Specific targeting of nasopharyngeal carcinoma cell line CNE1 by C225-conjugated ultrasmall superparamagnetic iron oxide particles with magnetic resonance imaging

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An accurate definition of clinical target volume (CTV) is essential for the application of radiotherapy in nasopharyngeal carcinoma (NPC) treatment. A novel epidermal growth factor receptor (EGFR)-targeting contrast agent (C225-USPIO) was designed by conjugating ultrasmall superparamagnetic iron oxide (USPIO) nanoparticles with cetuximab (C225), to non-invasively define the CTV of tumor. The immunobinding activity of C225-USPIO to NPC cell line CNE1 was confirmed by flow cytometry and immunofluorescence. The time-dependent accumulation of C225-USPIO in CNE1 cells was evaluated using Prussian blue staining. Targeted internalization and subcellular localization of C225-USPIO was confirmed by transmission electron microscope. The results indicated that C225-USPIO specifically bound to EGFR on the surface of CNE1 cells and was taken up into the cell. The uptake of C225-USPIO by CNE1 cells increased significantly with time, when compared with human IgG-USPIO. In addition, 4.7 T magnetic resonance imaging (MRI) revealed that C225-USPIO had a capacity to accumulate in the CNE1 cells, with a resultant marked decrease in MRI T2-weighted signal intensity over time. These findings imply that C225-USPIO has the potential as an MRI contrast agent and can be employed to non-invasively detect early-stage NPC with EGFR overexpression. This provides sufficient theoretical basis for commencing in vivo experiments with the compound.

Keywords cetuximab; ultrasmall superparamagnetic iron oxide; epidermal growth factor receptor; magnetic resonance imaging

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Introduction

Nasopharyngeal carcinoma (NPC) is a common malignant tumor among populations in southern China and southeast Asia, and has an annual incidence rate of 20–50 cases per 100,000 population [1]. Radiotherapy (RT) is the primary treatment modality for non-metastatic NPC. The 5-year overall survival rate of NPC patients treated by RT alone is ~70% [2]. A key to enhance the curative rate is to obtain an accurate definition of NPC boundaries, which constitutes a fundamental element for precise definition of RT target volume. This is particularly important in the treatment planning by intensity-modulated radiation therapy (IMRT), which requires highly conformed distribution patterns of the specified radiation dose. Accurate definition of the RT target volume, especially the clinical target volume (CTV) leads to an enhanced local control rate and reduces the incidence of RT-associated complication. However, identifying the right method to accurately define CTV constitutes a tough problem in the RT for NPC.

Compared with computed tomography, magnetic resonance imaging (MRI) has advantages, such as better soft tissue imaging, multiplanar capacity, and absence of radiation [3]. MRI has been applied widely for diagnosis and staging the RT in NPC [4,5]. However, conventional MRI has limited ability to accurately delineate the CTV. The development of MRI contrast agents which can accumulate specifically in the NPC might provide a solution to this problem.

With advances in molecular imaging, MRI utilizing specific molecular probes for target imaging is an important area that is under development. Several promising molecular probes have been applied to MRI in cancer. Among MRI contrast agents, ultrasmall superparamagnetic iron oxide (USPIO) may be a suitable tool for labeling molecular probes that target specific tumor-associated markers. The unique property of USPIO is its superparamagnetism that produces a decrease in signal intensity on T2-weighted images. Moreover, compared with the traditional contrast agent gadolinium, it exhibits higher sensitivity, lower toxicity, and longer half-life. In addition, it is also completely biodegradable. Therefore, USPIO has attracted extensive attention and has attained rapid development [6–8]. It has been reported that USPIO plays an important role in the
imaging diagnosis of malignant tumors [9–13]. USPIO-based target-specific MRI contrast agents are now primarily used in molecular MRI and are becoming increasingly popular. Several reports have indicated that targeted contrast agents conjugated using USPIO and different monoclonal antibodies can possibly maintain not only the immune reactivity of the monoclonal antibody, but also the superparamagnetism of USPIO. In addition, this contrast agent could reach specific tumor tissue, and in view of its good distribution and pharmacokinetics in vivo, it can thus substantially increase the accuracy and specificity of imaging [14–16].

