Original Article

A novel multifunctional nanocomposite C225-conjugated Fe₃O₄/Ag enhances the sensitivity of nasopharyngeal carcinoma cells to radiotherapy

Di Zhao, Xinchen Sun*, Jinglong Tong, Jun Ma, Xiaodong Bu, Ruizhi Xu, and Renhua Fan

Department of Oncology, Zhongda Hospital, School of Medicine, Southeast University, Nanjing 210009, China

*Correspondence address. Tel: +86-25-83275408; Fax: +86-25-8327206; E-mail: sunxch055@yahoo.com.cn

Radiotherapy is the major treatment for nasopharyngeal carcinoma, a malignant tumor of epithelial origin. In this process, a tracer with high sensitivity is pivotal for diagnostic imaging in radiotherapy. Here, we designed a novel multifunctional magnetic silver nanocomposite, Fe₃O₄/Ag conjugated to an epidermal growth factor receptor-specific antibody (C225), which can be potentially used for synchronous cancer therapy and diagnosis via magnetic resonance imaging. Characteristics of Fe₃O₄/Ag/C225 were determined by transmission electron microscopy, energy dispersive X-ray spectroscopy, ultraviolet spectra, and dynamic light scattering. The results demonstrated that Fe₃O₄/Ag/C225 nanoparticles were spherical and dispersed well in water. The activity of C225 was preserved ∼80% in the Fe₃O₄/Ag/C225 nanoparticles. Furthermore, we tested the cytotoxicity and radiosensitivity of the nanocomposite for human nasopharyngeal carcinoma cell lines (CNEs) in vitro. MTT analysis revealed that Fe₃O₄/Ag/C225 could inhibit the proliferation of CNEs in a dose- and time-dependent manner. The clonogenic assay indicated that Fe₃O₄/Ag/C225 combined with X-ray treatment could increase the sensitivity of CNEs to irradiation. In a summary, the novel multifunctional nanocomposite Fe₃O₄/Ag/C225 might be a potential radiosensitizer for treating malign tumors in the clinic.

Keywords radiotherapy; radiosensitivity; molecular targeted therapy; nasopharyngeal carcinoma cells

Introduction

Nasopharyngeal carcinoma, a malignant tumor of epithelial origin, is one of the most common malignant tumors in southern China. Radiotherapy is the main method in the treatment of nasopharyngeal carcinoma [1,2]. However, irradiation for nasopharyngeal carcinoma has a high treatment failure rate because of its rapid growth and invasive behavior [3]. Recently, with the rapid development of imaging and computer technology, radiotherapy can now be performed in four dimensions by image guide radiation therapy (IGRT) [4]. However, as no ideal medium for trace imaging in radiotherapy is available, IGRT cannot be used to its fullest potential.

Nanomaterials have been increasingly used in biomedical fields, especially in healthcare. Nanoparticles have enormous advantages in coupling with various antibody and luminescent labeling for its characteristic of small size (<100 nm) and surface paintability. Thus, nanoparticles can be a good tracer. Owing to the nanomaterial’s physicochemical properties, nanosized particulate system can be designed specifically in collaboration with molecular biology technique, which will combine cancer diagnosis with therapy [5,6]. As we all know, the Fe₃O₄ nanoparticles are qualified for serving as magnetic resonance imaging (MRI) probes to noninvasively monitor the molecular and cellular event in vivo. Fe₃O₄ nanoparticles also can couple together with proteins like some antibodies, which will induce the biological therapeutic effects [7,8]. By this means, diagnosis and treatment could be implemented synchronously.

Epidermal growth factor receptor (EGFR) is a tyrosine kinase receptor, which is important in the phosphatidylinositol 3-kinase (PI3K)/Akt signaling pathway [9]. The overexpression of EGFR correlates to cell proliferation, angiogenesis, and tumor growth, and may induce tumor cells to metastasize [10,11]. Clinical studies have shown that the patients with brain metastasis have the overexpression of human EGFR2 and/or EGFR [12,13]. EGFR inhibitors have been used to treat the advanced cancer patients by interrupting the PI3K/Akt-signaling cascades [14]. Several different EGFR antibodies including humanized monoclonal antibodies such as cetuximab (Erbitux, Merck Serono Ltd., Darmstadt, Germany) have been used to treat the head and neck tumor associated with radiotherapy, which can improve locoregional control and reduce mortality without increasing common toxic effects [15].

