Blocking transferrin receptor inhibits the growth of lung adenocarcinoma cells in vitro
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Keywords
Antibody blocking; cell proliferation; KRAS; lung adenocarcinoma; transferrin receptor.

Abstract
Background: Transferrin receptor (TfR) is expressed in most lung cancers and is an indicator of poor prognosis in certain groups of patients. In this study, we blocked cell surface TfR to inhibit lung adenocarcinoma (LAC) cell growth in vitro and investigated the associated molecular mechanisms to determine a potential therapeutic target in human LAC.

Methods: RNA interference and antibody blocking techniques were used to block the function of TfR in LAC cells, and cell proliferation assays were used to detect the results. Affymetrix microarray analysis was conducted using H1299 cells in which TfR was blocked with an antibody to investigate the molecular mechanisms involved.

Results: The cell proliferation assay demonstrated that H1299 cell proliferation was significantly inhibited after small interfering RNA knockdown or blocking of TfR. Mechanistic studies found that 100 genes were altered more than two-fold after TfR was blocked and that blocking TfR was accompanied by decreased expression of the oncogene KRAS.

Conclusion: Our data provide evidence that blocking TfR could significantly inhibit LAC proliferation by targeting the oncogene KRAS; therefore, TfR may be a therapeutic target for LAC. In addition, our results suggest a new method for blocking the signal from the oncogene KRAS by targeting TfR in LAC.

Introduction
Lung cancer remains the leading cause of cancer-related death worldwide. Non-small cell lung cancer (NSCLC) subtypes, including lung adenocarcinoma (LAC), account for approximately 85% of all lung cancers.1 Although tremendous efforts have been devoted to improving treatment procedures through novel chemotherapies combined with targeted agents, the overall survival rate remains low.2 Transferrin receptor (TfR) is a 190 kDa glycoprotein expressed on the cytoplasmic membrane of a wide variety of cells.3,4 One of its key roles is controlling cell growth through iron uptake. However, there is evidence that TfR has many other biological roles in addition to its primary function of facilitating iron transport and metabolism, such as its profound effect on mammalian cell growth and productivity.5,6 TfR expression is normally restricted to a limited number of sites in humans, including basal keratinocytes of the epidermis; islet cells of the pancreas; and parenchymal cells of the liver, testis, and pituitary gland.7 In healthy lung tissue, airway and alveolar epithelia and bronchial glands do not express TfR.7 However, TfR is expressed in most lung cancers and the presence of TfR in NSCLCs is an indicator of poor prognosis in certain groups of patients.7,10 In the present study we blocked cell surface TfR with the aim of inhibiting LAC cell growth in vitro, and investigated the associated molecular mechanisms to find a potential therapeutic target in human LAC.

Methods
Cell culture
Normal human bronchial epithelial (HBE) cells and LAC cell lines (H1299 and A549) were purchased from...
American Type Culture Collection (ATCC, Rockville, MD, USA). These cells were cultured in Dulbecco’s Modified Eagle medium (DMEM; Corning, Christiansburg, VA, USA) supplemented with 10% fetal bovine serum and a 1% antibiotic mixture (100 U/mL penicillin G and 100 μg/mL streptomycin sulfate; Beyotime Biotechnology, Shanghai, China), and incubated at 37°C in a humidified 5% CO₂ atmosphere. Upon reaching 90% confluence, the cells were dissociated using 0.25% trypsin (HyClone, Shanghai, China) and subcultured.

