

Cancer stem-like cell properties are regulated by EGFR/AKT/ β -catenin signaling and preferentially inhibited by gefitinib in nasopharyngeal carcinoma

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Keywords

cancer stem-like cells; EGFR; gefitinib; nasopharyngeal carcinoma; β -catenin

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(Received 23 September 2012, revised 22 February 2013, accepted 25 February 2013)

doi:10.1111/febs.12226

We report that the epidermal growth factor receptor (EGFR) pathway plays a critical role in regulating cancer stem-like cells (CSCs) in nasopharyngeal carcinoma (NPC), one of the most common malignant tumors in Southeast Asia. Effects of EGFR on maintaining CSCs are mainly mediated by AKT signaling, and β -catenin is responsible for governing CSC properties in response to EGFR/AKT activation. Significantly, CSCs are enriched by cisplatin and decreased by gefitinib in NPC xenograft models. Upon reimplantation in secondary mice, tumor cells derived from cisplatin-treated mice grew rapidly, whereas regrowth of tumor cells from gefitinib-treated mice was severely diminished. We further demonstrate that expression of EGFR correlates with expression of β -catenin and Nanog in primary tumor specimens from NPC patients. These findings provide mechanistic and preclinical evidence supporting the use of gefitinib alone or in combination with a chemotherapeutic agent in first-line therapy for patients with NPC. In addition, our results suggest that targeting β -catenin represents a rational clinical modality for patients whose tumors harbor activated EGFR or AKT.

Introduction

The epidermal growth factor receptor (EGFR) plays a critical role in regulating proliferation, differentiation and survival of epithelial cells and tumors of epithelial cell origin [1–3]. Previous studies have revealed that a single progenitor cell from either embryonic or adult mouse brain proliferates in response to epider-

mal growth factor (EGF) and generates undifferentiated cells with the properties of neuroepithelial stem cells [4,5]. EGFR signaling is also required for the differentiation and maintenance of neural progenitors from *Drosophila* embryos [6] and for self-renewal and differentiation of rat embryonic stem cells [7]. Fur-

Abbreviations

bFGF, basic fibroblast growth factor; CSC, cancer stem-like cell; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; NPC, nasopharyngeal carcinoma; PtdIns3K, phosphatidylinositol 3-kinase; shRNA, short hairpin RNA; siRNA, small-interfering RNA; SP, side population.

thermore, EGFR modulates a side population (SP) in human head and neck carcinoma cell lines, which exhibits stem-cell-like properties [8]. These findings suggest that EGFR may play an important role in regulating and maintaining human cancer stem-like cells (CSCs), a rare subpopulation of self-renewal cancer cells that could initiate tumors and promote cancer progression and may account for the failure of current therapies to eradicate malignant tumors [9,10].

Wnt/ β -catenin signaling pathway has been implicated in regulation of embryonic development, cell proliferation and self-renewal of CSCs in several types of tumors [11]. The canonical Wnt pathway consists of a series of events that eventually lead to the stabilization and translocation of β -catenin into the nucleus, where β -catenin accelerates expression of a broad range of Wnt target genes via binding to the TCF/LEF family of transcription factors. Recent studies revealed that the effect of AKT signaling on stem cells is also mediated by β -catenin [12–14]. Akt activates β -catenin and induces its nuclear translocation either by phosphorylation of the C-terminus of β -catenin at Ser552 [12], or indirectly through phosphorylation and inactivation of GSK3 β , resulting in hypophosphorylation of β -catenin at S33/S37/T41 [15].

Nasopharyngeal carcinoma (NPC) is a rare epithelial cancer in most parts of the world. However, it is one of the most common malignant tumors in Southeast Asia and southern China, with an incidence of 25–50 per 100 000, which is 25-fold higher than that observed in western countries [16]. The 5-year survival for stage IV NPC is only 30%, and poor survival is often associated with local, regional and systemic recurrences [17]. Because surgical approaches for NPC are limited due to the tumor's inaccessible anatomic nature and the fact that NPCs are sensitive to radiation, the primary treatment modality for NPC is radiotherapy with or without chemotherapy [18].

Recent studies showed that NPC contains a small fraction of cells with properties of CSCs; this tumor subpopulation plays a critical role in tumorigenesis and drug resistance [19–21]. In this study, we investigated the role of EGFR in the maintenance, self-renewal and tumorigenesis of CSCs. We found that activation of EGFR increased the number of CSCs, and this effect of EGFR was mediated by phosphatidylinositol 3-kinase (PtdIns3K)/AKT/ β -catenin signaling. In a NPC xenograft model using nude mice, CSCs were eradicated by treatment with gefitinib, whereas they were enriched by treatment with cisplatin. Thus, our findings reveal distinct effects of gefitinib and

cisplatin on CSCs versus the general tumor cell population, which may have important clinical implications for the treatment of NPC.

Results

EGFR expression in NPC cells and inhibition effects of gefitinib

EGFR is widely expressed in a variety of human tumors, and inhibition of EGFR has been exploited as a therapeutic strategy in several solid tumor types [1,22]. In NPC, EGFR is expressed in 50–80% of NPC specimens and represents a negative prognostic factor. Furthermore, EGFR expression was significantly linked to low overall survival and shorter time to progression [23]. To investigate the effect of EGFR on CSCs in NPC, we first examined expression levels of EGFR in NPC cell lines. Western blot analyses revealed that EGFR protein is expressed at various levels in seven of eight NPC cell lines analyzed (Fig. 1A). C666-1, the only cell line that did not express EGFR, exhibits mesenchyme-like morphology (data not shown). EGFR expression in primary tumor specimens from 22 NPC patients was assessed using immunohistochemical staining. Twelve samples (54.5%) showed detectable levels of EGFR expression. To determine the inhibitory effects of gefitinib on NPC cell viability, two cell lines, CNE1 and CNE2, were used in this study. CNE1 cells are differentiated and CNE2 cells are poorly differentiated NPC cell lines [24]. Compared with untreated cells, treatment with gefitinib for 72 h significantly inhibited cell viability of both cell lines, with an IC₅₀ of 2.63 and 3.11 μ M for CNE1 and CNE2, respectively (Fig. 1B).

Regulation of SP cells by EGFR signaling in NPC

SP cells are a small subpopulation of tumor cells that exhibit CSC properties in a variety of neoplasms [25]. A previous study showed that SP cells in human NPC cell line CNE-2 had stem-like cell characteristics *in vitro* and a strong ability to form tumors *in vivo* [19]. These cells are characterized by their high ability to efflux the fluorescent dye Hoechst 33342 from the cytoplasm through ATP-binding cassette transporters [26,27]. To investigate whether EGFR could be a regulatory factor for SP cells in NPC, logarithmically growing CNE1 and CNE2 cells were subjected to flow cytometric analysis. The untreated CNE1 and CNE2 cell lines contained 1.6 and 2.5% Hoechst 33342-dull SP cells, respectively. Treatment with EGF increased the SP by ~130% in

