Tetracysteine as a Reporter for Gene Therapy

CHEN-YU XU[#,*], YING GU[#,*,1], WANG-HENG HOU[#,*], YU-QIONG QUE[#,*], SHUANG-GUAN GAO[#], TONG CHENG[#,*], AND NING-SHAO XIA[#,*,1]

[#]*National Institute of Diagnostics and Vaccine Development for Infectious Diseases, School of Life Sciences, Xiamen University, Xiamen 361005, Fujian, China;* [*]*Key Laboratory of Ministry of Education for Cell Biology and Tumor Cell Engineering, School of Life Sciences, Xiamen University, Xiamen 361005, Fujian, China*

Objective To study the feasibility of using tetracysteine (TC) reporter in gene therapy. **Methods** Effects of TC reporter and conventional reporter genes encoding green fluorescence protein (GFP) and luciferase (Luc) on expression and function of the therapeutic gene $MGMT^{P140K}$ were compared. Cytotoxicity and drug resistance were studied by Western blot. TC reporter used in therapy was analyzed by flow cytometry (FCM). **Results** The TC reporter had no toxicity to cells and neither affected the expression or activity of therapeutic gene as compared to GFP and Luc. TC could be used in blood sample detection. **Conclusion** TC is a new kind of reporter gene for lentiviral vector in future gene therapy.

Key words: Tetracysteine; Lentiviral vector; Reporter; Gene therapy

INTRODUCTION

Genes encoding fluorescent proteins (FPs) and bioluminescence reporters (Luc) are commonly used reporters[1-3]. Although it is convenient to use these reporter genes, the expression and function of the chimeric gene and native target gene may be different[4-5]. This difficulty can be partially overcome by the use of internal ribosomal entry sites (IRES). The foot and mouth disease virus 2A sequence or 5'HS4 chromatin insulator of the chicken beta-globin locus allows the target and reporter genes to be expressed as separate proteins[6-9]. However, there is still no means to eliminate possible biological effects of the reporter protein on the target gene and/or host cells, and as a result wider application of these reporter genes in gene therapy is hindered.

Recently, the tetracysteine-biarsenical system for labeling proteins in living cells appears to be particularly promising[5,10-11]. It exploits the high binding affinity of the tetracysteine (TC) motif containing only 6 amino acids (Cys-Cys-Pro-Gly-Cys-Cys) of the thioarsolan groups of fluorescein (fluorescein arsenical hairpin binder, FlAsH) or resorufin (ReAsH). Importantly, TC motif is rarely seen in naturally occurring proteins, thus making this kind of specific fluorescent labeling an unique one. This technology has already been developed and commercialized by Invitrogen under the brand name Lumio™ in a convenient kit format for labeling TC-tagged proteins in living cells.

Although the TC system has been widely used, no reports are available on its use in gene therapy. We studied the feasibility of using TC as a reporter in gene therapy. The effects of TC, green fluorescence protein (GFP) and Luc reporters on a widely used therapeutic gene $MGMT^{P140K}$ mutant possessing more than 1000-fold increased resistance to the MGMT inactivator, O^6-benzylguanine (BG) were compared[12-16]. Therapeutic blood samples were detected with the TC system to testify its ability as a reporter in gene therapy.

In this study, TC was directly fused with C-terminal of $MGMT^{P140K}$ while GFP and Luc were linked by IRES. All genes were constructed in HIV-1 origin of a lentiviral vector, which is a potential gene therapy vector due to its maximum transfer efficiency in human non-dividing cells and stable integration into host cell genome[17-20].

[1]Correspondence should be addressed to Ying GU. Tel: +86-592-2184113; Fax: +86-592-2181258. E-mail: guying@xmu.edu.cn; Ning-Shao XIA. Tel: +86-592-2184110; Fax: 86-592-2181258. E-mail: nsxia@xmu.edu.cn

Biographical note of the first author: Chen-Yu XU, male, born in 1979, Ph D，School of Life Science, Xiamen University, major in molecular virology. E-mail: Xuchenyu@xmu.edu.cn

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MATERIALS AND METHODS

Plasmid Construction

The lentiviral vectors used in this study are the pBobi backbone containing a self-inactivating LTR deletion and an internal mPGK promoter. plasmid pBobi-M. pBobi-M3u was constructed as follows: $MGMT^{P140K}$ or $MGMT^{P140K}$-TC gene was inserted into the multiple cloning site of pBobi by flanked a 5' Xba I site and a 3' Xho I site. $MGMT^{P140K}$-TC gene inserting the TC tag into the C-terminal of $MGMT^{P140K}$ was obtained by overlap-extension PCR. A sub cloning step was required in construction of pBobi-MIG and pBobi-MIL. EGFP or Luc gene was first linked to the C-terminal of pIRES by flanked a 5' BamH I and 3' Xho I. Then IRES-EGFP (or IRES-Luc) fragment that was flanked by a 5' sal I and 3' Xho I was cloned into pBobi-M which was flanked by an Xho I. The lentiviral vectors pBobi-MIG and pBobi-MIL were obtained after the insertion direction was identified.