Cetuximab (C225), a human–mouse chimeric monoclonal anti-epidermal growth factor receptor (anti-EGFR) antibody, manifests high specificity for EGFR. It has been proved to have significant benefit in clinical treatments for metastatic colorectal cancer, advanced head and neck cancer, and advanced non-small cell lung cancer [17–19]. In addition, recent studies have demonstrated that EGFR was overexpressed in >80% of NPC [20–22]. Therefore, it is important to investigate the feasibility of applying C225-conjugated USPIO in tumor-targeted imaging.

However, to the best of our knowledge, there has been no report of a specific targeted magnetic imaging contrast agent that is capable of detecting NPC. This study aims at developing a novel MRI contrast agent for targeting EGFR that is expressed on NPC cells and evaluating its ability in detecting NPC with MRI in vitro.

Materials and Methods

Materials

CNE1, an NPC cell line that exhibits high expression of EGFR, was provided by Cancer Research Institute of Central South University (Changsha, China). Commercially available Fe3O4 nanoparticle-based TANBead®USPIO-101, coated with −NH3+, size 6–10 nm, Fe concentration 10 mg/ml (25 μM), and pH value 3.8 ± 0.5, was purchased from the Taiwan Advanced Nanotech Inc. (Taoyuan, China); C225, at a concentration of 5 mg/ml, was supplied by Merck Inc. (Darmstadt, Germany). Fluoresceine isothiocyanate (FITC)-labeled rabbit anti-human immunoglobin G (IgG), normal human IgG, and fluorescent dye Hoechst 33258 were all purchased from the Zhongshan Goldbridge Biotechnology Co., Ltd (Beijing, China); [1-ethyl-(3-dimethylaminopropyl)]-carbodiimide hydrochloride (EDC·HCl) was purchased from Jingchun Reagent Co., Ltd (Shanghai, China).

Preparation of C225-USPIO

A reagent bottle containing USPIO was oscillated on a vortex mixer for 1 min and 100 μl of USPIO was aliquoted into a 1.5 ml Eppendorf (EP) tube. The tube was placed on a magnetic separator for 3 min. The supernatant was removed and 376 μl of deionized water was added and blended with it. Then 376 μl of C225 (2 mg/ml) was mixed into the above solution, followed by the addition of 7.52 mg of EDC·HCl. The reaction was conducted for 4 h with constant stirring at room temperature. Thereafter, this tube was placed on the magnetic separator, the supernatant was removed, and the solution was washed using 1 ml of phosphate buffered saline (PBS) twice. The prepared contrast agent was finally dispersed into 1 ml PBS (pH 7.4). Similarly, normal human IgG was conjugated on to USPIO as a control (IgG-USPIO).

Cell culture

CNE1 cells were grown in RPMI 1640 culture medium (Genom Biopharmaceutical Technology Co., Ltd, Hangzhou, China) containing 10% newborn calf serum (Gibco, Grand Island, USA) in an incubator containing 5% CO2.

Flow cytometry

Cells (5 × 10⁵) were incubated with PBS, C225-USPIO (Fe concentration 10 and 100 μg/ml), and C225 for 30 min, respectively. Thereafter, 100 μl of normal rabbit serum was added for blocking and incubated for 30 min at 4°C. After incubation with FITC-labeled rabbit anti-human IgG for 30 min at 4°C, the cells were washed with PBS and examined by flow cytometry (FCM) analysis.

Immunofluorescence

Cells were fixed by iced methanol at −20°C for 10 min, washed with PBS, and blocked using normal rabbit serum at room temperature for 40 min. Then, cells were incubated with C225 (positive contrast), C225-USPIO, IgG-USPIO, and PBS (negative contrast) at 4°C overnight in a wet box, respectively. After that, the cells were incubated with FITC-labeled rabbit anti-human IgG for 1 h at room temperature, followed by staining with Hoechst 33258 for 30 min in a dark room. The cells were then washed with PBS and imaged by fluorescence microscope.

