ORIGINAL ARTICLE

DENDRITIC CELLS PULSED WITH GST–EGFR FUSION PROTEIN: EFFECT IN ANTITUMOR IMMUNITY AGAINST HEAD AND NECK SQUAMOUS CELL CARCINOMA

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Abstract: Background. Overexpression of epidermal growth factor receptor (EGFR) is common in head and neck squamous cell carcinoma (HNSCC). Targeting EGFR is an effective approach to treat EGFR-positive HNSCC. However, the clinical benefits of the present EGFR-targeting agents are still limited in HNSCC patients.

Methods. Recombinant glutathione-S-transferase (GST)–EGFR fusion protein was produced and purified. Dendritic cells (DCs) of C3H mice were pulsed with fusion protein. Mice were challenged with HNSCC cells before or after vaccination with these DCs, and the cytotoxic T-lymphocyte (CTL) response, interferon-γ (IFN-γ) secretion, tumor growth, and survival of mice were assessed.

Results. Significant in vitro and in vivo antitumor activities were observed for mice immunized with DCs pulsed with GST–EGFR fusion protein, compared with the control groups (p < .05).

Conclusion. The DCs pulsed with GST–EGFR fusion protein can provide not only preventive but also therapeutic antitumor activities against HNSCC in the animal model. © 2009 Wiley Periodicals, Inc. Head Neck 32: 626–635, 2010

Keywords: dendritic cell; epidermal growth factor receptor; head and neck squamous cell carcinoma; fusion protein; immunotherapy

Head and neck squamous cell carcinoma (HNSCC) is the sixth most common cancer worldwide. Classical treatment for HNSCC is surgery, with or without chemoradiation. Despite advances in surgical technique and the institution of novel chemoradiation approaches, there has been little improvement in the 5-year survival rate over the past 30 years.1

Epidermal growth factor receptor (EGFR) is a 170-kDa transmembrane growth-regulating glycoprotein, which has an intrinsic tyrosine-specific kinase activity. Ligand binding to EGFR
and receptor dimerization cause autophosphorylation and/or cross-phosphorylation of several tyrosine residues, which in turn initiate intracellular signaling cascade, ultimately resulting in increased proliferation and differentiation.\textsuperscript{2} Overexpression of EGFR has been frequently observed in many malignancies, such as HNSCC, nonsmall cell lung cancer, breast cancer, colon cancer, and pancreatic cancer.\textsuperscript{3–7} The level of EGFR expression is believed to be associated with prognosis and nodal status of these patients.\textsuperscript{8} Therefore, EGFR is considered to be an attractive molecular target for cancer therapeutics. EGFR monoclonal antibodies (mABs) and small-molecule EGFR tyrosine kinase inhibitors (TKIs) are 2 major therapeutic agents that target EGFR. They have been approved by the U.S. Food and Drug Administration. However, their clinical effectiveness is still limited in HNSCC patients when these agents are delivered as monotherapy.\textsuperscript{3,9}

Dendritic cells (DCs), which are the most potent and competent antigen-presenting cells (APCs), have the unique capability of sensitizing naïve T cells to protein antigens. The cytotoxic T-lymphocyte (CTL) responses elicited by DCs can kill the tumor cells directly, whereas the monoclonal antibodies (mAbs) and TKIs inhibit tumor growth mainly by blocking the EGFR signal-transduction pathway. The ability of DCs to present tumor antigens and thereby generate tumor-specific immunity has been demonstrated in several clinical trials.\textsuperscript{10–12}

Because current treatment of HNSCC has limited effectiveness in improving the survival rate, immunotherapy may be a promising strategy. However, the clinical effectiveness of the current immunotherapy agents such as EGFR mAbs and small-molecule EGFR TKIs is still limited in HNSCC patients. Different from them, DCs can elicit cytotoxic T-lymphocytes (CTLs) and kill the tumor cells directly. DC-based immunotherapy has been demonstrated in clinical trials with respect to other tumors. Moreover, as demonstrated earlier, EGFR is an attractive target. Therefore we postulate that immunotherapy using DCs pulsed with EGFR may be an effective approach and provide a novel strategy for HNSCC treatment in addition to current therapeutics. In the present study, DCs pulsed with glutathione-S-transferase (GST) and EGFR fusion protein were used to treat EGFR-positive HNSCC in a mouse model, and the preventive and therapeutic antitumor effects were observed.

