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Original Article

Combined effects of EGFR and hedgehog signaling blockade on inhibition of head and neck squamous cell carcinoma

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Abstract: Head and neck cancer, the sixth most common cancer, has poor prognosis and short survival. Anti-epidermal growth factor receptor (EGFR) therapies have been recently developed for the treatment of multiple cancer types. JK184, an inhibitor of Hedgehog pathway, prevents the growth of many tumor cell lines in several studies. Whether it enhances chemosensitivity to block EGFR expression by shEGFR plasmid and blocks the Hedgehog pathway by JK184 remains unclear in sinonasal tumors. The changes in cell apoptosis and proteins have been detected by flow cytometry and Western blotting, respectively. In vivo, the maxillary sinus model was established to detect the inhibition of tumor growth and tumor weight. A synergistic effect has been observed with JK184 combined with shEGFR, which is positively correlated with increased autophagy. The maxillary sinus model results demonstrated that the inhibitory rate of the combined therapy was higher than that of JK184 or shEGFR alone. Our findings suggest that JK184 in combination with shEGFR might have potential as a new therapeutic regimen against sinonasal tumors.

Keywords: Malignant sinonasal tumors, EGFR, Hedgehog, apoptosis, autophagy

Introduction

Head and neck squamous cell carcinomas (HNSCC), rising to more than 90% tumors in the head and neck, show poor cure rates with an overall 30-50% 5-year survival rate [1, 2]. The clinical treatment of sinonasal cancer mainly includes surgical approaches and radiotherapy. However, there are many problems in mutilation and aesthetic deformities, owing to the complex anatomical area of the sinonasal region and proximity to other structures such as eyes and brain, which is of special relevance to surgery and postoperative treatment.

Several studies have demonstrated that EGFR overexpression in about 40% of HNSCCs [3, 4]. Targeting EGFR has been as an important measure against HNSCC with rare EGFR activating mutations and widespread frequency of EGFR gene amplification [5-7]. However, in most preclinical and clinical studies, a lower local control after radiation therapy increases resistance after EGFR-targeted therapy in tumors overexpressing EGFR [8]. Furthermore, although high expression of EGFR has been shown to associate with a poor prognosis and resistance in HNSCC [9], in general, elevated EGFR expression has not been clearly identified to predict improved outcomes following EGFR-directed therapy [10]. Our growing knowledge of resistance pathways provides an opportunity to develop new mechanism-based inhibitors and combination therapies to prevent or overcome therapeutic resistance in tumors.

Recent findings have reported that the molecular mechanisms of cooperative Hedgehog (Hh)/GLI and EGFR signaling are two clinically relevant oncogenic pathways involved in the devel-

opment of many human malignancies [11]. In mammals, there are three specific extracellular Hh ligands (proteins) including DHH, (Desert Hedgehog), IHH, (Indian Hedgehog), and SHH (Sonic Hedgehog). Other components of the Hh signaling pathway include Patched protein 1 and 2, Smo FU, SUFU, KIF7, Gli1, Gli2, and Gli3 [12]. In The Hh signaling pathway genes play a crucial role in cell proliferation, differentiation, and tissue polarity during embryonic development [13, 14]. Furthermore, the Hh signaling pathway is a critical regulator in tumorigenesis and progression in several cancers. An increase in the Hh signal pathway has been linked to basal cell carcinomas, medulloblastomas, small-cell lung cancer, pancreatic adenocarcinomas, and prostate tumors [15]. A previous study reports that Gli1 is upregulated at the tumor-stroma intersection in HNSCC and is elevated by radiotherapy, leading to stromal-mediated resistance, and that Hh inhibitors offer rationale and strategies improve the resistance of HNSCC to radiotherapy [16]. Various components of the Hh signaling pathway including SHH, PTCH, SMO, GLI-1, GLI-2, and GLI-3 have showed dramatic overexpression in skin and HNSCC samples in immunohistochemistry analysis, and Shh overexpression has been significantly related to poor overall survival [12]. Overexpression of GLI1 [17] and GLI2 [18] have been shown in invasive and metastatic human melanoma cell lines. In addition, melanomas with GLI2 overexpression are more likely to develop bone metastasis [18].

The first evidence for the interaction between the vertebrate Hh/GLI and EGFR signaling pathways comes from *in vitro* studies of neocortical stem cells showing that Shh and EGFR cooperate in the stimulation of cell proliferation [19, 20]. In addition, overexpression of Shh in a human keratinocyte cell line grown in organotypic cultures leads to EGFR activation and increases levels of JUN and MMP9, thereby enhancing the invasive phenotype of the keratinocytes [21]. Because both EGFR and Hh/GLI pathways have been implicated in the pathogenesis of a considerable number of human cancers such as brain, skin, pancreatic, breast, colon, and liver cancers, it is tempting to speculate that cooperative interactions also occur in these malignancies and that combinatorial targeting may therefore provide a therapeutic benefit to a considerable number of sinonasal SCC patients.

JK184, animidazopyridine derivative, has been reported to specially inhibit Gli in the Hedgehog (Hh) pathway, which shows great promise for cancer therapeutics [22]. Here, we observe that JK184 and a shRNA plasmid targeting EGFR inhibit the growth of the FaDu cell line, and that JK184 and shEGFR plasmid have synergistic effects against tumor growth. Furthermore, our data suggest that the potential underlying mechanism of combined targeting of both pathways of Hh and EGFR enhances autophagy. Our findings challenge the traditional approaches of drug development for HNSCC.

Materials and methods

Reagents and antibodies

JK184 was synthesized according to previously published methods (HPLC normalization method) [23], with a purity of 99.2%. JK184 was dissolved in dimethyl sulfoxide (Sigma-Aldrich, St. Louis, MO, USA), stored at -80°C and diluted in fresh medium immediately before use. Polyclonal rabbit anti-human antibodies against LC3, Beclin 1, Bcl-2, Gli, mTOR, p-mTOR (Ser2448), Akt, and p-Akt (Ser473) were purchased from Cell Signaling Technology, Inc. (Beverly, MA, USA), and all secondary antibodies from Zhongshan Golden bridge Biotechnology Co., Ltd., Beijing, China.

