DNA into specific target sites. We quantified the numbers of GFP-expressing cells in the tumor sections under five different fluorescence microscope fields at ×100 magnification. The results showed that the number of positive cells significantly increased in the CombiMAG group at 24, 48, and 72 h post-injection (44.4 ± 6.0, 53.4 ± 8.6, 46.2 ± 9.5) compared with in the Lipo group (7.2 ± 1.9, 17.6 ± 6.2, 16.4 ± 7.0), respectively (P < 0.05) [Fig. 1(B)], which indicated that the targeted delivery efficiency via magnetofection is higher than that via lipofection.

Inhibition of IGF-1R expression in magnetofection tumors
To detect the effect of magnetofection on the expression of IGF-1R in the tumors, we performed western blot analysis. The results showed that the expression of IGF-1R significantly decreased in the CombiMAG group at 48 and 72 h after injection compared with in the Lipo group [Fig. 2(A)]. To further visualize the expression of IGF-1R in the tumors, immunohistochemistry assays were performed. DAB-stained cells were clearly visible in the Lipo group, but only a few positive cells were observed in the CombiMAG group [Fig. 2(B)], which demonstrated that magnetofection-mediated IGF-1R gene silencing was more effective than lipofection.

Magnetofection-mediated IGF-1R gene silencing inhibited tumor growth
In this study, the incidence of subcutaneous tumors derived from A549 cells was 100%. The time for tumorigenicity was 15 days, and the tumor volume was ~100 mm³. All of the mice survived for 45 days after inoculation. Then we explored whether the down-regulation of IGF-1R by CombiMAG suppressed tumor growth in these nude mice. On the 45th day, we euthanized all the animals and collected and weighed the tumors. The results showed that the tumor growth rate in the CombiMAG group was much lower than that in the other two groups (P < 0.05, Fig. 3(A)]. On the 45th day, the tumor size and weight in the CombiMAG-treated group were 789.32 ± 39.43 mm³ and 105.5 ± 6.1 mg, respectively, which were markedly lower than those in the Lipo group (893.83 ± 31.23 mm³ and 164.5 ± 9.1 mg, respectively) (P < 0.05; Fig. 3). No invasion was observed in any of the groups. These results suggested that magnetofection-mediated IGF-1R gene silencing efficiently inhibited tumor growth.

Figure 2 Inhibition of IGF-1R expression in magnetofection tumors
The tumors were removed from the mice and analyzed at 24, 48, and 72 h after injection. (A) The expression of IGF-1R protein in the tumors was detected by western blot analysis. Cellular protein was extracted from a 50 mg tumor tissue. (B) The expression of IGF-1R protein in the tumors was detected by immunohistochemistry assay (× 400).

Figure 3 Regression of tumor growth in nude mice by magnetofection-mediated IGF-1R gene silencing (n = 4)
A549 cell (5 × 10⁶) suspension was injected subcutaneously in nude mice. After 15 days, tail vein injections were performed. The magnet (400 mT) was held onto the subcutaneous tumor surface throughout the infusion. (A) Tumor sizes were measured and calculated as described in ‘Materials and Methods’. Data are shown as the mean ± SD of four animals from each group. (B) On the 45th day, the mice were sacrificed and the tumors were weighed. *P < 0.05, compared with Lipo group.
Treatment of CombiMAG did not induce any apparent toxicity

Finally, to evaluate the toxicity of CombiMAG injection into mice via the tail vein, we performed clinical manifestation, blood chemistry, and histopathological examination assays of the liver and kidneys in all groups. The clinical biochemistry tests revealed no significant change in the three groups (Table 1). Treatment with transfection did not induce any change in the absolute body weights (Table 1). The histopathological results of the kidney, liver, and lungs in the three different groups were identical (Fig. 4). Thus, the repeated administration of CombiMAG did not cause any apparent toxicity.

### Table 1 Body weight and serum biochemical parameters

<table>
<thead>
<tr>
<th>Item</th>
<th>Control (n = 4)</th>
<th>Lipo (n = 4)</th>
<th>CombiMAG (n = 4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight</td>
<td>26.5 ± 4.2</td>
<td>24.6 ± 5.8</td>
<td>25.1 ± 6.2</td>
</tr>
<tr>
<td>AST (U/l)</td>
<td>16.1 ± 6.3</td>
<td>22.1 ± 7.1</td>
<td>19.1 ± 7.3</td>
</tr>
<tr>
<td>ALT (U/l)</td>
<td>22.3 ± 7.8</td>
<td>29.3 ± 10.3</td>
<td>32.3 ± 11.5</td>
</tr>
<tr>
<td>Cr (μmol/l)</td>
<td>78.2 ± 11.5</td>
<td>88.2 ± 12.4</td>
<td>82.4 ± 10.8</td>
</tr>
<tr>
<td>Bun (mmol/l)</td>
<td>8.7 ± 3.2</td>
<td>7.7 ± 3.9</td>
<td>9.7 ± 4.5</td>
</tr>
<tr>
<td>Lac (mmol/l)</td>
<td>1.8 ± 0.4</td>
<td>1.5 ± 0.9</td>
<td>1.3 ± 0.8</td>
</tr>
<tr>
<td>Bil (μmol/l)</td>
<td>39.5 ± 12.7</td>
<td>33.9 ± 11.6</td>
<td>44.5 ± 10.3</td>
</tr>
<tr>
<td>Pro (g/l)</td>
<td>46.8 ± 9.3</td>
<td>48.9 ± 7.3</td>
<td>49.3 ± 7.1</td>
</tr>
</tbody>
</table>