### MATERIALS AND METHODS

**Cell Line and Laboratory Animals.** SCC VII is a spontaneously arising HNSCC of C3H mice and overexpresses EGFR.\textsuperscript{13} The SCC VII cells were a kind gift of Prof. Shi-Xi Liu (Department of Otolaryngology, West China Hospital of Medical College of Sichuan University), and were maintained in RPMI (Roswell Park Memorial Institute) 1640 medium supplemented with 10% fetal bovine serum (FBS), penicillin 100 U/mL, and streptomycin 100 μg/mL. The tumor cells were cultured in a 37°C incubator with a humidified 5% CO\textsubscript{2} atmosphere.

Six-week-old female C3H mice were purchased from Vital River Laboratories (Beijing, China) and housed in the Central Animal Facility at Zhejiang University. The mice were acclimated for at least 1 week before any of the experiments were undertaken. All studies involving mice were approved by the Institute’s Animal Care and Use Committee.

**Construction of the Expression Vector Encoding the Extracellular Domain of EGFR.** SCC VII cells were harvested and total RNA was isolated using Trizol reagent (Life Technologies, Carlsbad, CA). The cDNA corresponding to the extracellular domain (ECD) of EGFR was amplified by reverse transcription polymerase chain reaction (RT-PCR) using the RNA from SCC VII as a template. The upstream primer 5'-AGA TCT GTC TGC CAA GGC ACA AGT AAC-3' includes a Bgl II excision site. The downstream primer 5'-GCG GCC GCT CAC CTG CCT GTA CAG GTG ATG TTC-3' contains a Not I excision site and a stop codon. Total RNA was reverse transcribed using the specific downstream primer. PCR cycle parameters were 94°C for 30 seconds, 54°C for 30 seconds, and 72°C for 60 seconds, for a total 30 cycles, followed by a 10-minute final extension at 72°C. The RT-PCR product, a 1.6-kb fragment, was cloned into the pGEM-T Easy vector (Promega, Madison, WI) for sequence analysis. The fragment encoding for the ECD of EGFR was recovered using Bgl II/Not I enzymes and cloned into BamH I/Not I sites of the pGEX-4T-2 expression vector (GE Healthcare, Waukesha, WI), generating the pGEX-4T-2-EGFR plasmid.

All restriction enzymes were from New England Biolabs (Beverly, MA).
Expression and Purification of GST-EGFR Fusion Protein and GST Protein. The pGEX-4T-2-EGFR plasmid and pGEX-4T-2 plasmid were transformed into Escherichia coli XL 1-Blue (Stratagene, La Jolla, CA), respectively, and expression of GST-EGFR fusion protein or GST protein was induced with 0.1 mM isopropyl-β-D-thiogalactoside (IPTG) at 23°C overnight. The GST fusion protein used in our study was to facilitate the expression in E. coli and purification of the recombinant protein. Cells were harvested and kept at −80°C for later purification procedures. Expression of the proteins was identified by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and protein immunoblot (Western blot) with an anti-GST antibody (Becton Dickinson, San Jose, CA).

The MagneGST Protein Purification System (Promega) was used to purify the soluble GST-EGFR fusion protein and GST protein from the cell lysate. Briefly, cell pellets were suspended by adding MagneGST Binding/Wash Buffer (Promega). The suspensions were complemented with 90 μg/mL phenylmethylsulfonyl fluoride (PMSF) and sonicated on ice by 50 cycles (each cycle consisted of 10 seconds on followed by 50 seconds off). The cell lysate was added to the preequilibrated MagneGST particles (Promega) and incubated for 30 minutes at 4°C, followed by washing with MagneGST Binding/Wash Buffer. The proteins were eluted with buffer (50 mM glutathione, pH 7.0–8.0; 50 mM Tris-HCl, pH 8.1), and the purified proteins were examined by SDS-PAGE.