Construction of the short hairpin RNA (shRNA) expression vector

Plasmids expressing shRNAs were designed using the pGensil-2.1/U6 parental vector from Genesil Biotechnology Company (Wuhan, China). Small interfering oligonucleotides specific to EGFR were synthesized and annealed, and the sequence (corresponded to 2849-2866 nucleotides) was as follows: EGFR2849 [24], 5'-GCTGGATGATAGACGCAG-3'. The sequence was inserted into the parental plasmid pGensil-2.1/U6 to generate the plasmid named shEGFR. The empty expression plasmid shKB was designated as a control. Endotoxin-free plasmids were prepared using the Qiagen Endo-free Giga kit (Qiagen, Hilden, Germany).

Cell lines, cell culture, and transfection

Hep2 (ATCC CCL-23, laryngeal SCC) and FaDu (ATCC HTB-43, hypopharyngeal SCC) cell lines were obtained from the American Type Culture

Collection (ATCC, Rockville, MD, USA). The cells were cultured in RPMI-1640 (HyClone, Logan, UT, USA) supplemented with 10% fetal bovine serum (Gibco, Grand Island, NY, USA) and 100 U/mL penicillin/streptomycin at 37°C in a humidified atmosphere containing 5% CO_2. After cell growth reached 70-80% confluence in the bottom of the culture bottle, logarithmic phase cells were used for experiments. For *in vitro* gene transfection, 4×10^4 cells per well were plated into 96-well plates and cultured overnight. Plasmid shEGFR or control plasmid shKB was transfected into cells by FuGENE HD Transfection Reagent (Roche, Mannheim, Germany) according to the manufacturer's instructions.

Growth inhibition assays

For growth inhibition assays, 1×10^4 cells were plated into 96-well plates and after 24 h, cells were treated with different concentrations of JK184, shEGFR plasmid or both. Cells were then incubated with 20 μL MTT (5 mg/mL in PBS) for 4 h. Medium were removed and 150 μL DMSO was added to each well. Absorbance was determined at 540 nm using a Synergy HT Multi-Mode Microplate Reader (BioTek, Winooski, VT, USA). Whether the two drugs had synergistic or antagonistic effects was determined according to the method of Chou-Talalay equation [25].

Apoptosis detected by flow cytometry

Hep2 and FaDu cells (5×10^5/mL) were cultured in six-well plates for 24 h and then treated with JK184 (10 μM), shEGFR (2 μg/mL), or JK184 (10 μM) and shEGFR (2 μg/mL) for 48 h. Cells with no drugs added were used as the control. The Muse Annexin V/Dead Cell Assay Kit (Millipore, Darmstadt, Germany) was used for quantitative analysis of live, early/late apoptotic, and dead cells with a Muse Cell Analyzer (Merck Millipore, Billerica, MA, USA).

Colony formation assay

Hep2 and FaDu cells (500 cells/well) were seeded into 6-well plates. At 24 h later, cells were transfected with shEGFR plasmid, cultured with medium containing JK184, or treated with both shEGFR transfection and JK184 at the indicated concentrations. After 72 h, the drugs were removed, and cells were washed twice with PBS and allowed to grow in normal medium for 7 days. Finally, colonies were stained with 0.1% crystal violet in PBS for at least 30 min at room temperature, and the numbers of positive cells were counted. Colonies formed were expressed as the percentage of colony-forming units in treated cultures relative to the untreated controls. Each assay was performed in three replicates.

Western blot analysis

Hep2 and FaDu cells (5×10^5/mL) were cultured with JK184 (0.01 μmol/L), shEGFR (2 μg/mL), and JK184 (0.01 μmol/L) plus shEGFR (2 μg/mL). After 48 h, cells were lysed inRIPA buffer containing protease inhibitor cocktail. Equal amounts of cellular protein from each sample were applied to 8-12% SDS-PAGE gels, transferred to membranes, and membranes were probed with specific antibodies. The protein bands were normalized to the expression level of beta actin.

Maxillary sinus model

A mouse orthotopic sinonasal cancer model was established by implanting cells from a human sinonasal undifferentiated carcinoma into the maxillary sinus of mice as described [26]. Tumor cells were implanted via transcutaneous injection underneath the infraorbital muscle groups, with the needle angled towards the lateral wall of the maxillary sinus. We used a 1-mL tuberculin syringe (Hamilton) with a 30-gauge hypodermic needle for the injections. The absence of a fluid leak from the nasal cavity confirmed a successful injection. The mice were observed until the effects of anesthesia had resolved.

Tumor xenografts and treatment

Male Balb/c nude mice (aged 6-8 weeks) were provided by Beijing HFK Bioscience. Co., Ltd. All animal experiments were approved by the Sichuan Animal Care and Use Committee and strictly conducted in accordance with relevant guidelines. We injected experimental animals with Hep2 and FaDu cells (5×10^6) in the maxillary sinus to determine the optimal site of orthotopic tumor implantation. Tumor growth was monitored twice a week; tumor sizes were calculated using the formula 0.5 [length (mm)] × [width (mm)]^2. When tumors reached a volume of approximately 50 mm^3, animals were randomized to different treatment groups (six

