A novel treatment approach for retinoblastoma by targeting epithelial growth factor receptor expression with a shRNA lentiviral system

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ABSTRACT

Objective(s): Non-invasive treatment options for retinoblastoma (RB), the most common malignant eye tumor among children, are lacking. Epithelial growth factor receptor (EGFR) accelerates cell proliferation, survival, and invasion of many tumors including RB. However, RB treatment by targeting EGFR has not yet been researched. In the current study, we investigated the effect of EGFR down-regulation on RB progression using shRNA lentiviral vectors.

Materials and Methods: EGFR expression in Weri-Rb-1 cells was down-regulated by EGFR shRNA-bearing lentiviral vectors. Cell death proliferation, cell cycle as well as invasion after EGFR down-regulation were determined. Further signaling pathway analysis was done by Western blot. Results: Our results revealed that EGFR shRNA could specifically down-regulate EGFR expression and down-regulation of this protein promoted cell death. Further analysis on cell cycle demonstrated that EGFR down-regulation also suppressed cell proliferation by arresting cells at G1 phase. Invasion analysis showed that EGFR down-regulation suppressed cell invasion and was correlated with alteration in the expression of metalloproteinases 2 and 9. Further signaling pathway analysis revealed that EGFR down-regulation mediated BB progression was through PI3K/AKT/mTOR signaling pathway.

Conclusion: Our study revealed that EGFR down-regulation, through the PI3K/AKT/mTOR signaling pathway, could inhibit RB progression by promoting cell death while suppressing cell proliferation and invasion. The findings of our study indicated that down-regulation of EGFR using shRNA lentiviral vectors may offer a novel non-invasive treatment for RB.

Introduction

Retinoblastoma (RB), albeit rare among the whole population, is the most common malignant eye tumor among children, especially young children (1). Although the survival rate of this disease is high, the recovered patients may suffer from vision loss or in a worse scenario, eye removal. Currently, all available treatments for RB are invasive, from laser therapy or cryotherapy for small tumors to eye removal, radiation, and even stem cell transplantation for vision-loss or spread tumors (2-5). Therefore, novel less or non-invasive treatment strategies for this disease are still in need.

Epithelial growth factor receptor (EGFR), a member of the receptor tyrosine kinase family, has been reported to be involved in the progression of many forms of tumors including gastric, breast, cervical cancer, and RB (6). Previous studies have revealed that mutated and elevated EGFR expression could accelerate the growth, proliferation, and invasion of tumor cells through activation of downstream phosphatidylinositol 3 kinase (PI3K)/protein kinase B (PIK, also known as AKT) pathway (7-9). PI3K/AKT signaling pathway is a common anti-apoptosis pathway and is closely related to tumor cell biology. Following PI3K activation, AKT is phosphorylated and p-AKT can subsequently activate the mechanistic target of rapamycin (mTOR) through direct or indirect interactions, and activated mTOR (p-mTOR) can then regulate cell growth, proliferation, motility, and survival (10).

Given the significance of EGFR in cancer, treatments targeting EGFR using inhibitors or neutralizing antibodies have been investigated in some cancers including breast,

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colon, prostate, and renal cell carcinoma (11-13). However, the effect of EGFR-expression downregulation using shRNA systems on cancer progression remains to be investigated. In addition, EGFR-targeting strategies against RB have not yet been reported. In the current study, we, for the first time, investigated the efficacy of EGFR down-regulation using EGFR-specific shRNA carrying lentiviral vectors on RB treatment. Our results showed that this strategy could efficiently down-regulate EGFR expression, which consequently inhibited RB cell survival, growth, and invasion via the PI3K/AKT/mTOR signaling pathway.