In this study, we reported a novel multifunctional magnetic–particle–silver nanocomposite system. In this system, the core of the composite Fe₃O₄/Ag/C225 particle, Fe₃O₄, can
be used as a tumor tracer for radiation therapy, and silver has been previously described as a radiation sensitizer [16]. The EGFR-specific antibody (C225) has been successfully used as an anti-tumor treatment in clinical studies [15]. Then this magnetic silver nanocomposite could be potentially used for targeted cancer detection by MRI as well as for synchronous cancer therapy via a therapeutic antibody with pro-apoptotic effects. Furthermore, we found the composite particle Fe3O4/Ag/C225-inhibited CNEs proliferation and increased the sensitivity to irradiation. Therefore, Fe3O4/Ag/C225 might be a potential radiosensitizer for treating malign tumors in the clinic.

Materials and Methods

Chemicals and reagents
All chemicals used in this experiment were of analytical reagent grade. Ferrous sulfate (FeSO4•7H2O), oleic acid, dimethyl sulfoxide (DMSO), n-hexane, acetone, acetic acid, anhydrous sodium acetate, absolute alcohol, and 2,3-dimercaptosuccinic acid (DMSA) were purchased from Sinopharm Chemical Reagent Co. Ltd (Shanghai, China). Sodium borate and boric acid were reagents from Shanghai Lingfeng Chemical Reagent Co. Ltd (Shanghai, China). Tetramethylammonium hydroxide was supplied by Shanghai Zhourui Chemical Reagent Co. Ltd (Shanghai, China). 1-Ethyl-3-(3-dimethyllaminopropyl) carbodiimide hydrochloride (EDC) and 1-hydroxy-2,5-pyrrolidinedione (NHS) were purchased from Sigma Aldrich (St Louis, USA). Albumin bovine (BSA) was obtained from the Nanjing Bookman Biotechnology Ltd (Nanjing, China). Nimotuzu monoclonal antibody was from Beijing BaiTai Biopharmaceutical Co. Ltd (Beijing, china). Hydrodynamic size distributions of Fe3O4/Ag were assessed by dynamic light scattering (Beckman, Pasadena, USA). C225 (cetuximab) was purchased from Merck Serono (Darmstadt, Germany).

Preparation of DMSA-modified Fe3O4 nanoparticles and Fe3O4/Ag/C225 nanocomposites
Oleate-capped Fe3O4 nanoparticles were prepared. FeSO4•7H2O (3 mmol), oleic acid (10 ml), N(CH3)4OH (10 ml), and DMSO (35 ml) were mixed in a 100-ml three-necked bottle, and the mixture was refluxed at 140°C for 1 h with stirring in the presence of N2. A black precipitate was obtained via magnetic separation, washed with alcohol three times and dispersed in water. Fe3O4/Ag nanoparticles were synthesized by the reverse micelle method. One hundred microliters of 40 mM Fe3O4 nanoparticles were mixed with a W/O microemulsion containing 1.4 ml of Triton X-100, 1.4 ml of n-hexanol, and 7.5 ml of cyclohexane, with vigorous stirring. Then, 200 μl of 0.1 M AgNO3 was added. After 30 min, 200 μl of 0.2 M NaBH4 was added to the solution. The mixture was stirred at room temperature for 4 h. The black precipitate was obtained via magnetic separation, washed with alcohol three times and then centrifuged and repeatedly washed with ethanol and water to remove surfactant and unreacted materials. The obtained nanoparticles were suspended in water for future use. Conjugation of C225 to the Fe3O4/Ag by adding borax buffer (PH 9.0) to adjust pH value to 7.9, blocking the reaction by adding 4% BSA solution (400 μl) with continuous shaking at 37°C for 1 h, then adding C225 to Fe3O4/Ag with continuous shaking at 37°C for 30 min. Finally, Fe3O4/Ag/C225 nanoparticles were obtained by adding certain amount of purified water to resuspend nanoparticles after centrifugation at 11,742 g at 4°C for 1 h. The conjugated nanoparticles were purified by gel chromatography (GE sephacryl s-300, Pittsburgh, USA). The final Fe3O4/Ag/C225 composites were stored at 4°C. The particle size and morphology of the Fe3O4/Ag/C225 composites were characterized by transmission electron microscopy (TEM; JEOL JEM-2100, Tokyo, Japan). Photon correlation spectroscopy was used to determine the hydrodynamic size distribution using a Beckman Coulter N4 Plus Submicron Particle Analyzer.