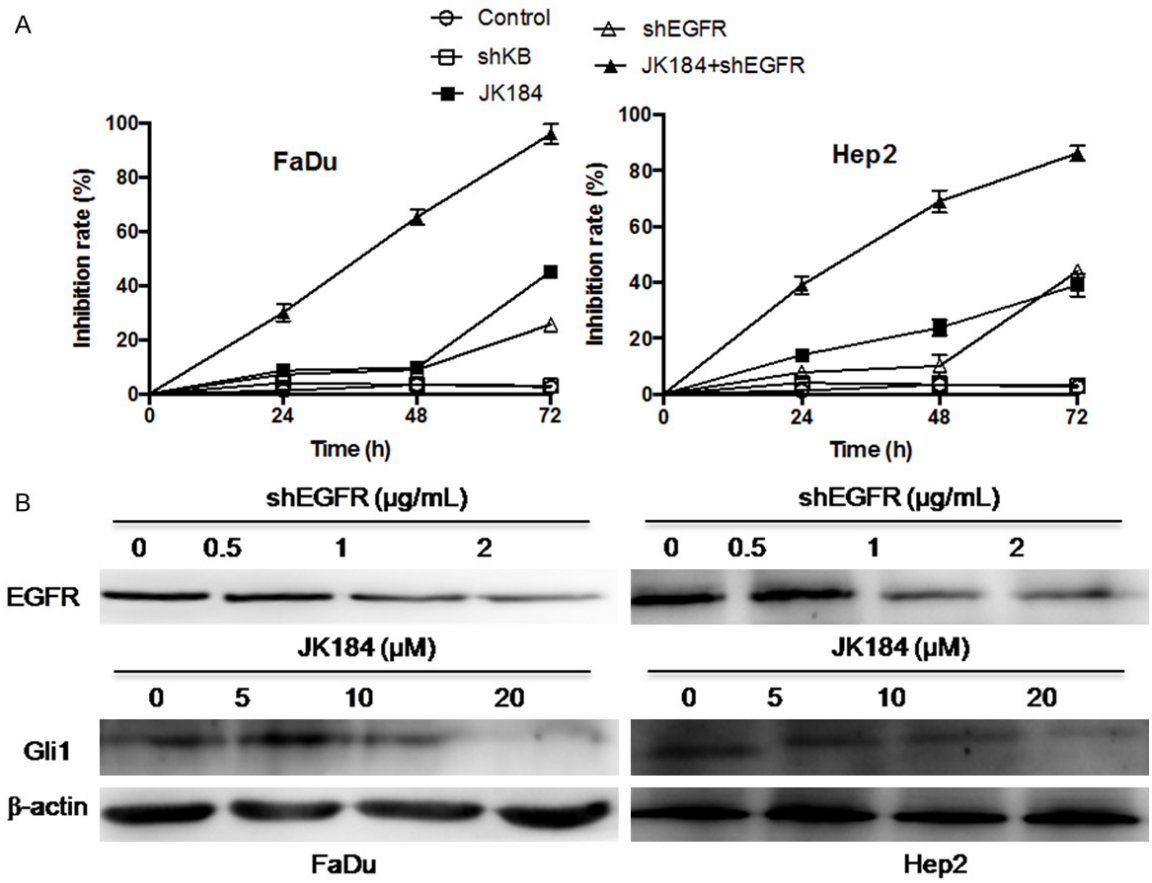

Figure 1. Growth inhibition in Hep2 and FaDu cells suppressed for EGFR expression or Hedgehog pathway. A. FaDu and Hep2 cells were treated with JK184 (10 μM), shEGFR (2 μg/mL), JK184 (10 μM) plus shEGFR (2 μg/mL) for 24, 48, and 72 h. Control group, no drugs; shKB, control plasmid. Cell viability was determined by MTT assay. The vertical bars indicate the means and standard errors (n=6). B. Fadu and Hep2 cells were treated with: (1) various concentrations (0.5 mg/mL, 1 mg/mL, 2 mg/mL) of shEGFR plasmid for 48 h; (2) various concentrations (5 μM, 10 μM, 20 μM) of JK184 for 48 h. β-actin, EGFR and Gli1 were analyzed by western blot in whole lysates. ShEGFR plasmid and JK184 significantly inhibited expression of EGFR and Gli1 respectively in a dose-dependent manner.

animals per group): (a) 5% dextrose solution; (b) 5 μg shKB/30 μg DOTAP: Chol complexes (liposome plasmid DNA complexes were prepared as described previously [24]); (c) 200 μL JK184 (10 mg/kg body weight); (d) 5 μg shEGFR/30 μg DOTAP: Chol complexes; and (e) 200 μL JK184 (10 mg/kg body weight) and 5 μg shEGFR/30 μg DOTAP: Chol complexes. Different treatment groups were injected into subcutaneous tumors three times a week (on Monday, Wednesday, and Friday) using a multisite injection manner.

Statistical analysis

Data were analyzed by SPSS version 13.0 software and were expressed as the mean ± SD. A single-factor analysis of variance was used to compare the differences between groups. For all analyses, $P<0.05$ was considered as the criterion to indicate a statistically significant difference.

Results

The effects of simultaneous inhibition of Hh and EGFR pathways on cell viability

To determine whether EGFR shRNA plasmid synergistically enhances the effect of Hedgehog antagonist JK184, Hep2 and FaDu cells were cultured with different concentrations of JK184 or shEGFR alone or in combination for 48 h, followed by the determination of cell viability using MTT assay. Combination of 2 μg/mL EGFR shRNA plasmid and 10 μM JK184 exhibited the most synergistic effect in reducing cell via-