Prussian blue staining

Cells were cultured for 24 h in six-well plates on glass coverslips. These cells were washed three times with PBS and incubated with C225-USPIO or IgG-USPIO (Fe concentration 50 μg/ml) for 15 min, 30 min, 1 h, and 2 h, respectively. Following incubation, cells were washed with PBS and subsequently fixed with ice-cold methanol at −20°C for 10 min. For Prussian blue staining, the fixed cells were incubated with 2% potassium ferrocyanide ferrocyanatum in 6% hydrochloric acid for 30 min and counterstained with nuclear fast red for 15 min.
Transmission electron microscope
Cells were incubated with IgG-USPIO or C225-USPIO (Fe concentration 50 μg/ml) at 37°C for 2 h. About $5 \times 10^6$ cells were collected and transferred to a 1.5 ml Eppendorf tube. Cells were fixed with glutaraldehyde for 2 h, then sequentially fixed with osmic acid, dehydrated with ethyl acetone in gradient, embedded, aggregated, sliced, stained by uranyl acetate and lead citrate, and finally processed for ultrathin sectioning. Micrographs were taken by transmission electron microscope (TEM) (FEI Tecnai G2 12 type, Eindhoven, The Netherlands).

MRI in vitro
Cells were incubated with C225-USPIO or IgG-USPIO (Fe concentration 50 μg/ml) for 15 min, 30 min, 1 h, and 2 h at 37°C prior to collection, respectively. Distilled water was used as the control. About $5 \times 10^6$ cells were collected following centrifugation and transferred into 1.5 ml Eppendorf tubes containing 4% liquid gelose. Following solidification of gelose, contrast-enhanced MRI of cells was obtained by a 4.7 T Small Animal MR Imaging System (Bruker Biospec, Ettlingen, Germany). Parameters for the imaging were as follows: T1: repetition time (TR) = 400 ms, echo time (TE) = 13.5 ms, number of acquisitions (NA) = 2, matrix = 128 × 128, slice thickness = 1 mm, and field of view (FOV) = 3.5 cm × 3.5 cm; T2: TR = 3500 ms, TE = 40–480 ms (12 echoes), NA = 2, matrix = 128 × 128, slice thickness = 1 mm, and FOV = 3.5 cm × 3.5 cm.

Results

Binding efficiency of C225-USPIO by FCM
To evaluate the binding efficiency of C225-USPIO, cells were analyzed by FCM. As shown in Fig. 1, C225 possessed the highest binding affinity to EGFR, which suggested that EGFR was overexpressed on CNE1 cells. C225-USPIO (Fe concentration 10 μg/ml) exhibited moderate binding affinity to CNE1 cells. With increasing Fe concentration, the binding rate of C225-USPIO to CNE1 cells also increased. These results imply that C225-USPIO possesses high binding affinity and good specificity for CNE1 cells overexpressing EGFR.

Immunobinding efficiency of C225-USPIO by immunofluorescence
To evaluate the immunobinding efficiency of C225-USPIO, cells were analyzed by immunofluorescence (Fig. 2). An intense background green fluorescence staining was detected in cells that were treated with C225, which suggested that EGFR was overexpressed on CNE1 cells. The green fluorescence staining, similar to that of C225, was detected in cells treated with C225-USPIO, indicating that C225-USPIO had good immune activities and bound to CNE1 cells specifically. However, only a dim green fluorescence staining, similar to that of negative contrast group, was detected in the IgG-USPIO group, thus indicating that the IgG-USPIO could not bind to CNE1 cells.

Targeted binding and uptake of C225-USPIO by Prussian blue staining
The uptake of C225-USPIO and IgG-USPIO was assessed histologically using Prussian blue staining. After 15 min of staining, there was no significant uptake noted for particles from both groups. However, after 30 min, 1 h, and 2 h of...
incubation, the differences in uptake of particles between cells treated with C225-USPIO and IgG-USPIO became prominent (Fig. 3). More blue-staining particles were observed in the C225-USPIO group with time point prolonged. The result indicates that the accumulation of these particles is specifically mediated by EGFR binding.

