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LABELING EFFICIENCY OF TB^{3+} CONJUGATED CD133 MONOCLONAL ANTIBODY NANOCOMPLEX TARGETING *IN VITRO* METASTATIC CANCER CELLS

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SUMMARY

Efficient cancer treatment remains a huge challenge worldwide. As reported by the World Health Organization, the number of cancer patients is estimated to be 24 million in 2035, which is a 70% increase compared to 14.1 million in 2012. The severity of cancer is due to the presence of cancer stem cells (CSCs), which are directly related to drug resistance, metastasis, and tumor relapse. Because of the unknown location of the primary tumor and/or the residency of CSCs, standard therapies deliver a high dose of drugs to the whole body, which can have negative effects and deadly consequences for patients undergoing treatment. Therefore, efficient luminescent materials for labeling and tracking CSCs are urgently needed to determine their distribution and target treatment. Herein, a fluorescent Tb^{3+} nano-ion and CD133 monoclonal antibody (mAb) were conjugated into a nano probe-complex (ET2). Tb^{3+} nano-ion is a rare-earth element and the CD133 mAb targets CD133, which is a CSC surface marker. The Tb^{3+} nanorods were surface treated with silica and activated with -NH_2 for functioning before being coupled with CD133 mAb. Strong fluorescent Tb^{3+} nanorods were used to decrease the toxicity of high-dose medicines, and the purpose of the CD133 mAb was to increase the specific binding capacity of CSCs to the ET2 nanocomplex. The luminescent properties of this coupled ET2 complex were determined and its ability to target and label CSCs was determined using the pluripotent human embryonic carcinoma cell line, NTERA-2. Fluorescence microscopy showed strong luminescent signals from ET2-exposed NTERA-2 cells. It was also demonstrated that the ET2 nanocomplex effectively labeled up to 97.74% of the tested NTERA-2 cells, but only 2.35% of CCD-18Co human colon normal cells. Therefore, these results show that the ET2 luminescent nanocomplex specifically targeted and labeled CSCs, and may be used for further applications in fundamental and clinical research.

Keywords: *Cancer stem cells, CD133 monoclonal antibody, ET2-luminescent nanocomplex, CCD-18CO; Ion Tb^{3+}, NTERA-2; Metastatic cancer cells*

INTRODUCTION

In previous studies, scientists recognized that there was a small group of cells called cancer stem cells (CSCs) or tumor-initiated cells in both *in vivo* tumors as well as *in vitro* continuously cultured cancer cell populations (Calvet *et al.*, 2014). These cells are directly related to drug resistance, metastasis, and tumor relapse, and significantly affect cancer treatment success (Clever, 2011; Gil *et al.*, 2008; Vinogradov, Wei, 2012). CSCs account for a small percentage of cells in tumors and can regenerate into various tumorous cell types causing growth and expansion of malignants. CSCs have drug-resistant ability and can overcome radiotherapy. CSCs that survive treatment allow the tumor to recur and spread throughout the body. Therefore, CSCs are considered promising targets and may lead to the discovery of more effective anticancer drugs or therapies. CSCs were characterized and found to have several specific surface markers. CD133, known as prominin-1, is a transmembrane glycoprotein that is a common surface marker of CSCs, which are found inside various cancer tumors. This transmembrane CD133

glycoprotein has an extracellular N-terminus and an intracellular C-terminus, and has been effectively used as a typical surface antigen to detect and isolate CSCs.

Traditional nanotechnological biomedicine has been used to enhance pharmacokinetics and reduce the systemic toxicity of chemotherapy by selectively targeting and effectively transporting anticancer drugs to tumors. The advantage of the use of nanoparticles in chemotherapy is that they increase the therapeutic index of distributed drugs enveloped inside or combined with nanoparticle surfaces. The selective supplies of nanotherapeutic platforms primarily depended on the tumor passive targets through rising of permeability and retention. Therefore, CSCs effectively labeling and tracking to evaluate cell distribution and homing for applications in fundamental research or in therapeutic treatment would be very helpful. A luminescent nanocomplex that combines a CSC-targeting monoclonal antibody (mAb) with a rare-earth element such as Tb^{3+} nano-materials or a cation terbi (III) emitting green fluorescence, would assist in the detection of CSCs and therefore cancer treatment (Hương *et al.*, 2012). Several studies on the effects of labeling cancer cells with nanomaterials have been reported (Arap *et al.*, 2015; Asha *et al.*, 2017). However, no studies have demonstrated the ability of rare-earth-based nanomaterials conjugated to CD133 mAb to selectively target tumor cells.

