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ORIGINAL ARTICLE

Antitumor Efficacy of SLPI Promoter-Controlled Expression of Artificial microRNA Targeting EGFR in a Squamous Cell Carcinoma Cell Line

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Abstract The purpose of this study was to develop a recombinant adenovirus with secretory leukoprotease inhibitor (SLPI) promoter-controlled expression for gene therapy of squamous cell carcinoma (SCC). An artificial microRNA targeting epidermal growth factor receptor (EGFR) was designed, and used to construct a replication-defective recombinant adenovirus with SLPI promoter-controlled expression. The silencing efficiency of this vector (Ad-SLPI-EGFRamiR) was detected in Hep-2 cells. Western blotting showed that the expression of 170 kD EGFR was significantly reduced in Hep-2 cells 72 h after infection with Ad-SLPI-EGFRamiR. At a multiplicity of infection (MOI) of 200 pfu/cell, proliferation of Hep-2 cells was highly inhibited by Ad-SLPI-EGFRamiR (inhibition rate: ~70%). The apoptosis rate of Hep-2 cells at 72 h after infection with Ad-SLPI-EGFRamiR at a MOI 35 pfu/cell was 32.8%. The adenovirus constructed was able to specifically inhibit the growth of SCC cells in vitro.

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Keywords EGFR · Gene therapy · microRNA · Recombinant adenovirus · SLPI

Introduction

Squamous cell carcinoma (SCC) can occur in any anatomical site covered by squamous epithelium including the skin, mouth, esophagus, urinary tract and parts of the female reproductive tract [1, 2]. Although great progress has been achieved in the treatment of head and neck squamous cell (HNSCC), current methods are still hindered by limited therapeutic effectiveness and adverse reactions [3–11]. Thus, novel therapies are needed to improve outcomes in these patients.

Epidermal growth factor receptors (EGFRs) have been implicated in the development and progression of a number of malignancies, including SCC, and EGFR-targeted therapies have been shown to be effective in a number of different cancers [12–16]. With development of molecular cloning techniques, viruses have been used as expression vectors in the RNA interference (RNAi) down-regulation of EGFRs, and have been used as a novel therapy for certain malignancies [17].

RNAi occurs widely, and refers to the double-stranded RNA induced specific degradation of homologous mRNA. Thus, RNAi may be employed to inhibit the expression of a specific gene. Based on RNAi, investigators have designed double-stranded small interference RNA (siRNA) or short hairpin RNA (shRNA) for cancer therapy [18]. Advances in technology have led to the development of artificial microRNA (second generation shRNA). As compared to siRNA and first generation shRNA, artificial microRNA not only preserves the specific sequence targeting a gene present in shRNA, but has a microRNA frame. Thus, it is not only efficient as an RNAi, but can be initiated by most promoters in mammals, which is its major advantage [19]. This advantage of artificial microRNA

makes the targeting regulation of EGFR expression via a specific promoter with RNAi possible.

Adenovirus vectors have high transduction efficiency and low pathogenicity, are easy to construct, and are convenient to prepare in solution with a high titer. Thus, they are widely used in gene therapy studies. However, adenovirus has a natural affinity for the liver [20], and thus it may not specifically kill targeted cells, which reduces the therapeutic efficacy and increases adverse effects. Thus, it is necessary to develop an adenovirus vector with improved targeting capability, which may increase therapeutic efficacy and safety.

Currently, the cell or tissue specific promoter controlled expression of a target gene is an effective method for targeted gene therapy [21]. Our previous study showed that the secretory leukoprotease inhibitor (SLPI) promoter at −650 to +22 has high transcription efficiency and is suitable to serve as a tissue specific promoter for the targeted gene therapy of laryngeal cancer [22]. To efficiently inhibit the survival and growth of laryngeal cancer cells, an artificial microRNA targeting EGFR was designed and used to construct adenovirus shuttle plasmids, which were then co-transfected with adenovirus backbone plasmids into human embryonic kidney cells (HEK293 cells). After site-specific homologous recombination, replication deficient adenovirus with expression of the target gene was successfully constructed, and recombinant adenovirus with a high titer was obtained after expansion and purification.