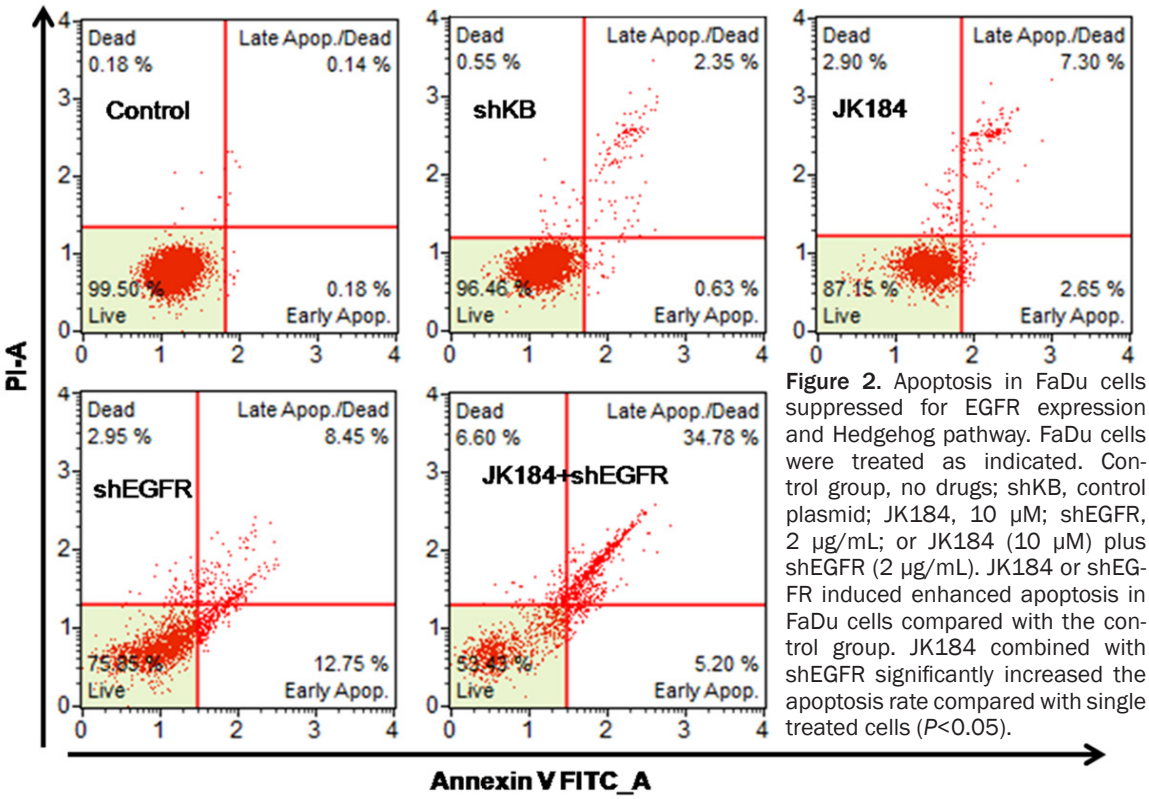

Figure 2. Apoptosis in FaDu cells suppressed for EGFR expression and Hedgehog pathway. FaDu cells were treated as indicated. Control group, no drugs; shKB, control plasmid; JK184, 10 μM; shEGFR, 2 μg/mL; or JK184 (10 μM) plus shEGFR (2 μg/mL). JK184 or shEGFR induced enhanced apoptosis in FaDu cells compared with the control group. JK184 combined with shEGFR significantly increased the apoptosis rate compared with single treated cells (P<0.05).

bility in both FaDu and Hep2 cell lines. Therefore, we decided to use these concentrations of EGFR shRNA plasmid and JK184 alone and in combination in all our subsequent experiments.

To investigate the role of Hedgehog and EGFR signaling in cell proliferation, we examined the effect of shEGFR, alone, and combined with JK184 on the growth of Hep2 and FaDu cells, cellular proliferation was monitored by MTT assay for 24, 48 and 72 h (**Figure 1**). We compared the results with our experimental combination therapy in Fadu cell line as well as in Hep2 cell line (**Figure 1A**). Treatment with shEGFR and JK184 alone or in combination resulted in a time-dependent inhibition of growth in both FaDu and Hep2 cell lines. And the inhibition of the combined treatment group was significantly greater than the single treatment group.

Meanwhile, treatments of both Fadu and Hep2 cell lines with increasing doses of shEGFR and JK184 alone were utilized, and the results of western blot demonstrated a dose-dependent decrease in EGFR and GLI1 protein levels, respectively (**Figure 1B**).

Apoptosis analysis of cells inhibited for Hh and EGFR pathways

Apoptosis in FaDu cells treated with JK184 and shEGFR was analyzed by flow cytometry. As shown in **Figure 2**, the percentage of apoptotic cells in JK184 (0.01 μmol/L)-treated FaDu cells and shEGFR (2 μg/mL)-treated FaDu cells was 7.30 ± 1.0% and 8.35 ± 0.8% (P< 0.01), respectively. However, the apoptosis rate in the combined treatment group was significantly higher (34.78% ± 3.1%) than that in the JK184 or shEGFR single treatment groups (P<0.01). These data indicated that the synergistic effects of JK184 and shEGFR induced more apoptotic cells than the single treatment groups in vitro. These results showed that the JK184 and shEGFR blocking of hedgehog and anti-EGFR signaling pathways resulted in inhibition of cell proliferation and enhanced apoptosis *in vitro*.

Colony formation analysis of cells with inhibited Hh and EGFR pathways

JK184 and shEGFR inhibited colony formation of FaDu cells. After 12 days of incubation, colo-