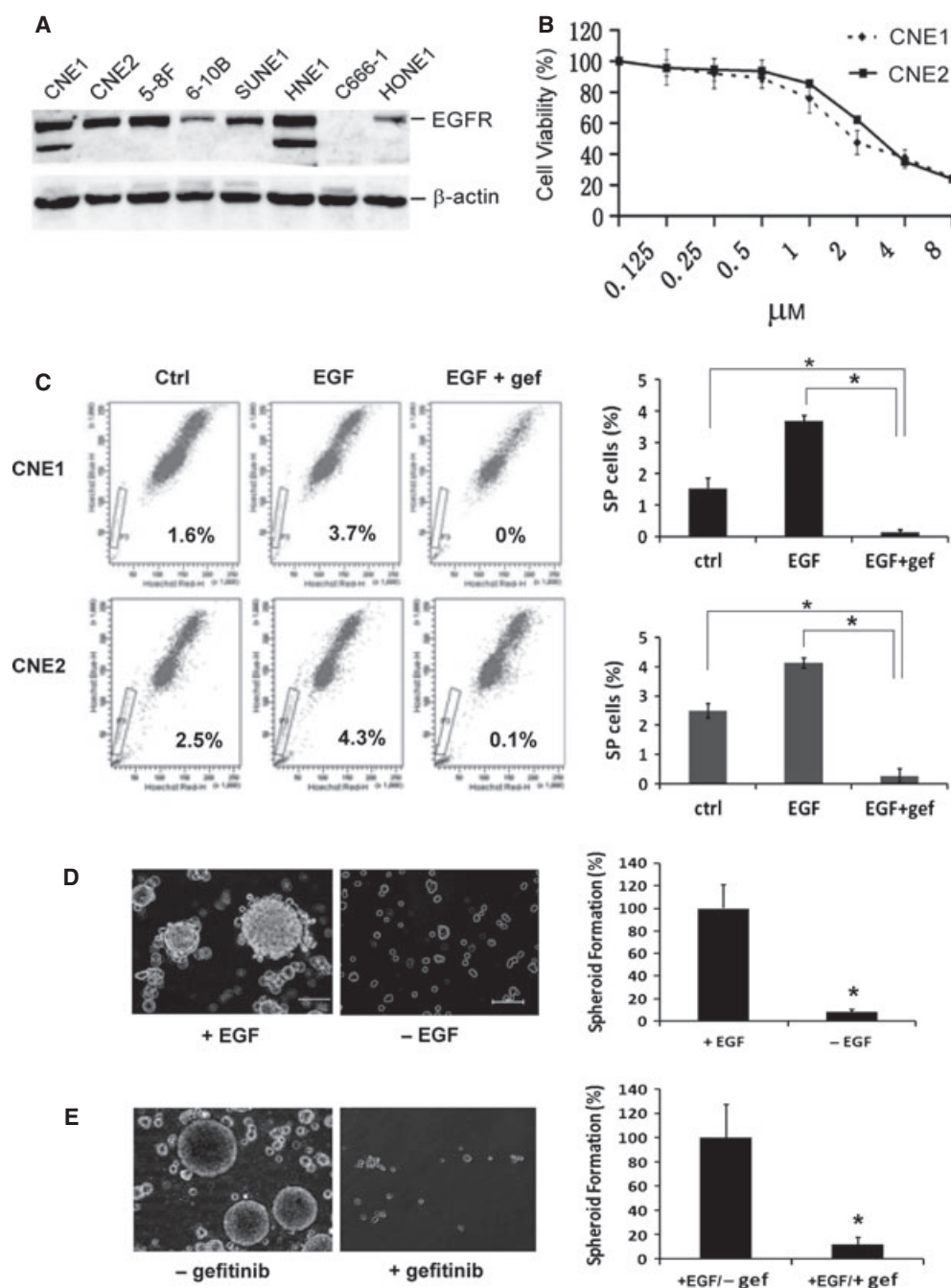


Fig. 1. Inhibition of CSCs by gefitinib in NPC. (A) Expression levels of EGFR in eight NPC cell lines. (B) Antiproliferative effects of gefitinib in CNE1 and CNE2 cell lines. Cells were treated with various concentrations of gefitinib for 72 h, and cell viability was determined by MTT assay. Error bars indicate standard deviations. The experiment was conducted three times with similar results. (C) Inhibition of SP by gefitinib (1 μ M) in NPC cells. Data are presented as mean \pm SD ($n = 3$). * $P < 0.01$. (D) EGF dependence of tumor spheroid formation of CNE2 cells. (E) Inhibition of tumor spheroid formation by gefitinib. CNE2 cells derived from primary tumor spheroids were cultured in suspension growth medium containing EGF and bFGF. Gefitinib was added at 2 μ M. Bar = mean \pm SD ($n = 3$). * $P < 0.01$. Scale bars, 100 μ m.

CNE1 cells and 70% in CNE2 cells, and this stimulatory effect of EGF was completely blocked by gefitinib (Fig. 1C). Similar results were observed when

gefitinib was replaced with another EGFR tyrosine kinase inhibitor PD153035 in these experiments (Fig. 2A).

