

# In-vivo imaging of oral squamous cell carcinoma by EGFR monoclonal antibody conjugated near-infrared quantum dots in mice

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**Objectives:** The purpose of this study was to investigate in-vivo visible imaging of oral squamous cell carcinoma (OSCC) by targeting epidermal growth factor receptor (EGFR) with near-infrared quantum dots.

**Materials and methods:** Quantum dots with an emission wavelength of 800 nm (QD800) were conjugated to monoclonal antibodies against EGFR, resulting in the probe designated as QD800-EGFR Ab. OSCC cell line (BcaCD885) expressing high levels of EGFR was transplanted subcutaneously into nude mice cheeks to develop an OSCC animal model. QD800-EGFR Ab containing 100 pmol equivalent of QD800 was intravenously injected into the animal model, and in-situ and in-vivo imaging of cheek squamous cell carcinoma was analyzed at 10 different time points.

**Results and conclusion:** In-vivo imaging and immunohistochemical examination of the tumors showed that intravenously injected QD800-EGFR Ab probe could bind EGFR expressed on BcaCD885 cells. Fluorescence signals of BcaCD885 cells labeled with QD800-EGFR Ab probe could be clearly detected, and these fluorescence signals lasted for 24 hours. The most complete tumor images with maximal signal-to-noise ratio were observed from 15 minutes to 6 hours after injection of the probe. To the best of the authors' knowledge, this is the first study that has obtained clear in-situ and in-vivo imaging of head and neck cancer by using QD800-EGFR Ab probe. The authors conclude that the combination of near-infrared quantum dots that are highly penetrating for tissues with EGFR monoclonal antibody has promising prospects in in-vivo imaging of OSCC and development of personalized surgical therapies.

**Keywords:** oral cancer, head and neck cancer, near-infrared fluorescence, visual in-vivo imaging, epidermal growth factor receptor, nanotechnology

## Introduction

Oral squamous cell carcinoma (OSCC) accounts for 90% of oral cancer, and currently surgery is one of the main treatments.<sup>1</sup> The most difficult part in surgery is to correctly define the boundary of the tumor and precisely determine the regions for surgical resection in order to improve survival rate and quality of life. Visualized methods to detect the tumor cells during surgery are currently not available. Clinical doctors estimate the tumor boundary for surgical resection by experience and the changes of the tumor tissue texture, which results in a 40% failure rate of complete removal of head and neck cancer<sup>2,3</sup> and greatly affects the survival rate of the patients. Therefore, development of methods for real-time identification of tumor cells during surgery and establishment of tailored surgical resection for each individual are one of the key factors in improving survival rate.

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Recently, quantum dots (QDs) were developed on the interdisciplinary advancement of nanotechnology, chemistry, and optics. The unique optical properties of QDs have shown promising prospects in the personalized surgical treatment for cancer patients.<sup>4,5</sup> QDs are nanocrystals (with a diameter of 2–10 nm) composed of elements belonging to group II-IV or group III-V. Compared with traditional fluorescence markers, QDs have narrow emission spectrum, wide excitation spectrum, high intensity of fluorescence, and good photochemical stability due to the quantum size and dielectric confinement effects. In addition, any emission spectrum from ultraviolet to near-infrared (or from blue to red) under the same excitation wavelength of light can be obtained by changing the particle size of QDs.<sup>6–8</sup> These optical characteristics of QDs are not possessed by any of the current fluorescent probes, including a variety of organic fluorescent dyes and fluorescent proteins. Particularly, the fluorescence of recently developed QDs with an emission of near-infrared wavelength from 700 nm to 900 nm has strong penetration in human tissues, which is extremely suitable for visible in-vivo medical imaging.<sup>9–11</sup> Currently, fluorescent probes have been developed by conjugating QDs with target molecules (eg, antibodies and peptides) and have been used for in-vivo visualization of cancer cells and tumor angiogenesis,<sup>4,12–14</sup> sentinel lymph node detection,<sup>15,16</sup> and imaging of drug targeting studies.<sup>17</sup> These studies have demonstrated that excellent optical properties of QDs have promising prospects in visualization of cancer development and personalized therapies. Because QDs are made of toxic heavy metal materials, previous studies have shown that toxicity of the QDs mainly comes from a quick release of heavy metal (such as Cd). In contrast, current biologically functionalized QDs have excellent biological compatibility and water solubility. More importantly, these biologically functionalized QDs do not have obvious side effects in humans at the required range of detection dosage and do not affect the growth, differentiation, and function of cells.<sup>7,8,18–20</sup>