In this study, we attempted to detect and effectively label NTERA-2 cells, a pluripotent human embryonic carcinoma cell line, using fluorescent $TbPO_4.H_2O$ conjugated to CD133 mAb nanocomplex (ET2).

MATERIALS AND METHODS

Materials

The pluripotent human embryonal carcinoma cell line (NTERA-2) as cancer stem cells and the human colon normal cells (CCD-18Co) as healthy cells were provided by Dr. Chi-Ying Huang, National Yang-Ming University and Dr. P. Wongtrakoongate, Mahidol University, Thailand.

Cultured medium *Dulbecco's Modified Eagle Medium* (DMEM), Fetal bovine serum (FBS), Trypsin-EDTA, antibiotics (antibiotics-antimycotics) were obtained from Invitrogen (Carlsbad, CA, USA). Human CD133 monoclonal antibody and human CD133 antibody conjugated with FITC (CD133-FITC) were from Miltenyi Biotec (Bergisch Gladbach, Germany). Other chemicals were from Sigma Aldrich (St. Louis, MO, USA).

In vitro Cell culture

Cells were cultured by strictly following the protocols from ATCC Cell Bank (American Type Culture Collection, USA). Accordingly, NTERA-2 and CCD-18Co cells were seeded in T75 flask with Dulbeco's Modified Eagle Medium (DMEM) supplemented with 2 mM L-glutamine, 10% fetal bovine serum – FBS, 1% antibiotic (Anti-Anti solution). The cells were subcultured after every 3-5 days with the ratio of 1:3 and incubated at 37°C, 5% CO_2 and 100% humidity.

Labeling cells with ET2 fluorescent nanocomplex

-Microscopic imaging cells using ET2 luminescent nanocomplex: NTERA-2 and CCD-18Co cells were seeded into 96-well plate with a concentration of 10000 cells/well and incubated at 37°C, 5% CO_2. After 24 hours of incubation, the culture medium was replaced with 10% formaldehyde in order to fix the cell for 10 minutes at room temperature (RT). Cells were then triple rinsed with Phosphate Buffered Saline (PBS) to totally remove formaldehyde. 10 µl of ET2 complex were dilluted with 190 µl of PBS to place into each well and incubated at 4°C for 1 hour. The unbound sample was discarded and triple rinsed with PBS; 100 µl PBS was then added into the wells before observation by the Olympus Scan^R fluorescence microscope (Olympus Europa SE & Co.KG, Hamburg, DE).

- Flowcytometry analysis of ET2 probed cells through CD133 surface marker: Cancer stem cells (NTERA-2), healthy cells (CCD-18Co) were seeded into 6-well plate and incubated at 37°C, 5% CO_2 overnight. After 24 hours of incubation, cells was harvested with trypsin -EDTA and collected into a falcon tube. Cells were re-suspended with DMEM medium containing 2% FBS, ET2 fluorescent nanocomplex or anti-CD133-FITC mAb, and incubated at 4°C for 10-15 minutes (protect from light). The number of labeled cells (10.000-12.000 counting cells) were measured and analyzed by Novocyte flowcytometry system and NovoExpress software (ACEA Bioscience Inc.).

Statistical analysis

The data was reported as mean ± standard deviation (SD), which were analyzed by the

GraphPad Prism 7 software using unpaired *t*-test. The *P*<0.05 was considered statistically significance.

RESULTS AND DISCUSSION

Probing NTERA-2 and CCD-18Co cells with ET2 luminescent nanocomplex

The results showed that NTERA-2 cells labeled with ET2 luminescent nanocomplex showed strong luminescence under fluorescence microscopy (Figure 1). The negative control (CCD-18Co) did not exhibit corresponding luminescence signals under the same conditions. The positive control (CD133-FITC antibody) also showed high luminescence. The results obtained in this study are consistent with our previous results.