Materials and Methods

Cell lines and shRNA lentiviral vectors

Human retinoblastoma cell line Weri-Rb-1 and Human Embryo Kidney cell line 293T were purchased from Cell Bank of the Chinese Academy of Sciences and American Type Culture Collection, respectively. Both cell lines were cultured in Dulbecco's Modified Eagle Medium (DMEM, Hyclone, GE Healthcare) supplemented with 10% fetal calf serum (Hyclone, GE Healthcare) and antibiotics ( Gibco, Thermo Scientific). Lentiviral vectors carrying EGFR-specific shRNA (TL320326) or negative control shRNA (TR30021) were purchased from origin and lentiviral particles were propagated on 293T cell line according to the manufacturer’s instructions.

EGFR knockdown

18–24 hr before assay, Weri-Rb-1 cells were seeded in a 6-well plate at a density of 1×10⁵ cells/well. And then cells were infected with lentiviral particles (dose ranging from 0.5 to 20 MOI) and incubated for up to 96 hr. EGFR knockdown efficiency and cell viability were determined by Western blot and MTT assay, respectively. A mock control (293T cell culture supernatant) and a negative shRNA control (lentiviral particles carrying non-specific shRNA) were introduced into the analysis for background assessment.

Cell apoptosis analysis

Cell apoptosis was analyzed using the Premix WST-1 Kit and performed according to the manufacturer’s instructions (TaKaRa). In brief, Weri-Rb-1 cells were infected with shRNA lentivirus or mock for up to 96 hr and then the culture medium was removed and Premix WST-1 was added and incubated for 2 hr in a humidified atmosphere. After incubation, the absorbance of the samples was measured using a microtiter plate reader at the testing wavelength of 440 nm and reference wavelength of 650 nm.

Cell cycle assay

Cell cycle assay was conducted as previously described (14). In brief, Weri-Rb-1 cells with or without shRNA lentivirus infection were harvested and fixed with ethanol and then stained with the PI/RNAse solution (ThermoScientific) for 30 min in the dark at room temperature. After staining the cells were analyzed without washing on an LSRFortessa cytometer (BD Biosciences).

Cell invasion assay

Cell invasion was evaluated using a Matrigel-based Transwell system as previously described with modifications (15). In brief, Weri-Rb-1 cells with or without shRNA lentivirus treatment were seeded onto the 1 mg/ml Matrigel-coated (Sigma-Aldrich) upper chamber of a Transwell plate (Coming) and incubated at 37˚C for 20 hr. After incubation, the Matrigel coating was fixed with 4% paraformaldehyde and then the cells invading into the Matrigel were stained with 1% crystal violet and counted under a microscope (Nikon).

Western blot

The expression of EGFR, matrix metalloproteinases (MMP) 2 and 9, and PI3K/AKT/mTOR signaling pathway proteins were measured by Western blot. Weri-Rb-1 cells with or without shRNA lentivirus infection were harvested, lysed, and separated by SDS-PAGE. Following electrophoresis separation, the proteins were transferred onto a PVDF membrane and blocked with 5% non-fat milk in PBST. After washes, the membrane was then probed with primary antibodies, anti-EGFR, MMP2, MMP9, PI3K, AKT, p-AKT, mTOR, p-mTOR, and internal control GAPDH followed by corresponding HRP-conjugated secondary antibodies for 2 hr and 1 hr at room temperature, respectively. Finally, the membrane was extensively washed with PBST and visualized using enhanced chemiluminescence Western blot substrate (Millipore) under a CCD camera (BioRad Imagelab). Semi-quantification of Western blot bands was performed with BioRad Imagelab.

Statistical analysis

All data in this study were expressed as mean ± standard deviation (SD) and analyzed using Graphpad Prism 4.00 (Graphpad). One-way ANOVA with SNK post hoc was adopted for statistical analysis and a P-value less than 0.05 was considered statistically significant.