Many studies have demonstrated that 90% of OSCCs and head and neck squamous cell carcinomas (HNSCCs) highly express epidermal growth factor receptor (EGFR).<sup>21,22</sup> Specific targeting of EGFR by EGFR antibodies has been widely used for the treatment of OSCC and HNSCC.<sup>22,23</sup> Therefore, visual imaging by targeting EGFR has broad applicability for OSCC and HNSCC. Visualization of in-situ and in-vivo imaging for OSCC and HNSCC by EGFR antibody conjugated QDs has not been reported. The authors of this paper attached EGFR monoclonal antibodies to QDs with a maximal emission wavelength of 800 nm to produce

a probe designated as QD800-EGFR Ab. OSCC animal model was developed by transplanting nude mice subcutaneously with human buccal squamous cell carcinoma cell line (BcaCD885). Finally, QD800-EGFR Ab was intravenously injected into the animal model for in-situ and in-vivo imaging of OSCC. The results obtained in this study provided fundamental bases for personalized surgical treatment of OSCC and HNSCC by using QDs.

## Materials and methods

### Main equipment and reagents

BcaCD885 was provided by West China College of Stomatology, Sichuan University (China). Qdot<sup>®</sup>800 Antibody Conjugation Kits were purchased from Invitrogen (Carlsbad, CA). A recombinant human/mouse chimeric anti-EGFR monoclonal antibody and Histostain<sup>™</sup>-Plus kits were purchased from Beijing Zhong Shan Company (China). A laser scanning confocal microscope (TCS-SP5) was purchased from Leica (Germany). The Maestro In-Vivo imaging system was purchased from CRi Inc (Woburn, MA).

### Experimental animals

Specific-pathogen-free level BALB/c (strain nu/nu) nude mice (n = 18, female, 6–8 weeks old, weight 20–25 g) were purchased from the Experimental Animal Center of Chongqing Medical University. The mice were bred in constant temperature and humidity conditions. Feeds, beds, and drinking water were sterilized. All the experimental procedures were approved by the Animal Administration Committee of the Institute of Chongqing University.

### Preparation and purification of QD800-EGFR Ab probe

Preparation of QD800-EGFR Ab probes was performed according to instructions of the Qdot Antibody Conjugation Kits. Briefly, 14  $\mu$ L of the dual function SMCC (succinimidyl-4-(N-maleimidomethyl)cyclohexane-1-carboxylate) solution (10 mM) was mixed with 125  $\mu$ L of QD800 solution (4  $\mu$ M) for 1 hour at room temperature. After 1-hour activation, the mixture was loaded onto an NAP<sup>™</sup>-5 (GE Healthcare, Little Chalfont, UK) column and a total of 500  $\mu$ L elution (Elution 1) was collected. A total of 6.1  $\mu$ L of dithiothreitol solution (1 M) was added to 300  $\mu$ L of EGFR monoclonal antibody (1 mg/mL). After 30 minutes of reduction reaction at room temperature, the dye indicators were added. The mixture was loaded onto an NAP-5 column, and a total of 500  $\mu$ L elution (Elution 2) was collected. Elution 1 and Elution 2 were mixed. After 1-hour coupling reaction at room

temperature, 3  $\mu$ L of 2-mercaptoethanol (10 mM) was added for 30 minutes of inactivation. The coupled and inactivated solution obtained above was added into an ultra-filtration tube and was centrifuged for 15 minutes at 7000 rpm. The solution in the inner membrane of the ultra-filtration tube was collected and chromatography was conducted to obtain purified QD800-EGFR Ab probe. Finally, the concentration of QD800-EGFR Ab was calculated using the following formula:  $A = \epsilon cl$ , where A represented absorbance,  $\epsilon$  represented the extinction coefficient, c represented the concentration, and l represented the optical path.

