Quantum-dot-based immunofluorescent imaging of Cancer Marker P53 Detection in Breast Cancer

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Abstract. The study is to design a biomarker based on hydrophilic quantum dots(QDs) can be bound to the P53 protein for diagnoses of breast cancer (BC). To achieve the purpose, we use the water-soluble quantum (QDs) AgInS₂/ZnS connected with P53 monoclonal antibody to design a novel kind of biomarker as a new detection of breast cancer. Significantly, this targeted quantum dots not only have good target recognition ability, but also have low toxicity and strong light stability. Moreover, confocal laser scanning microscopy (CLSM) showed that the localized fluorescence would be detected around the nucleus of human breast cancer cell MCF7 (high expression of P53) compared with that of T47D (low expression of P53). In summary, we believe that this QDs probe will provide a broad platform for clinical diagnosis and treatment of breast cancer and in vitro studies in future.

Introduction

Breast cancer is the most common malignant tumor in women, and attributed to increased understanding of molecular pathology mortality rate of it has decreased[1]. The tumor suppressor P53 is the transcription factor that controls the cellular fate[2,3]. Functionally, the P53 protein acts as a transcription factor and the mutated P53 was related to the occurrence of many cancers, including breast cancer, esophageal cancer, colorectal cancer, lung cancer, brain tumors and osteosarcoma[4-6].

Quantum dots (QDs) are fluorescent semiconductor nanocrystals, whose basic structure is composed of a core semi-conductor, typically CdSe or CdTe, enclosed in a shell of another semiconductor, such as zinc sulfide (ZnS)[7-9]. Relevant experimental data has shown that the QDs' quantitative analysis revealed its new optical properties, such as low level of antigen detection samples, which has important significance for the diagnosis and treatment of cancer[10-15]. They have potential applications ranging from medicine to energy [16,17]. Compared with traditional organic fluorophores and fluorescent proteins, QDs are superior in fluorescence brightness, photobleaching resistance, and fluorescence emission tuneability [18].

Here we focused on a new synthesized compound, which could turn QDs into hydrophilic molecules and conjugated with P53 protein via chemical experiments. We then investigate whether this newly synthesized QDs-P53 could combine with the mutational P53 in the breast cancer cells effectively and specifically. Finally we evaluate the in vitro cytotoxicity of the probe to determine the safety in biological applications.

Materials and methods

Materials

Human breast cancer cells T47D and MCF-7 were obtained from the China-Japan Union Hospital, Jilin University (Changchun, China). The SP1 clone rabbit anti-human P53 polyclonal antibody was the product of Zhongshan Golden Bridge Bio Company (Zhongshan Golden Bridge

Bio Co., Ltd, Beijing, China). IgG (from goat serum) and bovine serum albumin (BSA) were purchased from Sigma–Aldrich. AgInS₂/ZnS hydrophilic QDs ($\lambda_{\text{ex}} = 605\text{nm}$) (QDs encapsulated with PASP-Na-g-PEG-DDA) were supplied by Alan G. MacDiarmid Institute of Jilin University. N-ethyl-N-(N-dimethylaminopropyl) carbodiimide (EDC) were purchased from Thermo Scientific Company (Thermo Scientific, Rockford, IL, USA). MTT were purchased from Beyotime Institute of Biotechnology (Nantong, China). Bisphenol A (BPA), HCl, and NaOH were purchased from Sinopharm Chemical Reagent Co. Ltd. (Shanghai, China) and Aladdin Ltd. (Shanghai, China). Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were purchased from Sunshine Biotechnology Company (Nanjing, China).

Aqueous quantum dots conjugation

AgInS₂/ZnS QDs were synthesized by a modified method based on the reported procedure [19]. EDC as a coupling agent, dissolved in phosphate buffer solution (PBS) buffer (pH 7.4), and then SP1 clone rabbit anti-human P53 polyclonal antibodies were first activated by incubating 500 μL of 0.1 mg/L-1 antibody solution with 500 μL mixture of freshly prepared EDC + sulfo-NHS(0.1 mmol/L-1) for 15 min at room temperature (RT, $25 \pm 1^\circ\text{C}$). The reaction mixture of QDs were incubated with SP1 clone rabbit anti-human P53 polyclonal antibodies (50 nmol) for 24 h at $^\circ\text{C}$. The EDC and uncombined P53 protein were removed, and ultimately we obtained the P53-targeted QDs (QDs-P53).