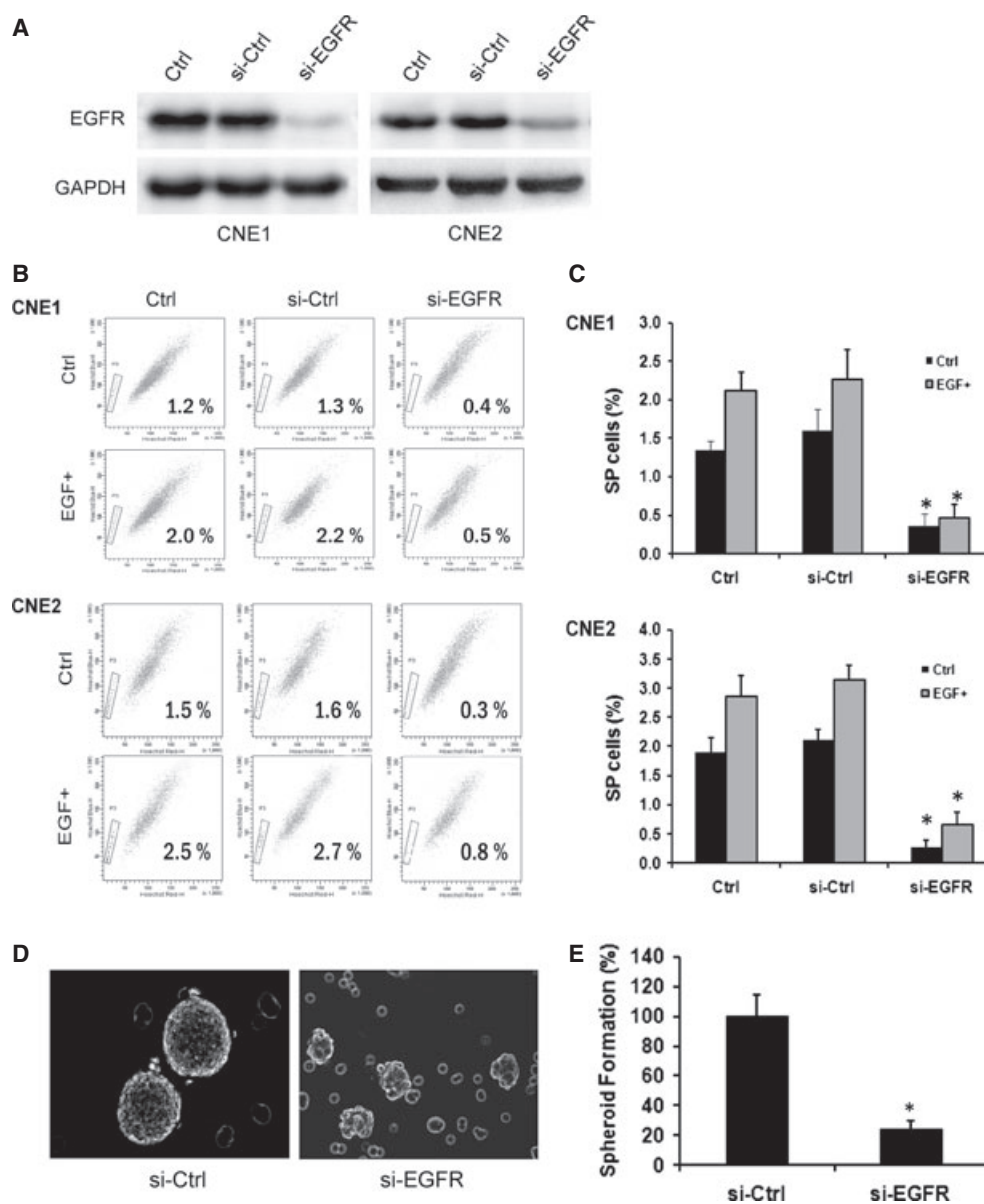


Fig. 2. Knockdown of *EGFR* with siRNA diminishes CSC properties. (A) CNE1 and CNE2 cells were transfected with control siRNA or siRNA targeting *EGFR* followed by western blot analysis. In both cell lines, exposure to *EGFR* siRNA substantially reduced EGFR expression. (B, C) After transfected with control siRNA or *EGFR* siRNA, CNE1 and CNE2 cells were treated with or without EGF ($50 \text{ ng}\cdot\text{mL}^{-1}$). Knockdown of *EGFR* reduced the percentages of the SP of CNE1 and CNE2 at both basal levels and after EGF stimulation. Data are presented as mean \pm SD ($n = 3$). $*P < 0.01$. (D, E) Depletion of EGFR suppressed tumor spheroid formation of CNE2 cells. Bars are means \pm SD ($n = 3$). $*P < 0.01$.

EGFR signaling is essential for tumor spheroid formation and proliferation

CSCs from several types of cancer have been isolated and propagated as nonadherent 3D tumor spheroids that are known to closely mimic phenotypes of *in vivo* tumors [28]. To examine whether EGFR signaling is essential for tumor spheroid formation in NPC, CNE2 cells were cultured in serum-free medium containing

basic fibroblast growth factor (bFGF). When EGF was supplemented at $10 \text{ ng}\cdot\text{mL}^{-1}$, floating tumor spheroids were observed after 7 days of cultivation. In the absence of EGF, however, tumor spheroid formation was significantly inhibited (Fig. 1D). To further determine the role of EGFR signaling in the self-renewal of CSCs, tumor spheroids that formed in the presence of EGF were dissociated enzymatically to

obtain a single-cell suspension and then replated in tissue culture dishes. The cells formed secondary tumor spheroids in the above growth medium supplemented with EGF. However, when gefitinib was present in the medium, tumor spheroid formation was markedly reduced, and most cells died by day 7 (Fig. 1E).

Knockdown of EGFR diminishes the CSC phenotype

To further validate the effect of EGFR signaling on the CSC phenotype, we used small-interfering RNA (siRNA) to knockdown *EGFR* expression in CNE1 and CNE2 cells (Fig. 2A). Compared with untransfected cells, CNE1 and CNE2 cells transfected with *EGFR* siRNA displayed reduced percentages of SP, whereas cells transfected with control siRNA had no obvious effect. When cells were grown in $50 \text{ ng}\cdot\text{mL}^{-1}$ EGF, the percentage of SP cells was increased in untransfected cells and in cells transfected with control siRNA, but not in cells transfected with *EGFR* siRNA (Fig. 2B,C). Next, we measured the effect of *EGFR* knockdown on tumor spheroid formation. CNE2 cells were cultured in serum-free medium containing EGF and bFGF. Knockdown of *EGFR* significantly reduced the number of tumor spheroids (Fig. 2D,E).

EGFR/PtdIns3K/AKT pathway regulates CSC phenotypes in NPC

Because EGFR signaling exerts its biological functions mainly through Raf/Mek/ERK and PtdIns3K/AKT pathways, we asked which pathway mediates the regulatory function of EGFR on CSC phenotypes. To this end, cells were treated for 24 h with EGFR inhibitor gefitinib ($1 \mu\text{M}$), PtdIns3K inhibitor LY294002 ($15 \mu\text{M}$) or Mek inhibitor PD0325901 ($10 \mu\text{M}$). Treatment with EGF increased the percentage of SP cells, and this effect was blocked by gefitinib or PD153035. When gefitinib was replaced by LY294002, the PtdIns3K inhibitor abolished the SP. However, the Mek inhibitor PD0325901 had only a modest effect on the percentage of SP cells (Fig. 3A).

We next examined the effects of gefitinib on EGFR activity and downstream effectors using phospho-specific antibodies and western blot analysis. As shown in Fig. 3B, treatment of NPC cells with EGF for 24 h resulted in marked increases in the phosphorylation levels of EGFR, AKT and ERK1/2, indicating activation of EGFR and its downstream effectors by EGF. When gefitinib or another EGFR inhibitor PD153035 was added, the effects of EGF were diminished substantially. The molecular target specificity of

LY294002 and PD0325901 was also validated: LY294002 abolished phosphorylation of AKT, but not EGFR or ERK. Likewise, PD0325901 inhibited phosphorylation of ERK, but not EGFR or AKT.

The effect of EGFR on CSCs is mediated through downstream β -catenin signaling

The AKT kinase family regulates a variety of cellular processes including proliferation, survival and

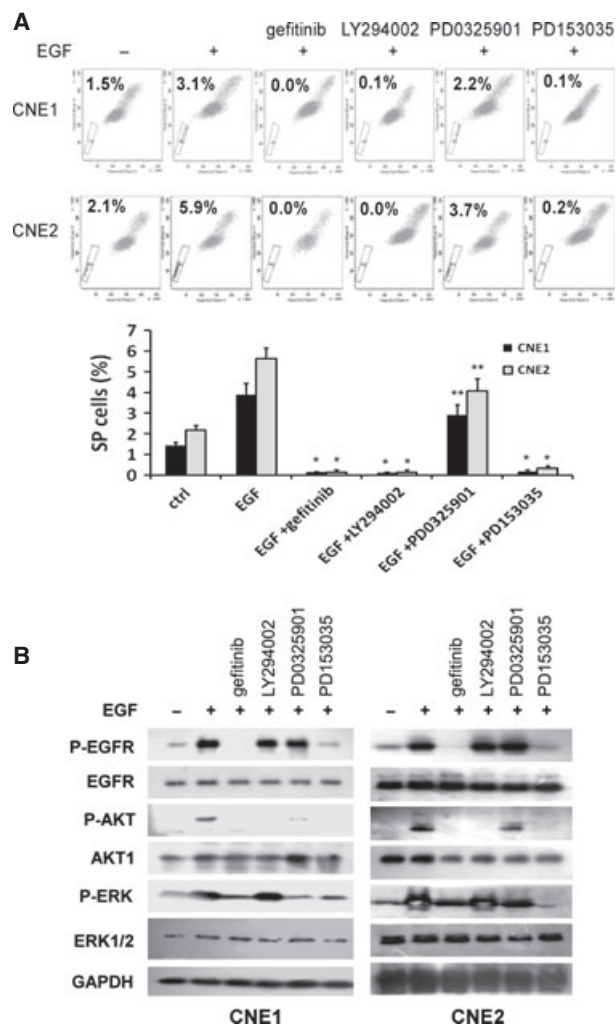


Fig. 3. EGFR/PtdIns3K/AKT pathway regulates CSC phenotype in NPC. (A) CNE1 and CNE2 cells were treated with indicated inhibitors for 24 h followed by FACS SP analyses. EGF-induced ($50 \text{ ng}\cdot\text{mL}^{-1}$) SP cells were diminished by EGFR inhibitor (either $1 \mu\text{M}$ of gefitinib or $0.5 \mu\text{M}$ PD153035) or PtdIns3K inhibitor LY294002 ($15 \mu\text{M}$), but not by Mek inhibitor PD0325901 ($10 \mu\text{M}$). Data are presented as mean \pm SD ($n = 3$). * $P < 0.01$; ** $P > 0.05$. (B) Cells were treated as described in (A) followed by western blot analysis. EGF-induced phosphorylation of EGFR, AKT and ERK1/2 was blocked by gefitinib or PD153035.