### Immunohistochemical detection of EGFR

One milliliter of BcaCD885 cells at a concentration of  $2 \times 10^4$  cells/mL were seeded into each well of a 24-well plate containing a glass coverslip. After 24 hours of culture, the glass coverslip was taken out and fixed with 4% paraformaldehyde for 30 minutes. Streptavidin peroxidase (SP) immunohistochemical staining was performed according to instructions of the Histostain-Plus kits. Briefly, the coverslips were incubated with 3%  $H_2O_2$  deionized water for 10 minutes, washed with distilled water, and soaked in phosphate buffered saline (PBS) for 5 minutes. Consequently, the coverslips were incubated with normal goat serum at room temperature for 15 minutes before EGFR monoclonal antibody (1:100) was added. After incubation overnight at 4°C, the coverslips were washed with PBS three times and then biotin-labeled goat anti-rabbit immunoglobulin G and streptavidin conjugate of horseradish peroxidase were added. Finally, diaminobenzidine was added for color development. Negative control was performed by replacing the primary antibody with PBS. Positive control was provided by the Histostain-Plus kits. Appearance of brown-yellow granules within the cells indicated positive.

### In-vitro detection of the binding between EGFR and QD800-EGFR Ab probe

BcaCD885 cells at a concentration of  $5 \times 10^4$  cells/mL were seeded onto three 35 mm-culture dishes with glass bottoms (1 mL in each dish). After 24 hours of culture, the cells were washed twice with PBS, and the cells were divided into three groups. The experimental group was added with 100  $\mu$ L of QD800-EGFR Ab probe (100 nM). The control group I was added to 100  $\mu$ L of QD800 (100 nM). The control group II was added to 200  $\mu$ L of EGFR monoclonal antibody (1  $\mu$ g/mL) for 2 hours of blocking. Subsequently, the cells were washed twice with PBS and then an equimolar amount of QD800-EGFR Ab probe was added to the experimental

group. The cells in the three groups described above were incubated for 30 minutes at 37°C and then washed with PBS three times. Distribution of QD800 fluorescence within the cells was analyzed by confocal microscopy. All the experiments were repeated three times.

### In-vivo imaging of BcaCD885 cells by QD800-EGFR Ab probe in the OSCC mice models

BcaCD885 cell suspension ( $1 \times 10^6$ ) in 0.1 mL PBS was transplanted subcutaneously into the right cheek of the nude mice (n = 18) to develop the BcaCD885 cheek squamous cell carcinoma model. Three weeks post-injection, the maximal diameter of the tumor reached 0.8–1.1 cm. The mice were divided into the experimental group (n = 6), control group I (n = 6), and control group II (n = 6). All the animals were anaesthetized by intraperitoneal injection of 2% pentobarbital (40 mg/kg). Subsequently, for the experimental group, 100  $\mu$ L of QD800-EGFR Ab probe containing 100 pmol equivalent of QD800 was injected into the mice through the tail vein. For control group I, 100  $\mu$ L of QD800 (100 pmol) was injected. For control group II, 250  $\mu$ L of EGFR monoclonal antibody (1 mg/mL) was injected and 24 hours later an equimolar amount of QD800-EGFR Ab probe was injected. In-vivo imaging detection was performed 15 and 30 minutes, and 1, 2, 3, 4, 6, 8, 10, and 24 hours post-injection of QD800-EGFR Ab probe and QD800 using the Maestro in-vivo imaging system. Images were analyzed by Maestro 2.10.0 software.