CCD-18Co cells were also stained with luminescent materials and examined under the same conditions. However, as mentioned above, luminescence was not observed in this cell line. The positive control (CD133-FITC) showed stronger fluorescence than ET2-probed cells. In this experiment, the luminescence from the ET2 luminescent nanosystem was not observed in healthy cells (Figure 2). Therefore, the ET2 nanocomplex is a specific probe that targets metastatic cancerous cells without affecting normal cells.

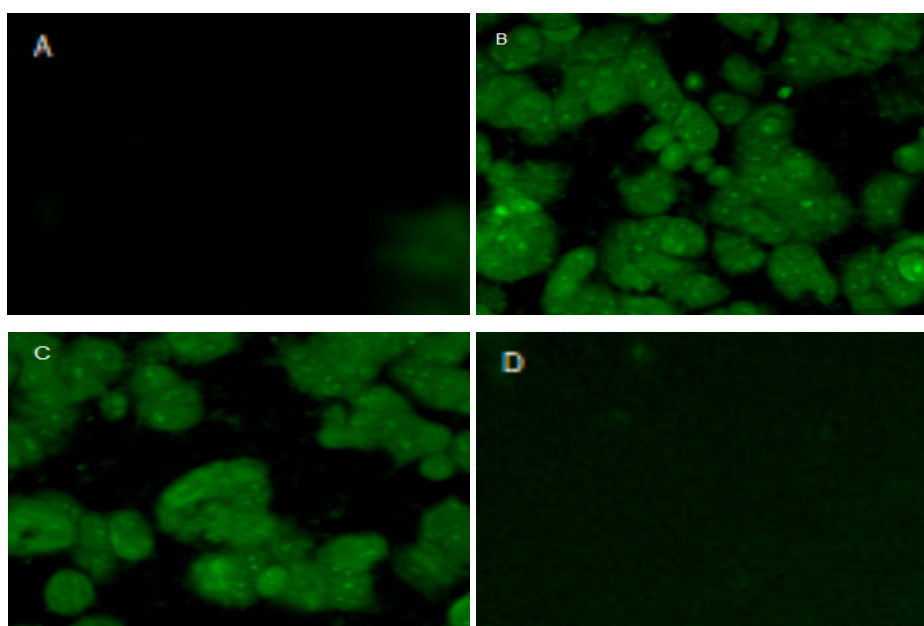

Figure 1. NTERA-2 cells was probed after 1 hour of incubation with (A) $TbPO_4.H_2O$.silica-NH_2; (B) ET2 luminescent nano system; (C) CD133-FITC and (D) unstained control, observed by fluorescence microscopy Olympus Scan^R.

Fluorescent labeling performance using the nano luminescence system ET2

To evaluate the specificity of the nanoluminescence system ET2, we used flow cytometry. The CD133-FITC served as a positive control. The results are shown in Table 1 and Figure 3.

The results showed that the ET2 luminescent nanocomplex probed 97.74% of NTERA-2 cells, which is higher than the number of cells (92.12%) stained with the positive control antibody, CD133-FITC. In addition, $TbPO_4.H_2O$@silica -NH_2 as well as the positive control (CD133-FITC) could not label and distinguish CCD-18Co normal cells. Thus, the ET2 luminescent nano system has been shown to be effective for labeling metastatic cancerous cells. These preliminary results also need to be further studied *in vivo* and clinically verified.