Cell culture
CNE cells were purchased from the the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). Cells were cultured in a humidified atmosphere containing 5% CO2 in RPMI 1640 medium (Gibco, Carlsbad, USA) supplemented with heat-inactivated fetal bovine serum (15% by volume), penicilllin G (50 U/ml), and streptomycin (50 μg/ml). The cell line was maintained in the exponential growth phase and provided with fresh medium every 2–3 days.

Enzyme-linked immunosorbent assay
We used a double antibody sandwich enzyme-linked immunosorbent assay (ELISA) to test the level of C225 in the nanocomposite, Fe3O4/Ag/C225. CNEs were seeded in a 96-well plate in 300-μl medium at a density of 0.8 × 104 cells/well and then cultured for 2 days. Then the cells were washed with phosphate buffered saline (PBS) four times, and fixed with pre-cooled 0.125% pentyl glycol for 30 min at 4°C. After that the cells were incubated in 1% BSA at 37°C for 1 h to block non-specific binding, and
then washed twice. The serially diluted C225 and different concentrations of Fe$_3$O$_4$/Ag/C225 were added to the 96-well plate. Horseradish peroxidase (HRP)-conjugated anti-mouse immunoglobulin G (IgG) (1:2000, Zhongshan Goldbride Ltd., Beijing, China) was added, followed by the TMB (Horseradish Peroxidase Color Development Solution). Positive and negative control wells were included to quantify protein levels for all samples. The absorbance was measured at 450 nm using a microplate spectrophotometer (SpectraMax Molecular Devices, Sunnyvale, USA).

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay
To measure the cytotoxicity of Fe$_3$O$_4$/Ag/C225, cells were seeded in 96-well plates at a density of 2.5 x 10$^4$ cells/well and allowed to adhere for 24 h at 37°C. The cells were cultured in the presence of different concentration of Fe$_3$O$_4$/Ag/C225 (0, 50, 100, 200, 400, and 800 µg/L) for 24–48 h, followed by 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma) treatment for 4 h at 37°C. Then DMSO was added to each well to dissolve the dark blue crystal product. The absorbance was measured at 488 nm using a microplate reader (Bio-Rad, Hercules, USA).

Clonogenic assay
Exponentially growing cells were irradiated at the doses of 0, 1, 2, 4, 6, and 8 Gy at room temperature, followed by being treated with or without Fe$_3$O$_4$/Ag/C225 treatment for 24 h. Then the cells were seeded in a 24-well plate at a density of 200 cells/well, and cultured for 10–14 days. Plates were washed in PBS and the colonies were fixed with 95% ethanol. Staining was carried out with 0.1% crystal violet solution. Colonies of > 50 cells were counted to calculate the surviving fraction. Six parallel samples were scored for each treatment condition.

Western blot analysis
Treated and untreated cells were lysed in extraction buffer [1% sodium dodecyl sulfate (SDS), 1 mM Na$_3$VO$_4$, 0.1 M Tris (pH 7.4)], plus protease inhibitor mixture (Roche, Indianapolis, USA), and phosphatase inhibitor mixture (Upstate Biotechnology, Lake Placid, USA) at 4°C. The protein concentration was measured using a protein assay kit according to the manufacturer’s instructions (Bio-Rad). Proteins (40 µg/lane) were running on a 5% or 10% SDS-polyacrylamide gel, and then transferred to polyvinylidene difluoride membranes (Millipore, Bedford, USA). The membranes were blocked with 5% non-fat dried milk for 4 h in PBS—0.1% Tween (PBST) and probed with C225 (1:1000) at 4°C overnight. After three times washing with PBST, the membranes were incubated with HRP-conjugated goat anti-rabbit IgG (1:100) (Promega, Madison, USA) for 2 h, and were visualized using ECL chemiluminescent substrate (GE Healthcare Life Sciences, Fairfield, USA).