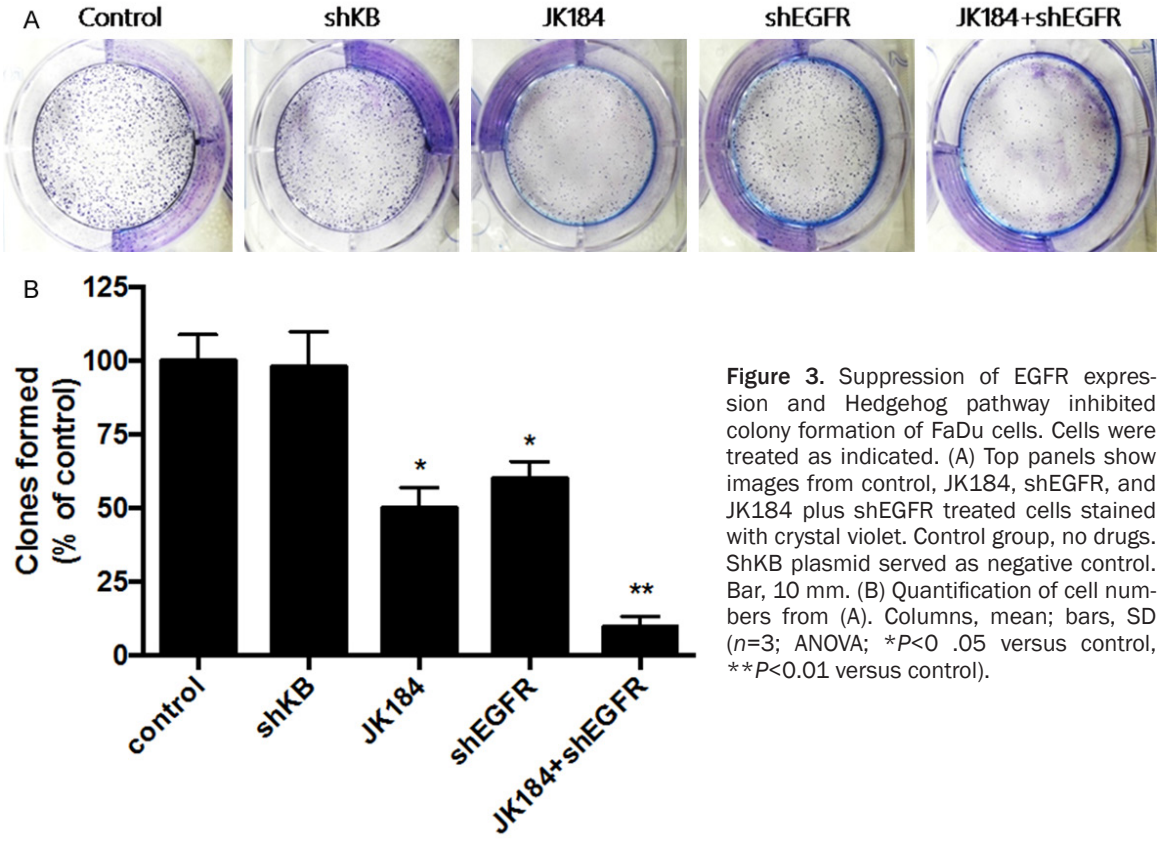

Figure 3. Suppression of EGFR expression and Hedgehog pathway inhibited colony formation of FaDu cells. Cells were treated as indicated. (A) Top panels show images from control, JK184, shEGFR, and JK184 plus shEGFR treated cells stained with crystal violet. Control group, no drugs. ShKB plasmid served as negative control. Bar, 10 mm. (B) Quantification of cell numbers from (A). Columns, mean; bars, SD (n=3; ANOVA; *P<0 .05 versus control, **P<0.01 versus control).

ny formation in the JK184 and shEGFR groups were reduced compared with controls (**Figure 3A**). Furthermore, a marked decrease in the number of colonies was observed in FaDu cells both transfected with shEGFR and treated with JK184 compared with single treated cells. Quantitative determination of colony formation confirmed that JK184-treated, shEGFR-transfected, and both JK184- and shEGFR-treated groups showed a significant decrease in the number of colonies by 50% (P<0.05), 60% (P< 0.05), and 10% (P<0.01), respectively, compared with the control group (**Figure 3B**).

Western blot analysis of apoptotic factors in cells with inhibited Hh and EGFR pathways

We next examined whether shEGFR treatment downregulates GLI1, an Hh signaling factor, and used the Hh pathway inhibitor JK184 as control. The results showed that treatment of FaDu cells with JK184 significantly suppressed GLI1, while shEGFR demonstrated no significant change in GLI1 content at 48 h (**Figure 4B**). Using shEGFR combined with JK184, we observed partial GLI1 suppression to levels similar to that with JK184 treatment (**Figure 4B**).

We then sought to further delineate the mechanisms that underlie the combined effects of JK184 and shEGFR on FaDu cell apoptosis by examining the expression of the major anti-apoptotic protein Bcl-2. JK184 or shEGFR single treatment significantly reduced the level of Bcl-2 expression (P<0.05) compared with controls. Furthermore, JK184 combined with shEGFR significantly reduced the level of Bcl-2 expression compared with single treatment groups (P<0.01) (**Figure 4B**).

Combined inhibition of Hh/GLI-EGFR induced autophagy factors in FaDu cells

In cells undergoing autophagy, the microtubule-associated LC3 protein is post-translationally translocated into the membranes of autophagosomes, which is tracked by the conversion of LC3-I to LC3-II [27]. As shown in **Figure 4A** and **4B**, FaDu cells treated with shEGFR, JK184, or the combination of both agents induced processing of LC3-I (18 kDa) to LC3-II (16 kDa).