metabolism. AKT kinases control these processes through phosphorylation-mediated regulation of numerous substrates [29]. As shown above, we have demonstrated that the effect of EGFR signaling on the CSC phenotype is mediated by AKT, and β -catenin was reported to be responsible for mediating the effect of AKT signaling on stem cells [12,13]. Given these observations, we next investigated whether β -catenin plays a role in the EGFR/AKT pathway governing CSCs in NPC. We first examined the effect of EGFR signaling on the subcellular localization of β -catenin in CNE2 cells (Fig. 4A). In untreated cells, β -catenin was located predominantly at the plasma membrane, with faint staining distributed in the cytoplasm. When cells were treated with EGF, β -catenin staining shifted from the cytoplasmic membrane to the nucleus. Inhibition of EGFR with gefitinib or inhibition of PtdIns3K with LY294002 reversed the accumulation of β -catenin in the nucleus. To confirm the results of immunofluorescent staining, we performed western blot analysis with an antibody specific for the active form of β -catenin, dephosphorylated on Ser37 or Thr41 (Fig. 4B). In response to EGF stimulation, expression level of active form of β -catenin was increased. This effect of EGF was blocked by gefitinib or LY294002. Importantly, we further found that EGFR signaling regulates expression of c-Myc oncogene that is a critical target of β -catenin [30] as well as an essential factor for reprogramming adult cells into induced pluripotent stem [31]. Nanog, a critical stem cell maker, was also regulated by EGFR signaling, which is in agreement

with a previous report demonstrating that β -catenin upregulates Nanog expression in embryonic stem cells [32]. Thus, the above experiments reveal a functional EGFR/PtdIns3K/AKT/ β -catenin axis that regulates the CSC phenotype in NPC cells.

Subsequently, we investigated the requirement of β -catenin in the maintenance of CSCs in NPC. To this end, β -catenin was knocked down with lentivirus-mediated shRNAs (Fig. 5A). Compared with cells infected with the control short hairpin RNA (shRNA) (sh-green fluorescent protein) lentivirus, cells infected with sh- β -catenin lentivirus exhibited decreased percentage of SP cells at both basal levels and after EGF stimulation (Fig. 5B). Likewise, knockdown of β -catenin also markedly reduced tumor spheroid formation in the absence or presence of gefitinib (Fig. 5C). Taken together, these results provide mechanistic evidence for a role of β -catenin in maintaining the CSC population and indicate that β -catenin is a crucial mediator of a signal transduction pathway downstream of EGFR/PtdIns3K/AKT.

Gefitinib inhibits CSCs in NPC xenografts

To further evaluate the importance of EGFR signaling in the maintenance of CSC self-renewal and evaluate the anti-CSC efficacy of gefitinib *in vivo*, we performed experiments in xenograft models with nude mice. CNE2 cells were subcutaneously implanted into the flanks of mice. After 2 weeks, mice were treated with saline, gefitinib or cisplatin, the latter being the most

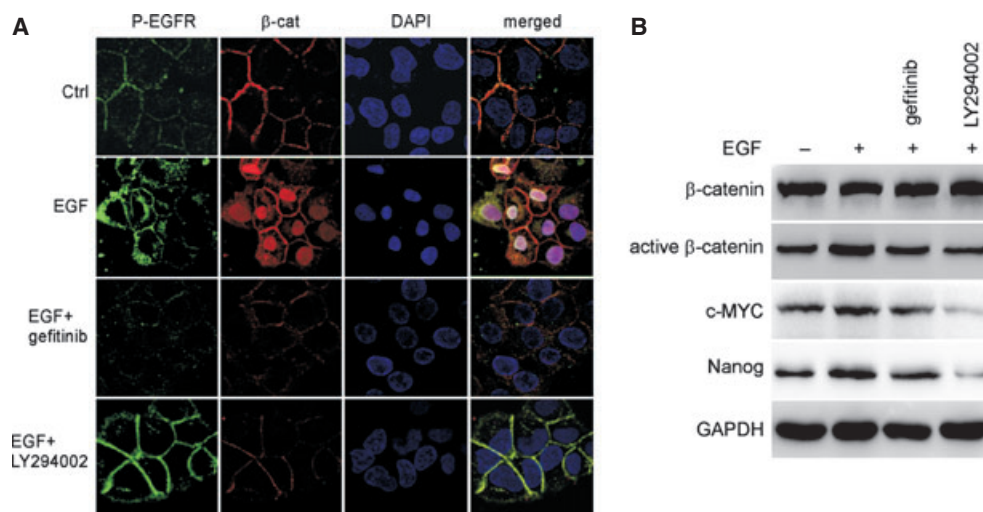


Fig. 4. The effect of EGFR on the CSC phenotype is mediated through β -catenin signaling. (A) In untreated CNE2 cells, β -catenin was located in cytoplasmic membranes and cytoplasm. Treatment with EGF (50 ng-mL⁻¹) resulted in increased β -catenin nuclear staining, which was reversed by either gefitinib (1 μ M) or LY294002 (15 μ M). (B) Western blot analysis showed that elevated expression of active β -catenin, c-myc and Nanog in response to EGF (50 ng-mL⁻¹) can be reversed by either gefitinib (1 μ M) or LY294002 (15 μ M).