Immunofluorescence staining
HBE, H1299, and A549 cells were harvested from flasks and washed with phosphate buffered saline. Each sample contained 100 000 cells and was resuspended in 10 μL of a 50-fold-diluted human Fc receptor binding inhibitor (eBioscience, San Diego, CA, USA) for 5 minutes on ice, followed by staining with 2 μL of either a phycoerythrin-labeled human TfR antibody (OKT9, eBioscience) or a PE-labeled mouse immunoglobulin G1 (IgG1) isotype control antibody (P3.6.2.8.1, eBioscience) for 30 minutes at 4°C in the dark. The cells were then washed three times and resuspended in 400 μL of ice-cold phosphate buffered saline/2 mM ethylene-diamine-tetraacetic acid. The median fluorescence intensity (MFI) of the samples was quantified using an Attune cytometer with a 488 nm excitation laser and a 574/26 nm emission filter (Life Technologies, Waltham, MA, USA). Cell populations were gated according to the forward and side scattering channel, and 10 000 events were collected. Data analysis was performed using Attune cytometer software (Life Technologies). Experiments were conducted three times.

RNA interference
A transfection mix consisting of 1 μL of RNAiMAX (Invitrogen, Carlsbad, CA, USA) and 12 nM TfR siRNA (Silencer Select small interfering RNAs [siRNAs] 107 046, 107 047, and 107 048) purchased from Ambion in Opti-Mem (Invitrogen) was mixed with suspended cells for 20 minutes at room temperature prior to seeding at 5000 cells per well in 96-well plates. The medium was changed to DMEM after 24 hours. Total cellular RNA was extracted using 1× Nucleic Acid Purification Lysis Solution (Applied Biosystems, Foster City, CA, USA).

Antibody blocking of transferrin receptor (TfR)
An antihuman TfR monoclonal antibody (M-A712) used for blocking was purchased from BD Biosciences Pharmingen (San Diego, CA, USA).11 Cells were incubated with 25 μg/mL isotype control or the TfR antibodies. After 24, 48, 72, and 96 hours, cellular RNA was harvested using 1× Nucleic Acid Purification Lysis Solution (Applied Biosystems).

Proliferation assay
Cell proliferation was measured using colorimetric 3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium, inner salt (MTS) assay (CellTiter 96 AQueous One Solution Cell Proliferation Assay; Promega, Madison, WI, USA). H1299 cells (2 × 10⁴ cells/mL) were incubated with 25 μg/mL anti-TfR antibody in complete medium at 37°C and 5% CO₂. The proliferation assay was performed at 24, 48, 72, and 96 hours after antibody blocking. The MTS assay was performed as previously described.12 Each experiment was performed three times, and the typical results are shown.

Microarray analysis
Cellular RNA from H1299 cells was extracted using TRizol (Invitrogen), and quality control was performed as directed by the Affymetrix expression technical manual. RNA (50 ng) was used to produce biotin-labeled complementary RNA, which was hybridized to the Affymetrix GeneChip Human Genome U133 Plus 2.0 array (Affymetrix, Inc., Santa Clara, CA, USA). Array washing, scanning, and probe quantification protocols were performed according to the manufacturer’s instructions using Affymetrix GeneChip Operating Software (GCOS) (http://www.affymetrix.com).13 Genes that displayed two-fold or greater expression changes between the control and antibody blocking groups were filtered using the Student’s t-test with a significance threshold of P < 0.05. Hierarchical clustering with average linkage was performed only for the differentially expressed genes (DEGs), using Cluster 3.0. The cluster data were visualized using Java TreeView software (Microsoft, Redmond, WA, USA). Gene ontology (GO) and pathway analysis of the DEGs were performed using CapitalBio Molecule Annotation System (MAS) version 3.0 (http://bioinfo.capitalbio.com/mas3/), which can integrate multiple biological databases with the MAS core database. The information required for pathway analysis was obtained from three pathway databases, the Kyoto Encyclopedia of Genes and Genomes (KEGG), Biocarta, and GenMAPP, which are the major authoritative pathway databases.14