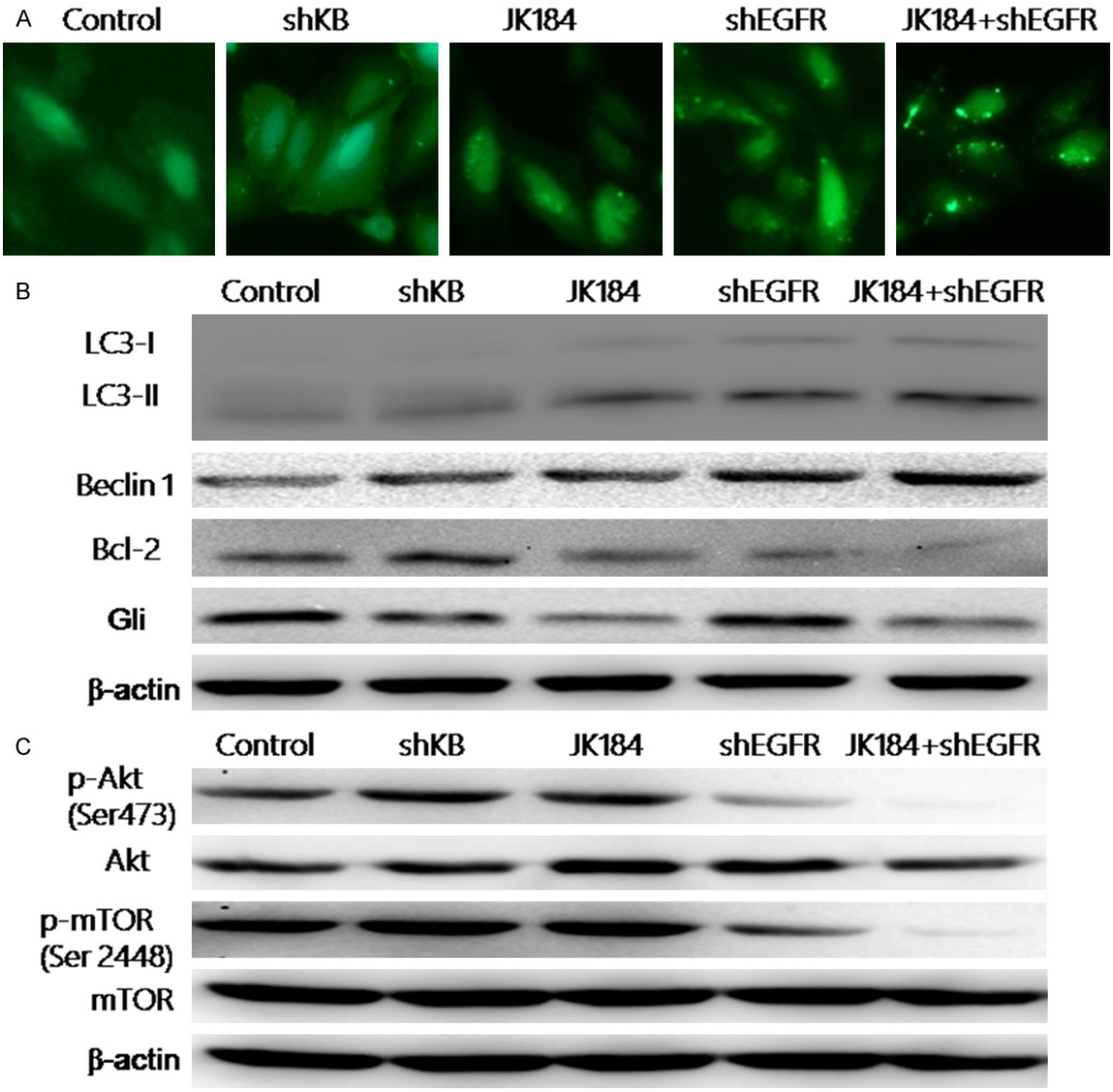

Figure 4. The synergistic effect of shEGFR and JK184 induced LC3 turnover and activated autophagy-related genes in Fadu cells. (A) FaDu cells were transiently transfected with pEGFP-LC3 plasmid alone or cultured with JK184 (10 μM), or shEGFR (2 μg/mL), or JK184 (10 μM) plus shEGFR (2 μg/mL) for 48 h. More than 100 randomly fields for each condition were defined as that cells have GFP-LC3 punctate dots. The appearance of GFP-LC3 positive puncta is indicative of the induction of autophagy. Western blot analysis of signaling pathway proteins in FaDu-treated cells. Squamous carcinoma (FaDu) cells were cultured with JK184 (10 μM), shEGFR (2 μg/mL), or JK184 (10 μM) plus shEGFR (2 μg/mL) for 48 h. Control group, no drugs. ShKB plasmid served as negative control. Cell lysates were analyzed by western blot analysis for (B) anti-LC3, anti-Beclin 1, and anti-Bcl-2, and (C) anti-p-Akt, anti-Akt, anti-p-mTOR, and anti-mTOR. β-actin was used as a loading control.

Furthermore, LC3-II was accumulated in shEGFR- or JK184-treated FaDu cells, and the accumulation was more prominent with the combination of shEGFR and JK184. We observed similar results with the expression of Beclin 1. Together these data indicate that the combination of shEGFR and JK184 may induce autophagy.

Combined inhibition of Hh/GLI-EGFR down-regulated the Akt-mTOR signaling pathway in FaDu cells

The Akt-mTOR signaling pathway is a critical negative regulator of autophagy induction [28]. Therefore, we investigated whether phosphorylation of both Akt and mTOR was involved in

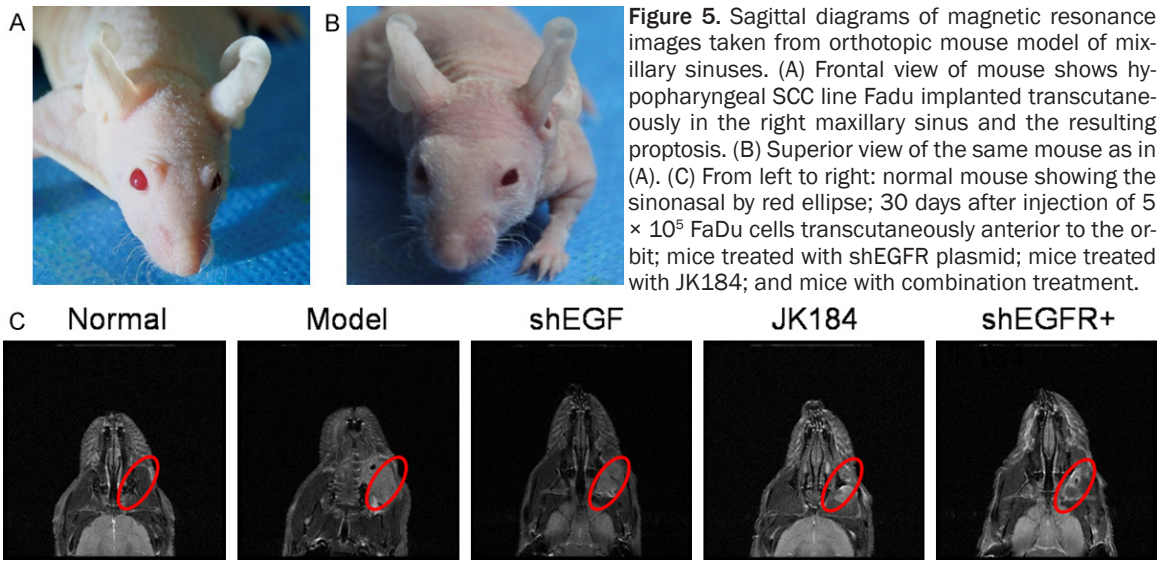

Figure 5. Sagittal diagrams of magnetic resonance images taken from orthotopic mouse model of mixillary sinuses. (A) Frontal view of mouse shows hypopharyngeal SCC line Fadu implanted transcutaneously in the right maxillary sinus and the resulting proptosis. (B) Superior view of the same mouse as in (A). (C) From left to right: normal mouse showing the sinonasal by red ellipse; 30 days after injection of 5 × 10^5 FaDu cells transcutaneously anterior to the orbit; mice treated with shEGFR plasmid; mice treated with JK184; and mice with combination treatment.

