Electronic Supplementary Material (ESI) for Journal of Materials Chemistry B.

Supporting information

Transferrin-functionalized chitosan-graft-poly(L-lysine) dendrons as a high-efficiency gene delivery carrier for nasopharyngeal carcinoma therapy

Tao Liu [1,*], Shaohua Chen [1], Siyi Zhang [1], Xidong Wu [2], Peina Wu [1], Beiping Miao [3], Xiang Cai [4]

[1] Department of Otolaryngology, Guangdong General Hospital, Guangdong Academy of Medical Sciences, Guangzhou, 510080, China

[2] Department of Pharmacology, Jiangxi Testing Center of Medical Instruments, Nanchang 330029, China

[3] Department of Otolaryngology, the First Affiliated Hospital of Shenzhen University, Shenzhen second people hospital, Shenzhen 518035, China

[4] Department of Light Chemical Engineering, Guangdong Polytechnic, Foshan 528041, China

*Corresponding author: Tao Liu, Tel.: +86 20 83827812, E-mail: taoliu18@126.com.

1. Material and methods

1.1. Cell culture

NPC cell lines (HNE-1, CNE-1, CNE-2, HONE-1 and SUNE-1) and normal nasopharyngeal epithelial cell line (NP-69) were obtained from Guangdong Academy of Medical Sciences. The NPC cells were cultured in RMPI-1640 medium with 10% fetal bovine serum (Biological Industries, Israel) and 1% streptomycin and penicillin (Gibco, USA) in a 37˚C and 5% CO_2 incubator. The NP-69 was cultured in K-MSF medium (GIBCO, USA) with bovine pituitary extract (BPE) and epidermal growth factor (EGF).

1.2. Western blot analysis

Procedures were performed using the Whole Cell Lysis Assay Kit and Western blot related reagents (KeyGEN BioTECH, China). Briefly, all cells were freshly lysed in RIPA buffer. The cell lysates were separated by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and transblotted on the PVDF membranes (Millipore 0.22um, USA). Then, the membranes were incubated with TFRCC Rabbit mAb (1:1000, Sigma, USA) overnight at 4˚C, following incubation with the anti-Rabbit-HRP (1:2000, Biosharp, China). The membranes were washed and proteins were visualized using enhanced chemiluminescence kit (PerkinElmer Inc., Waltham, MA, USA). Western blotting results were semi-quantitative analyzed by Image Pro Plus software.

1.3. Immunohistochemical analysis

In this assay, 42 NPC paraffin tissues and 9 chronic nasopharyngitis tissues (Zhujiang Hospital, Southern Medical University, in Guangzhou, China, from January 2009 to September 2016) were obtained in pathology department. All nasopharyngeal cancer tissues and chronic nasopharyngitis tissues were embedded in paraffin then cut into sections with a thickness of 4 μm and heat at 60˚C for 2 hours. Sections were then dewaxed in xylene and rehydrated through a series of ethanol solutions. Subsequently, high-temperature antigen retrieval was performed in high-pressure drum with EDTA solution (PH = 8), followed by incubation in 3% H_2O_2 for 8 min. Then, the slides were incubated with TFRC1 Rabbit mAb (1:400) at 4˚C overnight. Then, sections were incubated with the secondary anti-rabbit antibody for 30 min at 37˚C. Subsequently, the sections were stained with DAB/haematoxylin, mounted in neutral gum and analysed under the microscope. Two pathologists

evaluated the intensity and distribution of specific staining independently. The results were evaluated by the HSCORE method [1], distribution scores (D) of SGK3 expression were recorded as percentages of positive stained cells in each of four intensity categories, which were scored as 0 (no staining), 1 (weak staining), 2 (distinct staining) and 3 (strong staining). The HSCORE in each tissue was derived by summing the four digitals of distribution scores (D) at each category multiplied by intensity scores (i), HSCORE=ΣD(i). Where D varies from 0 to 100% and i = 0, 1, 2, 3. The maximum of HSCORE is 300, and the minimum is 0. HSOCRE = 75 was taken as the cut-off point to distinguish positive and negative expression.

1.4. Flow cytometry analysis

To measure the TFRC1 expression on different kinds of NPC cells, they were cultured in 100 mm dishes till the cells were grown at least 1×10^6 with RMPI-1640 (10% FBS). The cells were washed with PBS three times, followed by detached using trypsin. Then each kind of cells were suspended in 1ml PBS and incubated with anti-TFRC labelled with FITC for 2 hours. After three times of wash with PBS and suspended, the cells were used for flow cytometry. Data were analyzed with FlowJo software and the mean fluorescence intensity of the cells was measured.