Real-time PCR
Total RNA was extracted and then reverse transcribed into complementary DNA (cDNA) using a PrimeScript RT reagent kit (TaKaRa Biotechnology, Dalian, China) according
to the manufacturer’s instructions. The PCR reactions were carried out in a 50-μL reaction mixture with a 96-well format using SYBR Premix Ex Taq II (TaKaRa Biotechnology) in a 7300 real-time PCR system (Applied Biosystems). Three biological replicates were created for each gene. The results were normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH), and the amount of each transcript was calculated using the formula $2^{-\Delta\text{CT(target gene)} - \Delta\text{CT(GAPDH)}}$, where the cycle threshold value indicates the time at which the fluorescence rose appreciably above the background fluorescence.14 The following primers were used: TfR, 5′-CA GCCCAGCAAGACCTT-3′ and 5′-CCAAGAACCCTT-TATCCAG-3′; KRAS, 5′-GACTGAATAAATCCTTG-GTAGTTGACCCT-3′ and 5′-TCCTCTTGACCTGCTGTGT CG-3′; and GAPDH, 5′-CAAGATCATCAGCAATGCCT-3′ and 5′-AGGGATGATGTCTGGAGAG-3′. 

**Western blot**

Total cellular proteins were isolated from cultured cells using lysis buffer and assayed by Western blot according to previously described procedures.15 Proteins were detected according to the manufacturer’s instructions. Briefly, equal amounts of protein were denatured, electrophoresed (SDS-PAGE gel kit, Beyotime), and transferred to polyvinylidene fluoride membranes (Millipore, Billerica, MA, USA). The membranes were then incubated overnight with primary antibody, washed with tris-buffered saline plus tween 20, and then incubated with a secondary antibody. The proteins were detected using ECL chemiluminescence (Millipore). The primary antibodies were mouse anti-TfR (1:1000, M-A712, BD Pharmingen), and mouse anti-KRAS (1:1000) and mouse anti-GAPDH (1:1000; Cell Signaling Technology, Danvers, MA, USA). The secondary antibody was horseradish peroxidase-conjugated rabbit anti-mouse IgG (1:10 000; Cell Signaling Technology).

**Statistical analysis**

The results are presented as the mean ± standard deviation. Proliferation assay and real-time PCR data were analyzed using paired t-tests. Statistical analysis was conducted using SPSS version 13.0 statistical software (SPSS Inc., Chicago, IL, USA). A two-tailed P value of < 0.05 was considered statistically significant.

**Results**

**TfR expression**

The TfR expression levels in the normal HBE cells and lung adenocarcinoma cell lines (H1299 and A549) were measured via flow cytometry. The cells were immunostained with a fluorescent-labeled anti-TfR antibody or an isotype control antibody. As shown in Figure 1a, the MFI across three cell lines was unchanged when stained with the isotype antibody, indicating that little fluorescence was caused by non-specific antibody binding. By contrast, H1299 and A549 cells demonstrated a significantly higher MFI after being stained with the anti-TfR antibody compared to the HBE cells and the MFI in H1299 cells was higher than in A549 cells (Fig 1a). Similarly, H1299 and A549 cells demonstrated markedly higher levels of TfR protein expression compared to the HBE cell line, as determined by Western blot analysis (Fig 1b). Additionally, the TfR protein expression level in H1299 cells was higher than in A549 cells (Fig 1b). Therefore, the H1299 cells were considered the TfR overexpressing cell model for our study.

**TfR knockdown using small interfering RNA inhibits H1299 cell proliferation**

As an initial experiment to determine whether TfR is functionally involved in H1299 cell proliferation, we assessed the effect of TfR knockdown. H1299 cells were transfected with an irrelevant control siRNA or a TfR-specific siRNA 24 hours after cells were seeded. Real-time PCR confirmed the knockdown efficiency of the TfR-siRNA. TfR messenger RNA (mRNA) expression was significantly decreased ($P = 0.004$ vs. the negative control; $P = 0.009$ vs. the mock transfection) (Fig 1c) 96 hours after transfection in H1299 cells following the silencing of TfR by its specific siRNA, resulting in downregulation of the TfR protein (Fig 1d).

After transfection, the cells were incubated in complete medium for an additional 24, 48, 72, or 96 hours. MTS assay results showed that transfection with the TfR siRNA resulted in significantly decreased cell viability compared to the negative control at each time point (Fig 2a).

