Abstract. The aim of this study was to explore a novel gene vector for targeting gene therapy. Materials and Methods: We conjugated a peptide ligand (named GA3) for endothelial TEK tyrosine kinase (Tie2) with polyethylenimine (PEI) to construct a GA3-PEI complex and used the vector to transfer reporter and therapeutic gene in vitro and in vivo respectively. Results: The results demonstrated the vehicle was able to transfer reporter genes specifically into lung cancer SPC-A1 cells and SPC-A1 xenografts highly expressing Tie2 and epithelial cells of bronchus, but not in heart, liver, spleen, kidney, lung alveolar and vascular tissues. In the gene therapy study, tumor growth was significantly inhibited in SPC-A1 xenograft-bearing mice treated with GA3-PEI/p53 complexes compared with control groups (p<0.05). Conclusion: Our results indicated that GA3-PEI is an efficient gene delivery system targeting Tie2.

Current approaches such as surgery, chemotherapy and radiotherapy are still insufficiently effective in treating tumors because of their invasive, aggressive growth profile, as well as the complex mechanisms involved in cancer development. New anticancer strategies are thus urgently required. Targeted cancer gene therapy is of unquestionable importance for improving therapeutic efficacy and minimizing adverse effects (1, 2). However, current gene therapy is mainly limited by the handicap of producing an efficient gene delivery vehicle. Receptor-mediated gene delivery systems have been developed as an attractive approach to targeting genes into defined cells overexpressing cellular membrane receptors (3, 4). It is particularly interesting that this approach has the potential to circumvent the main disadvantage of viral vectors (5). The first successful gene transfer with a receptor-mediated gene transfer system was shown by Wu and Wu (6) who developed methods to introduce foreign genes into the liver via the asialoglycoprotein (ASGP) receptor. ExGen 500 is a linear (22 kDa) polyethylenimine (PEI) that was shown to be efficient in gene transfer in vitro (7, 8). PEI conjugated with the epidermal growth factor receptor (EGFR) peptide ligand facilitated 10- to 100-fold higher gene expression levels in tumor tissue than in other tissues (9). Recently, small molecular peptide ligands have been pursued as potential tumor-targeting agents for selective delivery of therapeutic genes to tumors because of advantages of their being readily diffusible, having less immunogenecity, and higher target-to-background ratios compared with natural macromolecular ligands (10, 11).

The endothelial cell-restricted receptor tyrosine kinase, Tie2, has been described as a novel marker of microvasculature of solid tumors (12, 13). It plays a pivotal role in tumor angiogenesis. Gene therapy targeting Tie2 has been proposed as a potentially powerful approach for the treatment of cancer (14, 15). In our previous study, we identified a peptide of 23 amino acids, named GA3, which is able to bind to Tie2 as its ligand. Here, we conjugated the peptide ligand with PEI to construct a Tie2-mediated non-viral gene delivery vector and used the vector to transfer the reporter gene in vitro and in vivo. Mutated p53 has been found in nearly 50% of cases with lung cancer, and is associated with poor prognosis (16). PEI/wt p53 transfection can inhibit the growth of human head and neck squamous cell carcinoma xenografts with mutated p53 in mice (17).
this study, the experimental gene therapy was carried out in mice bearing human lung carcinoma xenografts by transferring wild-type p53 gene with the GA3 gene delivery vector.