### Cellular and histological examination of the BcaCD885 tumors

Two mice from the experimental group, control group I, and control group II were euthanized 3 hours after injection of QD800-EGFR Ab probe and QD800. The remaining four mice from the experimental and control groups were euthanized 24 hours after QD800-EGFR Ab probe injection. The subcutaneous cheek tumors were removed from the euthanized mice and embedded in optimal cutting temperature compound. The embedded samples were frozen, and cryo-sections with a thickness of 7  $\mu$ m were continuously cut under  $-20^\circ\text{C}$  conditions. One of every two continuous sections was conducted with hematoxylin and eosin (H&E) staining for observation of the tumor growth. The nucleus in another section was stained blue with Hoechst 33342 (0.5  $\mu$ g/mL) for 5 minutes and then washed with PBS three times. Distribution of QD800 fluorescence in the tumor tissues was observed by confocal microscopy.

## Statistical analysis

The values were expressed as the mean  $\pm$  standard error. Statistical analysis was performed using Student's *t*-test. A *P*-value  $< 0.05$  was considered to be statistically significant. All statistical analyses were performed using the SPSS Statistics (IBM Corporation, Somers, NY) software package.

## Results

### Preparation and purification of QD800-EGFR Ab probe

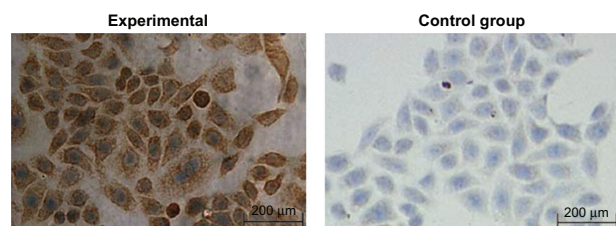
Based on the extinction coefficient of QD800 at 550 nm ( $\epsilon_{550} = 1.7 \times 10^6 \text{ (mol/L)}^{-1} \text{ cm}^{-1}$ ), the concentration of the purified QD800-EGFR Ab probe was calculated to be 2  $\mu\text{M}$ .

### QD800-EGFR Ab probe specifically binds EGFR of BcaCD885 cells in vitro

SP immunohistochemical analysis indicated that EGFR was expressed on the membrane and in the cytoplasm of BcaCD855 cells (Figure 1). Direct immunofluorescence examination with QD800-EGFR Ab probe showed that red fluorescence was present on the membrane of BcaCD885 cells in the experimental group. In contrast, red fluorescence was not observed in the BcaCD885 cells in control groups I or II (Figure 2). These results demonstrated that QD800-EGFR Ab probe cannot label EGFR of BcaCD885 cells that had been blocked by EGFR antibody. In contrast, QD800-EGFR Ab probe can recognize and bind efficiently with unblocked EGFR of BcaCD885 cells in vitro.

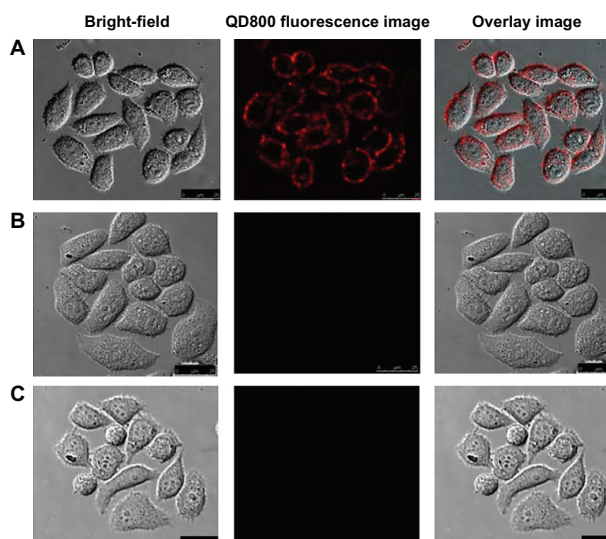
### Visible in-situ and in-vivo imaging of OSCC by QD800-EGFR Ab probe