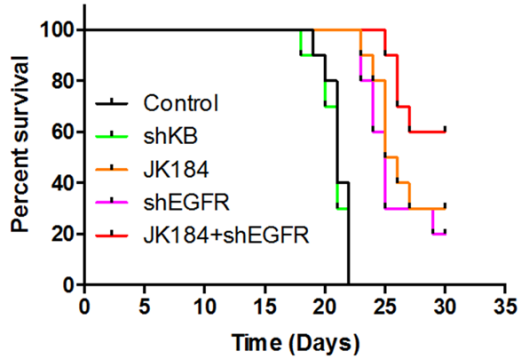

Figure 6. Survival rate of a mouse orthotopicsinonasal cancer model treated by EGFR knockdown and Hedgehog antagonist JK184. 30 days after injection of 5 × 10^5 FaDu cells transcutaneously anterior to the orbit; mice treated with shEGFR plasmid; mice treated with JK184; and mice with combination treatment. The survival rate of a mouse orthotopicsinonasal cancer model showing statistically significant differences in survival times between mice treated with shEGFR and JK184 alone and in combination.

autophagy induced by JK184 combined with shEGFR in FaDu cells. As shown in **Figure 4C**, shEGFR treatment resulted in a noticeable inhibition of both Akt (S473) and mTOR (S2448) phosphorylation, while JK184 had no impact. Furthermore, reduced phosphorylations of Akt and mTOR were more pronounced with the combination of JK184 and shEGFR treatment. Taken together, our results clearly indicate that tumor inhibition by combination of JK184 and shEGFR is through inactivation of PI3K/AKT/mTOR signaling pathway.

Orthotopic implantation is technically feasible and can be used with a spectrum of human tumor xenografts

To examine the possible anti-tumor effects of combination anti-EGFR treatment in a more clinical scenario, we established an orthotopic tumor model of maxillary sinus in nude mice. The *in vivo* behavior accurately reflected the invasive character of maxillary sinus tumors in patients. Representative MRI images for each group are shown in **Figure 5**. The normal maxillary sinus is shown in the red ellipse. Model mice showed high rates of regional invasion in the maxillary sinus within 30 days, indicating a successful orthotopic implantation and tumor growth. Multiple tumor invasion sites include nasal cavity, basilar region, and through the lamina papyraceaintotheorbital cavity. Mice with either EGFR blockade or JK184 inhibition showed hindered tumor growth in the maxillary sinus but still showed invasion into both maxillary and nasal cavities. Tumors were restricted to local invasion and no regional metastasis was observed in the combination treatment group.

Combination therapy improves survival in head and neck cancer maxillary sinus

Successful tumor formation was achieved with implantation of 5 × 10^5 FaDu cells into the maxillary sinus of nude mice. As shown in **Figure 6**, high death rates were seen within 23 days in control mice with tumors from cells implanted

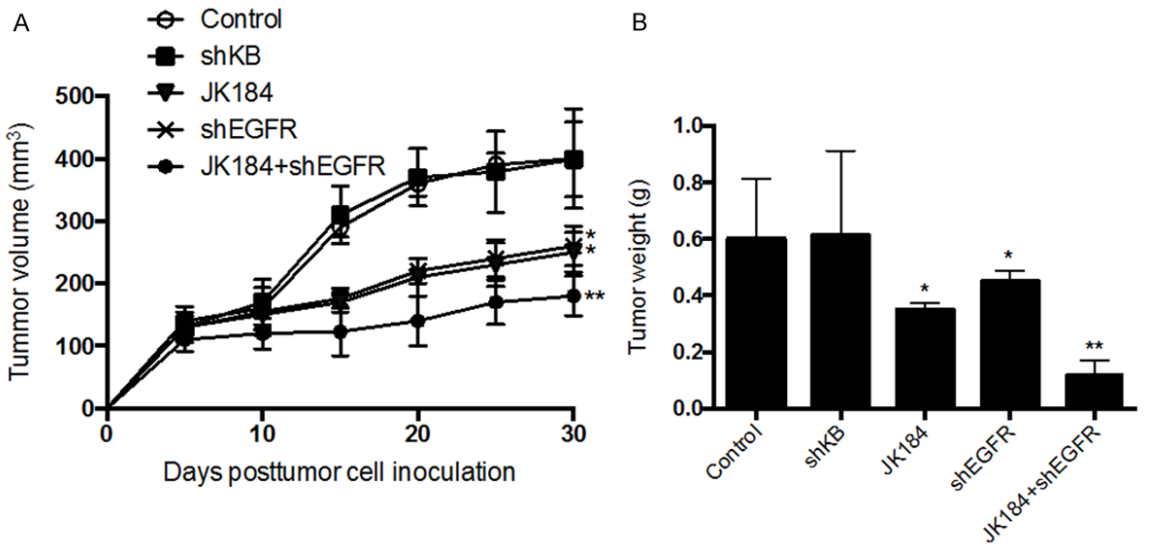

Figure 7. Antitumor effects of EGFR knockdown and Hedgehog antagonist JK184 on cancer models. Models were established by subcutaneous implantation of 5 × 10^6 FaDu cells into the right flank of 6-8 week-old male nude mice. Four groups of six mice each were examined as indicated, including control (5% dextrose solution), JK184 (5 μg shKB/30 μg DOTAP: Chol complexes), and shEGFR (5 μg shEGFR/30 μg DOTAP: Chol complexes). DOTAP: Chol-based nanoparticles were delivered by intravenous injection. A. Tumor sizes were measured by calipers every 5 days. B. Tumor weights were measured for each group. Treatment with shEGFR resulted in significant inhibition of tumor growth and tumor weight versus vehicle control (n=6; ANOVA; $^{**}P<0.01$).

in the maxillary sinus. To determine whether inhibition of Hedgehog and EGFR signaling in head and neck cancer model mice improved survival rates, we examined tumor-bearing mice implanted with cells treated with JK184 and shEGFR alone or JK184 and EGFR together. Although mice in single-treated groups showed better survival rates than controls, a statistically significant difference was shown in mice with combination therapy of JK184 and EGFR compared with mice treated with JK184 (P=0.0451) or shEGFR (*P*=0.0353) only.

Antitumor effect of Hh blockage and EGFR knockdown in tumor xenograft models

The FaDu mouse model single treatment groups with JK184 or shEGFR showed a considerable inhibition in tumor growth as demonstrated by reduced tumor size (**Figure 7A**) and tumor weight (**Figure 7B**), indicating a significant anti-tumor ability by JK184 and shEGFR *in vivo*. More importantly, we observed an increased reduction in tumor size and tumor weight in the JK184 and shEGFR combination treatment group. Tumor growth in the JK184 treatment, shEGFR treatment, and JK184 combined with shEGFR treatment groups was decreased by 37.3%, 16.4%, and 63.4% compared with the control, respectively.