In our pilot study, the activity of common specific promoters (SLPI, squamous cell carcinoma antigen [SCCA], and survivin) used in squamous cell carcinoma was investigated in a laryngeal squamous cell carcinoma cell line (Hep-2 cells) in which a human umbilical vein endothelial cell line (HUVEC) served as a normal control. The results showed the SLPI promoter had a better specificity in Hep-2 cells as compared to SCCA and survivin promoters [22].

Thus, in the current study we designed an artificial microRNA targeting EGFR which was used to construct a replication-defective recombinant adenovirus with SLPI promoter-controlled expression. The virus was transduced into Hep-2 cells, and the silencing efficiency of this vector was detected in Hep-2 cells in vitro.

Materials and Methods

Generation of Ad-SLPI-EGFRamiR, Ad-SLPI-GFP

The experimental procedure followed the instructions supplied in the user manual of the packaging system. The AdMax™ System adenovirus packaging system (Microbix Biosystems, Canada) was used for virus packaging in which the adenovirus shuttle plasmids with expression of exogenous gene and adenovirus backbone plasmids expressing adenoviral genomic DNA were co-transfected into HEK293 cells. The replication deficient adenovirus with expression of the target gene was successfully constructed after site-specific homologous recombination. The shuttle strategy for plasmid construction was based on the method of Chen et al. [22], and is illustrated in Fig. 1.

Hep-2 cells and HUVEC cells in the logarithmic growth phase were seeded into 6-well plates at a density of 5×10^5 cells/well and maintained in RPMI1640 containing 10% calf serum for 24 h. Hep-2 cells and HUVEC cells was transduced with Ad-SLPI-GFP at a multiplicity of infection (MOI) of 50 plaque forming units (pfu)/cell. After 48- and 72-h of transduction, green fluorescent protein expression was observed under a fluorescence microscope.

Western Blot Assay

After virus transduction, Hep-2 cells were collected from 6-well plates, and were treated with Ad-SLPI-EGFRamiR or Ad-SLPI-GFP at a MOI of 50 pfu/cell for 72 h. An equal of protein cell lysate (40 μg) was used for analysis. Anti- EGFR (1:100, 000) (Cell Signaling, MA, USA) and anti- GAPDH (1:5000) (Santa Cruz, CA, USA) antibodies were used for protein detection.

MTT Assay

Recombinant adenovirus Ad-SLPI-EGFRamiR and control adenovirus Ad-SLPI-GFP were independently diluted to MOIs of 400, 200, 100, 50, and 0 pfu/cell. Then, both viruses were independently used to transduce Hep-2 cells and HUVEC cells for 72 h. The MTT assay was performed to detect cell proliferation by measuring optical density (OD) based on the manufacturer's instructions, and the cell proliferation inhibition rate was calculated.

Flow Cytometry

Hep-2 or HUVECs transduced Ad-SLPI- EGFRamiR or Ad-SLPI-GFP at a MOI of 35 pfu/cell were cultured for 72 h and harvested. Cells were stained using an ApoScreen Annexin V Apoptosis kit (SouthernBiotech, AL, USA) and analyzed by flow cytometry. Cells stained by 7-aminoactinomycin D (7-AAD) or annexin V were identified as dead cells.

Statistical Analysis

The OD value of Hep-2 cells and HUVEC cells transducted by Ad-SLPI-EGFRamiR or Ad-SLPI-GFP at MOIs of 0, 50, 100, 200, and 400 pfu/cell was determined and the difference in mean of of OD values were compared using one-way ANOVA with post-hoc comparisons buy Bonferroni test. All statistical assessments were two-tailed, and a value of $p < 0.05$

was considered to indicate statistical significance (Results were not shown in Tables or figures). The inhinitory rate was calculated as the ratio of the measured OD value relative to that at a MOI level of 0 pfu/cell. The mean inhibitory rate was presented as a bar graph. All statistical analyses were carried out with IBM SPSS statistical software version 22 for Windows (IBM Corp., New York, USA).