In-vivo imaging analysis showed that the size of the fluorescence signal equivalent to the size of the tumors can be detected 15 minutes after injection of QD800-EGFR Ab probe in the experimental group. The size of fluorescent



**Figure 1** Immunohistochemical examination of EGFR expression on the membrane and in the cytoplasm of BcaCD885 cells in the experimental group and control group (the primary antibody was replaced by PBS) (SP  $\times 200$ ).

**Abbreviations:** BcaCD885, human buccal squamous cell carcinoma cell line; EGFR, epidermal growth factor receptor; PBS, phosphate buffered saline; SP, streptavidin peroxidase.



**Figure 2** In-vitro labeling of BcaCD885 cells with QD800-EGFR Ab probe. Detection of the fluorescence was conducted after 30 minutes of labeling. The excitation wavelength was 405 nm, and the emission wavelength was 750–800 nm. (A) Experimental group: the cells were labeled with QD800-EGFR Ab probe (100 nM) at 37°C for 30 minutes. (B) Control group I: the cells were added with QD800 (100 nM) at 37°C for 30 minutes. (C) Control group II: the cells were blocked with 200  $\mu\text{L}$  of EGFR monoclonal antibody (1  $\mu\text{g}/\text{mL}$ ) for 2 hours before addition of QD800-EGFR Ab probe (100 nM) at 37°C for 30 minutes.

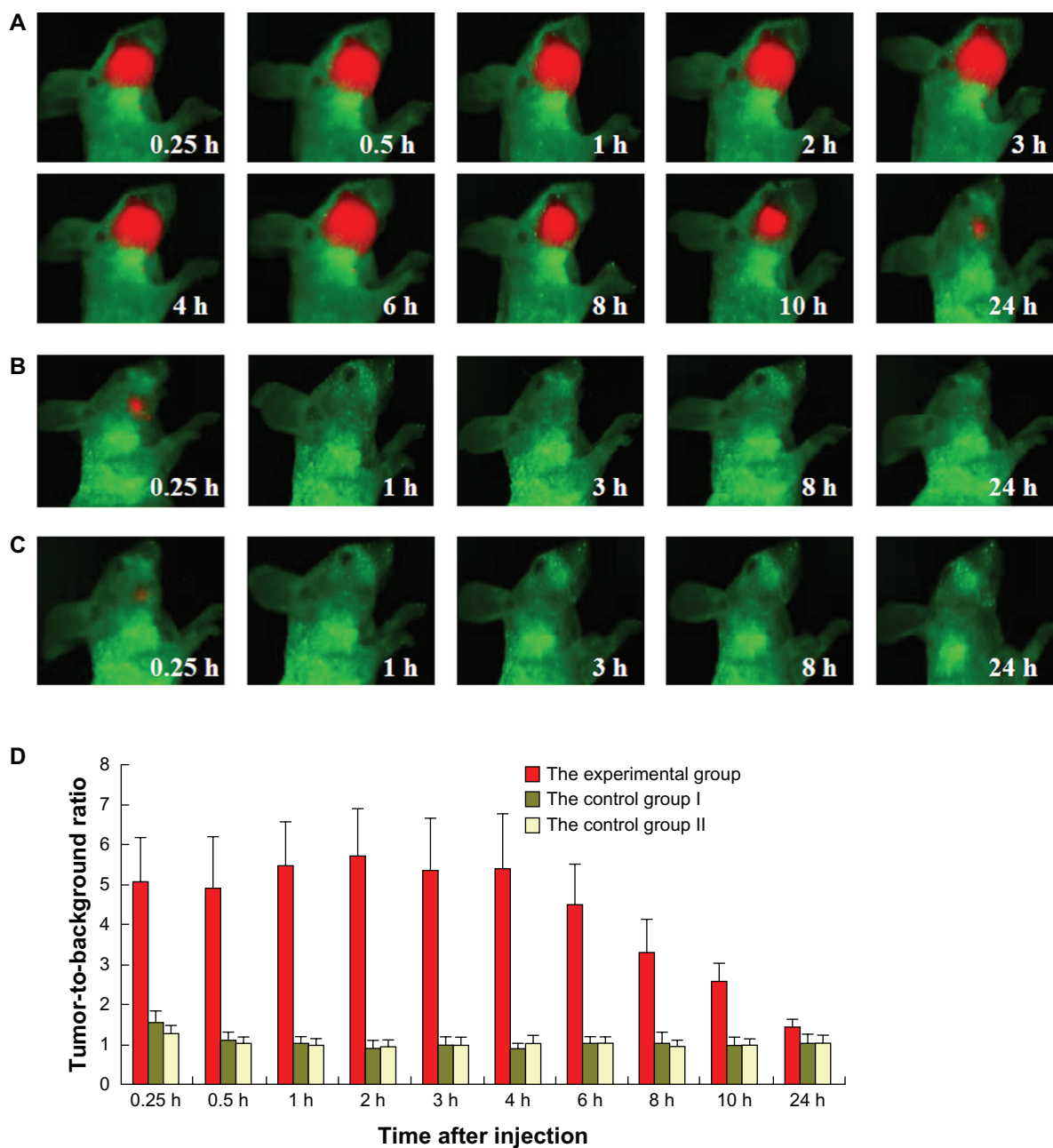
**Abbreviations:** Ab, antibodies; BcaCD885, human buccal squamous cell carcinoma cell line; EGFR, epidermal growth factor receptor; QD800, quantum dots with a maximal emission wavelength of 800 nm.

