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Original Article

Synergistic cooperation of EGFR/Stat3/Smad3 axis induces human nasopharyngeal carcinoma cells apoptosis

Shitao Zhang, Minghui Zhou, Dong Dong, Jia Wang, Yulin Zhao

Department of Rhinology, The First Affiliated Hospital of Zhengzhou University, Zhengzhou, China

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Abstract: Tumor growth factors-β (TGF-β) signaling is associated with regulation in various types of cancer cells apoptosis. Epidermal growth factor receptor (EGFR) signaling is involved in the progression of human nasopharyngeal carcinoma (NPC). Whether there is a crosstalk between TGF-β and EGFR signaling that regulating NPC cells apoptosis remains unclear. Here, we report agonist-mediated TGF-β signaling activation induced Smad2/3 phosphorylation leading to human NPC CNE1 cells apoptosis *in vitro*. Antagonist-mediated EGFR inhibition synergistically promotes TGF-β activation-induced CNE1 cells apoptosis. Inhibition of EGFR expression by siRNA led a reduction on Stat3 phosphorylation, which results in over expression of TGF-β-activated Smad3 phosphorylation in CNE1 cells. These results show that EGFR inhibition synergistically promotes TGF-β-induced human NPC cells apoptosis through EGFR/Stat3/Smad3 Signaling cooperation. The novel molecular network potentially gives insights to the molecular-targeted therapeutics development for human nasopharyngeal carcinoma.

Keywords: EGFR, TGF-β, Stat3, human nasopharyngeal carcinoma, apoptosis

Introduction

Nasopharyngeal carcinoma (NPC) is one of the common head and neck malignancies in Southern China and Southeast Asia. Despite the higher sensitivity to radiation, local recurrence is still a clinical challenge in 80% patients after radiation treatment [1]. Although modern anticancer techniques and strategies have provided opportunities to improve patient overall survival and prognosis in recent years, underlie molecular mechanisms of NPC pathogenesis remained understood. Thus, better understanding the precise molecular mechanisms of NPC pathogenesis are essential for exploring the novel molecular-targeted therapies for NPC treatment.

It has been reported that transforming growth factor β (TGF-β) signaling pathway plays a key role in the regulation of development, tissue homeostasis, tumorigenesis and metastasis [2]. It signals through the Smad2 and Smad3 proteins phosphorylation by TGF-β ligand-receptor interaction. Phosphorylated Smad2 and Smad3 transfer in the nucleus and regulate numerous genes transcriptions including growth arrest and apoptosis induction [3]. TGF-β/Smad2/3 signaling exerts the opposite roles in cancer including tumor promotion effect [4, 5] or tumor suppression effect [6, 7] in different type tumors. In NPC patients, hyperactivation of TGF-β signaling were associated with lymph node metastasis, suggesting that TGF-β signaling has a role in the anticancer therapy for NPC [8].

Recently, epidermal growth factor receptor (EGFR) gene has emerged as a new target for NPC therapy. It reported that EGFR overexpression occurred in over 80% NPC patients and correlated with shorter survival after radiation therapy [9]. Previous studies also showed that EGFR tyrosine kinase inhibitor (TKI) increased the cell sensitivity to radiation and inhibited human NPC cells proliferations [10]. However, whether there is a crosstalk between TGF-β and EGFR signaling that cooperatively regulate NPC cells apoptosis remain unclear. In current study, we studied the pro-apoptotic effects of TGF-β

signaling on human NPC cells *in vitro*, and further explored the potential crosstalk between TGF-β and EGFR signaling that synergistically induce human NPC cell apoptosis.

Materials and methods

Experimental drugs

TGF-β and LY2109761 were purchased from Sigma-Aldrich (St Louis, MO, USA), AEE788 and SIS3 were from MedChem Express LLC (NJ, USA). The human nasopharyngeal carcinoma cell line CNE1 was purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA).

Cell culture

CNE1 was cultured in RPMI1640 (Gibco, USA) containing 10% FBS (Hyclone, China), supplemented with 100 U/ml penicillin and 100 μg/ml streptomycin (Life Technologies, Grand Island, NY, USA). The cells were maintained at 37°C in a humidified 5% CO_2 incubator.

CCK-8 assay

Cell Counting Kit-8 (Sigma-Aldrich, St Louis, MO, USA) was chose to determine cell viability following the manufacturer's instructions. Briefly, CNE1 cells were plated in 96-well cell culture plates at the concentration of 5×10^3 cells per well. The medium was removed 24 h later and replaced with fresh medium without or with various doses (0-20 ng/ml) of TGF-β for 24, 48, and 72 h respectively. At various time points, 10 μl of CCK-8 solution was added into each well of the plate and the plates were incubated for 4 h in the incubator, and then measured at 450 nm and the absorbance is directly proportional to cell viability.