Statistical analysis
Data were expressed as the mean ± standard error (SE). SPSS version 16 (SPSS, Chicago, USA) was used to analyze one-way analysis of variance followed by a Student–Newman–Keuls test. $P < 0.05$ was considered statistical significant.

Results
The characteristics of Fe$_3$O$_4$/Ag/ C225 nanocomposites
Figure 1(A–D) shows the morphology of Fe$_3$O$_4$, AgNPs, Fe$_3$O$_4$/Ag and Fe$_3$O$_4$/Ag/C225 nanoparticles determined by TEM. The majority of magnetic nanoparticles were spherical or nearly spherical. The Fe$_3$O$_4$/Ag/C225 particle sizes were small in a range between 25 and 35 nm, which is larger than that of Fe$_3$O$_4$ and Ag. And there is no significant difference in size compared with Fe$_3$O$_4$/Ag under TEM.

To confirm the composition of the composites, EDS spectrum in situ composition analysis was collected. The result showed the presence of silver, iron, and oxygen in the composites [Fig. 2(A)]. The diffraction peaks of silver, iron, and oxygen exist in the pattern, which indicated that Fe$_3$O$_4$/Ag nanocomposites have been successfully synthesized. The ultraviolet spectra of the nanocomposite indicated that the absorption peak of Fe$_3$O$_4$/Ag at 423 nm gradually increased after binding to Fe$_3$O$_4$, with a red shift from 390 to 423 nm [Fig. 2(B)]. The hydrodynamic size distributions of Fe$_3$O$_4$/Ag and Fe$_3$O$_4$/Ag/C225 as assessed by dynamic light scattering were 35.98 ± 15.15 nm and 102.34 ± 42.12 nm, respectively [Fig. 2(C)]. The larger hydrodynamic size of magnetic nanoparticles was attributed to the presence of the antibody C225 in the hydration layer and the particle aggregations in water. The results of in vitro release rate testing revealed that the nanocomposites were released slowly and steadily [Fig. 2(D)].

The activity of the EGFR antibody is preserved
in Fe$_3$O$_4$/Ag/C225 nanoparticles
The results of ELISA showed that the activity of the EGFR antibody in the nanocomposites Fe$_3$O$_4$/Ag/C225 was preserved ~80%. Furthermore, when the concentration decreased the preserved activity increased. When the concentration of the pure C225 antibody was 500 µg/L, the activity of the C225 in the Fe$_3$O$_4$/Ag/C225 was 76.82% ± 2.12%. However, when the concentration of C225 was 72.5 µg/L, the preserved activity was 83.17% ± 3.23%.

Fe₃O₄/Ag/C₂₂₅ enhances the sensitivity of CNEs to radiotherapy

Figure 1 TEM of Fe₃O₄/Ag/C₂₂₅ nanoparticles  
(A) The morphology of Fe₃O₄ nanoparticles. (B) The morphology of AgNPs. (C) The morphology of Fe₃O₄/Ag nanoparticles. (D) The morphology of Fe₃O₄/Ag/C₂₂₅ nanoparticles.

Figure 2 Characteristics of Fe₃O₄/Ag/C₂₂₅ nanoparticles  
(A) EDS spectrum of Fe₃O₄/Ag composites. (B) The absorption peak of Fe₃O₄/Ag at 423 nm. (C) Hydrodynamic size distributions of Fe₃O₄/Ag and Fe₃O₄/Ag/C₂₂₅ nanoparticles were assessed by dynamic light scattering. (D) The release rate of Fe₃O₄/Ag/C₂₂₅ under in vitro conditions. (E) The activity preservation of C₂₂₅ in the nanocomposite Fe₃O₄/Ag/C₂₂₅ was analyzed by ELISA.
Thus, C225 activity in the Fe₃O₄/Ag/C225 nanocomposite was well preserved [Fig. 2(E)].

Fe₃O₄/Ag/C225 inhibits the proliferation of CNE cells
When CNEs were treated with Fe₃O₄/Ag/C225 at different concentrations (0, 50, 100, 200, 400, 800 μg/L) for 24 h, the morphology of the cells changed from polygonal to round, and the cell numbers also decreased [Fig. 3(A)]. MTT assays showed that Fe₃O₄/Ag/C225 inhibited cell growth in a concentration-dependent manner with an IC₅₀ of 350.49 ± 3.14 μg/L and 262.53 ± 4.47 μg/L for 24 and 48 h exposure, respectively (P < 0.05) [Fig. 3(B)].