Results

Construction of Recombinant Adenoviruses for Ad-SLPI-EGFRamiR and Ad-SLPI-GFP

The recombinant adenoviruses were successfully constructed. After amplification, purification, and concentration, the titers for Ad-SLPI-EGFRamiR and Ad-SLPI-GFP reached 1×10^{10} pfu/ml and 6.3×10^{9} pfu/ml, respectively, which was sufficient for further studies.

Strong green fluorescence signals were seen in a large number of Hep-2 cells, but not in HUVECs 72 h after transduction with Ad-SLPI-GFP (Fig. 2), indicating the specificity of the SLPI promoter toward SCC cells. Western blotting showed that the expression of 170 kd EGFR was significantly reduced in Hep-2 cells 72 h after infection with Ad-SLPI-EGFRamiR (Fig. 3). These results indicated that the SLPI promoter could effectively downregulate the expression of EGFR in SCC cells.

Cytotoxicity of Ad-SLPI-EGFRamiR to Human SCC by MTT Assay

Recombinant adenovirus Ad-SLPI-EGFRamiR had a potent inhibitory effect on the proliferation of Hep-2 cells, and the inhibition rate increased with increasing MOI. However, the adenovirus had a poor inhibitory effect on the proliferation of HUVEC cells (Fig. 4). Ad-SLPI-GFP transduction failed to significantly inhibit the proliferation of both Hep-2 cells and HUVEC cells. The proliferation of Hep-2 cells was inhibited by Ad-SLPI-EGFRamiR after 72 h at a MOI of 50 pfu/cell (inhibition rate: ~20%), while no significant inhibition was observed in HUVEC cells (inhibition rate: −10%). These results suggest the safety of this potential anti-SCC therapy.

Flow Cytometry for Quantitative Analysis of Cell Death

Flow cytometry results are presented in Fig. 5. The apoptosis rate of Hep-2 cells at 72 h after infection with Ad-SLPI-EGFRamiR at a MOI 35 pfu/cell was 32.8%, and after infection with Ad-SLPI-GFP was 10.2%. For HUVEC, 72 h infection with Ad-SLPI-EGFRamiR and Ad-SLPI-GFP at a MOI of 35 pfu/cell the apoptosis rates were 11.2% and 9.2%, respectively.

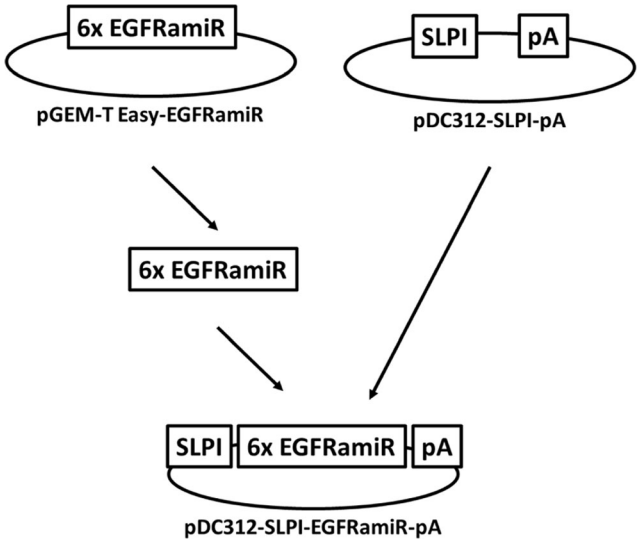

Fig. 1 Shuttle strategy of pDC312-SLPI-EGFRamiR-pA construction. The EGFRamiR sequence is unpublished data

Discussion

In this study, we successfully constructed a recombinant adenovirus (Ad-SLPI-EGFRamiR) with laryngeal cancer specific SLPI promoter controlled expression of artificial microRNA targeting EGFR, as well as control adenovirus (Ad-SLPI-GFP). The titer of virus constructed was high, and Western blotting confirmed that transduction with this virus was able to down-regulate EGFR expression in a SCC cell line. These findings provide support for the application of recombinant adenovirus in SCC therapy.