Apoptosis detection

For apoptosis assay, CNE1 were collected and washed with cold PBS twice after the indicated drug treatment. Then the cells were resuspended in 1× binding buffer [0.01 mmol/l HEPES (pH 7.4), 0.14 mol/l NaCl, and 2.5 mmol/l $CaCl_2$] at a concentration of 2×10^6 cells/ml. 100 μl of cell solution was transferred into a 5 ml culture tube, and 5 μl of Annexin V-FITC and 5 μl Propidium iodide (Beyotime Biotechnology, Shanghai, China) were added. After gentle vortex and incubation for 15 minutes at room temperature in the dark, 400 μl of the 1× binding buffers was added into each tube, and cells were analyzed by flow cytometry within 1 hour.

Western blot

Cells were collected into 1.5 ml EP tubes and were lysed with lysis buffer (Beyotime, Shanghai, China) on ice for 20 min. The supernatant of lysates were transferred into a new EP tube after 12000 g centrifugation at 4°C for 10 min. The tube contained protein lysates were add β-mercaptoethanol with a ratio of 20:1, followed by incubation at 100°C for 10 min. Total proteins were separated by 10% SDS-PAGE gels. Proteins were then electrophoretically transferred onto PVDF membranes and incubated with the primary antibodies Rabbit anti human p-Smad2 (sc-135644), Smad2/3 (sc-376928), p-Stat3 (sc-135649), Mouse anti human EGFR (sc-377229), Bad (sc-8044), caspase-3 (sc-65496), GAPDH (sc-365062) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, US); Mouse anti human Bcl-2 (ab77567), p-Smad3 (ab63403) were purchased from Abcam (Cambridge, UK) after blocking in 5% fat-free milk dissolved in Tris-buffered saline with Tween-20 (TBST). The HRP-conjugated anti-rabbit or anti-mouse second antibodies (Beyotime, Shanghai, China) were applied subsequently, and a chemiluminescent substrate system was used to detect the signals. Band intensity was analyzed using ChemiDoc XRS software (Bio-Rad Laboratories, Hercules, CA, USA).

EGFR-siRNA transfection

CNE1 cells were transfected with EGFR siRNA using LipofectamineTM 2000 (Invitrogen, USA) according to the manufacturer's instructions. The transfection complexes, prepared by adding EGFR siRNA transfection reagent, were incubated for 25 min at room temperature, and then the cells were incubated with transfection complexes for 6 h before cultured in corresponding mediums. EGFR protein levels were then determined by Western blot for transfection efficacy.

Statistical analysis

The paired-samples t-test was applied to check the significance in cell lines experiments by the Graph Pad Prism 5 software (San Diego, CA,