signal persisted for 6 hours post-injection, and after 8 hours the size of the signal began to become significantly smaller (Figure 3A). Twenty-four hours after the probe injection, the fluorescent signal was very weak (Figure 3A). The signal-to-noise ratio (the fluorescence intensity ratio between tumor and background) was high from 15 minutes to 6 hours post-injection. This signal-to-noise ratio began to decrease significantly after 8 hours and was close to the baseline level after 24 hours (Figure 3C). In control group I, QD800 was not conjugated with EGFR Ab. In control group II, EGFR was blocked before injection of QD800-EGFR Ab probe, and thus only a very weak fluorescence signal was detected on the tumor sites 15 minutes post-injection in control groups I and II. This weak signal could be due to the nonspecific phagocytosis of QD800-EGFR Ab probe and QD800 by BcaCD885 cells. However, the fluorescence signal above the background level was never detected 30 minutes to 24 hours post-injection in control groups I and II (Figure 3B).

### Cellular and histological detection of BcaCD885 tumors

H&E staining of the tumor sections from the mice 3 and 24 hours post-injection showed a large amount of tumor cells, and the tumors grew well (Figure 4). The nucleus of tumor



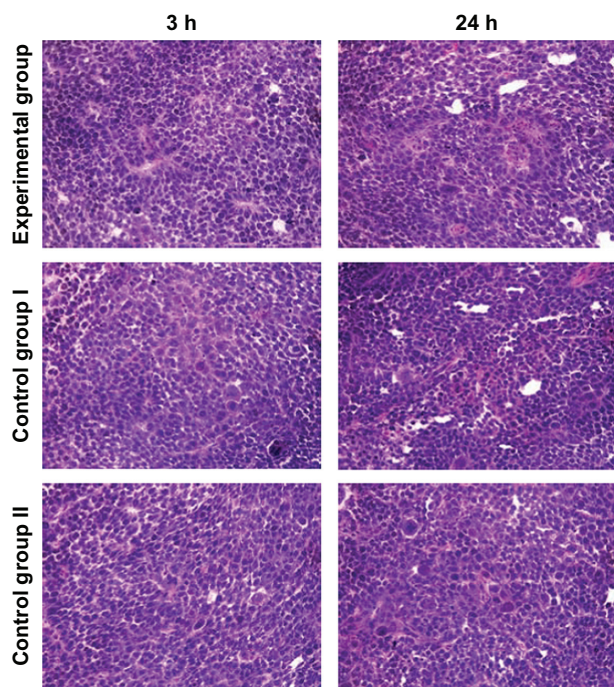


**Figure 3** Visible imaging of BcaCD885 cells in the cheek squamous cell carcinoma animal model by QD800-EGFR Ab probe at different times (excitation/emission 630/800 nm; exposure time 50 ms; collection time 10 seconds,  $2 \times 2$  Binning  $1024 \times 1024$  pixels). **(A)** Visible images of BcaCD885 cheek squamous cell carcinoma mice after intravenous injection with QD800-EGFR Ab probe containing 100 pmol equivalent of QD800 in the experimental group. **(B)** Visible images