Fe₃O₄/Ag/C225 enhances the sensitivity of CNE cells to X-ray irradiation
The effects of Fe₃O₄/Ag/C225 on the cytotoxicity of X-ray irradiation in CNE cells were investigated using clonogenic assay (Fig. 4). Cells were first irradiated at different doses of X-rays (0, 1, 2, 4, 6, and 8 Gy), then incubated with 50 μg/L Fe₃O₄/Ag and 30 μg/L Fe₃O₄/Ag/C225 (~1/9 concentration of IC₅₀) for 24 h. This concentration of Fe₃O₄/Ag and Fe₃O₄/Ag/C225 and exposure time were not cytotoxic (data not shown). Cell survival curves were plotted with a single-hit multitarget model to yield values of the relative parameters. The parameter D₀ was used to characterize the radiosensitivity in the linear (high dose) region, and the value of parameter Dq indicated the cells ability to repair potentially lethal damage in the shoulder (low dose) region. According to the Fig. 4, for X-ray irradiated CNE cells without Fe₃O₄/Ag and Fe₃O₄/Ag/C225 treatment, the D₀ and Dq values were 2.852 ± 0.074 and 2.124 ± 0.121 Gy, respectively. For the cells synchronously treated under irradiation and Fe₃O₄/Ag or Fe₃O₄/Ag/C225, the D₀ values were 1.923 ± 0.012 Gy (for Fe₃O₄/Ag) and 1.261 ± 0.023 Gy (for Fe₃O₄/Ag /C225), and the Dq values were 1.792 ± 0.004 (for Fe₃O₄/Ag) and 1.704 ± 0.032 Gy (for Fe₃O₄/Ag /C225), respectively. Therefore, Fe₃O₄/Ag/C225 potentiates the cytotoxicity of X-rays in CNE cells compared with Fe₃O₄/Ag alone (~1.525 fold (D₀ for Fe₃O₄/Ag compared with D₀ for Fe₃O₄/Ag/C225). The effect was observed at 30 μg/L Fe₃O₄/Ag/C225 for 24 h. Colony-forming efficiency was determined 10–14 days later, and the survival fractions of Fe₃O₄/Ag- or Fe₃O₄/Ag/C225-treated cells were calculated by normalizing to the colony-forming efficiency of untreated cells.

Fe₃O₄/Ag/C225 down-regulates the expression of EGFR
To explore the molecular mechanism of Fe₃O₄/Ag/C225-sensitizing CNE cells to irradiation, we further detected the expression of EGFR in CNE cell lines when treated with Fe₃O₄/Ag/C225 combined with X-ray irradiation. Western blot analysis showed that Fe₃O₄/Ag/C225 or irradiation alone decreased the expression levels of EGFR,
whereas Fe$_3$O$_4$/Ag/C225 inhibited the expression of EGFR more dramatically when combined with irradiation (Fig. 5), which indicates that Fe$_3$O$_4$/Ag/C225 treatment might increase the radiosensitivity of CNE cells by inhibiting the expression of EGFR.

**Discussion**

Radiation therapy is one of the main treatments for malignant tumors, and ~70% of cancer patients require radiation therapy [17]. IGRT is used to accurately target tumors, especially in head and neck malignant tumors. Meanwhile, IGRT allows to optimize the dose of irradiation according to the target tumor volume [18]. Therefore, IGRT can reduce the radiation dose on the surrounding normal tissue considering the three-dimensional shape and density of the tumor. An imaging tracer with high sensitivity, specificity, and safety is important for acquiring the high-resolution imaging. However, currently it is hard to find an ideal tracer in the clinical imaging. Although radionuclide imaging methods can evaluate the function of transplanted cells, the sensitivity and spatial resolution are low, and the safety also is an issue [19–21]. In addition, fluorescence-imaging technology can provide images with low background noise and high sensitivity, but so far they only have been used in animal studies. Furthermore, the spatial resolution is poor, anatomical details are not apparent, and the fluorescent light only can image a shallow depth, which will limit its clinical application [22–24].