Body weight of mice and the serum biochemical parameters were measured at the end of treatment. Control: mice treated with PBS via tail vein injection; Lipo: mice treated with pGFPshIGF-1R:Lp2000 complex via tail vein injection; CombiMAG: mice treated with pGFPshIGF-1R:CombiMAG:Lp2000 complex via tail vein injection and under a magnetic field within 400 mT. There is no significant difference among the three groups.

**Discussion**

The feasibility of magnetic fields to enhance superparamagnetic iron oxide nanoparticle-mediated transfection in vivo has been described by Scherer et al. [14] and Xenariou et al. [15]. The present results further confirmed that liposomal magnetofection can deliver plasmid DNA into diverse mouse organs. This approach can be used to deliver the gene into targeted tissues and organs in vivo under the control of a magnetic field [16]. Liposomal magnetofection is a process wherein nucleic acids are associated with magnetic nanoparticles combined with cationic lipids to form magnetic lipoplexes. These complexes are then concentrated onto the surface of targeted cells under the influence of a magnetic field. Figure 1(A) shows that plasmid DNA was found in almost all organs, and their concentration in the tumor tissue of the CombiMAG group was significantly higher than that of the Lipo group. As expected, the magnetofection efficiency of pGFPshIGF-1R into subcutaneous tumors via vein injection was superior to lipofection at three different time points. During liposomal magnetofection, the magnetic field forms a translational force on the particles and causes a rapid sedimentation of magnetic lipoplexes over the cell surface. As a result, magnetofection enhances transfection efficiency by applying static magnetic fields to increase the local concentration of particles on the cell surface, thereby promoting uptake rather than activating cellular uptake mechanisms [14,17].

Magnetofection-mediated pGFPshIGF-1R delivery significantly reduced the expression of IGF-1R in the tumors compared with controls (Fig. 2). Our data showed that shRNA delivered by CombiMAG in vivo significantly down-regulated the targeted gene expression at 24, 48, and 72 h post-intravenous administration compared with lipofection. In the magnetofection group, the inhibition effect of shRNA on the IGF-1R gene became obvious with

![Figure 4](https://academic.oup.com/abbs/article-abstract/44/7/591/1152)
increased exposure time to pGFPshIGF-1R. However, the same effect was not significant in the lipofection group. These results provide a very important insight into the kinetic parameters of magnetofection in vivo, and will benefit further studies on therapeutic gene delivery as well as targeted gene therapy via magnetofection in vivo.

The delivery of pGFPshIGF-1R to tumors has previously been shown to promote apoptosis in an intraperitoneal route-mediated delivery system [3], and also increases apoptosis via intratumoral injections [4]. In the present study, we evaluated the target tumor suppression of magnetofection via CombiMAG-carrying plasmids expressing pGFPshIGF-1Rs in tumor-bearing mice. This report is the first targeted gene delivery via the vein system for the gene therapy in lung cancer. The accumulation and delivery of pGFPshIGF-1R within the tumor mediated by nanoparticles were correlated with a significant reduction in the growth of tumors compared with controls from the 3rd day to 30th day post-intravenous administration (Fig. 3). Our data showed that magnetofection contributed to the targeted delivery of cancer therapeutic genes in vivo. pGFPshIGF-1R delivered by magnetofection significantly reduced the gross tumor volume and weight of xenograft tumors compared with the lipofection group. This result indicated that tumor targeting using magnetic vectors under an external magnetic field increased the site specificity and selectivity of the therapy. Similar results have also been reported by Krotz et al. [18].

In gene transfection, magnetic nanoparticles have been demonstrated to exhibit very good specificity and efficiency. However, the lack of information on the toxicity of manufactured nanoparticles poses serious problems. To obtain information on toxicity, we performed serum biochemical, blood chemistry, and histopathological examinations. Injection of the therapeutic dose did not have obvious side effects on the host within a short period of time based on the analyses of body weight, as well as liver and kidney pathological changes.

Overall, we successfully applied magnetofection-mediated gene delivery to significantly inhibit lung cancer xenograft growth in vivo. The nanoparticles did not cause clear toxicity under the current conditions, providing a novel method for targeted and therapeutic gene delivery in gene therapy. These findings further strengthened the potential use of CombiMAG as a possible anti-cancer agent for clinical application.

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References