EGFR over-expression is common in SCC cells and has been confirmed to promote the malignant growth of cancer cells. For these reasons EGFR has been a focus in the biotherapy of SCC

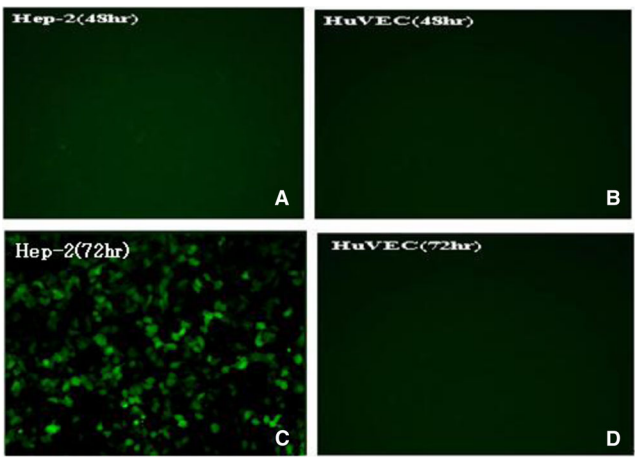

Fig. 2 Specificity of the SLPI promoter toward squamous cell carcinoma cell lines. GFP expression in Hep-2 cells and HUVEC cells after transfection with Ad-SLPI-GFP for 48 h and 72 h. **a** Hep-2 cells transfected with Ad-SLPI-GFP for 48 h. **b** HUVEC cells transfected with Ad-SLPI-GFP for 48 h. **c** Hep-2 cells transfected with Ad-SLPI-GFP for 72 h. **d** HUVEC cells transfected with Ad-SLPI-GFP for 72 h

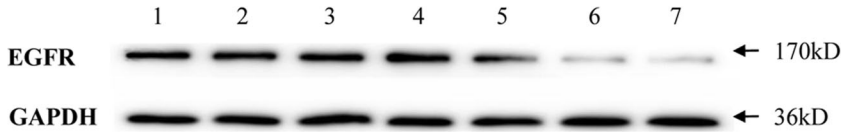

Fig. 3 Knockdown of EGFR expression in Ad-SLPI-EGFRamiR transduced HEP-2 cells. 1) Hep-2 cells maintained for 72 h in the absence of transfection. 2) Hep-2 cells transfected with Ad-SLPI-GFP (MOI = 50) for 72 h. 3) Hep-2 cells transfected with Ad-SLPI-GFP (MOI = 100) for 72 h. 4) Hep-2 cells transfected with Ad-SLPI-GFP (MOI = 200) for 72 h. 5) Hep-2 cells transfected with Ad-SLPI-EGFRamiR (MOI = 50) for 72 h. 6) Hep-2 cells transfected with Ad-SLPI-EGFRamiR (MOI = 100) for 72 h. 7) Hep-2 cells transfected with Ad-SLPI-EGFRamiR (MOI = 200) for 72 h

[23]. However, few gene therapies targeting EGFR have been conducted. In the current study, transduction with Ad-SLPI-EGFRamiR at a low MOI (50) for 72 h was able to significantly inhibit the growth of Hep-2 cells, but had a poor inhibitory effect on the growth of HUVEC cells. This indicates that Ad-SLPI-EGFRamiR has specificity for SCC cells. However, in control group Ad-SLPI-GFP at the same MOI (50) failed to significantly inhibit the growth of Hep-2 cells, indicating the adenovirus itself has a low toxicity to cells. In addition, the inhibition rate of Ad-SLPI-EGFRamiR increased with increasing MOI, a finding not observed in the control group further confirming the safety of Ad-SLPI-EGFRamiR. Of note, however, at an MOI of 400 pfu/cell transduction with Ad-SLPI-EGFRamiR also significantly inhibited the growth of HUVEC cells, and the inhibition rate of Ad-SLPI-GFP was also increased in Hep-2 cells. Thus, the safety of Ad-SLPI-EGFRamiR at a high MOI is challenged: on one hand, the SLPI promoter may initiate the gene expression in normal cells, and on the other hand the adenovirus vector itself has cytotoxicity.