Generating and Pulsing Dendritic Cells. Murine DCs were generated following the protocol described by Inaba et al. Briefly, bone marrow cells were prepared from the femora and tibiae of normal C3H mice, were depleted of erythrocytes with ammonium chloride (17 mM Tris, 144 mM NH₄Cl, pH 7.2), and were then cultivated in RPMI 1640 medium supplemented with 10% FBS, 100 U/mL penicillin, 100 μg/mL streptomycin, 10 ng/mL granulocyte-macrophage colony-stimulating factor (GM-CSF) (R&D Systems, Minneapolis, MN), and 10 ng/mL interleukin 4 (IL-4; R&D Systems) at 37°C and 5% CO₂. On day 3, the nonadherent granulocytes and T and B cells were gently removed, after which fresh medium was added. The remaining adherent cells were guided to differentiate into DCs. On day 5, 50% of the medium was replaced with fresh culture medium containing 10 ng/mL GM-CSF and 10 ng/mL IL-4, and to pulse with antigen, DCs were incubated with GST-EGFR fusion protein (50 μg/mL) or GST protein (15 μg/mL) for 48 hours. As a control, unpulsed DCs were also cultured for 48 hours.

Identification and Characterization of Dendritic Cells. To characterize the populations of CD11c-positive DCs, cells were collected on day 5 and incubated with fluorescein isothiocyanate labeled (FITC-labeled) CD11c mAB (eBioscience, San Diego, CA) for 30 minutes at 4°C in phosphate-buffered saline (PBS; 0.137 M NaCl, 0.003 M KCl, 0.008 M Na₂HPO₄, 0.001 M NaH₂PO₄, pH 7.3). After being washed 3 times, the cells were resuspended in PBS. Analysis was performed on a fluorescent-activated cell sorting flow cytometer (FACScan, Becton Dickinson).

To evaluate maturation of DCs, cells were collected 48 hours after being pulsed with antigen proteins, and stained with FITC-labeled CD80 (eBioscience), FITC-labeled CD40 (eBioscience), phycoerythrin (PE)-labeled CD86 (eBioscience), or PE-labeled I-Ak (Invitrogen, Carlsbad, CA).

Animal Tumor Models. After being pulsed with the GST-EGFR, 1 × 10⁶ DCs were subcutaneously injected into the left flank of C3H mice 3 times at an interval of 1 week. Two control groups were set as follows: a group of mice vaccinated with GST-pulsed DCs and another group of mice vaccinated with DCs alone. Each group consisted of 10 mice. To determine preventive antitumor activity, at 1 week after the third vaccination, 1 × 10⁶ SCC VII tumor cells were subcutaneously injected into the right flank of the animal. To observe therapeutic antitumor activity, mice were challenged with 1 × 10⁶ SCC VII tumor cells 2 days before the first vaccination. Tumor dimensions were measured with calipers, and tumor volumes were calculated according to the formula [length × width² × 0.5].

Enzyme-Linked Immunosorbent Assay for Interferon-γ. One week after the last immunization, splenocytes were extracted from each group and were passed over nylon wool fiber columns to remove B cells and macrophages. Subsequently, T cells were separated and enriched. These T cells were plated into flat-bottom plates and restimulated with GST-EGFR (50 μg/mL) or GST (15 μg/mL) for 5 days at 37°C. The amount of interferon-γ (IFN-γ) from
the culture supernatants was measured by the enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems) according to the manufacturer’s instructions. Briefly, the culture supernatants were plated into anti-IFN-mAb–coated flat-bottom ELISA plates and incubated for 90 minutes at 37°C. The plates were then washed and incubated with biotin-labeled anti-IFN-mAb at 37°C for 60 minutes. After being washed, they were added with horseradish peroxidase–conjugated streptavidin and incubated for 30 minutes at 37°C. Then the plates were incubated with tetramethylbenzidine substrate solution, and the optical density was determined at 450-nm wavelength with an ELISA reader.

**Cytotoxic T-Lymphocyte Assay.** T cells were separated and enriched from each group of mice as described earlier. These T cells were plated into flat-bottom plates and restimulated with GST–EGFR (50 µg/mL) or GST (15 µg/mL) for 5 days at 37°C. One hundred microliters of effector cells (T cells) and target cells (SCC VII) were assigned at different effector-to-target ratios to each well of plates and incubated for 4 hours at 37°C. Samples of culture wells were then harvested, and the cytotoxicity of the T cells against SCC VII was determined by using CytoTox96 Nonradioactive Cytotoxicity Assay (Promega). The CytoTox96 Assay quantitatively measures the lactate dehydrogenase (LDH), a stable cytosolic enzyme that is released upon cell lysis. Released LDH in culture supernatants was measured with a 30-minute coupled enzymatic assay. Wavelength (490 nm) absorbance data were collected using a standard 96-well plate reader. The percentage of specific lysis was calculated as \[
\left(\frac{\text{experimental} - \text{effector spontaneous}}{\text{target maximum} - \text{target spontaneous}}\right) \times 100.
\]