**Internalization and subcellular localization of C225-USPIO by TEM**

TEM provided insight into the subcellular localization of the particles in CNE1 cells following incubation with C225-USPIO or IgG-USPIO for 2 h. Large amounts of C225-USPIO were seen to be internalized and accumulated in the cellular endosomes and some moieties were attached to the cell membrane, but most of the IgG-USPIO was deposited outside the cells (Fig. 4).

**MRI T2 signal intensity change by 4.7 T MRI**

Figure 5 presents representative T2 images of cells in agarose. After 15 min of incubation, there was no significant change in signal intensity between cells treated with C225-USPIO and IgG-USPIO. However, the T2 signal intensity in cells treated with C225-USPIO decreased significantly and the intensity in cells treated with IgG-USPIO decreased slightly with time as noted for different time intervals (30 min, 1 h, and 2 h). These results indicate that CNE1 cells can specially internalize C225-USPIO instead of IgG-USPIO. Therefore, C225-USPIO can be used for in vitro MRI study of CNE1 cells overexpressing EGFR.

**Discussion**

RT is the standard therapy for NPC and may or may not be combined with adjuvant chemotherapy. The emergence of IMRT is an important milestone in the development of RT, and has not only improved the survival of NPC patients significantly, but also reduced the rate of RT-induced complications [23]. A major problem faced by IMRT is the accurate definition of CTV. Conjugation of USPIO with different antibodies generates the possibility of targeted MRI for a specific molecular target at cellular and subcellular levels. It is expected that molecular imaging technology will facilitate the precise delineation of CTV in NPC based on EGFR overexpression. In this study, C225-USPIO was developed and its ability to bind specifically to the EGFR on CNE1 cells was investigated in vitro.

In contrast to most previous molecular MRI approaches that used dextran-coated particles, the USPIOs used in this study were coated with NH$_3^+$+. This coating provides a significant advantage wherein the presence of amino groups on the USPIO surface facilitates conjugation with a specific ligand and that the coating layer can be kept very thin [24]. This surface facilitates an uptake of USPIO in specific cells, increases the half-life of USPIO in the blood by preventing its uptake by the reticular endothelial system and

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**Figure 3** Prussian blue staining analysis of targeted binding and uptake of C225-USPIO

Prussian blue-stained and nucleus fast red-counterstained CNE1 cells were incubated, respectively, with IgG-USPIO and C225-USPIO (50 μg Fe per milliliter of growth medium) for different time intervals (15 min, 30 min, 1 h, 2 h). Control cells were not incubated with USPIO. Higher uptake of C225-USPIO (blue particles) compared with IgG-USPIO (blue particles) is linearly indicated in a time-dependent manner. Original magnification, 200×.

**Figure 4** TEM analysis of internalization and subcellular localization of C225-USPIO

TEM images of CNE1 cells respectively incubated with IgG-USPIO and C225-USPIO (50 μg Fe per milliliter of growth medium) for 2 h were shown. Although C225-USPIO was already internalized and accumulated in endosomes, IgG-USPIO was predominantly located outside the cells. Bar = 0.5 μm.

**Figure 5** 4.7 T MRI analysis of MRI T2 signal intensity change after incubation with C225-USPIO

T2-weighted MR images of CNE1 cells in agarose after 15 min, 30 min, 1 h, and 2 h incubation (50 μg Fe per milliliter of growth medium), respectively, with C225-USPIO, IgG-USPIO, and distilled water as the control were shown. Compared with the cells incubated with IgG-USPIO, the T2 signal intensity decreased significantly in cells treated with C225-USPIO at different time intervals (30 min, 1 h, and 2 h).
improves the magnetic targeting ability of nanoparticles in vivo.

USPIO particles generate a reduction of the T2 signal intensity, which results in increased sensitivity of MRI when compared with gadolinium-containing ones. The notion of applying targeted MRI contrast agents comprising superparamagnetic iron oxide particles that are conjugated to different antibodies is not novel. Early work in this field explored the capability of these agents to accumulate in targeted tumor tissues and then applied them successfully in molecular imaging for tumors [25–27]. However, a contrast agent that is able to specifically accumulate in NPC tissue has not been developed as yet. The conjugation of USPIO nanoparticles with an antibody that specifically targets tumors offers the possibility of MR molecular imaging.