Chimeric Lentivirus Production

Chimeric lentiviral particles were generated by co-transfection of helper plasmids pMDL (HIV-1 gag/pol), pRev (HIV-1 rev) and pVSVG (VSV-G envelope glycoprotein) and the gene transfer vector pBobi (or pBobi-M, pBobi-M3u, pBobi-MIG, pBobi-MIL) in HEK 293FT cells (Invitrogen). Culture supernatants were collected 2 days after transfection, centrifuged at 500 ×g for 15 min to remove cell debris, filtered through 0.22 μm PVSF filter and concentrated by ultracentrifugation at 25 000 rpm for 90 min at 4 ℃. Viral stocks were then re-suspended in serum-free RPMI 1 640 (Invitrogen) and stored at -70 ℃, which were correspondingly named L-N, L-M, L-M3u, L-MIG, and L-MIL. Titers of chimeric lentivirus were determined by real-time PCR as previously described[21-22] .

Cell Culture and Infection

CHO cells were cultured in Dulbecco's modified Eagle medium (DMEM, Invitrogen) supplemented with 10% (v/v) fetal bovine serum (FBS, Hyclone). HEK293FT cells were cultured in DMEM with 10% FBS and 0.1 mmol/L MEM non-essential amino acid (Invitrogen). Cells were cultured at 37 ℃ in a 5% CO_2 incubator and infected with lentivirus in the presence of polybrene (Sigma, 6 μg/mL) and centrifuged at 600 ×g for 60 min. The infected cells were then washed after 12 hours and cultured in new media.

Cytotoxicity Analysis

The cytoxicity caused by reporters was tested by comparing the proliferation of cells detected with a cell counting kit-8 (Dojindo, CCK-8). The CCK-8 values of samples detected by ELISA (450 nm / 630 nm) after incubation with 10 μL CCK-8 solution for 1 h, which depended on the amount of produced water-soluble formazan, could represent the number of cells in samples. Each sample was plated in 45 single holes (500 cells/hole) after infection. Among them, sample blank was a medium only representing the reaction background. Then each sample (5 single tests/day) was detected from day 1 to day 9.

Western-blot Analysis

Three days after lentiviral infection, cells were collected and lysed in an ice-cold lysis buffer (10 mmol/L Tris pH 7.5, 1 mmol/L EDTA, 1% NP-40) for Western-blot analysis. The expression of β-Actin and MGMT proteins in infected cells was assessed using mouse anti-human β-actin (Sigma) or mouse anti-human MGMT (BD) first antibody and HRP-conjugated goat anti-mouse second antibody. All data were collected and analyzed with Odyssey v1.1.

Drug-resistant CFU

BG/carmustine (BCNU)-resistant CFU showing the drug-resistant ability of $MGMT^{P140K}$ was carried out as previously described[23-24]. Briefly, cells were pretreated 24 h after infection with BG (100 μmol/L) in a serum-free medium for 2 h, treated with BCNU (80 μmol/L) and BG (100 μmol/L) for 1 h, left in a serum-free culture for another 16 h, and plated in a medium with 10% FBS plus BG (10 μmol/L) at a density of 100 cells/ 60-mm dish for 10 days. Cell colonies were counted by ImmuoSpot (C.T.L.) after staining with 0.15% methylene blue. Colony-forming efficiency was expressed as the number of cells surviving the treatment with drugs.

Isolation of Lymphocytes

Human peripheral blood mononuclear cells (PBMCs) were isolated form blood sample by Ficoll-plus (Amersham Pharmacia). The isolated PBMCs were then infected with L-M3u and mixed with blood sample from the same donor in different ratios (1.0×10^6-1.25×10^5 infected cells into 1 mL blood) 3 days after infection. PBMCs used in latter experiments were isolated from all mixed blood samples by Ficoll-plus after 24 h.