In this study, we synthesized a new multi-functional composite nanoparticle, Fe$_3$O$_4$/Ag/C225, which could be potentially used for cancer therapy and diagnosis at the same time. The Fe$_3$O$_4$/Ag/C225 formulation, using superparamagnetic Fe$_3$O$_4$ nanoparticles, had good characteristics for MRI [25,26]. Aggregates of magnetic nanoparticles can produce magnetic kernels to enhance the sensitivity of MRI. Thus, the use of Fe$_3$O$_4$ nanoparticles as MRI agent might provide new and highly effective imaging tracers for *in vivo* tumor cell radiotherapy. Our previous studies have demonstrated that AgNPs could enhance the inhibitory effect of radiation on tumor cells, and could be used as radiosensitizers for improving the efficiency of radiotherapy [16]. C225 is the specific monoclonal antibody of EGFR and has been used in clinics to inhibit tumor cell growth. C255 also has been proved to be a radiation-sensitizer. As EGFR is highly expressed in most solid tumors and the antibody possesses a high affinity for its antigen, C225 in the Fe$_3$O$_4$/Ag/C225 formulation might play an important role as an imaging tracer *in vivo* and *in vitro* by binding to the EGFR.

We first conjugated Fe$_3$O$_4$ core with AgNPs by EDC/NHS coupling chemistry, followed by coupling C225 by surface chemical bonds. Then we confirmed the successful synthesis of the nanocomposite by TEM, EDS, ultraviolet spectra, and dynamic light scattering (Fig. 2). The curve of nanocomposite release showed that the Fe$_3$O$_4$/Ag/C225 was stable *in vitro*. As nanocomposites are important for biological applications, we tested the activity preservation of the antibody by ELISA. The results showed that the activity preservation of C225 in the Fe$_3$O$_4$/Ag/C225 nanocomposites was increased when the concentration of C225 decreased. Furthermore, we explored the effect of Fe$_3$O$_4$/Ag/C225 on CNE cells *in vitro*. MTT assay indicated that Fe$_3$O$_4$/Ag/C225 had an inhibitory effect on CNE cells in a dose-dependent manner (Fig. 3). Meanwhile, Fe$_3$O$_4$/Ag/C225 and X-ray treatment could synergistically improve the efficiency of radiotherapy (Fig. 4). Fe$_3$O$_4$/Ag/C225 treatment enhanced the cytotoxicity of X-rays in CNE cells ~2.262 fold, which indicated that Fe$_3$O$_4$/Ag/C225 might be a useful radiosensitizer for improving the outcome of cancer radiotherapy.

EGFR play an important role in cancer development and progression, especially in a variety of human malignancies including lung, head and neck, colon, breast, ovary, and glioma cancer [27]. The majority of head and neck cancers is squamous cell carcinomas, and commonly over-expresses EGFR [28]. In addition, the high expression or activation of EGFR correlates with the radiosistance of cells and tumors [29–31]. In order to investigate the mechanisms of Fe$_3$O$_4$/Ag/C225-induced inhibition in CNEs when combined with X-rays, we measured the expression of EGFR proteins. Western blot results showed...
that Fe$_3$O$_4$/Ag/C$_{225}$ combined with X-ray irradiation treatment remarkably inhibited the expression level of EGFR compared with X-ray treatment alone (Fig. 5). Thus, Fe$_3$O$_4$/Ag/C$_{225}$ might enhance the sensitivity of CNEs to irradiation by inhibiting EGFR expression.

In conclusion, we synthesized a multi-functional Fe$_3$O$_4$/Ag/C$_{225}$ nanocomposite, which may be used as a potent radiosensitizer and imaging tracers for treating malignant tumors in radiotherapy. In the future, in vivo MRI will be performed in order to further explore the sensitivity of Fe$_3$O$_4$/Ag/C$_{225}$ composites as a tracer in vivo. And the effect of Fe$_3$O$_4$/Ag/C$_{225}$ composites on the radiotherapy in vivo also will be confirmed. The novel multifunctional nanocomposite Fe$_3$O$_4$/Ag/C$_{225}$ might be a potential radiosensitizer for treating malignant tumors in the clinic. Fe$_3$O$_4$/Ag also can be coupled with other antibodies, which will provide a wide application in cancer therapy.

**Funding**

This work was supported by the grant from National Natural Science Foundation of China (30970792).

**References**