of BcaCD885 cheek squamous cell carcinoma mice after intravenous injection with 100 pmol QD800 in control group I. **(C)** Visible images of BcaCD885 cheek squamous cell carcinoma mice in control group II (250  $\mu$ L of EGFR monoclonal antibody (1 mg/mL) was injected, and 24 hours later QD800-EGFR Ab probe containing 100 pmol equivalent of QD800 was injected). **(D)** The signal-to-noise ratio in the BcaCD885 squamous cell carcinoma model after different times of probe injection in the experimental group.

**Abbreviations:** Ab, antibodies; BcaCD885, human buccal squamous cell carcinoma cell line; EGFR, epidermal growth factor receptor; QD800, quantum dots with a maximal emission wavelength of 800 nm.

sections was stained blue by Hoechst 33342 under confocal microscope. QD800 fluorescence signal was not detected in the tumor section of the mice in control groups I and II 3 and 24 hours post-injection. In contrast, in the experimental group, QD800 fluorescence signal was detected outside of most of the nucleus in the tumor section of the mice 3 hours

post-injection (Figure 5). Twenty-four hours post-injection, a fluorescence signal was detected only outside a small number of nuclei (Figure 5). The fluorescence signal was distributed in the cytoplasm and on the membrane. These results indicated that QD800-EGFR Ab probe can specifically target and bind EGFR of BcaCD885 cells.



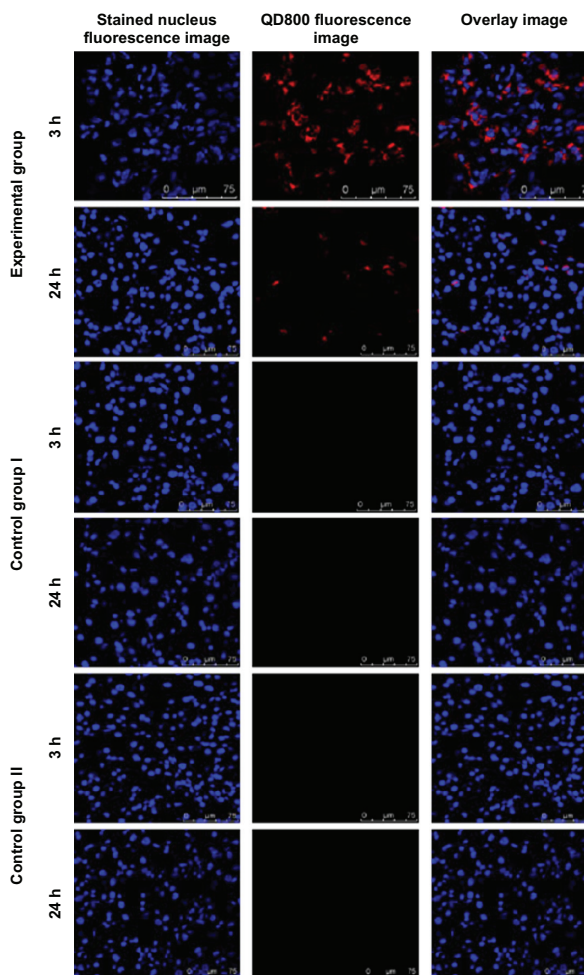
**Figure 4** H&E staining of tumor section from the experimental group (intravenous injection of QD800-EGFR Ab probe), control group I (intravenous injection of QD800), and control group II (preinjection of EGFR monoclonal antibody 24 hours before QD800-EGFR Ab probe injection) of BcaCD885 squamous cell carcinoma 3 and 24 hours after probe injection ( $\times 200$ ).