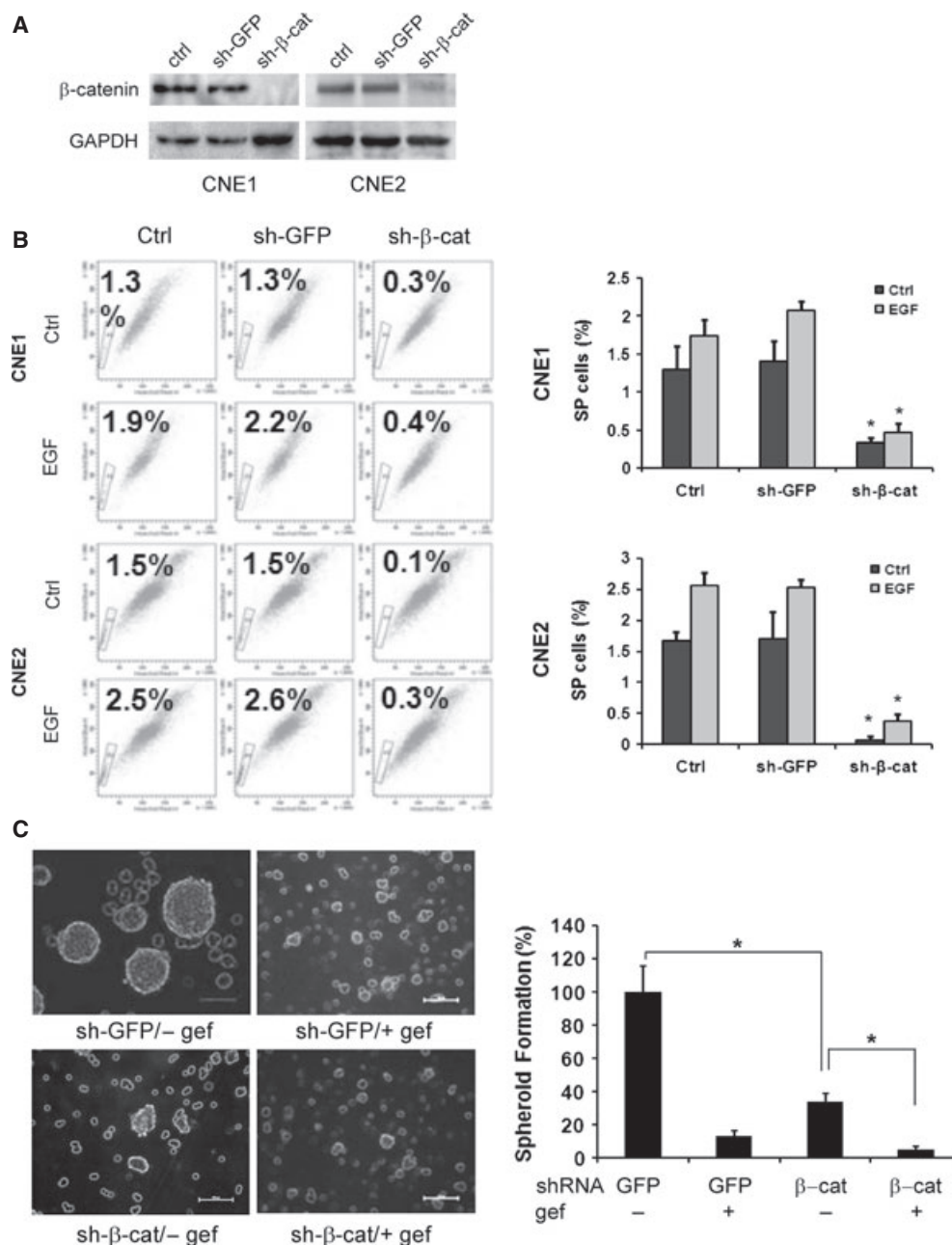


Fig. 5. Knockdown of β -catenin inhibits CSC properties. (A) CNE1 and CNE2 cells were infected with control shRNA lentivirus or β -catenin shRNA. In both cell lines, β -catenin protein was markedly reduced following expression of β -catenin shRNA. (B) Infection with β -catenin shRNA lentivirus reduced the percentage of SP cells at the basal level and after EGF ($50 \text{ ng}\cdot\text{mL}^{-1}$) stimulation. Bar = mean \pm SD ($n = 3$). $*P < 0.01$. (C) Compared with cells infected with the control shRNA (sh-green fluorescent protein) lentivirus, cells infected with sh- β -catenin lentivirus exhibited reduced tumor spheroid formation in the absence or presence of gefitinib. Bar = mean \pm SD ($n = 3$). $*P < 0.01$. Scale bars, 100 μm .

commonly prescribed chemotherapeutic drug for patients with NPC. Mice were killed 2 weeks later, and tumor size was measured. Treatment with gefitinib resulted in a 53% decrease in the mean tumor size, whereas cisplatin dramatically reduced the tumor size

by 82% when compared to the control treatment (Fig. 6A). Drug toxicity was evaluated by measuring body weight of the mice. Gefitinib-treated mice showed only a modest (7%) weight loss. By contrast, cisplatin treatment resulted in a 30% reduction in

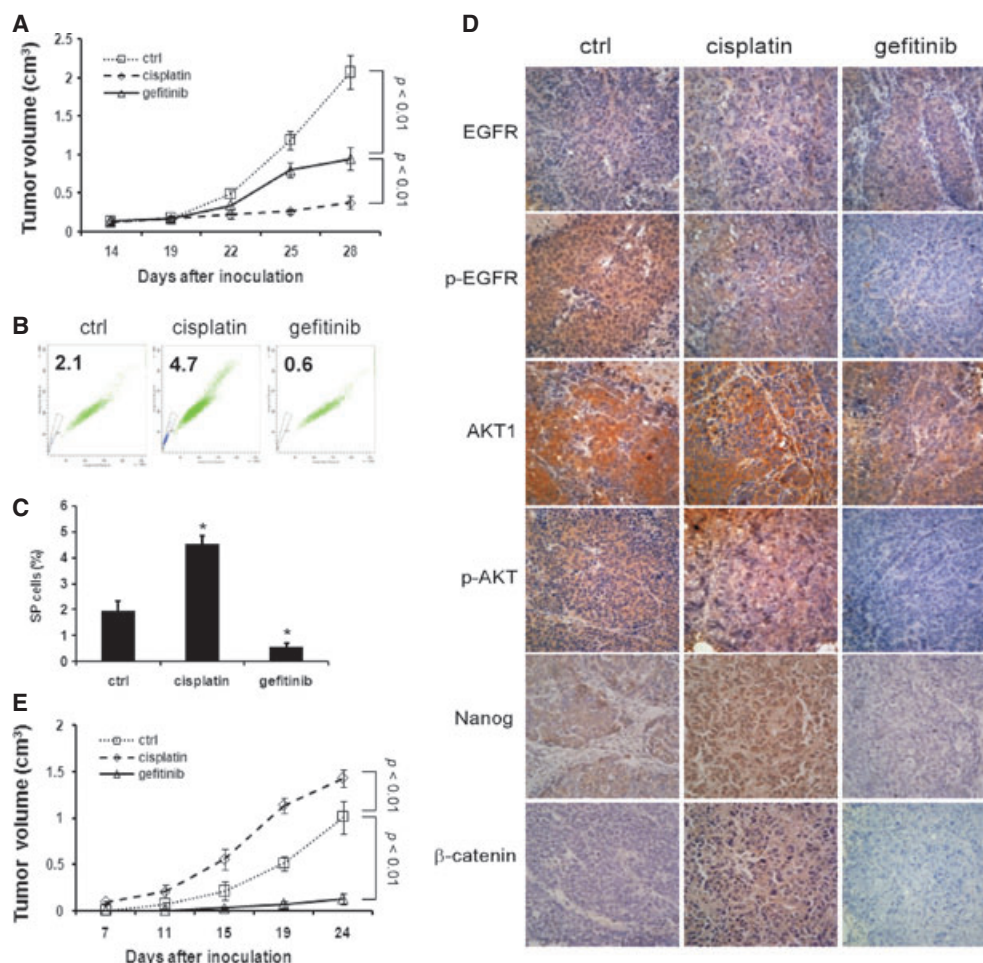


Fig. 6. Inhibition of CSCs by gefitinib in NPC xenografts. (A) Nude mice harboring CNE2 cell xenografts were treated with saline, gefitinib, or cisplatin for 2 weeks as described in Materials and methods. Graphs represent mean volume \pm SD ($n = 6$). $P < 0.01$. (B, C) Cells dissociated from primary xenografts were subjected to FACS analyses. Percentages of SP cells were significantly decreased by gefitinib and increased by cisplatin. Bar = mean \pm SD ($n = 3$). $*P < 0.01$. (D) Immunohistochemical staining of primary tumors from the mouse model. Photographs were taken at $\times 200$ magnification. (E) Cells from primary xenografts were reimplanted into nude mice for development of secondary tumors. Gefitinib abrogated tumor regeneration in secondary mice. Graphs represent mean volume \pm SD ($n = 6$).

body weight (data not shown), underscoring the differential toxicities of the two drugs.