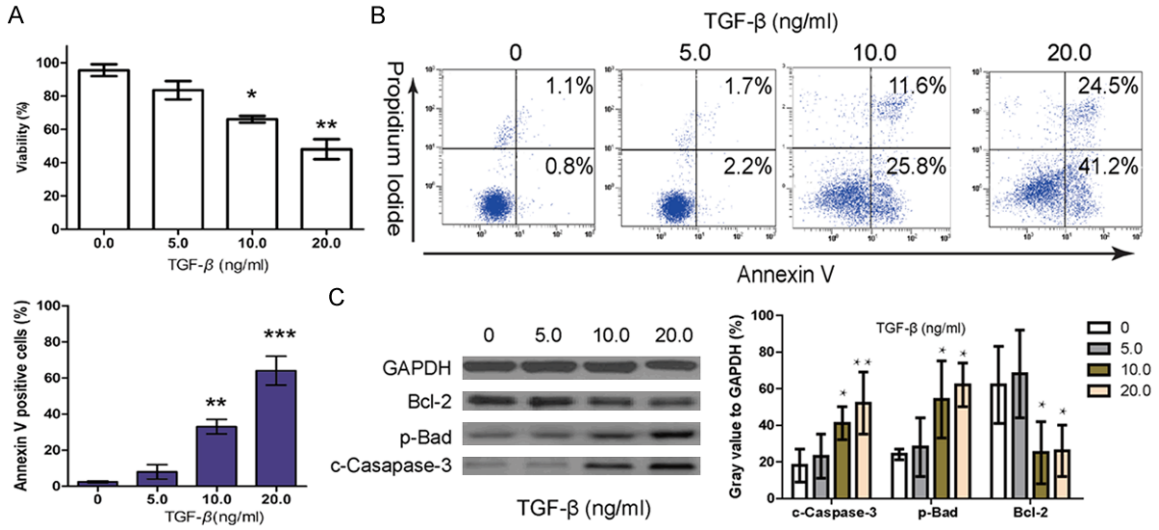

Figure 1. TGF-β promotes human NPC cell apoptosis. Human NPC cell lines CNE1 was cultured with (0-20 ng/ml) TGF-β for 48 hs. A. Representative histogram of CNE1 viability analyzed by CCK-8 after 48 hs TGF-β treatment; B. Graphic cytometry scatter dots representative of CNE1 cells apoptosis analyzed by Annxin V/PI staining. Quantitation data from three separate experiments of CNE1 cells apoptosis analyzed by Annxin V/PI staining. C. The amount of cleaved Caspase 3 (c-Caspase 3), Bad and Bcl-2 proteins in lysates from CNE1 analyzed by western blot. The quantification of the amount of c-Caspase 3, Bad and Bcl-2 proteins were from three independent experiments. Results are showed as the mean ± SEM. *P<*0.05*, **P<0.01, ***P<0.001; compared with the control.

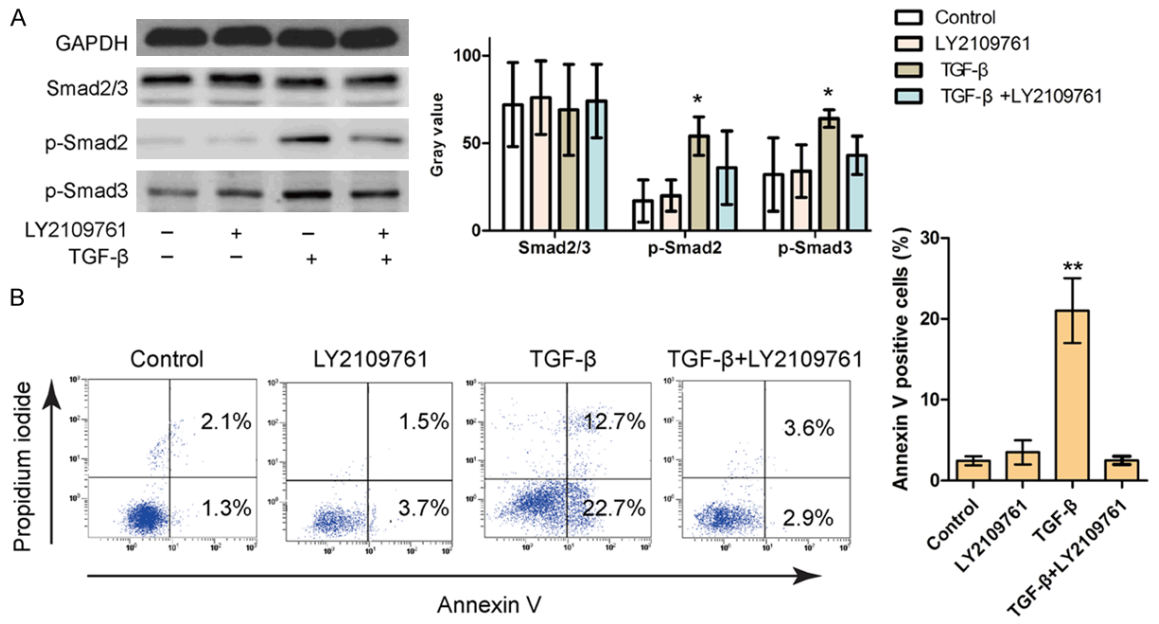

Figure 2. Smad2/3 phosphorylation is involved in TGF-β-induced NPC cells apoptosis. A. Human NPC cell lines CNE1 were treated with 5 μM LY2109761 for 4 hs, and then washed by cold PBS and followed by 10 μM TGF-β treatment for 48 hs, respectively. CNE1 were treated with the same volume of DMSO for 48 hs as negative control. The amount of Smad2/3 and phosphorylated Smad2 (p-Smad2), phosphorylated Smad3 (p-Smad3) proteins in lysates from CNE1 were analyzed by western blot after 48 hs treatment. The quantification of Smad2/3 and p-Smad2, p-Smad3 proteins were from three independent experiments. B. The CNE1 cells were analyzed by Annexin V/PI staining after 48 hs treatment mentioned above. The quantification of the apoptotic rate of Annexin V-positive cells was from three independent experiments. Results are showed as the mean ± SEM. n = 3, *P<0.05, **P<0.01; compared with the control.