**Blocking cell surface TfR inhibits H1299 cell proliferation**

Because TfR knockdown appeared to inhibit H1299 cell proliferation, we investigated whether blocking surface TfR had a similar effect. Initially, we performed blocking experiments in which antibodies were added to the cells 24 hours after cell seeding. H1299 cells were incubated with irrelevant mouse IgG negative control or the anti-TfR. The cells were then incubated in complete medium for an additional 24, 48, 72, or 96 hours. MTS assay results showed that blocking TfR also resulted in significantly decreased cell viability compared to the negative control at each time point (Fig 2b).
Differentially expressed genes

To investigate the molecular mechanisms of blocking TfR inhibiting cell proliferation, cDNA microarray analysis was utilized to identify regulated gene expression 72 hours after TfR was blocked. A total of six microarrays were used, and three biological replicates were created for each condition (anti-TfR vs. the negative control group). Transcriptome analysis between the negative control and anti-TfR groups revealed that 100 DEGs with a cut-off threshold of $P < 0.05$ showed a correct hybridization signal on the six arrays. Of the 100 differentially expressed mRNAs between the negative control and anti-TfR groups, 4 (4.0%) were highly expressed in the anti-TfR groups and 96 (96.0%) were highly expressed in the negative control group. Hierarchical clustering is presented in Figure 2c.

GO analysis of the 100 DEGs revealed that blocking TfR regulates genes that are involved in cell proliferation, apoptosis, cell cycle, transcription, translation, biosynthetic processes, metabolic processes, protein and nucleic acid binding, proteolysis, signal transduction, and ion transmembrane transport regulation, among others (Table S1). Table 1 shows the DEGs associated with cell proliferation, apoptosis, the cell cycle, and transcription. Pathway analysis was successfully performed to associate differentially regulated genes with known specific biological pathways using the CapitalBio MAS platform. This pathway analysis revealed the central regulatory role played by KRAS (Fig 3).
Validation of microarray data by real-time PCR and Western blot

To validate the microarray results, we performed real-time PCR and Western blot analysis on the negative control and anti-TfR H1299 cells. KRAS was relevant to the biological function of proliferation and could partially illuminate the molecular mechanisms of cell proliferation induced by blocking TfR (Table 1). Because the pathway analysis showed that KRAS plays a central regulatory role after TfR is blocked (Fig 3), KRAS was selected for further analysis. To observe the dynamic trend of the KRAS gene after blocking TfR, we performed real-time PCR on control and anti-TfR H1299 cells 24, 48, 72, and 96 hours after blocking TfR. The results were analyzed by comparing relative gene expression using the 2−ΔΔCt method.14 KRAS mRNA expression levels in the anti-TfR group were significantly lower than in the negative control from 24 to 96 hours after blocking (P < 0.05) (Fig 4a). We then determined whether blocking TfR affected the expression of endogenous KRAS at the translational level. As shown in Figure 4b, Western blot analysis revealed that the level of KRAS protein expression in the anti-TfR group was significantly inhibited compared to that in the negative control group 96 hours after blocking.

Discussion

Transferrin receptor is a membrane protein expressed at low levels in most cells and can bind to the iron transport glycoprotein Tfn, which consequently triggers fast internalization.16–19 Our investigation of the functions and properties of TfR in iron transport indicate that TfR plays a greater biological role than previously thought.5,20,21 Xie et al. reported that H1299 cells demonstrate significantly higher TfR expression compared to A549 and H460 cells.22 Therefore, we chose to use A549 and H1299 cell lines to locate a TfR overexpressing cell. In this study, we observed upregulated protein levels of cellular TfR in lung adenocarcinoma H1299 cells (Fig 1a,b). After siRNA knockdown or TfR blocking, we observed inhibition of H1299 cell
proliferation, demonstrating that TfR plays a role in LAC growth (Fig 2a,b). Mechanistic studies suggest that blocking TfR may exert an effect by inhibiting KRAS (Figs 3, 4).