To determine if CSCs are affected by these treatments, tumor tissues were isolated from animals and disaggregated into single cells. CSCs were evaluated by SP analyses (Fig. 6B). Compared with the control, gefitinib reduced the percentage of SP cells by 71%, whereas cisplatin increased the SP-positive populations by 123%. To further determine the effects of gefitinib and cisplatin on EGFR/AKT/ β -catenin signaling, primary tumor sections were stained immunohistochemically with antibodies against different components of this pathway (Fig. 6D). Although neither cisplatin nor gefitinib had an effect on the expression levels of EGFR or AKT1/2, gefitinib, but not cisplatin, markedly inhibited phosphorylation of EGFR and AKT.

Importantly, whereas tumors from control mice exhibited faint and diffused staining of β -catenin, treatment with cisplatin resulted in strong nuclear accumulation of the protein that was barely noticeable in tumors treated with gefitinib. Furthermore, the stem cell maker Nanog was reduced by gefitinib and moderately increased by cisplatin.

To determine tumorigenic potential of the residual cancer cells spared by drug treatments, we performed a more definitive assay to evaluate their self-renewal ability in nude mice [33]. For this purpose, 1×10^5 living cells dissociated from primary xenografts were reimplanted into nude mice to assess the development of secondary tumors. Analysis of the resulting tumors from each group revealed that cancer cells from cisplatin-treated mice grew rapidly and formed visible

tumors by day 7, whereas cells from the control group produced tumors by day 11. By contrast, the regrowth capability of cells from gefitinib-treated mice was severely diminished in the recipient animals. At 24 days, tumors derived from xenografts of cisplatin-treated mice reached an average size of $1.42 \pm 0.09 \text{ cm}^3$, 1.4 times larger than those from the control mice ($1.01 \pm 0.18 \text{ cm}^3$) (Fig. 6E). The above xenograft experiments were performed in duplicate, with similar results obtained. In summary, our *in vitro* and *in vivo* experiments indicate that gefitinib preferentially targets CSCs and eliminates tumor cell regrowth, whereas cisplatin predominantly kills the bulk population of the tumor, leading to enrichment of CSC and fostering tumor regrowth.

Expression of EGFR, β -catenin and Nanog is correlated in human NPC samples

Our *in vitro* and *in vivo* experiments demonstrate that CSCs are regulated by EGFR signaling. To determine whether the same effect of EGFR occurs in primary tumors from NPC patients, we performed an immunohistochemical analysis using a tumor tissue microarray consisting of paraffin-embedded NPC samples derived from 22 patients using antibodies against EGFR, β -catenin and Nanog. In addition, the microarray slides also contain cores from normal nasopharyngeal epithelial tissues, used as negative controls (Fig. 7A). Positive immunostaining for EGFR was found in 12 of 22 tumors (54.5%). Eleven of the 12 (92%)

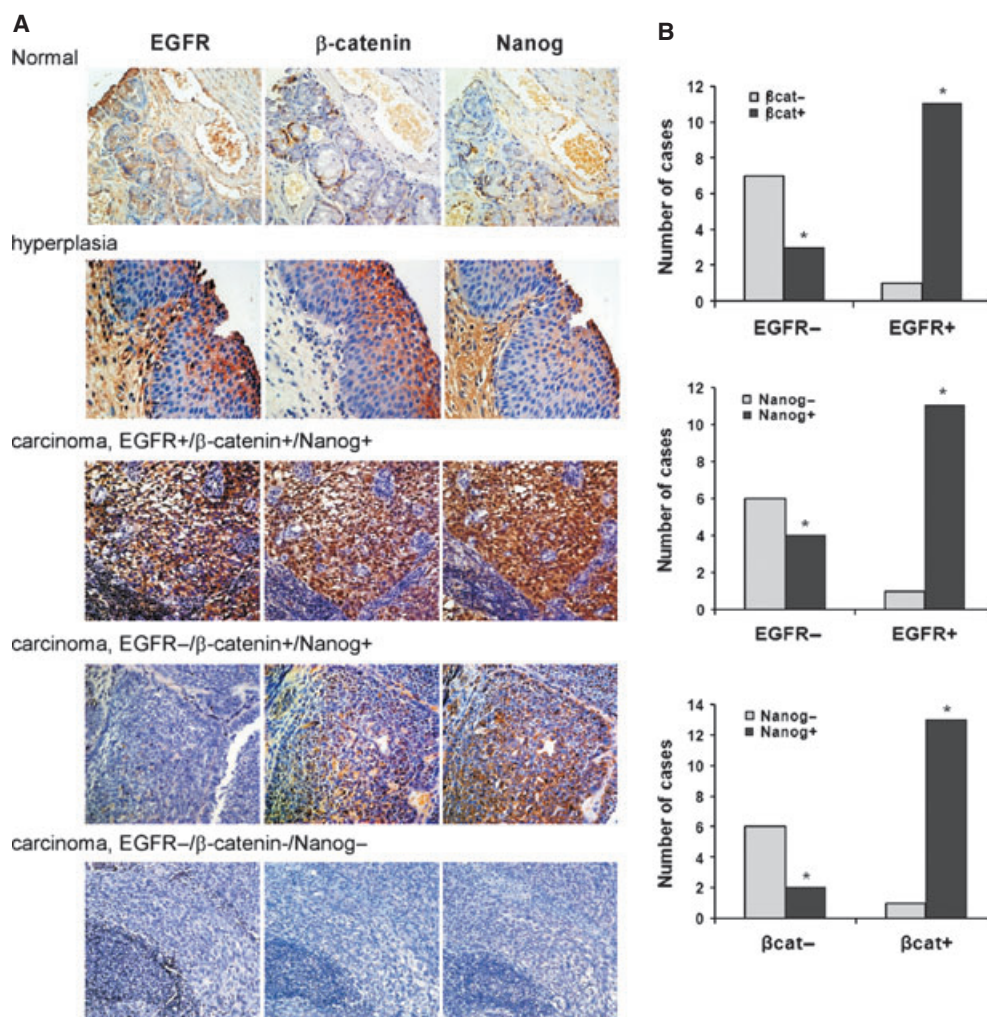


Fig. 7. Correlation of expression of EGFR, β -catenin, and Nanog in human NPC samples. (A) Immunohistochemical studies of representative NPC specimens showing staining of EGFR, β -catenin and Nanog. Normal and hyperplastic nasopharyngeal tissues were included as controls. Photographs were taken at $\times 200$ magnification. (B) Expression of EGFR is associated with that of both β -catenin ($P < 0.01$) and Nanog ($P < 0.05$), and immunoreactivity of β -catenin is associated with that of Nanog ($P < 0.01$).

specimens that stained positively for EGFR also displayed both β -catenin and Nanog immunoreactivity. Among the 10 EGFR-negative specimens, three displayed both β -catenin and Nanog staining, whereas six showed neither β -catenin nor Nanog staining, and one stained only for Nanog (Fig. 7B, upper and middle graphs). Importantly, both β -catenin and Nanog displayed nuclear staining patterns in cancer tissues, whereas β -catenin showed cell membrane localization and Nanog exhibited cytoplasmic distribution in hyperplasia specimens. Statistical analysis of these results revealed that EGFR positivity is significantly associated with that of β -catenin and Nanog ($P < 0.01$ and $P < 0.05$, respectively). Intriguingly, immunoreactivity of β -catenin also correlated significantly with that of Nanog ($P < 0.01$), with 12 of 22 tumors (58%) tumors displaying both β -catenin and Nanog staining, and 7 of 22 (34%) tumors displaying neither β -catenin nor Nanog staining (Fig. 7B, lower graph).