Secretory leukocyte protease inhibitor (SLPI) is mainly expressed in human respiratory epithelium, and macrophages and neutrophils also have SLPI expression [24]. SLPI overexpression has been found in multiple cancers, and its promoter has tumor specificity. Thus, SLPI has been successfully used in the gene therapy of non-small cell lung cancer and ovarian cancer [25, 26]. Our previous study also indicated that the SLPI promoter had a high specificity in laryngeal cancer. However, the current study showed that normal cells expressed the target gene after transduction with adenovirus at a high MOI, leading to cell growth inhibition. These findings are consistent with those of a study investigating Ad-SLPI controlled expression of caspase-3 [22]. Thus, as with other tissue specific promoters, the specificity of SLPI promoter in laryngeal cancer is only relative and cannot be administered at a high virus MOI.

The selection and construction of expression vectors is a key element of gene therapy, and targeting capability and efficient transduction are crucial for effectiveness. Recombinant adenovirus vectors have been widely used and have a number of advantages. 1) Adenovirus vectors have a large capacity, and in the current study a first generation recombinant adenovirus was used and it can load an exogenous sequence of as long as 8 kb. Thus, it is suitable for general gene manipulation. 2) The vectors can infect a wide range of host cells, and may infect cells in the mitotic of a non-mitotic phase. 3) The infection is transient, which means that it may integrate into the genome of host cells and not cause tumorigenesis or focal malformation due to gene mutation. 4) It is easy to prepare a virus solution with high titer; each infected cell can package about 10^4 viral particles and the final titer can be as high as $10^{12\text{-}13}$ pfu/ml. 5) Adenovirus is a DNA virus, and its DNA stability is higher than that of RNA viruses.

However, the wide application of adenovirus vectors had disclosed a number of disadvantages. 1) The host cell range of adenoviruses is large, and infection can occur in the respiratory tract, gastrointestinal tract, and liver. 2) Recombinant adenovirus vectors may still express adenovirus-related proteins, and repeated use may cause an immune reaction reducing the

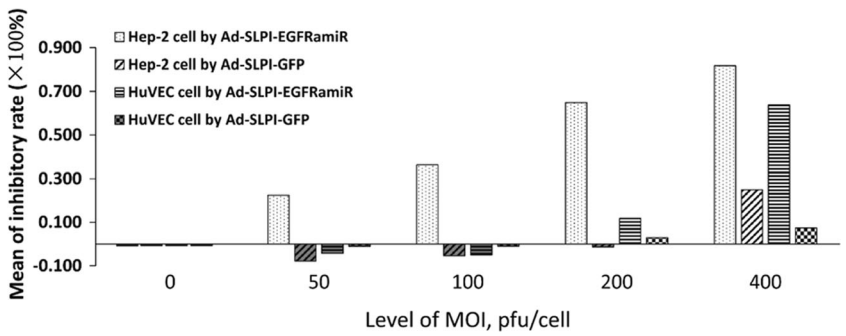

Fig. 4 Inhibition of cell growth at 72 h post infection at different MOI. Recombinant adenovirus Ad-SLPI-EGFRamiR and control adenovirus Ad-SLPI-GFP were independently diluted to MOI of 400, 200, 100, 50, and 0 pfu/cell. Both viruses were independently used to transfect Hep-2 cells and HUVEC cells for 72 h. The MTT assay was performed to detect the cell proliferation by measuring optical density, and the cell proliferation inhibition rate was calculated