1.5. Statistical analysis

SPSS 13.0 was used for statistical analysis. The data were expressed as the mean±standard deviation. Independent two sample t-test and X^2 test were used to compare continuous and categorical variables. All tests were two-tailed and $P < 0.05$ was considered significant differences.

2. Results

2.1. TFRC1 Expression in NPC cells

TFRC1 protein was measured in different NPC cell lines. The result of western-bolt showed that all NPC cells had the higher expression of TFRC1 compared to NP-69 cells (Fig.1). The flow cytometry also showed that all NPC cell lines had the higher TFRC1 expression than NP-69 cells (Fig. 2).

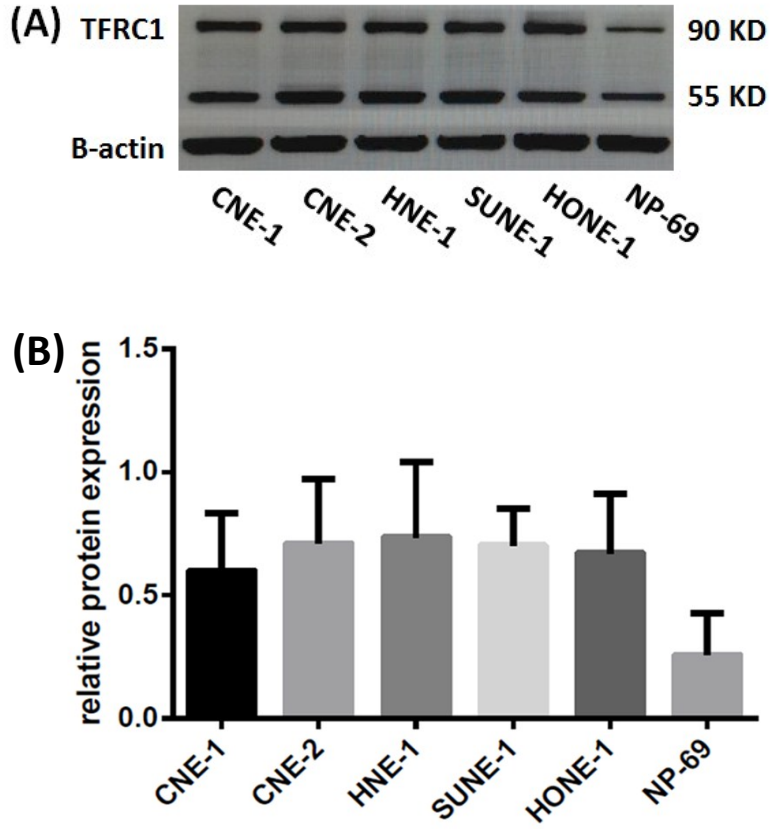

Figure 1. (A) Representative TFRC1 protein expression in NPC cells and NP-69 cell determined by Western-blot analysis. (B) Analysis of light intensities of TFRC1 protein expression as the ratio of TFRC1 to β-actin (n = 3).

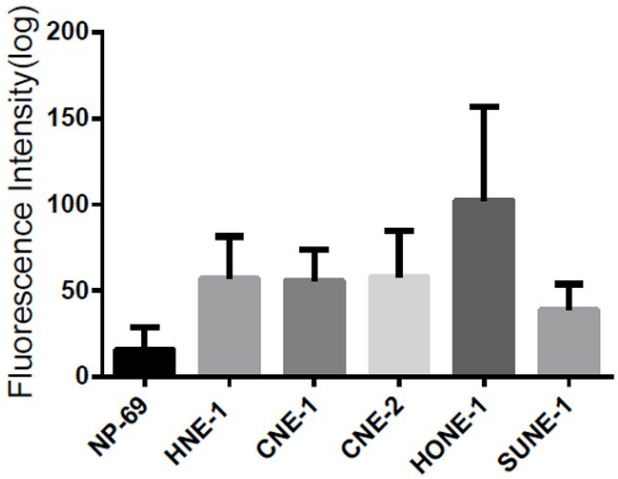

Figure 2. Results of fluorescence intensities of TFRC1 protein expression in NPC cells and NP-69 cell (n = 5).

2.2. TFRC1 expression in NPC tissues

The average HSCORE in 42 NPC tissues and 9 chronic nasopharyngitis tissues was 130 and 26 respectively (Fig. 3), and the positive expression rate of TFRC1 in NPC tissues and chronic nasopharyngitis tissues was 83.33% and 0.00% respectively. The HSCORE and positive rate of TFRC1 expression in NPC were much higher than that in chronic nasopharyngitis ($P < 0.05$).