Discussion

We have shown that the EGFR pathway plays an important role in regulation of CSC properties in NPC. We found that CSC numbers are increased by EGF and suppressed not only by the EGFR specific inhibitors gefitinib or PD153035, but also by siRNA-mediated knockdown of EGFR. It has been reported previously that gefitinib inhibits ABC transporters involved in the generation of the SP phenotype [34,35]. Our experiments with siRNA-mediated knockdown of EGFR suggested that EGFR signaling is essential for the CSC phenotype and inhibition of EGFR by gefitinib contributes significantly to suppression of SP cells. We further demonstrated that regulation of the CSC phenotype by EGFR is dependent mainly on the PtdIns3K/AKT pathway. β -Catenin is a key effector of Wnt signaling and is also a substrate of AKT. AKT stimulates β -catenin nuclear translocation and activation either directly through phosphorylation of β -catenin at S552 [12], or indirectly through phosphorylation and inactivation of GSK3 β , resulting in hypophosphorylation of β -catenin at S33/S37/T41 [15]. By immunofluorescent staining and western blot analysis, we have elucidated a functional EGFR/PtdIns3K/AKT/ β -catenin axis that regulates the CSC phenotype in NPC cells. In response to EGF, β -catenin is translocated from the plasma membrane/cytoplasm to the nucleus. Treating cells with either gefitinib or LY294002, however, prevented EGF-induced β -catenin nuclear localization. We also showed that genetic inhibition of β -catenin expression via lentiviral shRNA blocks the effect of EGF stimulation. In NPC xenograft models, we found that residual CSCs

spared by drug treatment were reduced in gefitinib-treated mice and enriched in cisplatin-treated animals. These results reveal profound differences between the effects of gefitinib and cisplatin on CSCs versus the bulk tumor cell population of a NPC. Importantly, our study also provides clinical evidence that expression of EGFR correlates significantly with that of β -catenin and Nanog in primary tumor specimens from NPC patients. Thus, our results demonstrate for the first time a critical role of β -catenin in mediating the effect of EGFR/PtdIns3K/AKT signaling on CSCs in NPC.

The generation of a continuously growing tumor is a fundamental property of CSCs. In NPC xenograft models, we determined that CSCs among the residual tumor cells spared by drug treatment in 'first-generation' mice were reduced by gefitinib but not by cisplatin. In fact, treatment with cisplatin actually increased the proportion of residual cell with CSC properties. In the secondarily transplanted mice, cells transplanted from the cisplatin-treated first-generation mice grew more rapidly and formed larger tumors compared with the cells from a control group. By contrast, cells transplanted from the gefitinib-treated mice developed tumors that were barely noticeable in the second generation animals. These results reveal distinct effects of gefitinib and cisplatin on CSCs versus the bulk tumor cell population.

Our findings have potential clinical implications. Gefitinib was the first targeted drug to enter clinical use for the treatment of nonsmall cell lung cancer [36]. Subsequently, it has been evaluated in patients with different epithelial cancers with variable responses [36]. Several multicenter phase III clinical trials in patients with nonsmall cell lung cancer demonstrated that patients with activating *EGFR* mutations have significantly longer progression-free survival if they are treated with gefitinib as a first-line therapy than if treated with cisplatin [37–39]. In a phase II study of NPC patients who had recurrent or metastatic tumors after prior platinum-based chemotherapy, gefitinib had little activity as a monotherapy [40]. However, the failure of this clinical trial may be explained by the inability of gefitinib to target CSCs that had accumulated more mutations under the selection pressure of the first-line chemotherapeutic agents such as cisplatin. In accord with our assumption, a recent phase II study of NPC employing the EGFR inhibitor cetuximab, in combination with other therapies, showed significant and encouraging improvement over previous modalities. The trial, consisting of concurrent cetuximab-cisplatin and radiotherapy in NPC, resulted in a 2-year distant-metastases-free survival of 92.8% and a loco-regional failure-free rate of 93% [41]. In this study, we establish

potent anti-CSC activity of gefitinib in xenograft models of NPC. Our results provide not only strong pre-clinical evidence, but also a mechanism supporting the use of gefitinib in combination with a chemotherapeutic agent in first-line therapy for patients with NPC.

Resistance to gefitinib or other EGFR tyrosine kinase inhibitors represents a major therapeutic challenge. Several clinical studies have shown that despite initial responses to gefitinib, all patients will eventually develop resistance to this agent. The *EGFR* T790M point mutation, c-Met oncogene amplification, and PtdIns3K/AKT activation account for most of the acquired resistance seen in the clinic [42,43]. In line with these observations, we have provided evidence that the EGFR/AKT/ β -catenin axis regulates CSCs that are preferentially targeted by gefitinib, and modulation of various components of the pathway was able to alter the effects of gefitinib on CSC properties. This suggests that resistance to gefitinib or other EGFR inhibitors in NPC may result from aberrant activation of any component of the EGFR/AKT/ β -catenin pathway. Since we showed that AKT, but not ERK, is the major mediator of EGFR in regulating the CSC phenotype, our results provide the rationale for targeting AKT to overcome gefitinib resistance conferred by either *EGFR* mutations or by EGFR-independent activation of AKT such as c-Met amplification, ErbB3 overexpression, or loss of PTEN. In addition, our findings also suggest that activation of β -catenin is sufficient to confer resistance to gefitinib and that targeting β -catenin may represent an effective modality for patients whose tumors harbor activated EGFR or AKT.

EGFR and Notch pathway interaction have fundamental roles in regulating stem and progenitor cell signaling in *Drosophila*, *Caenorhabditis elegans*, and mammals [44]. Recently it was found that β -catenin activates Notch signaling through inducing Hes1 and downregulating Atoh1 [45]. Interestingly, a more recent study with NPC cell lines found that Notch signaling was highly activated in SP cells and Notch inhibition resulted in depletion of SP [46]. Here we showed that effects of EGFR signaling on SP and tumor spheroids are mediated by β -catenin. SP cells possess CSC properties and are regulated by drug transporters ABCG2, ABCB1 and ABCC1–5 [25]. Our results suggest that β -catenin may represent a missing link connecting EGFR and Notch pathways. Thus, Notch signaling could be activated in response to EGF stimulation in our system and this possibility is currently under investigation.

In conclusion, our results show that EGFR signaling plays a crucial role in the regulation of stem cells of nasopharyngeal cancer. EGFR exerts these effects

through the downstream effectors AKT/ β -catenin. Importantly, the EGFR tyrosine kinase inhibitor gefitinib preferentially targets the CSC population. By contrast, the chemotherapeutic agent cisplatin predominantly eliminates the general tumor cell population. These findings provide preclinical evidence supporting the use of gefitinib alone or in combination with a chemotherapeutic agent in first-line therapy for patients with NPC. In addition, our results suggest that targeting β -catenin represents a rational clinical modality for patients whose tumors harbor activated EGFR or AKT.