**Statistical Analysis.** Data were presented as means ± SD. The significance of differences between the values of different groups was evaluated by Wilcoxon’s rank-sum test, and \( p \leq .05 \) was considered statistically significant.

**RESULTS**

**Construction of the Expression Vector Encoding the Extracellular Domain of EGFR.** EGFR–ECD cDNA was obtained from SCC VII tumor cells by RT-PCR. The product was separated by 1% agarose gel electrophoresis and stained with ethidium bromide. The expected length was 1.6 kbp (Figure 1A). The sequence of the ECD of EGFR was confirmed, by dideoxy nucleotide sequencing analysis, to be identical to those previously reported. Then the EGFR–ECD cDNA was successfully cloned into the pGEX-4T-2 expression vector as previously described. The pGEX-4T-2-EGFR plasmid was verified by Pst I digestion. The product was separated by 1% agarose, and 2 expected fragments appeared (4 and 2.4 kbp) (Figure 1B).

**GST–EGFR and GST Expression and Purification.** Expression of the soluble GST-EGFR or GST was analyzed by SDS-PAGE and Western blot (Figures 2A and 2B). As expected, an 87- or 26-kDa protein band was displayed in induced cells but not in uninduced ones. The proteins were purified by the MagneGST Protein Purification System and confirmed by SDS-PAGE, showing again the 87- or 26-kDa protein band (Figure 2C). The purity of the achieved proteins was >90%.

**Characterization of Dendritic Cells.** The degree of expression of CD11c on the DCs’ surface was evaluated by flow cytometry after 5 days of culture. The result showed that the CD11c-positive cells were 59.86%, which indicated the purity of DCs, and was approximately the same as the result when using the conventional Inaba method. Expressions of CD40, CD80, CD86, and I-Ak were analyzed by flow cytometry after a 48-hour stimulation with proteins. The positive cells among the DCs pulsed with GST–EGFR were 58.82%, 59.88%, 89.42%, and
FIGURE 2. GST–EGFR and GST expression and purification. (A) Expression of GST–EGFR and GST was analyzed by 10% and 12% SDS-PAGE gels with Coomassie blue staining, respectively. Uninduced cells were used as control. (B) Expressed proteins were further confirmed by western blot using an anti-GST antibody. Uninduced cells were used as control. (C) The purification of GST–EGFR and GST was afforded by MagneGST Protein Purification System, and the purified proteins were confirmed by 10% and 15% SDS-PAGE gels with coomassie blue staining respectively. GST, glutathione-S-transferase; EGFR, epidermal growth factor receptor; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.
90.28%, respectively, and among the DCs pulsed with GST were 54.32%, 55.02%, 88.36%, and 89.04%, respectively. These results were greater than those of DCs unpulsed with protein, in which the positive cells were 17.87%, 42.84%, 81.07%, and 80.36%, respectively (Figures 3A and 3B).

**Antitumor Activity.** To evaluate the preventive antitumor response induced by GST–EGFR-pulsed DCs, C3H mice were injected subcutaneously with SCC VII cells (1 × 10⁶) at day 7 after the third immunization, and tumor growth and survival were observed. We found that SCC VII tumor grew progressively in the 2 control groups of mice that were immunized with GST–DC or DCs alone, whereas in the group treated with GST–EGFR–DC, the tumor growth was significantly inhibited (p < .05) (Figure 4A). On day 26 after tumor inoculation, the mean tumor volumes in 3 groups were 8453.35 ± 2049.26, 9309.32 ± 3493.99, and 3911.5 ± 1656.45 mm³, respectively. The survival of the mice immunized with GST–EGFR–DC was also significantly longer than that of the control mice treated with GST–DC or DCs alone (p < .05) (Figure 4B). The

![Figure 3](image_url)

**FIGURE 3.** DCs mature when cocultured with proteins. Expressions of CD40, CD80, CD86, and I-Ak on either the GST–EGFR-pulsed DCs’ surface (A) (filled peaks) or the GST-pulsed DCs’ surface (B) (filled peaks) were higher than that on unpulsed DCs’ surface (open peaks). DCs, dendritic cells; GST, glutathione-S-transferase; EGFR, epidermal growth factor receptor.