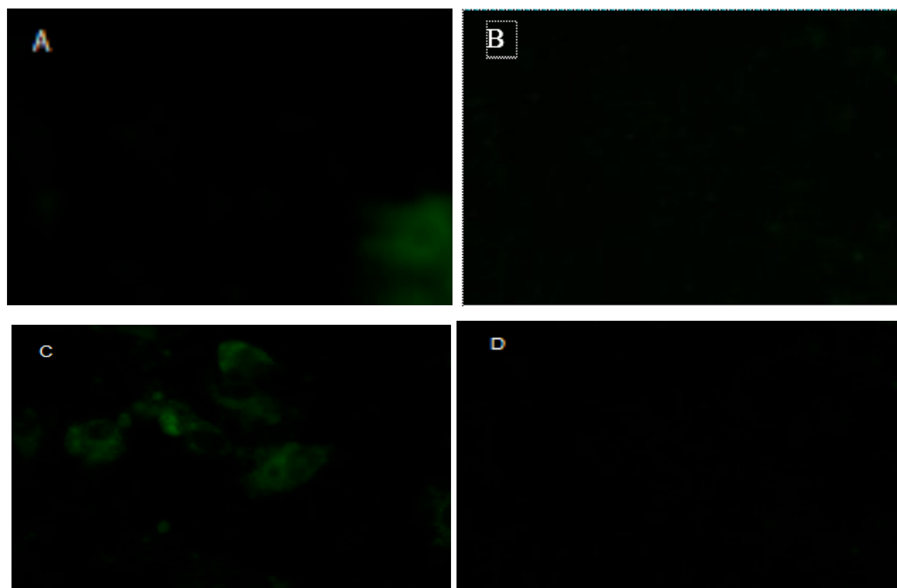

Figure 2. CCD-18Co cells was probed after 1 hour of incubation with (A) $TbPO_4.H_2O$.silica-NH_2; (B) ET2 luminescent nano system; (C) CD133-FITC and (D) unstained control, observed by fluorescence microscopy Olympus Scan^R.

Table 1. Cancer stem cells and healthy cells probing performance.

Samples	The number of $CD133^+$ and fluorescent labeled cells (%)	
	Cancer stem cells (NTERA-2)	**Healthy cells (CCD-18CO)**
ET2 luminescent nano system	97.74 ± 5.36	2.35 ± 0.22
$TbPO_4.H_2O$@silica -NH_2	1.11 ± 0.06	-
CD133-FITC	92.12 ± 4.83	1.82 ± 0.16
Unstained control	0.10± 0.02	0.20 ± 0.04

Note: (-) not examined

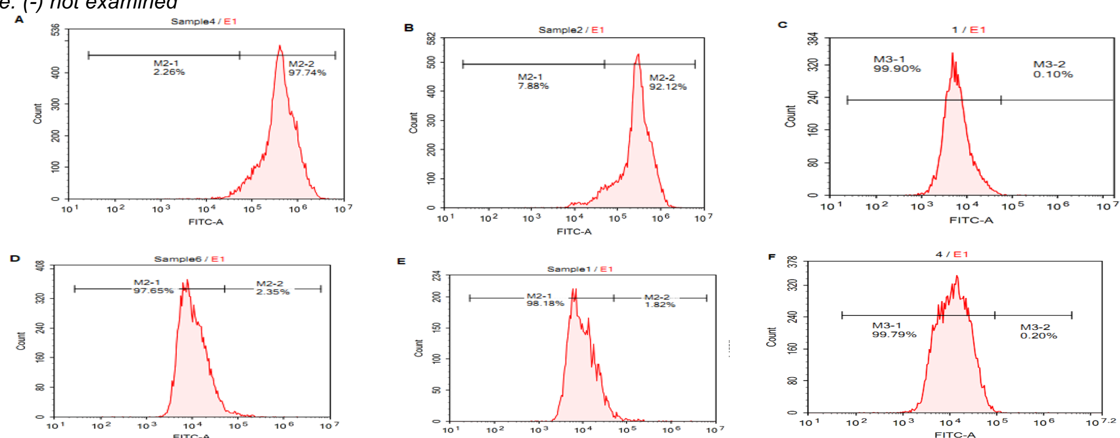

Figure 3. Flow-cytometry analysis to determine the number of fluorescent NTERA-2 cells incubated with various materials: (A) ET2 luminescent nano system (B) CD133-FITC ,(C) unstained control; Flow-cytometry analysis to determine the number of fluorescent and $CD133^+$ CCD-18Co cells incubated with various materials: with ET2 luminescent nano system (D); CD133-FITC (E) and unstained control (F).