Transferrin receptor plays a crucial role in the cellular uptake of iron, and cellular iron deficiency arrests cell growth, leading to cell death.23 TfR is expressed more abundantly in malignant tissues than in normal tissues because cancer cells require copious amounts of iron to maintain their high proliferation rates.24 Therefore, TfR is an attractive target for immunotherapy and for the delivery of cytotoxic agents because of its increased expression in malignant compared to normal cells.25–27 TfR is expressed in most lung cancers, and the presence of TfR in NSCLCs may be an indicator of poor prognosis in certain groups of patients.7 Herein, we showed that TfR itself could possibly be a therapeutic target for LAC, with the anti-human TfR-specific antibody described in this manuscript. The mechanism behind the selective cancer cell killing ability of the anti-TfR antibody has not yet been elucidated. However, it is speculated that cancer cells require more iron than normal cells to sustain their abnormally rapid growth rates, thus reducing the intracellular iron concentration by preventing TfR-mediated cellular iron uptake may cause growth inhibition and cell death in cancer cells.28,29 In LAC, the anti-TfR antibody is suspected to block the internalization of transferrin into cancer cells, and the resulting transferrin deficiency might block the cell cycle and/or induce apoptotic cell death because of the potentially abnormal transferrin requirements of cancer cells. In addition, Tf, which is the ligand for TfR, can act as a growth factor in most cells in tissue culture, as a neurotrophic factor during neural stem cell development, and as an angiogenic factor to promote endothelial cell migration.5 Blocking TfR results in the inhibition of these Tf functions. In this study, we observed that the proliferation of H1299 cells was significantly inhibited after siRNA knockdown or after TfR was blocked (Fig 2a,b). These results indicate that TfR is potentially a therapeutic target for lung adenocarcinoma. In addition, the small-molecule iron transport inhibitor ferristatin/NSC306711 promotes TfR degradation, which may be used in LAC therapy in the future.17,30

As previously reported, TfR expression is significantly upregulated in cancer, increasing several 100-fold in various tumor cell lines and malignant tissues.36 Although the receptor has an established physiological function within the tissue, its aberrant stimulation and overexpression in cancer could act as an attractive targeting molecule for cancer therapy. A number of studies have shown the capacity of anti-transferrin receptor antibodies to limit cell growth in cultured, normal, and malignant cells.36 However, no relevant study has reported whether blocking TfR will have any adverse effects on other normal tissues or organs. This issue requires further exploration in future research.

Complementary DNA microarray analysis showed that 100 genes were altered more than two-fold after TfR was blocked. We grouped the 100 regulated genes into

| Function   | Gene ID | Gene title                        | Gene symbol | Fold change (Anti-TfR vs. control groups) | P       |
|------------|---------|-----------------------------------|-------------|-----------------------------------------|---------|
| Proliferation | 8678    | Beclin 1, autophagy related       | BECN1       | 0.4585                                  | 0.0006  |
|            | 1296    | Collagen, type VIII, alpha 2     | COL8A2      | 0.3775                                  | 0.0437  |
|            | 2100    | Estrogen receptor 2 (ER beta)    | ESR2        | 0.3821                                  | 0.0053  |
|            | 3845    | Kirsten rat sarcoma viral oncogene homolog | KRAS    | 0.4682                                  | 0.0053  |
|            | 5697    | Peptide YY                       | PYY         | 0.4656                                  | 0.0424  |
|            | 9481    | Solute carrier family 25, member 27 | SLC2A27    | 0.3886                                  | 0.0392  |
| Apoptosis | 23 604  | Death-associated protein kinase 2 | DAPK2       | 0.4978                                  | 0.0199  |
| Cell cycle | 3537    | Cytoskeleton associated protein 2 | CKAP2       | 0.4244                                  | 0.0160  |
|            | 54 737  | M-phase phosphoprotein 8         | MPHOSPH8    | 2.1859                                  | 0.0297  |
|            | 4772    | Nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 1 | NFATC1    | 0.4236                                  | 0.0018  |
|            | 126 520 | Polo-like kinase 5               | PLK5        | 0.4779                                  | 0.0004  |
|            | 5933    | Retinoblastoma-like 1            | RBL1        | 0.4931                                  | 0.0361  |
|            | 6714    | SRC proto-oncogene, non-receptor tyrosine kinase | SRC      | 0.3405                                  | 0.0157  |
| Transcription | 1386    | Activating transcription factor 2 | ATF2        | 0.4413                                  | 0.0174  |
|            | 2303    | Forkhead box C2 (MFH-1, mesenchyme forkhead 1) | FOXC2     | 0.4735                                  | 0.0075  |
|            | 136 259 | Kruppel-like factor 14           | KLF14       | 0.4533                                  | 0.0070  |
|            | 7849    | Paired box 8                     | PAX8        | 0.4337                                  | 0.0344  |
|            | 862     | Runt-related transcription factor 1; translocated to, 1 (cyclin D-related) | RUNX1T1    | 0.3855                                  | 0.0130  |
|            | 147 949 | Zinc finger protein 583          | ZNFS83      | 0.4020                                  | 0.0107  |