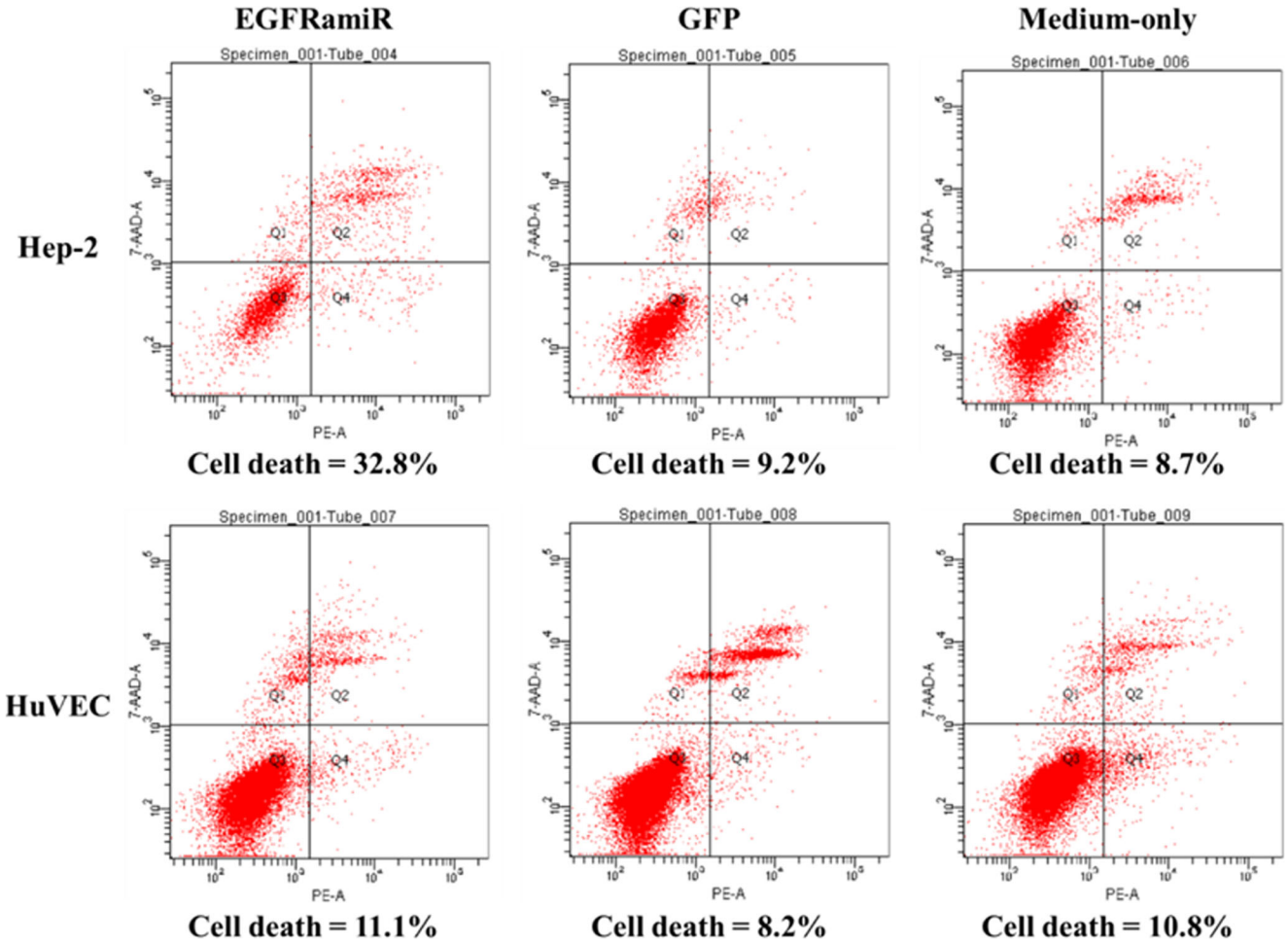

Fig. 5 Flow cytometry. Severe cell death was noted in Ad-SLPI-EGFRamiR transduced HEP-2 cells. Recombinant adenoviruses Ad-SLPI-EGFRamiR and Ad-SLPI-GFP were independently transfected into Hep-2 cells or HUVEC cells at a MOI of 35 pfu/cell. At 72 h after transfection, dead cells were detected with ApoScreen Annexin V Apoptosis kit

therapeutic efficacy. 3) Adenoviruses recognize target cells via the Cosakie and Adenovirus Receptor (CAR). If tumor cells have no CAR, efficient transduction will not occur, and this factor limits their wide application in clinical practice [27, 28]. Thus, the targeting capability and efficient transduction are crucial for the preparation of adenovirus vectors.