As reported, EpCAM, a surface antigen epithelial cell adhesion molecule, which is also a transmembrane protein, plays important functions in cellular signal transmission during migration, proliferation, and differentiation. This protein is a target marker for isolating and probing CSCs. In one report by Chen, the EpCAM mAb was combined with nano vesicles composed of 3-component copolymer (triblock copolymer-poly (ethylene oxide) -block-poly [2- (diisopropylamino) ethyl methacrylate] - block-poly (acrylic acid) (PEO43-b-PDPA76-bPAA17) to transport drugs or small interfering RNAs to EpCAM-expressing cells (Chen *et al*., 2015). However, this nanocarrier lacked luminescent properties for simultaneously probing cancer cells. In another report, a nanomaterial was created from five rare-earth elements to enhance radiation sensitivity, which can increase the effectiveness of brain cancer treatment *in vitro* (Lu *et al*., 2019). In this study, the rare-earth element nano-ion Tb^{3+} was conjugated with anti-CD133 mAb to form an ET2 luminescent nanocomplex. This complex showed promising results for its specific targeting capacities and ability to label CSCs. It might be the first rare-earth-based nanomaterial in the development stage and has valuable applications in cancer diagnostics and treatment.

CONCLUSIONS

The ET2 luminescent nanocomplex is a complex that combines a rare-earth-based Tb^{3+} nanorod with CD133 mAb. The luminescent properties of this ET2 nanocomplex were assessed in cancer stem cells (NTERA-2) and healthy colon cells (CCD-18Co). NTERA-2 cells exhibited strong signals from the ET2 complex when observed with fluorescence microscopy. Flow cytometry showed that the labeling efficiency of NTERA-2 cells with ET2 was 97.74 ± 5.36%. By contrast, healthy cells were very weakly probed (2.35 ± 0.22%). In conclusion, the ET2 luminescent nanocomplex was highly effective for targeting metastatic cancerous cells *in vitro*.

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HIỆU QUẢ ĐÁNH DẤU HƯỚNG ĐÍCH *IN VITRO* TẾ BÀO UNG THƯ GÂY DI CĂN CỦA PHỨC HỢP VẬT LIỆU NANO TỪ Tb^{3+} LIÊN KẾT VỚI KHÁNG THỂ ĐƠN DÒNG KHÁNG CD133

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TÓM TẮT

Ung thư là căn bệnh khó điều trị dứt điểm, WHO dự báo số bệnh nhân ung thư mới phát hiện trong năm 2035 sẽ là 24 triệu, gia tăng 70% so với năm 2012 là 14,1 triệu. Mức độ nguy hiểm của ung thư là do tế bào gốc ung thư (CSCs) gây ra. CSCs được cho là chịu trách nhiệm cho sự kháng thuốc, di căn, tái phát của ung thư. Do không xác định được chính xác vị trí của CSCs nên các liệu pháp chữa trị còn chưa hiệu quả, gây tác động trên toàn thân, nhiều tác dụng phụ độc hại, có thể dẫn tới tử vong cho bệnh nhân. Vì vậy, việc tìm kiếm những vật liệu phát quang hiệu quả để phát hiện và đánh dấu CSCs là rất cấp thiết. Trong nghiên cứu này, phức hợp ET2 phát quang hướng đích CSCs đã được tạo ra nhờ sự tổ hợp thanh nano ion đất hiếm Tb^{3+}được bọc silica, hoạt hóa bằng $-NH_2$, với kháng thể đơn dòng kháng CD133 - một dấu ấn bề mặt đặc trưng của CSCs. Phức hợp ET2 đã được kiểm chứng khả năng gắn kết, phát quang đánh dấu đặc hiệu CSCs dòng NTERA-2. Kết quả cho thấy tế bào NTERA-2 sau khi ủ với phức hệ ET2 đã phát quang mạnh khi quan sát bằng kính hiển vi huỳnh quang. Phân tích bằng kĩ thuật flowcytometry cũng cho thấy có tới 97,74% tế bào NTERA-2 đã được phát hiện nhờ đánh dấu bằng ET2, trong khi chỉ có 2,35 % tế bào CCD-18Co (không ung thư) bị đánh dấu. Như vậy, phức hệ nano phát quang ET2 đã cho thấy sự hiệu quả trong việc phát huỳnh quang đánh dấu hướng đích CSCs và mở ra những ứng dụng tiềm năng trong nghiên cứu cơ bản và lâm sàng.

Từ khóa:*Tế bào gốc ung thư, kháng thể đơn dòng CD133, phức hệ nano phát quang ET2, CCD-18CO; Ion* Tb^{3+}*, NTERA-2*