![Figure 4](image_url)

**FIGURE 4.** Preventive antitumor activity in SCC VII HNSCC animal model. Mice were challenged with 1 × 10⁶ SCC VII cells subcutaneously 7 days after the third immunization. (A) Tumor volume (mm³) in mice treated with GST–EGFR–DC and controls. (B) The mean survival time of treated mice. SCC VII, a spontaneously arising HNSCC of C3H mice; HNSCC, head and neck squamous cell carcinoma; GST, glutathione-S-transferase; EGFR, epidermal growth factor receptor; DC, dendritic cell. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]
mean survival term of the mice in 3 groups was 52.75 ± 3.3, 41.6 ± 5.37, and 43.75 ± 5.74 days, respectively.

To evaluate the therapeutic antitumor activity induced by GST–EGFR-pulsed DCs, mice were challenged with SCC VII cells 2 days before DC immunization. Significant antitumor effects similar to those in the preventive model were found in the group treated with GST–EGFR–DC, compared with 2 control groups treated with GST–DC or DCs alone (p < .05) (Figure 5A). On day 28 after tumor inoculation, the mean tumor volumes in 3 groups were 8257.1 ± 4254.2, 17345.18 ± 2916.22, and 15692.16 ± 4410.53 mm³, respectively. Meanwhile, prolonged survival in the group treated with GST–EGFR–DC was also found, compared with the group treated with GST–DC (p = .053) or DCs alone (p = .058) (Figure 5B). The mean survival terms in 3 groups were 44 ± 6.38, 37.17 ± 3.13, and 36.43 ± 4.24 days, respectively.

**Interferon-γ Secretion.** By 7 days after the third immunization, splenic T cells were isolated from each group of mice for the evaluation of IFN-γ production. The secretion of IFN-γ was higher in the group treated with GST–EGFR–DC (250.36 ± 4.83 pg/mL) than in the control group with DCs alone (66.17 ± 18.49 pg/mL) (Figure 6A). The difference between them was statistically significant (p < .05). In another experiment, the amount of IFN-γ production of T cells in mice treated with GST–DC (84.47 ± 12.96 pg/mL) was similar to that in the control group.
Cytotoxic T-Lymphocyte Assay. CTL assay was performed at different effector-to-target ratios of 200:1, 150:1, 100:1, and 50:1. The splenic T cells isolated from GST–EGFR–DC-immunized mice exhibited significantly higher cytotoxicity than those from DC-alone immunized mice \((p < .05)\). By released LDH measurement, the percentages of specific lysis of the former were 81.71 ± 7.21%, 64.05 ± 5.9%, 72.21 ± 5.73%, and 70.51 ± 15.73% for each effector-to-target ratio, respectively (Figure 7A). Compared with this, the percentages of specific lysis of the latter were 7.69 ± 2.32%, 10.47 ± 2.75%, 8.93 ± 0.67%, and 9.13 ± 1.09% for each effector-to-target ratio, respectively (Figure 7A). However, in another experiment, the CTL response obtained by immunization with GST–DC was similar to that of DCs alone. The percentages of specific lysis of the former were 9.47 ± 4.42%, 10.22 ± 4.28%, 6.76 ± 3.53%, and 13.55 ± 8.54%, respectively; the latter were 8.99 ± 5.07%, 10.74 ± 6.19%, 8.24 ± 3.55%, and 9.93 ± 1.4%, respectively (Figure 7B).

Potential Toxicity. The mice immunized with GST–EGFR–DC were particularly investigated for potential toxicity. No obvious adverse effects were observed such as weight loss; life span; and pathologic changes of liver, lung, and kidney.