Results

EGFR shRNA lentivirus efficiently downregulated EGFR expression with high specificity

Since EGFR overexpression could lead to RB progression, the down-regulation of this protein may offer an approach to treating this disease (16). Consequently, in the current study, we investigated the effect of EGFR down-regulation on RB progression. First, we tested the knock-down effect of EGFR shRNA lentivirus. Weri-Rb-1 cells were firstly treated with 293T culture medium (mock control), negative control shRNA lentivius (5 MOI), or EGFR shRNA lentivirus (5 MOI) for 48 hr; and then EGFR expression was measured. As shown in Figures 1A and B, EGFR expression was sharply reduced in the EGFR shRNA lentivirus group while its expression remained unchanged in the mock treated or
negative control shRNA lentivirus groups. These data indicated that the EGFR shRNA lentivirus used in our study could specifically target to and knock-down EGFR expression.

**EGFR down-regulation promoted RB cell death**

Increased EGFR expression could enhance RB cell survival, therefore, we next determined whether the down-regulation of the protein could reverse this enhancement and promote tumor cell death. In order to obtain effect of EGFR down-regulation on RB cell death in more detail, infection dose and infection time assays were performed.

As shown in Figure 2A, in the EGFR shRNA group, cell survival rate declined in a lentivirus infection dose manner. Of note, in the negative control shRNA group, cell viability also showed a decrease when infection dose was more than 10 MOI, indicating that high infection dose may have a cytotoxic effect or non-specific down-regulation of genes. Unlike these two groups, the mock control group did not show an apparent impact on cell survival. These data here indicated that the optimal infection dose for EGFR shRNA lentivirus was 5 MOI. At an infection dose of 5 MOI, EGFR shRNA also affected cell survival in an infection time-dependent manner, while the two controls showed a very slight effect on cell survival (Figure 2B). Since at the infection dose of 5 MOI and infection time of 48 hr, cell death in EGFR shRNA group was significantly higher than the two control groups, this dose and time condition was adopted for the downstream analysis.

**EGFR down-regulation arrested RB cells at G1 phase**

Uncontrolled cell proliferation is one of the most important characteristics of tumor cells. Since EGFR down-regulation enhanced RB cell death, we presume that it might also have an impact on cell proliferation. To test our hypothesis, cell cycle of EGFR shRNA treated Weri-Rb-1 cells was analyzed. As shown in Table 1, in the mock control group, cells at G1, S and G2 phases were 65.78%, 24.38%, and 9.84%, respectively. Cells in control shRNA group showed similar distribution; cells at G1, S, and G2 phases were 66.42%, 23.89%, and 9.69%, respectively. Surprisingly, after EGFR shRNA treatment, cells were arrested at the G1 phase (83.39%), while those at S and G2 phases were significantly decreased (10.30% and 6.31%, respectively). Taken together, these data indicate that EGFR down-regulation could inhibit RB cell proliferation by causing cell G1 phase arrest.

**EGFR down-regulation suppressed RB cell invasion**

Cancer cell invasion is an indicator of cancer progression, therefore we next investigated whether EGFR down-regulation could also suppress Weri-Rb-1 cell invasion. Our results demonstrated that a sharp
Figure 3. EGFR down-regulation suppresses RB cell invasion. (A and B) Weri-Rb-1 cells were first infected with shRNA lentiviruses and then cell invasion was determined by a Matrigel-based Transwell system. (A) Representative data is shown. (B) Data shown are mean±SD of three independent experiments. **, *P < 0.01. (C) Weri-Rb-1 cells were first infected with shRNA lentiviruses and then the expression of MMP2 and MMP9 was determined by Western blot. Representative result is shown.

Figure 4. EGFR down-regulation mediated RB inhibition is through the PI3K/AKT/mTOR signaling pathway. Weri-Rb-1 cells were first transfected with shRNA lentiviruses and then the expression of PI3K, p-AKT, AKT, p-mTOR, and mTOR was determined by Western blot. (A) Representative result is shown. (B) Grayscale analysis. Data shown are mean±SD of three independent experiments. *, *P<0.05; **, *P<0.01.
expression and consequently lowered the activation levels of AKT and mTOR.