**Abbreviations:** Ab, antibodies; BcaCD885, human buccal squamous cell carcinoma cell line; EGFR, epidermal growth factor receptor; H&E, hematoxylin and eosin; QD800, quantum dots with a maximal emission wavelength of 800 nm.

## Discussion

Development of methods to visualize tumor cells during surgery and determine the precise boundary of the tumors for personalized surgical resection is the key to improve the survival rate of cancer patients. Traditional methods (eg, computed tomography [CT] and magnetic resonance imaging [MRI]) that can provide good imaging detection of tumors are not suitable for visible detection of tumor cells during surgery. Optical examination is clinically the safest, simplest, and most economical method. QDs have unique optical properties as mentioned earlier. More importantly, QDs can be easily modified and conjugated by other biological molecules. As nanoparticles, QDs with good photochemical stability can easily penetrate tumor angiogenesis and access cancer cells. Therefore, QDs have shown unique advantages in the surgical treatment of individual cancer patients.<sup>6-11</sup>

In this study, we intravenously injected QD800-EGFR Ab probe to target EGFR, which is highly expressed by OSCC. In-vivo imaging and tumor immunohistochemical analysis showed that QD800-EGFR Ab probe can specifically bind BcaCD885 cells with high level expression of EGFR. Thus, the fluorescence of QD800 can represent the presence of



**Figure 5** Laser scanning confocal microscope analysis of tumor section from the experimental group (intravenous injection of QD800-EGFR Ab probe), control group I (intravenous injection of QD800), and control group II (preinjection of EGFR monoclonal antibody 24 hours before QD800-EGFR Ab probe injection) of BcaCD885 squamous cell carcinoma 3 and 24 hours after probe injection. Nucleus was stained by Hoechst 33342.

**Abbreviations:** Ab, antibodies; BcaCD885, human buccal squamous cell carcinoma cell line; EGFR, epidermal growth factor receptor; QD800, quantum dots with a maximal emission wavelength of 800 nm.

BcaCD885 cells and can be visualized from outside of the body; toxicity of the QDs in the mice was not observed in this study. The complex metabolic process of QD800-EGFR Ab probe in the body is currently unknown. However, we obtained the most complete fluorescence images and highest signal-to-noise ratio 15 minutes to 6 hours post-injection, indicating that the optimal time period for personalized surgery is between 15 minutes and 6 hours after injection of QD800-EGFR Ab probe. Previous studies<sup>4,10</sup> also show that  $10^4$  cancer cells can be visibly detected after QD800 labeling in the presence of skin barrier. This sensitivity was 100-fold higher than that of CT and MRI. In addition, the sensitivity can be further enhanced when the tumor is exposed during the surgery. Gao et al predicted that in-vivo visible imaging

can detect as low as 10–100 QD-labeled cancer cells.<sup>24</sup> With the continuous advancement in imaging techniques, and synthesis of QDs with stronger penetration abilities for tissues, the authors of this paper believe that visible imaging detection can reach sensitivity at single-cell level under the wound exposure condition. In the future, clinical doctors will only need to wear a very small excitation source and near-infrared light receiver to perform personalized and tailored surgical removal of real tumors.

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## Disclosure

The authors declare that they have no competing interests.

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