Discussion

Despite recent reports on the progress of cancer therapy, the disease is still considered incurable in many cases and identifying precisely effective targeting therapies for cancer cells is a major challenge for oncology research. Perhaps the most promising approach to significantly improving patient survival is to attack cancer cells from multiple sides using defined combinations of targeted anti-cancer drugs alone or together with chemotherapy, surgery, and/or radiation therapy. To meet these challenges, the development of rational drug combinations for novel efficacious targets will therefore be a key to the success of such multimodal anti-tumor strategies. Several *in vitro* studies suggest that the combination of specific EGFR and Hh signaling inhibitors may provide a synergistic therapeutic benefit. For instance, metastatic prostate cancer cell lines or putative prostate cancer stem cells treated with a combination of the selective EGFR inhibitor gefitinib, the SMO antagonist cyclopamine, and/or the chemotherapeutic drug docetaxel, which can inhibit cell growth, induce apoptosis, and/or interfere with invasiveness [29, 30].

EGFR inhibition would be of benefit to patients with HNSCC, but its effect is limited [31]. Hh

signaling has been shown to be a key driver of tumor growth and metastasis in multiple cancers [32]. In our study, we observed cooperative Hh-EGFR signaling in HNSCC cells through blocking EGFR and Hh signaling by shEGFR plasmid and JK184, respectively.

First, we defined how inhibition of EGFR and Hh, alone and in combination, regulates signaling in HNSCC cell lines. As previously described [33], we observed that cross-talk between the EGFR and Hh pathways might occur through increasing autophagy level in HNSCC. Inducing apoptosis against tumor development is one of the classic strategies [34, 35] and it had been reported that carcinomas from epithelial cells were associated with an increased malignant tumor phenotype and a poorer patient outcome with EGFR activation [36, 37]. However, a great many of anti-EGFR agents have induced minimal response in epithelial cancer [38]. Simultaneous activation of the Hh signaling pathway is involved in human cancers. Varnat *et al.* reported that blocking Hh-GLI signaling affected growth, recurrence, and the metastasis of human colon cancer epithelial cells [39], but the development of antagonists for many types of devastating cancers could lack support [40]. We found blockage of EGFR and Hedgehog signaling in FaDu cells could increase apoptosis level compared with silencing EGFR by shEGFR plasmid or JK184 treatment. Secondly, we also observed the presence of synergistic interactions between shEGFR and JK184 in FaDu cells in the growth inhibition of colony formation and significantly reduced cell growth. Furthermore, our studying results showed that combined inhibition of EGFR and Hh signaling could enhance the efficacy of the anti-EGFR therapy.

Until now, the mechanisms underlying the synergistic effects of silencing EGFR and blocking HH-GLI1 signaling against tumors were not clear, although some evidence suggested the effects may involve autophagy. The anti-EGFR monoclonal antibody panitumumab was reported to affect colon cancer cell proliferation independent of KRAS mutation status, possibly through the induction of autophagy [41]. Our findings showed that EGFR/Hh blockade upregulated Beclin 1 and decreased Bcl2 expression, which decreased Beclin 1-associated class III phosphatidylinositol-3 kinase activity, followed by induction of autophagy. We suggested that dual EGFR/Hh blockade results in a synergistic anti-tumor effect through autophagy. As previously described in epithelial cancer cell lines [42, 43], inhibition of EGFR and Hh, both pharmacologically and genetically, augmented tumor inhibition by increasing activation of both MEK/ERK and PI3K/AKT [33]. This dual EGFR/Hh blockade approach may inhibit tumor development and retard tumor growth and spread through the Akt-mTOR signaling pathways. We also found that the expression of p-mTOR and p-Akt in the shEGFR treatment group was lower than the Hh blockade treatment. Synergic treatment of EGFR and Hh blockades reduced levels of p-mTOR and p-Akt to the lowest levels. Akt can be activated by EGFR [44] and thus represents a possible molecular link between the mTOR and the EGFR pathways. However, whether Hh helps anti-EGFR lower than the levels of p-mTOR and p-Akt through other pathways requires further study.

Our findings support the use of a mouse orthotopic sinonasal cancer model that better mimics the human environment. Achieving effective drug concentrations is one of the difficult issues in cancer therapy. Furthermore, during the *in vivo* delivery process, one challenge of siRNA is reaching target cells and the siRNA off-target effect. Moreover, cellular uptake of siRNA is very difficult to cross the plasma membrane of a target cell, due to the negative charge and large size of a naked siRNA [45]. To avoid these problems, here we targeted EGFR with plasmid-based shRNA to overcome siRNA off-target effects and targeted Hh with JK184 through local injection using effective concentrations based on currently available EGFR inhibitors. Delivery is still the therapeutic bottleneck *in vivo*. Various routes of administration of nucleic acids are available. However, the safety of recombinant virus vectors for human gene therapy must be considered. Retroviral vectors, the best tool currently available for stable genetic modification, insert at random positions in the cellular genome and may cause malignant phenotypes. Although intravenous delivery of naked plasmids has been used to block tumor growth in xenograft models owing to benefits of safety and simplicity, the effect was limited probably owing to the short half-life of the plasmids *in vivo*. To increase intravenous DNA delivery, we used DOTAP: Chol liposomes to form complexes with DNA, which resulted in

enhanced expression of targeted genes in most tissues examined. The choice of DOTAP: Chol liposomes mainly depended on several parameters, such as biodegradation, biodistribution, and toxicity [24].

Our study showed that the combined inhibition of EGFR and HH/GLI signaling in a mouse orthotopic sinonasal cancer model efficiently increased the survival rate *in vivo* as did combined inhibition of GLI and JUN [11]. We showed that a combination of shEGFR and JK184 treatment induced tumor shrinkage 2-4 fold compared with shEGFR or JK184 treatments in our *in vivo* model. Because both the EGFR and Hh/GLI pathways have been implicated in the pathogenesis of a considerable number of human cancers, it is tempting to speculate that these malignancies also harbor these cooperative interactions and that combinatorial targeting may provide a therapeutic benefit to cancer patients.

These provoking results provided the rationale for the clinical translation of this novel combinatory paradigm.

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Disclosure of conflict of interest

None.

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