FlAsH Labeling

Cells were labeled by Lumio Green in-cell kit

(Invitrogen). In three days after infection, the medium was removed and the cells were washed once with Opti-MEM (Invitrogen) to remove the dead cells and cell debris. Then Opti-MEM containing 2 μmol/L FlAsH and 100 μmol/L EDT2 (Sigma) was added at 37 ℃ for 30 min. The staining solution was removed and the cells were washed once with Opti-MEM to remove the residual dye for FCM analysis.

FCM Analysis

FCM analysis was carried out for the detection of cellular TC/FlAsH reaction by Coulter Epics XL (Beckman) using a 530/30 nm band pass filter (FL1 channel) following excitation with an argon ion laser source at 488 nm. The data were analyzed with EXPO32 ADC analysis software. For each sample, 20 000 events were collected.

RESULTS

Vector Construction and Chimeric Lentivirus Production

The vector containing $MGMT^{P140K}$ or $MGMT^{P140K}$ fuse with reporters were obtained by fusing TC to the target gene directly while the GFP or Luc gene was linked via IRES (Fig. 1). Chimeric lentiviruses were obtained by co-transfection of the recombinant plasmids with helper plasmids into 239FT cells, named L-N, L-M, L-M3u, L-MIG, and L-MIL correspondingly. Letiviral stocks were all high, up to 10^7 infectious units (UI) per mL (data not shown).

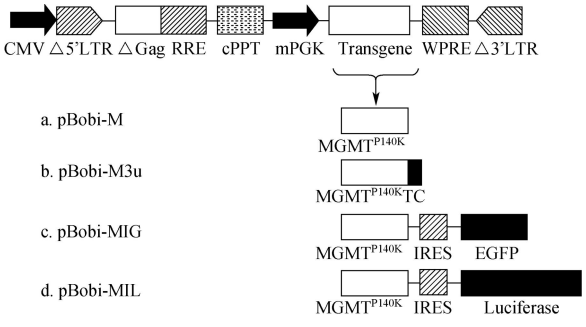

FIG. 1. Schematic diagrams of the expression cassettes of pBobi-based lentiviral vectors. a. pBobi-M: MGMTP140K gene was inserted into the vector and guided by mPGK promoter; b. pBobi-M3u: TC sequence was directly fused to the C-terminal of MGMTP140K; c and d. GFP or Luc gene was linked to the MGMTP140K by IRES.

Influence of Introducing Reporters

The growth rate of transduced cells was detectd, cells infected with L-M were the controls (Fig. 2). After 6 days of culture, the growth rate of cells infected with L-MIG was lower than the control (P<0.05). While that of the cells infected with L-M3u or L-MIL was not significantly different to the control.

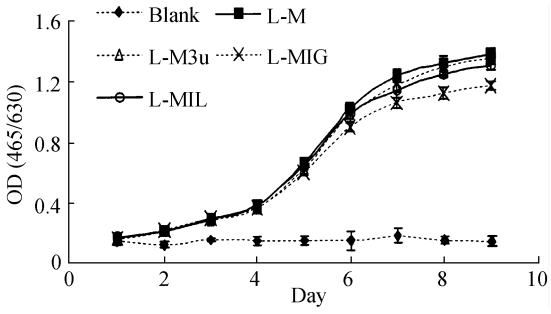

FIG. 2. Cytotoxicities of different reporters. Proliferation of infected cells was determined by ELISA using a CCK-8 kit from day 1 to day 9 post-infection. All samples were infected with different lentiviruses at the same MOI. Blank sample containing only a medium represented the reaction background. All data were shown as $\bar{x} \pm s$ from five independent experiments.

The effects of the reporters on $MGMT^{P140K}$ gene expression were evaluated by Western-blot. The cells were infected with different recombinant viruses at the same MOI (MOI=5). The β-actin expression levels in all samples were almost the same while the MGMT expression levels were different (Fig. 3). Although the cells infected with TC had a comparable level of MGMT expression with L-M, both GFP and Luc decreased the expression of MGMTP140K gene significantly (P<0.01), by more than 3 folds.

The effects of reporter genes on the function of $MGMT^{P140K}$ gene was measured by BG-resistance, we caculated the number of survival colonies of BG/BCNU resistant cells which infected by therapeutic lentivirus at a series of MOI. As indicated in Fig. 4I, no colony was found in the L-N group that means cells we used in this test were susceptible to drugs. At the same time the number of drug resistant colonies increased in line with the MOI of L-M. Drug resistance caused by $MGMT^{P140K}$ was markedly decreased in the presence of GFP or Luc, but not affected by TC. Repeated assays showed that CFU of L-MIG (18) or L-MIL (23) in series d (MOI=10) was nearly equal to that of L-M (19) or L-M3u (20) in series b (MOI=1.25) (Fig. 4II), indicating that the capacity of target gene conferring drug resistance was decreased about 4 folds by GFP or Luc (P<0.01).