**Materials and Methods**

**Regents.** GA3 (CHPIETLVDFQYEYQPDEIEYIFKPSVPLMRP) was synthesized in an Applied Biosystems A430 Peptide Synthesizer (Foster, CA, USA). All the oligopeptides were purified by high performance liquid chromatography (HPLC) with >95% purity (GL Biochem Company, Shanghai, P.R. China). PEI of 22 kDa (ExGen 500, Fermentas, Hanover, USA) was purchased from Sigma (St. Louis, MO, USA). Protamine sulfate with molecular weight of 7 kDa was purchased from Shanghai First Biochemistry Medicine Company P.R. China. Succinimidyl-3-(2-pyridyldithio)-propionate (SPDP), dithiothreitol (DTT), 5-bromo-4-chloro-3-indolyl-β-D-galactosidase (X-gal) were purchased from Sigma. Sephadex G-50 (500, Fermentas, Hanover, USA) was purchased from Sigma (St. Louis, MO, USA). Protamine sulfate with molecular weight of 7 kDa was purchased from Shanghai First Biochemistry Medicine Company P.R. China. Succinimidyl-3-(2-pyridyldithio)-propionate (SPDP), dithiothreitol (DTT), 5-bromo-4-chloro-3-indolyl-β-D-galactosidase (X-gal) were purchased from Sigma. Sephadex G-50 was purchased from Pharmacia (New Jersey, USA). The human p53 gene and β-gal gene were inserted into the pCPE vector (Invitrogen, Shanghai, P.R. China), which was driven by cytomegalovirus promoter.

**Cell culture and tumor model.** SMMC7721, a human hepatic carcinoma cell line that lacks expression of Tie2 (18), was a gift from the Second University of Military Medicine (Shanghai, P.R. China). SPC-A1, a human lung adenocarcinoma cell line expressing Tie2 (18), was bought from the American Type Culture Collection (Manassas, VA, USA). These cells were cultured at 37°C in medium consisting of 90% Dulbecco’s modified Eagle’s medium (GIBCO-BRL, Grand Island, NY, USA) containing 10% fetal calf serum (FCS) in a humidified 5% CO2 atmosphere. Nude mice with subcutaneously s.c. transplants derived from lung adenocarcinoma SPC-A1 cells were provided by Shanghai Cancer Institute. Nude mice were injected s.c. with 1×106 cells and ready for in vivo assay when the tumor size reached 0.5 cm in diameter.

**Preparation of GA3-PEI/DNA complexes.** PEI was conjugated with GA3 at the molar ratio of 1:1. The conjugation procedure of GA3-PEI was largely the same as that described previously (19). GA3-PEI and plasmid DNA were sterilized by filtration through 0.22 μm filters (Millipore, Billerica, USA). The DNA/GA3-PEI complex was prepared by mixing DNA with GA3-PEI. GA3-PEI was largely the same as that described previously (19). GA3-PEI was conjugated with plasmid DNA at a rate ≥3, suggesting that GA3-PEI was capable of forming polyplexes with different DNA.

**In vitro transfection studies.** In the in vitro gene delivery assay, 4×104 SMMC7721 and SPC-A1 cells were seeded into 0.5 ml medium in each well of a 24-well plate (Falcon, St. Louis, MO, USA). After cells reached a confluence of approximately 50%, the medium was removed and washed with 0.5 ml phosphate buffered saline (PBS), then replaced with 0.5 ml serum-free media containing GA3-PEI/pBK-CMV-luciferase polyplexes or PEI/DNA with the quantity equivalent to 1 μg DNA. The cells were incubated for another 24 h, then the medium was removed from each well again and the cells were washed with 0.5 ml/well cold PBS. Ice-cold lysis buffer (100 μl; 100 mM Tris-HCl, 2 mM EDTA, 1% Triton X-100, pH 7.8) was added to each well and eluates incubated on ice for 15 min. The lysate was transferred into 0.5 ml centrifuge tubes and centrifuged at 13,000 rpm for 15 min. The activity of luciferase was measured in a Turner Designs luminometer (Promega, Madison, Wisconsin, USA) immediately after mixing 20 μl of cell lysate with 100 μl of luciferase substrate (Promega, Madison, Wisconsin, USA) in terms of relative light units per milligram protein (RLU/mg). All experiments were performed in triplicate.