Cell culture and fixation

Human breast cancer cells T47D and the MCF-7 were respectively cultured in DMEM high glucose medium supplemented with 10% fetal bovine serum, 1% penicillin and streptomycin at 37°C with 5% CO₂. Then two cell lines were fixed with 4% formaldehyde. We used 2% wt./vol. albumin bovine serum to block two cell lines for 30 min at room temperature to minimize nonspecific binding. Finally, the cells were incubated in 0.3% Triton X-100 for 10 min.

Fluorescence imaging of QDs labeled cells

T47D and the MCF-7 cells were incubated with 100 μL QDs-P53 probes (0.2 mg/mL) or mock conjugated QDs (0.2 mg/mL) for control groups at 37°C for 1 h. Add DAPI to the cells for nucleus staining and leave 15 min for the reaction at 37°C . Mount the coverslips onto the glass slides and observe the cells by confocal laser scanning microscope (CLSM, Olympus Fluoview FV1000, Japan). Image analysis was performed using FV10-ASW 3.1 Viewer software (Olympus, Japan).

Photostability comparison of QDS-based IHC detection

QD-based IHC staining was adapted from previously described protocols [20,21]. The fixed and blocked two cell lines were incubated with the QDs-P53 for 4h at 37°C . And then the cells were cultured with either secondary antibody or Alexa Fluor 488-labeled goat anti-rabbit IgG for 2h at 37°C after another blockade with 2% BSA for 30 min. As a negative control, primary antibody was replaced by PBS. All cell nucleus were stained with 1 $\mu\text{g/mL}$ DAPI. Images were captured with CLSM camera at 1 min intervals for each color automatically with the same excitation wavelength for 10 min. The same microscope settings were used for both treated and control samples.

In vitro cytotoxicity studies

Cell viability after QDs exposure was tested by using MTT assay. Human breast cancer T47D and MCF-7 cells were first cultured until cell counts were 4×10^3 in each well. Then the culture medium was replaced with 100 μL of DMEM containing QDs-P53 at different doses (0.1 mg/mL, 0.2 mg/mL, 0.3 mg/mL to 0.4 mg/mL). After 24h, 48h and 72h of reaction, MTT assay was performed according to the manufacturer's instructions. After removing MTT, 150 μL of DMSO was added. The resulting mixture was shaken about 10 min at room temperature and the colorimetric measurements were performed at 490nm using the ELx800 Absorbance Microplate Reader (BIO-TEK Instruments, USA).

Results and Discussion

Synthesis and properties of QDs-P53

Fig.1 is a schematic diagram of the QDs-P53. The average size of the synthesized QDs-P53 is about 605 nm. It has been widely proved that single domain antibodies-quantum dot conjugates have been shown to be promising candidates for detection of breast cancer biomarkers [22]. Since the time when the term “quantum dot” has been coined by Reed et al.[23], quantum dots (QDs) have been commonly perceived as inorganic semiconductor nanocrystals. They can exert their interactions with biological structures at the near atomic scale [24]. “Classical” semiconductor QDs have display unique optical characteristics due to the above mentioned quantum confinement effect. To study the spectral properties of the QDs-P53, the UV absorption, photoluminescence (PL) emission and excitation spectra were acquired from the aqueous dispersion of QDs-P53s as illustrated in Fig.2. In Fig.2(a), the characteristic peaks of the QDs which conjugated with the P53 protein still appeared, and the shape of the characteristic peak still existed after the reaction. As shown Fig.2(b), the ultraviolet absorption spectrum of the QDs-P53 could also show the characteristic peak of the QDs. These results show that this chemical reaction does not affect the structure of the QDs. The QD wavelength was 620 nm. This wavelength enables researchers to observe the fluorescent signal under fluorescence microscope.

Fig.1 The schematic diagram of the QDs-P53

Fig.2 (a) PL spectra of QDs and QDs-P53, (b)UV-Vis absorption spectra of QDs and QDs-P53

In vitro cell imaging

Breast cancer cell lines MCF7 (high expression of P53) and T47D (low expression of P53) were reacted with QDs-P53 probes, and observed with CLSM. Fig.3 shows the fluorescence microscopy images of MCF-7 cells after treating with 0.2 mg/mL QDs-P53. Apparently, QDs-P53s entered into the MCF-7 cells and displayed bright photo luminescence, while the shape and viability of cells show no substantial change, indicating the QDs-P53s can label the cells of MCF-7[Fig. 3(a)]. In contrast, weak nonspecific binding was observed in T47D cells which had a weak expression of P53 [Fig. 3(b).]. On the control group, in the blank control groups, no fluorescence signal were observed in neither MCF7 nor T47D [Fig. 3(c)]. In conclusion, the fluorescent markers of QDs-P53 probe which we obtained may be applied to in nanotechnology.