Table 1 Genes associated with proliferation, apoptosis, cell cycle, and transcription
functional categories that included cell proliferation, apoptosis, cell cycle, transcription, translation, biosynthetic processes, metabolic processes, protein and nucleic acid binding, proteolysis, signal transduction, and ion transmembrane transport regulation, among others (Table S1).

Pathway analysis showed that KRAS plays a central regulatory role, and was thus selected for further analysis (Fig 3). Real-time PCR and Western blot analysis validated the array findings for the KRAS gene (Fig 4).

KRAS is an oncogene that functions as a critical molecular switch for various biological processes, including cell proliferation, survival, differentiation, and death. KRAS is associated with increased cell growth and differentiation in many cancers. In addition, Yoon et al. showed that KRAS plays a key role in signal transduction in NSCLC. Activating KRAS mutations are present in more than 80% of all NSCLCs. This mutation leads to constant receptor phosphorylation and

Figure 3 The differentially expressed genes were analyzed using CapitalBio MAS version 3.0. The blue lines indicate that the information regarding the gene relationships was obtained from the KEGG database, and the green lines indicate that the information was obtained from the GenMAPP database. Pathway analysis revealed that KRAS (colored red) has a greater number of connections with other genes.
the activation of downstream cascade pathways (such as the RAS, PI3K, and AKT signaling pathways) that are important for the regulation of cell proliferation and growth.\(^4\)\(^0\) It has been shown that activating KRAS mutations lead to subsequent activation of the RAS–RAF pathway.\(^4\)\(^0\) Inhibiting KRAS results in the inhibition of cancer cell proliferation and growth; however, direct KRAS targeting has proven to be difficult.\(^4\)\(^1\)–\(^4\)\(^3\) Some strategies for interrupting oncogenic KRAS signals, such as inhibiting post-translational modification and Ras protein membrane association, have been employed, but unfortunately no clinical benefits using these methods have been determined.\(^4\)\(^1\) In this study, we observed that blocking TfR resulted in the downregulation of KRAS mRNA and protein levels in lung adenocarcinoma H1299 cells (Figs 3, 4). Our results suggest a new method for blocking the oncogenic KRAS signal by targeting TfR in LAC. The mechanisms of TfR suppression of KRAS expression are unknown. TfR acts as a growth factor in most cells in tissue culture,\(^5\)\(^6\) thus we hypothesize that this may be related to the non-facilitating iron transport of TfR and intend to conduct further research.

In conclusion, we found that blocking TfR could significantly inhibit LAC proliferation by targeting the oncogene KRAS. These results indicate that blocking TfR may suppress LAC progression and imply its potential application in LAC therapy. We plan to initiate a clinical trial to determine the usefulness and benefits of TfR as a therapeutic target for LAC.

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Disclosure

No authors report any conflict of interest.

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Supporting Information
Additional Supporting Information may be found in the online version of this article at the publisher’s website:
Table S1 Genes associated with various functions.