**In vivo gene delivery studies.** In in vivo studies, GA3-PEI/pCMV-β-gal polyplexes were injected into peri-tumoral regions at a dose equivalent to 0.2 μg DNA/mouse, PEI/β-gal gene was used as control. The mice were sacrificed at 24 h after injection and tumor xenografts, heart, liver, spleen, lung and kidney were dissected and washed three times with 0.1 M PBS (pH 7.4), fixed and dyed with X-gal. The detailed method was reported previously (7). All experiments were carried out in experimental groups containing at least six mice bearing tumor. PEI/pCMV-β-gal complexes were used as control.

**In vivo targeted gene therapy experiments.** Human lung cancer SPC-A1 cells (1×106) were inoculated subcutaneously into 4-week-old female athymic mice (BALB/c). When tumor size reached about 0.5 cm in diameter, mice were randomly divided into the following five groups with six mice in each group: normal saline (NS); GA3; wt p53; PEI/wt p53; and GA3-PEI/wt p53. Mice were injected subcutaneously into peri-tumoral regions with GA3-PEI/wt p53 complexes and other agents every two days for a total of eight times. Tumor growth was monitored by measuring the tumor dimensions with a vernier caliper three times weekly until necrobiosis appeared in the tumor xenograft. Tumor volume was calculated according to the formula (V=πab2/6), where a=tumor long dimension and b=tumor short dimension.

**Statistical analysis.** One-way ANOVA followed by the two-tailed Student’s t-test was used for statistical evaluation of differences. Results are represented as the mean±SE.

**Results**

**Construction of GA3-PEI/DNA complexes.** The capability of GA3-PEI to package DNA was determined by gel retardation assay. The N/P ratio of GA3-PEI/DNA electrostatic complex reflects the overall positive to negative charge ratio. The Figure 1 shows that GA3-PEI retarded the mobility of plasmid DNA at a rate ≥3, suggesting that GA3-PEI was capable of forming polyplexes with different DNA.

**In vitro gene delivery assay.** To assure the targeting ability of GA3-PEI vector, an in vitro gene transfection study was conducted in SPC-A1 cells and SMMC7721 cells using GA3-PEI/luciferase cDNA complex. The results in Figure 2 show that the level of luciferase activity detected in groups treated with GA3-PEI/luciferase cDNA 24 hours later in SPC-A1 cells was significantly higher than that of Tie2-
negative SMMC7721 cells (p<0.05). But the luciferase activity detected in SPC-A1 cells treated with PEI/luciferase cDNA 24 hours later was not significantly different from that of SMMC7721 cells.

In vivo gene delivery assay. To determine the ability of GA3-PEI to mediate targeted gene delivery in vivo, mice bearing SPC-A1 xenografts expressing Tie2 were injected with GA3-PEI/β-gal gene complex subcutaneously into peri-tumoral regions. The results reveal that SPC-A1 xenograft treated with PEI/β-gal gene complex as control had no detectable blue dye (Figure 3A) but blue staining was found in SPC-A1 xenograft transfected with GA3-PEI/β-gal gene complex (Figure 3B). Little β-gal activity was detected in heart, liver, spleen and kidney with the exception of lung bronchial mucous membrane transfected with GA3-PEI/β-gal gene complex (Figure 4).

Targeted gene therapy assay. In vivo gene therapy experiments showed that the difference between the experimental and control groups (naked p53, PEI/p53, NS) was significant (p<0.05). By comparing the control group with the NS group, the inhibition rate of GA3-PEI/p53 was 61.29%. As shown in Figure 5, injection of peri-tumoral regions with GA3-PEI/wt p53 complexes inhibited tumor xenograft growth. But no difference was observed in tumor growth between untreated mice and control mice treated with irrelevant PEI/DNA complex.