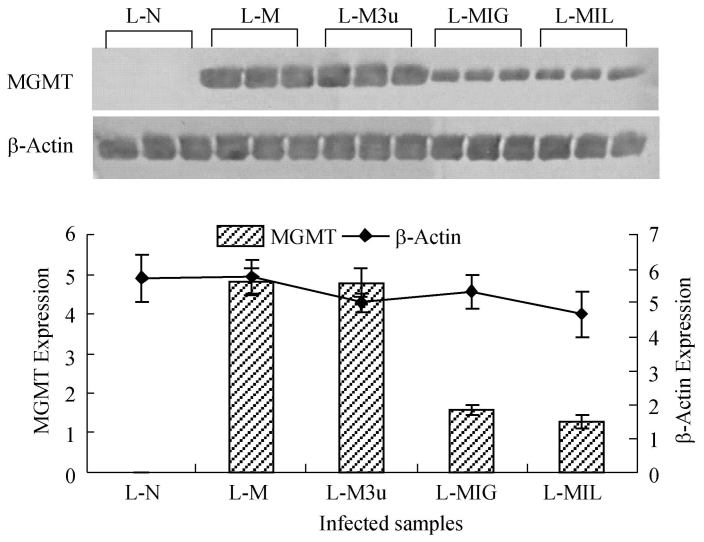

Fig. 3. Western-blot analysis using anti-βactin or anti-MGMT. Lane a: L-N; lane b: L-M; lane c: L-M3u; lane d: L-MIG; lane e: L-MIL. All samples were infected at the same MOI. All expression data were collected and analyzed by Odyssey v1.1. All data were shown as mean ± SD from three independent experiments.

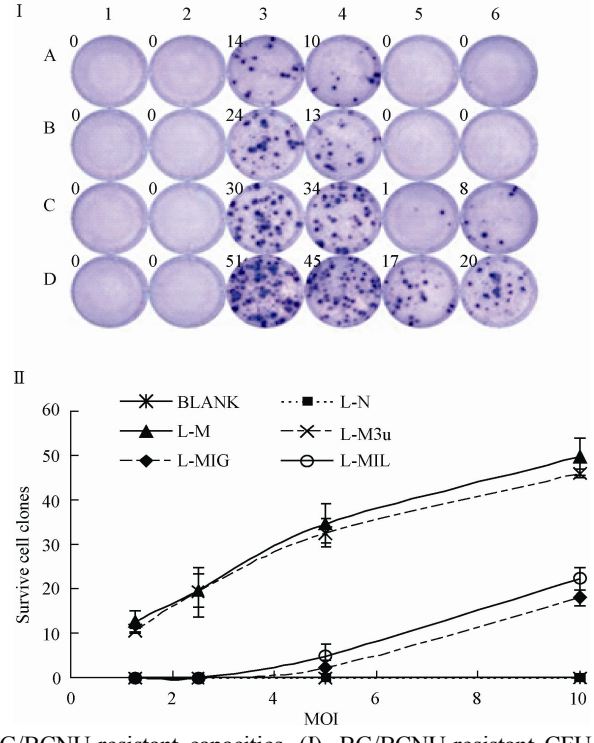

Fig. 4. Comparison of BG/BCNU-resistant capacities. (I). BG/BCNU-resistant CFU. Cells were infected with different lentiviruses at a series of MOI and then treated with BG/BCNU. Drug resistance was detected as the number of survival CFU. Cell colonies were counted by ImmuoSpot (C.T.L.) after staining with methylene blue. MOI series: a. 1.25; b. 2.5; c. 5; d. 10. Sample series: 1. Blank; 2. L-N; 3. L-M; 4. L-M3u; 5. L-MIG; 6. L-MIL. Blank sample which infected no virus was to testify the ability of BG/BCNU killing. (II). Comparison of obtained CFU. All data were shown as $\bar{x} \pm s$ from three independent experiments.

Application of TC as a Reporter in Gene Therapy

L-M3u infected PBMCs were mixed into blood samples at different proportion. All samples were isolated and detected by FCM after incubation with a Lumio Green kit. In this experiment, the infection ratio of PBMCs was 38%. There is a linear dependence between mixed percentage of infected

cells and the TC-positive ratio of all samples (Fig. 5).