Materials and methods

Cell lines and reagents

All cell lines were preserved in our laboratory. The NPC cell lines CNE1, CNE2, HNE1, HONE1, SUNE1, C666–1 and 5–8F were maintained in RPMI–1640 medium supplemented with 10% FBS. A normal human nasopharyngeal epithelial cell line, NP69, was maintained in keratinocyte–serum-free medium (SFM) supplemented with bovine pituitary extract and recombinant EGF (Invitrogen, Carlsbad, CA, USA). Gefitinib (AstraZeneca, Wilmington, DE, USA) was purchased from the Pharmacy of Nanfang Hospital of South Medical University. Cisplatin, Hoechst 33342, propidium iodide and LY294002 were from Sigma-Aldrich (St. Louis, MO, USA). PD0325901 and PD153035 were from Calbiochem (Billerica, MA, USA). Antibodies against the following proteins were used: EGFR, phospho-EGFR and phospho-AKT (Cell Signaling Technology, Danvers, MA, USA); AKT, ERK1/2, phospho-ERK1/2, β -catenin, Nanog and GAPDH (Santa Cruz Biotechnology, Santa Cruz, CA, USA); active β -catenin (Millipore, Temecula, CA, USA).

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide cell viability assay

The inhibitory effect of gefitinib on NPC cell viability was evaluated by using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT; Sigma-Aldrich). CNE1 or CNE2 cells were seeded on a 96-well culture plates at a density of 1500 cells per well and treated with increasing concentrations of gefitinib as indicated in the figure. MTT reagent was added to the plate 72 h after treatment and absorbance was measured at 590 nm.

Tumor spheroid formation assay

Tumor spheroid culture was performed as described previously [28]. Single cells were plated in Ultra Low Attachment plates (Corning, Acton, MA, USA) in serum-free Dulbecco's modified Eagle's medium–F12 supplemented

with 10 ng·mL⁻¹ bFGF, 10 ng·mL⁻¹ EGF and B27 (all from Invitrogen). Under these conditions, the cells grew in suspension as spherical clusters. To assess whether spheroid formation is EGF-dependent, cells were cultured in the above medium with or without EGF. Primary spheroids were dissociated enzymatically with trypsin to obtain a single-cell suspension and then replated to evaluate self-renewal by formation of secondary tumor spheroids.

SP analysis

CNE1 and CNE2 cells in logarithmic growth phase were serum-starved for 24 h followed by 16 h treatment with EGF (50 ng·mL⁻¹) in the absence or presence of gefitinib (1 μM). Subsequently, cells were suspended in Dulbecco's modified Eagle's medium plus 2% FBS at 1 × 10⁶ cells·mL⁻¹ and stained with Hoechst 33342 dye (5 μg·mL⁻¹) for 90 min at 37 °C with continuous mixing. Following incubation, cells were washed with ice-cold NaCl/P_i, stained with propidium iodide (1 μg·mL⁻¹) and maintained at 4 °C for flow cytometric analyses and for sorting of SP fraction using a FACSAria Flow cytometer (Beckton Dickson, Franklin Lakes, NJ, USA). The Hoechst dye was excited with an UV laser at 351–364 nm, and its fluorescence was measured with a 515-nm side population filter (Hoechst blue) and a 608 EFLP optical filter (Hoechst red). A 540 DSP filter was used to separate the emission wavelengths.

Western blot analysis

Cells were lysed, and then equal amount of protein were subjected to electrophoresis on a SDS/PAGE gel. The separated proteins were transferred to poly(vinylidene difluoride) membranes (Millipore) and probed with appropriate primary antibodies. Protein bands were detected by using enhanced chemiluminescence reagents (Pierce Biotechnology, Rockford, IL, USA).

Immunohistochemistry

For immunohistochemistry, paraffin-embedded sections were deparaffinized in xylene and rehydrated in a graded alcohol series. Antigen retrieval was performed by boiling the slide preparations in 10 mM sodium citrate buffer, pH 6.0. Staining was carried out using an EliVision Plus Kit (Maixin Bio, Beijing, China), according to the manufacturer's protocol. DAB was used as a substrate for peroxidase, and expression levels were evaluated following criteria reported elsewhere [47].

Immunofluorescent staining

For immunofluorescent staining, cells were grown on the surface of cover slides and fixed with 4% paraformaldehyde.

After rehydration in NaCl/P_i, the fixed cells were incubated with respective primary antibodies at room temperature for 1 h or at 4 °C overnight. Fluorescein isothiocyanate-conjugated secondary antibodies were incubated for 30 min at room temperature. The nuclei were stained with 2,6-diaminopimelic acid. Sections were examined with a Nikon Eclipse 80i fluorescent microscope (Nikon Instruments, Inc., Melville, NY, USA).

Lentiviral constructs and infection of NPC cells

Both pLKO.1 lentiviral shRNA vector and control shRNA targeting GFP were from Sigma-Aldrich. The β-catenin targeting sequence, GCTTGGAAATGAGACTGCTGAT, has been described previously [48]. The sense and antisense oligonucleotides were annealed and ligated into pLKO.1 lentiviral vector. The viruses were then packaged in 293T cells according to standard protocols. Viral production and infection of target cells were previously described [49]. Infected cells were selected using 2 μg·mL⁻¹ puromycin.

In vivo tumorigenicity assay

Animal studies were conducted in strict accordance with the principles and procedures approved by the Committee on the Ethics of Animal Experiments of Southern Medical University. Nude mice (BALB/C nu/nu) were fed autoclaved water and laboratory rodent chow. A volume of 100 μL of culture medium mixed with Matrigel (BD Biosciences, San Jose, CA, USA) containing 3 × 10⁶ CNE2 cells was transplanted into the flanks of mice by subcutaneous injection. Two weeks after implantation, the mice were randomly separated into different treatment groups (six mice per arm) and subjected to one of the following treatments: group 1, control mice receiving either intraperitoneally injected or orally administrated control solvent; group 2, mice treated with gefitinib administrated orally at 150 mg·kg⁻¹ each every day; and group 3, mice treated with cisplatin injected intraperitoneally twice weekly at 3 mg·kg⁻¹. The animals were monitored daily, and tumor volumes were measured every 3 days using a caliper slide rule. Tumor volume was calculated as follows: $V = 1/2 (\text{width}^2 \times \text{length})$.

After treatment for 2 weeks, animals were humanely killed and tumors harvested. To obtain a single-cell suspension, tumors were minced using scalpels and incubated in RPMI-1640 medium containing collagenase/hyaluronidase at 37 °C for 60 min. The tissues were further dissociated by pipet trituration and then passed through a 40-μm nylon mesh to produce a single-cell suspension used for subsequent experiments. In the secondary tumor experiment, 1 × 10⁵ cells dissociated from first-generation tumors were implanted into the flanks of recipient mouse (six mice per group).

Statistical analysis

Data were expressed as means \pm SD. Significant differences between groups were determined by analysis of variance and by Student's *t*-test. Graphs summarizing immunohistochemical staining results were analyzed using the Fisher's exact test. Differences were considered significant when the $P < 0.05$.

Acknowledgements

We thank Dr Craig Menges for helpful discussion. This study was supported by the National Basic Research Program of China (973 Program, No 2010CB529401) and a Natural Science Foundation of China grant (81072205). JRT's work on AKT is supported, in part, by NCI Grant CA77429.

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