Taken together, our results in the current study revealed that down-regulation of EGFR using shRNA lentivirus could inhibit RB progression by promoting cell death and suppressing cell proliferation and invasion. This inhibitory effect was through regulation of the PI3K/AKT/mTOR signaling pathway. The findings of our study indicated that down-regulation of EGFR using shRNA lentiviral vectors may offer a novel non-invasive treatment for RB.

Discussion

RB is one of the most common malignant eye diseases among children while current effective treatments for this malignancy are all invasive. Therefore, the development of non- or minimally-invasive treatment procedures for RB is essentially required. In the current study, we have revealed that down-regulation of EGFR could promote RB cell death and suppress cell proliferation and invasion. Further, we have also shown that this RB inhibition effect by EGFR down-regulation was through the PI3K/AKT/mTOR signalling pathway. Taken together, our results imply a novel RB treatment strategy by targeting EGFR expression. The translation of the study to the animal model and preclinical level is warranted.

Previous studies have demonstrated that EGFR plays important roles in the progression of multiple tumor types and the expression level of EGFR is correlated with poor prognosis (19, 20). Given the importance of EGFR in tumor progression, many studies have investigated the anti-tumor effect of EGFR inhibitors in various tumors including lung cancer, breast cancer, and colorectal cancer (21-24). All these studies have proved that inhibition of EGFR showed certain degrees of efficacy in cancer treatment. However, in the field of RB, an investigation of treatment targeting EGFR has not been previously described. Our current study, for the first time, has demonstrated that targeting EGFR was also an applicable approach in RB treatment. Moreover, we have also shown that targeting EGFR by down-regulation of its expression instead of functional inhibition could also inhibit RB progression.

Previous studies have shown that repression of cancer cell proliferation is caused by cell G1 phase arrest (14, 25, 26). Similarly, previous studies targeting EGFR using neutralizing monoclonal antibodies also caused cancer cell G1 phase arrest (27, 28). In agreement with previous findings, our current study showed that downregulating EGFR expression using EGFR-specific shRNA lentiviral system also caused RB cancer cell arrested at G1 phase, resulting in a significant decrease in S and G2 phase cells (Table 1).

In vitro knockdown of gene-of-interest using shRNA or shRNA lentivirus has already been widely adopted in various fields of research. Comparing to conventional shRNA or siRNA transfection, lentivirus-based shRNAs can achieve much better knock-down efficiency, which renders this technology increasingly popular (29, 30). However, the translation of this technique to clinical and preclinical level as a treatment strategy is relatively new. Since shRNA lentiviral vectors are artificially modified virus particles, debates regarding the safety of this technology in treatment have been discussed. After years of evaluation, these lentiviral vector-based technologies have been proved to be safe in use in clinical applications (31-33). Consequently, the EGFR shRNA lentiviral system adopted in our current study is safe to be translated to preclinical and clinical studies. Of note, even though the shRNA lentiviral system is believed to be a safe approach for gene expression manipulation, appropriate dosage should be tested since low dosage may not be able to achieve desired efficacy while overdosage may cause side effects. In fact, our current study showed that control shRNA lentiviral vector could cause cell death when used in a dose above 10 MOI, indicating that high infection dose may have a cytotoxic effect or non-specific down-regulation of genes (Figure 2A).

Taken together, our results in the current study revealed that down-regulation of EGFR using shRNA lentivirus could inhibit RB progression by promoting cell death and suppressing cell proliferation and invasion. This inhibitory effect was through regulation of the PI3K/AKT/mTOR signaling pathway. The findings of our study indicated that down-regulation of EGFR using shRNA lentiviral vectors may offer a novel non-invasive treatment for RB.

Conclusion

Our study revealed that EGFR down-regulation, through the PI3K/AKT/mTOR signaling pathway, could inhibit RB progression by promoting cell death while suppressing cell proliferation and invasion. The findings of our study indicated that down-regulation of EGFR using shRNA lentiviral vectors may offer a novel non-invasive treatment for RB.

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Conflict of Interest

The authors declare no conflict of interest.

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