Discussion

We obtained a peptide, GA3, which showed specific binding capability to Tie2 in our previous study (18). It was designed according to the putative binding region of Ang-2 to its receptors. It maintained the capability of binding Tie2 and was smaller than Ang-2 (496 amino acids). It would also be less likely to undergo intramolecular conformational alteration and has lower immunogenicity as compared with using whole growth factor macromolecule as targeting element. In this study, we showed that the GA3 gene delivery system was able to transfer exogenous gene to cells with Tie2 expression both in vitro and in vivo. This is most likely due to the generally rapid diffusion and clearance of PEI/DNA polyplexes from the circulation and the fact that most normal organs express Tie2. In addition, in the in vivo gene therapy assay, the inhibition rate of the GA3-PEI/p53 group was 61.29% compared with that of the NS group. This indicated

![Figure 1](https://example.com/figure1.png)

**Figure 1.** Retardation of DNA migration by GA3-PEI/DNA complexes was examined by 1% agarose gel electrophoresis. GA3-PEI was mixed with plasmid (pCMV-β-gal, pCMV-luciferase) DNA and analyzed by agarose electrophoresis. Each sample had 0.2 μg DNA loaded into the sample well. A: pCMV-β-gal retardation of different complexes using PEI as backbone. Lane M: DNA marker, Hind III. B: pCMV-luciferase retardation of different complexes using PEI as backbone.

![Figure 2](https://example.com/figure2.png)

**Figure 2.** In vitro gene delivery assay using GA3 peptide as a targeting agent. Different types of cells were transfected with GA3-PEI/luciferase complex for 24h after transduction, luciferase activity was measured in relative light units (RLU/mg protein). Twenty-four hours after transduction, luciferase activity was higher in SPC-A1 cells than in SMMC7721 cells (p<0.05), but no difference was found between SPC-A1 and SMMC7721 cells when transfected with PEI/DNA or naked DNA.

Figure 4. Expression of β-galactosidase gene in different organs transfected with GA3-PEI/β-gal gene complex. Little β-gal activity was detected in heart (A), liver (B), spleen (C) and kidney (E), but was detected in lung bronchial mucous membrane (D) (×400).
that PEI was essential for the full biological function of the composite vector system for gene delivery. PEI interacts with DNA by electrostatic binding to form a soluble complex which enables the endocytosed polypeptide/DNA complex to escape lysosome fusion and subsequent degradation. GA3, as ligand for Tie2, can target the DNA-PEI-ligand complex in cells by binding to the receptor and being endocytosed. This also demonstrates that GA3-PEI might be an efficient novel gene delivery vector for targeting Tie2.

PEI is one of the most promising non-viral vectors that has been developed (20, 21). Studies showed that linear PEI with low molecular weight was the most efficient in transfection and the least cytotoxic. The advantages associated with these non-viral vectors include their large-scale manufacture, their low immunogenic response, and their capacity to carry large inserts. However, the transfection efficiency of non-viral vector is still lower than that of its viral counterpart. A number of alterations (e.g. ligand attachment) could improve non-viral vectors to become more promising gene delivery systems and allow them to be modified to make systemic delivery possible. Goula et al. used linear low molecular weight PEI (22 kDa)/DNA complexes for systemic application and showed efficient gene delivery, with high gene expression in lung and lower expression in a variety of organs including heart, liver, spleen, and kidney (22). Li et al. reported that GE11, a small peptide ligand of epidermal growth factor receptor (EGFR), conjugated with PEI vector can efficiently transfer genes into EGFR overexpressing cells and tumor xenografts (7).

However, the complicated mechanism of Tie2-mediated endocytosis remains to be further elucidated. The data presented in this context would possibly provide some indirect evidence to support the notion that the GA3 system may transfer genes via a Tie2-mediated mechanism. Taken together, these data show the present GA3 system can target genes in tumor vascular endothelial cells and tumor cells. Studies are underway in order to further improve the Tie2-mediated gene delivery system for future clinic trials of cancer gene therapy.

Acknowledgements
We thank Mrs. Yuyan Zhang for her technical assistance, Dr. Wenxin Qin for offering pCDNA3.0 plasmid, and Dr. Rong Wang (Medical School of Oregon State University, Corvallis, USA) for useful suggestion.

References