There are a number of ways to improve the targeting capability of adenovirus vectors. 1) An adapter protein may be used to bridge the interaction between adenovirus and host cells in a CAR independent manner [29–31]. This, however, increases the complexity of the system and can lead to unforeseen consequences. 2) Modification of virus genes or insertion of virus surface fibrin may be used to alter the high affinity to CAR [32, 33]. However, because the virus genes are modified the targeting capability is reduced. 3) A tumor specific promoter can be used to regulate expression of the target gene. Compared to the other methods, use of a tumor specific promoter is relatively convenient and easy, and progress has been achieved with this method. For example, an alfa fetoprotein (AFP) promoter has been used in liver cancer, a prostate specific antigen (PSA) promoter has been used in prostate cancer, and a survivin promoter has been used in cervical cancer [21]. In our previous study, detection of dual luciferase activity was employed to compare the activity of SLPI, SCCA, and survivin (3 promoters commonly used in SCC) in Hep-2 cells with HUVEC as the normal control. The results showed the SLPI promoter had a higher specificity in Hep-2 cells as compared to SCCA and surviving, indicating that the SLPI promoter may serve as a promoter specific to laryngeal SCC. Further investigation also revealed that replication-defective adenovirus with an SLPI promoter could efficiently transduce into Hep-2 cells, but not HUVEC cells, confirming the specificity of a recombinant adenovirus with the SLPI promoter in laryngeal SCC [22].

The classic SCC-EGFR was used as the target gene in the present study for a number of reasons. 1) A variety of studies have confirmed EGFR over-expression in SCC, which is closely related to the growth, metastasis, and prognosis of SCC [34]. 2) The structure, ligand, signaling pathway, and mechanism of action of EGFR as a classic cancer-related molecule have been extensively investigated [12]. 3) Drugs

targeting EGFR (such as monoclonal antibodies including cetuximab, and small molecule inhibitors including gefitinib) have been shown to be effective and have been approved for clinical use: In 2006 cetuximab was approved by the Food and Drug Administration (FDA) for treatment of HNSCC [11]. Small molecule inhibitors, however, have not been shown to be effective for HNSCC [5]. Although monoclonal antibodies are superior to small molecule inhibitors and may be used as adjunctive therapy following traditional radiotherapy and chemotherapy [6, 7], the response rate is generally poor, there is a high secondary resistance rate, and skin and gastrointestinal adverse effects are common. These disadvantages (especially the high resistance rate) significantly limit their wide application [8–10, 35]. Both small molecule inhibitors and monoclonal antibodies act at the protein level, and the complex quaternary structure of proteins and interactions between proteins may affect their long-term effectiveness.

In the present study, RNAi was employed to inhibit EGFR synthesis at mRNA level. To assure that this inhibition was under the control of the laryngeal cancer specific SLPI promoter, artificial microRNA (second generation shRNA) was prepared. The artificial microRNA is not controlled by the U6 promoter, and its expression can be initiated by most secondary promoters (including SLPI) [19]. The microRNA designed may form functional short single-stranded RNA after intracellular metabolism, which matches the junction between the untranslated region (UTR) of mRNA and the first codon. Theoretically, it may degrade the mRNA to down-regulate EGFR protein synthesis, which was confirmed by Western blotting in this study.

Although the adenovirus is cytotoxic, its cytotoxicity is lower than that of Ad-SLPI-EGFRamiR. The adenovirus used in this study was a first generation with E1 and E3 gene knock-out, but there may still be expression of some wild-type protiens [27]. Thus, adenovirus at a high MOI may cause cell apoptosis.

Conclusions

Taken together, the recombinant adenovirus Ad-SLPI-EGFRamiR was successfully constructed in the present study, and its anti-tumor effect was explored in Hep-2 cells. The results confirmed that this adenovirus is able to specifically inhibit the growth of SCC cells in vitro. However, whether this adenovirus has a similar anti-tumor effect and a high safety in vivo remains to be determined.

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Compliance with Ethical Standards

Disclosure of Potential Conflicts of Interest All of the authors declare no conflicts of interest.

Research Involving Human Participants and/or Animals This study does invlolve any human participants or animals.

Informed Consent This study does invlolve any human participants.

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