To be an antibody-based targeted MRI contrast agent, it must not only maintain the immunoreactivity to the monoclonal antibody of biomarkers, but also maintain the superparamagnetism of USPIO. As indicated by FCM, when the Fe concentration in C225-USPIO was 10 μg/ml, C225-USPIO demonstrated a moderate binding rate with the EGFR on CNE1 cells. However, with the increase of Fe concentration, the binding rate of C225-USPIO to EGFR increased gradually and fluorescence intensity increased as well (Fig. 1); this might be related to the higher dose of C225 that was conjugated. Reynolds et al. [28] reported similar results that when MES-1-USPIO was incubated with Chinese hamster ovary (CHO) cells expressing mouse E-selectin, the concentration-dependent surface binding was demonstrated by FCM. In the present study, similar results were observed through immunofluorescence detection (Fig. 2). These results obtained from FCM and immunofluorescence indicate that C225-USPIO manifests good immunobinding capacity. In this study, we observed that with higher Fe concentration in the contrast agent, the contrast agent became conglobate more easily. In order to maintain the binding activity of the contrast agent without affecting the proliferative activity of cells, an Fe concentration of 50 μg/ml was selected in this study; this is lower and safer than the Fe concentration in the contrast agents that have been reported in previous studies [14,16,28].

To ensure successful magnetic resonance molecular imaging, sufficient quantities of USPIO particles must accumulate in the specific tumor tissue. Accordingly, the selected biomarker should be overexpressed in targeted tumor cells and the complex formed by conjugation of USPIO with antibody should be taken up by the cells. Therefore, the CNE1 cell line with capacity for high expression of EGFR was chosen in this study. Prussian blue staining demonstrated that iron nanoparticles were indeed present, as shown by the MRI results. Larger amounts of C225-USPIO accumulated in the tumor than IgG-USPIO. The difference in the uptake of C225-USPIO and IgG-USPIO by CNE1 cells became remarkable with increasing time (Fig. 3). The significantly higher uptake of C225-USPIO can more probably be attributed to a strong specific uptake compared with IgG-USPIO, which points to a receptor-mediated endocytosis mechanism [29]. However, there was only a little amount of IgG-USPIO being taken up by cells even after longer time duration, and this slight transport can be explained by pinocytosis [30]. Both the non-specific and the receptor-mediated specific uptake contributed to the accumulation of iron nanoparticles in cells. TEM provided insight into the subcellular localization of the particles in CNE1 cells following incubation with C225-USPIO and IgG-USPIO (Fig. 4). The results obtained in this study suggest that C225-USPIO can specifically bind with EGFR and undergo uptake by the cells through a receptor-mediated endocytosis, which forms the basis of MR molecular imaging.

To detect the variation of MRI T2 signal intensity in CNE1 cells following incubation with C225-USPIO and IgG-USPIO, 4.7 T MRI was performed. The T2 signal intensity in cells treated with C225-USPIO decreased significantly and the intensity in cells treated with IgG-USPIO decreased slightly with increasing time (Fig. 5). These findings can be attributed to the targeted delivery of contrast agent into CNE1 cells by the mechanism of receptor-mediated endocytosis. It is apparent from these results that C225-USPIO can specifically bind to CNE1 cells that highly express EGFR and undergo uptake into the cells. This finding provides the potential to develop a novel targeted MRI contrast agent for tumor overexpressing EGFR.

The present study had certain limitations. The various iron concentrations and method of incubation of C225-USPIO need further investigation. In addition, ‘signal to noise’ ratio should also be considered. The questions raised above have actively been explored in our laboratory. In conclusion, our findings indicate that it is possible to non-invasively detect early-stage NPC with EGFR overexpression by using an MRI contrast agent that can target EGFR. Thus, molecular imaging of EGFR can provide insight into the molecular profile of tumor boundary and has potential in accurate definition of CTV in RT for NPC.

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