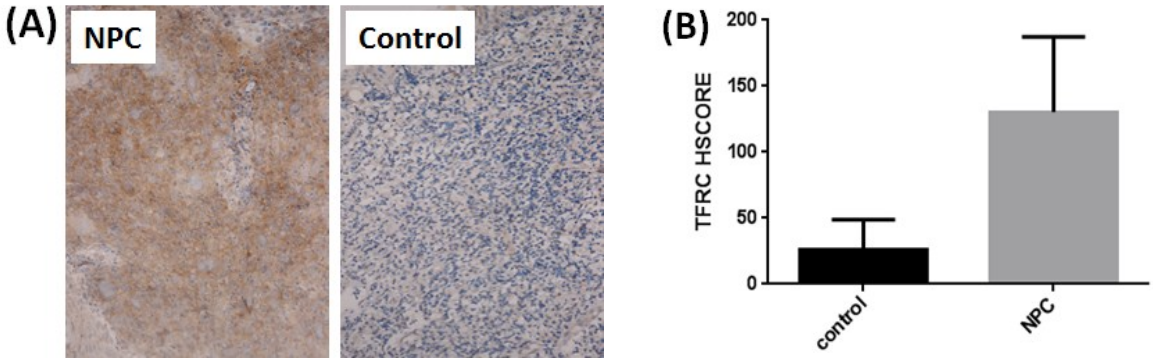

Figure 3. (A) Immunohistochemical staining in NPC tissues and nasopharyngitis tissues (×400). (B) TFRC1 scores in NPC tissues and nasopharyngitis tissues.

3. Discussion

It has been reprted that TFRC mediates the endocytosis of transferrin. TF/TFRC1 had been affirmed as the major route for iron transmembrane transport [2]. Thus, the TFRC1 was expressed in plenty of human cells. Because of highly iron intake of cancer cells, most solid tumor shows an overexpression of TFRC1.

In our research, different NPC cells had higher expression of TFRC1 than normal epithelial cells. The quick proliferation of cancer cells need highly intake of iron [3,4], which lead to TFRC1 overexpression in cancer cells. It's also obvious that the NPC tissues had higher expression of TFRC1 than chronic nasopharyngitis. Because overexpression of TFRC1 in the surface of tumor cells can enhance the endocytosis of transferrin [5], a new strategy for cancer therapy is to conjugate transferrin with smal molecule anticancer drugs [6,7], peptides, proteins or genes [8] to enhance the intake of cancer cells.

References

[1] McCarty KS Jr, Szabo E, Flowers JL, Cox EB, Leight GS, Miller L, Konrath J, Soper JT, Budwit DA and Creasman WT: Use of a monoclonal anti-estrogen receptor antibody in the immunohistochemical evaluation of human tumors, *Cancer Res* 46 (1986), 4244-4248.

[2] Aisen, P, Transferrin receptor 1. *The International Journal of Biochemistry & Cell Biology* 36 (2004), 2137-2143.

[3] Trowbridge IS and Omary MB, Human cell surface glycoprotein related to cell proliferation is the receptor for transferrin, *Proc Natl Acad Sci USA* 78 (1981), 3039-3043.

[4] Sutherland R, Delia D, Schneider C, Newman R, Kemshead J and Greaves M, Ubiquitous cell-surface glycoprotein on tumor cells is proliferation-associated receptor for transferrin. *Proceedings of the National Academy of Sciences* 78 (1981), 4515-4519

[5] Qian ZM, Li H, Sun H and Ho K, Targeted drug delivery via the transferrin receptor-mediated endocytosis pathway, *Pharmacol Rev* 54 (2003), 561-587.

[6] Singh M, Transferrin As A targeting ligand for liposomes and anticancer drugs, *Current Pharmaceutical Design* 30 (1999), 443-451.

[7] Kovár M, Strohalm J, Ulbrich K and Ríhová B, In vitro and in vivo effect of HPMA copolymer-bound doxorubicin targeted to transferrin receptor of B-cell lymphoma 38C13, Journal of Drug Targeting 10 (2002.), 23-30.

[8] Sato Y, Yamauchi N, Takahashi M, Sasaki K, Fukaura J, Neda H, Fujii S, Hirayama M, Itoh Y, Koshita Y, Kogawa K, Kato J, Sakamaki S and Niitsu Y, In vivo gene delivery to tumor cells by transferrin-streptavidin-DNA conjugate, *Faseb Journal Official Publication of the Federation of American Societies for Experimental Biology* 14 (2000), 2108-2118.