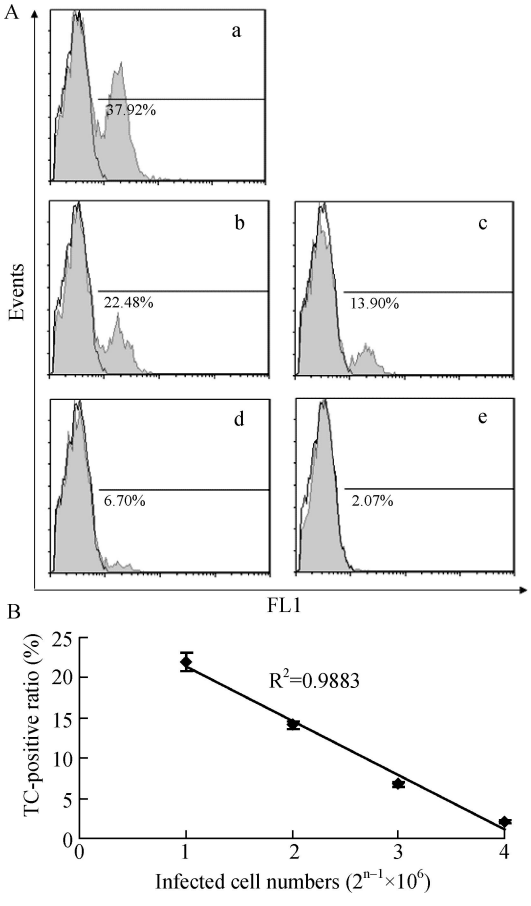

FIG. 5. TC-positive cell ratio in mixed blood samples. A. TC-positive cell ratio analyzed by FCM. a. Infected PBMC samples; b-e. PBMC isolated from blood samples in different mixed ratio (1.0×10^6-1.25×10^5 infected cells into 1 mL blood). B. TC-positive ratios in mixed samples and infected cell number.

DISCUSSION

TC motif contains only a few amino acids. In this study, we evaluate its feasibility as a reporter in gene therapy by comparing with GFP and Luc. We choosed a widely used gene $MGMT^{P140K}$, which possesses more than 1 000-fold increased resistance to the MGMT inactivator, O6-benzylguanine (BG) than wild type MGMT, as therapeutic gene[12-16]. All these report genes were conducted into lentiviral vector, a popular gene therapy vector.

The result of Western-blot showed that using GFP or Luc would decreased the expression levels of the therapeutic gene. The reasons could be less expression efficiency once added IRES or the overexpression of reporters. The drug-resistance assay also indicated the same effects to the function of the therapeutic gene while employed GFP or Luc, but not TC. In that the $MGMT^{P140K}$ and GFP (or Luc) were expressed respectively, the structure of $MGMT^{P140K}$ would not be interfered when introducing IRES. That suggested that this difference was mostly due to the average expression level of therapeutic genes, which might explain why the drug resistant difference was almost equal to that of MGMT expression.

When employed to location *in vitro* or *in vivo* model, the report gene should no effect the growth and function in cells. GFP shows detectable cytotoxicity to infected cells growth, while TC and Luc had no significant effects on cell proliferation. These results confirmed that GFP was somewhat toxic to host cells[25-26]. The most possible reason was because of its easy over-expression in cells. That would cause an accumulated toxicity which might explain why it needed 6 days to produce significant cytotoxicity, or would interfere with the expression of normal protein related to cell metabolism.

FCM analysis indicated that TC would be sensitive enough to be used as a reporter in PBMC monitor *in vitro*. suggesting that TC could be used as a reporter in gene therapy. With the improvement of the TC system, particularly lots of research continued modifying its sequence to strengthen the signal for detection, it would be more practicable to use TC as a reporter in future therapy[27-29].

The overall findings of the present study show that TC was non-cytotoxic and did not affect the expression or function of the target gene. The GFP, in contrast, was cytotoxic. Both GFP and Luc decreased the expression level of target gene, thereby compromising the ability of their target gene to produce drug resistance, which would decrease the probability of successful therapy. Besides, the side-effects caused by cytotoxicity of the reporter gene during therapeutic intervention would be increased and the higher molecular weight of foreign protein used would increase the risk in treatment, indicating that TC would be a relatively safe and effective choice in gene therapy.

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