Fig.3 Confocal microscopy images of MCF7/ T47D cells stained with QDs-P53 and QDs. Cell nuclei were counterstained with DAPI and shown in blue. (a) MCF7 with QDs-P53; (b) MCF7 cells with QDs; (c) MCF7 cells with PBS as blank control.

The optical properties, which deservedly positioned QDs as an outstanding class of fluorescent probes for biomedical imaging, include broad absorption spectra, very narrow and size-tuneable light emission bands along with high resistance to photo bleaching [19-20]. The design of QDs for specific targets is achieved via their functionalization with biologically active molecules, such as antibodies, drugs, peptides and small molecular moieties, micelles, etc.. In our study, QDs-P53 were able to offer an opportunity to investigate the fine organization of human breast cancer cells at the cytoplasmic and subcellular organelle levels. Evaluation of tumor markers has long been an important part of clinical practice guideline in breast cancer. QDs were recently used for IHC detection in the non-small cell lung cancer clinical samples by Qu et al. The technology of QD-based immunofluorescence for detection of tumor markers in breast cancer had been developed for decades and these studies had the obvious advantages of QDs-based detection than other tradition methods. Chuang Chen et al. successfully conducted a quantitative determination of HRs and HER2 by QD-IHC [21]. Liu et al. used the color technology of quantum dots via simultaneous imaging of HER2 and type IV collagen in cancer tissues. Since the P53 gene is frequently mutated or inactivated in several different cancer types, it is a highly attractive therapeutic target for treating the disease, and P53 mutations are the most frequent genetic alterations in breast carcinomas, reaching 30% of them [22]. Several reports have mentioned a link between p53 status and response to anthracyclin-based regimen, showing either a better response or a worse response [38]. The photology stability of compound was essential. The QDs were also found to be responsive to the temperature changes [23]. In this study, we compare the fluorescent stability of organic fluorescent dyes-Alexa Fluor 488 with QDs-P53. The intensity of the QDs-P53 probes had a much more stability in the whole process. By contraries, the light stability of Alexa Fluor 488 decreased rapidly from the beginning of the experiment. We can get the conclusion that the probe of the quantum dot is more stable than the traditional materials in the photology. There are some studies show that the fluorescence of the QDs could still be detected even after one month. Thus, the quantum dot probe is not only suitable for the study of the body, but also in vitro molecular and cell study, for example, long-term observation.

In vitro cytotoxicity

In general, all types of QDs should show minimal signs of toxicity if applied in vivo or introduced into human organism following an unintentional exposure⁴⁰. Therefore, T47D and MCF-7 cells were incubated with QDs-P53 for 24h. T47D and the MCF-7 cells were exposed to the different concentration of QDs-P53 (0.1 mg/mL, 0.2 mg/mL, 0.3 mg/mL to 0.4 mg/mL) at 24 h, 48 h and 72 h, then we observed and collected the viability data. Our study showed that after 24 h of incubation, no significant cytotoxicity was registered with QDs-P53 concentrations of up to 100 mg/ml, which are in good agreement with those in literature [23]. After 48 h the cell viability of T47D cells changed to 92.3% (0.1 mg/mL), 93.2% (0.2 mg/mL), 90.3% (0.3 mg/mL), and 90.5% (0.4 mg/mL), and that of MCF-7 cells changed to 94.7% (0.1 mg/mL), 96.3% (0.2 mg/mL), 92.3% (0.3 mg/mL), and 91.5% (0.4 mg/mL). After 72 h the viability of T47D and MCF-7 cells decreased by 13% and 17% respectively when the concentrations of QDs-P53 reached up to 0.4 mg/mL. The QDs featured low toxicity, were retained within the labelled cells population over days and were redistributed into dividing daughter cells. It was proved that QDs-P53 probes have no obvious cytotoxicity on T47D and MCF-7 cells. Our result is consistent with the literatures that illustrated the dose of QDs had low cytotoxicity and good biocompatibility for cell labeling and imaging after incubating 24 h[24]. At the concentrations typically used in bioimaging applications (<200 g/mL), the cytotoxicity of the QDs was found to be marginal.

Fig.4. Cell viability of QD-P53 in T47D cells and MCF7 cells.

Conclusion

In this study, we developed a new QDs-P53 probe. This new probe may enhance our ability to assess breast tumor markers in specimens sensitively. The vitro cytotoxicity experiments also proved that the QDs-P53 probes had no obvious cytotoxicity. Therefore, we have reason to believe that this new type of probe in the future will become an significant technology of diagnosis and treatment of tumor.

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