**DISCUSSION**

Among DC-based antitumor immunotherapeutics, tumor-specific antigens offer theoretical advantages. The immune response induced by such antigens would target tumor cells specifically, and limit the risk to normal tissues. Nevertheless, the immunogenicity of these antigens may be limited by the number of epitopes, and these antigens may vary between individuals within the same tumor type. Tumor-associated antigen is another immunotherapy target. Although most T cells capable of recognizing self-proteins are presumably deleted in the thymus, many tumor-associated antigens have been shown to stimulate T-cell responses in vitro when presented by DCs. EGFR is a tumor-associated antigen of HNSCC. The percentage of tumors overexpressing EGFR in head and neck is >80%. Despite this, the critical pathways that contribute to HNSCC formation are still unknown, although EGFR has been implicated in HNSCC development and progression. According to Fong et al, a favorite target antigen should be universal to some tumor type and be required for tumor survival and growth. Therefore EGFR is an ideal target of DC-based antitumor immunotherapy of HNSCC. The study by Hu et al showed that the ECD of EGFR-pulsed DCs can induce antitumor immunity against murine Lewis lung carcinoma and mammary carcinoma. However, to our knowledge, no other studies concerning DC-based approaches targeting EGFR have been reported. For SCC, the effectiveness of this approach still remains unknown.
In the present study, the stronger CTL response against SCC VII and the greater IFN-γ secretion were found in the group immunized with GST–EGFR–DC, compared with control groups. CTLs are believed to be critical effectors of antitumor immune responses, whereas the CD4+ T cell, characterized by the secretion of IFN-γ, is primarily responsible for activating and regulating the development and persistence of CTL. Previous studies in mice have shown that the in vivo induction of CTL responses, especially those induced through cross-priming of exogenous antigens by DCs, is dependent on a CD4+ T-cell response. Moreover, CD4+ T cells are also essential for the activation of memory CTLs into tumor killer cells. Therefore, our in vitro observations indicated that GST–EGFR-pulsed DCs could induce effective therapeutic and preventive antitumor immunity against SCC VII.

In our in vivo animal models, both therapeutic and preventive antitumor activities were observed. The results showed that either in the preventive model or in the therapeutic model the tumor growth was significantly inhibited, and the mean survival term of the mice was longer in the group treated with GST–EGFR–DC than in the control group treated with GST–DC or DCs alone. In a previous study by Son et al, DCs pulsed with apoptotic tumor cells were used to induce the antitumor immunity against SCC. Significant preventive antitumor activities were found, but no therapeutic activities were observed when using the DC vaccine alone. However, in the other study using EGFR-pulsed DCs to induce antitumor immunity, only the preventive antitumor activities were evaluated. Moreover, a recent study of DC-based antitumor immunotherapy against SCC VII, which used DCs pulsed with apoptotic SCC VII cells, also assessed just the preventive effect. Similar to the results of all these studies, effective preventive antitumor activities were also found in our study. In addition to this, effective therapeutic antitumor activities of the GST–EGFR-pulsed DCs vaccine against SCC were confirmed in the present study. In some studies of DC-based antitumor immunotherapy, complete tumor rejection was observed in animal models. In the present study, however, such a result was not observed.

In many DC-based immunotherapeutics, tumor-derived protein extracts or RNA was used to pulse DCs. When these approaches are used, the vaccine contains multiple antigens, thus increasing the probability of inducing immunity to >1 tumor-associated antigen. Nevertheless, on the other hand, there is increased potential for the induction of a destructive autoimmune response to antigens expressed on normal tissues. In the study by Tamaki et al, autoimmune hepatic inflammation was found in the mice vaccinated with DCs loaded with tumor cells. EGFR, a tumor-associated antigen, also expresses on normal tissues. Some studies have found that abrogation of EGFR in vitro or in vivo inhibits HNSCC proliferation without affecting the growth of normal mucosal epithelial cells. This may suggest that EGFR is critical for cancer cell growth, but not for the growth of normal cells, and in normal cells EGFR expression can be decreased through a negative regulation. Observations of side effects in our study are consistent with the other study of EGFR-pulsed DC vaccine. In both studies, no obviously adverse consequences were observed. These findings indicate that the GST–EGFR-pulsed DCs vaccine may be a safe vaccine.

In summary, our study indicates that GST–EGFR-pulsed DCs could effectively elicit therapeutic and preventive antitumor activities against EGFR-positive HNSCC in animal models, and this provides a promising approach for increasing antitumor activity of EGFR-targeted therapy of HNSCC.

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