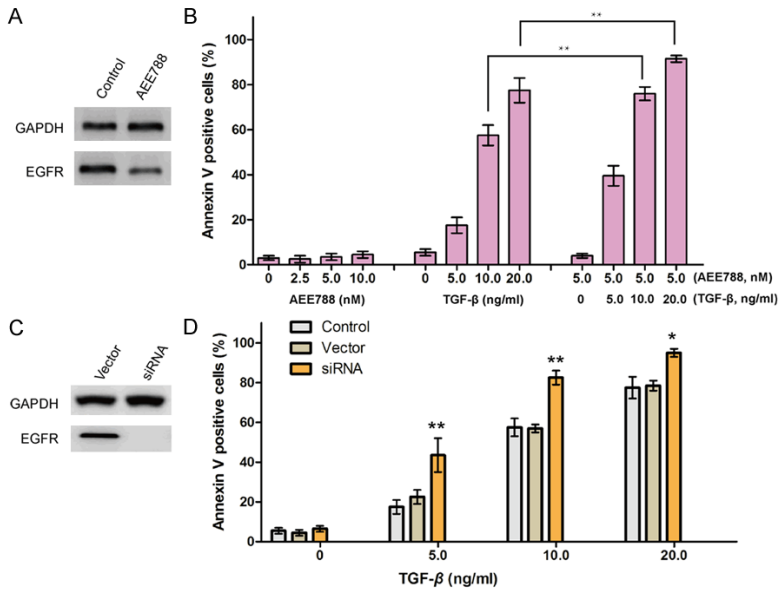

Figure 3. EGFR inhibition synergistically induces TGF-β-mediated NPC cells apoptosis. A. The protein expressions of EGFR were detected by Western blot in CNE1 after 5 nM AEE788 treatment for 4 hs, compared with the control. B. Human NPC cell lines CNE1 were treated with 5 nM AEE788 for 4 hs, and then washed by cold PBS and followed by (0-20 ng/ml) TGF-β for 48 hs, respectively. Annexin V-positive cells were analyzed by Annexin V/PI staining after 48 hours treatment. Results are showed as the mean ± SEM., **P<0.01; C. The protein expressions of EGFR were detected by Western blot in CNE1 cells transfected with EGFR siRNA or vectors. D. Normal CNE1 cells (Control), EGFR siRNA-transfected (siRNA) and vector-transfected (Vector) cells were treated by (0-20 ng/ml) TGF-β for 48 hs, respectively. Annexin V-positive cells were analyzed by Annexin V/PI staining after 48 hours treatment. Results are showed as the mean ± SEM., *P<0.05, **P<0.01; the quantification of the apoptotic rate of Annexin V-positive cells was from three independent experiments.

UAS). For cell lines experiments, histograms show mean values of the indicated number of experiments, with error bars corresponding to SEM values.

Results

TGF-β promotes human NPC cell apoptosis

TGF-β/Smads signaling is involved in mediating apoptosis process in several cell types [11, 12]. To address the effects of TGF-β signaling on human NPC cells *in vitro*, the human NPC cell lines CNE1 were treated with various doses of TGF-β (0-20 ng/ml). CCK-8 assay showed that TGF-β led a dose-dependent decrease in CNE1 cell viability *in vitro* (**Figure 1A**). Next, we performed the Annxin V/PI staining to explore whether the decreased viability were contributed by cell apoptosis. As is showed in **Figure 1B**, TGF-β treatment led a significant increase on Annxin V-positive cells in a dose-dependent manner. TGF-β treatment led a significant increase of phosphorylated Bad and caspase-3 expressions, but decrease on Bcl-2 expression (**Figure 1C**).

Smad2/3 phosphorylation is involved in TGF-β-induced NPC cells apoptosis

To define whether Smad2 and Smad3, the two of major downstream effectors of TGF-β signaling, directly participate in the TGF-β-induced NPC cells apoptosis *in vitro*, we detected the expressions of phosphorylated Smad2 (p-Smad2) and Smad3 (p-Smad3) in NPC cells after TGF-β treatment. It showed that the level of p-Smad2 and p-Smad3 in CNE1 were significantly unregulated after TGF-β signaling activation by TGF-β treatment compared with the control (**Figure 2A**). Next, we used the TGF-β receptor I/II (TβRI/II) inhibitor LY21097-61 to block TGF-β signaling activation for further confirmation. It showed that the expressions of p-Smad2 and p-Smad3 were significantly downregulated by LY2109761 compared with that in TGF-β treatment group. It further supported by the Annexin V staining results that TGF-β-induced cells apoptotic changes were inhibited by the TGF-β signaling inhibitor LY2109761 (**Figure 2B**). It indicates that Smad2/3 phosphorylation is directly involved in TGF-β-mediated human NPC cells apoptosis *in vitro*.

EGFR inhibition synergistically induces TGF-β-induced NPC cells apoptosis

It has been reported that robust expression of epidermal growth factor receptor (EGFR) is involved in NPC metastasis and short survival [13]. Therefore, the role of EGFR in NPC cells apoptosis *in vitro* was detected. As shown in **Figure 3A** and **3B**, EGFR tyrosine kinase inhibitor AEE788-mediated EGFR inhibition did not induce significant apoptotic change on CNE1 cells by Annexin V/PI staining. Next, we found that combined treatment of TGF-β and AEE788

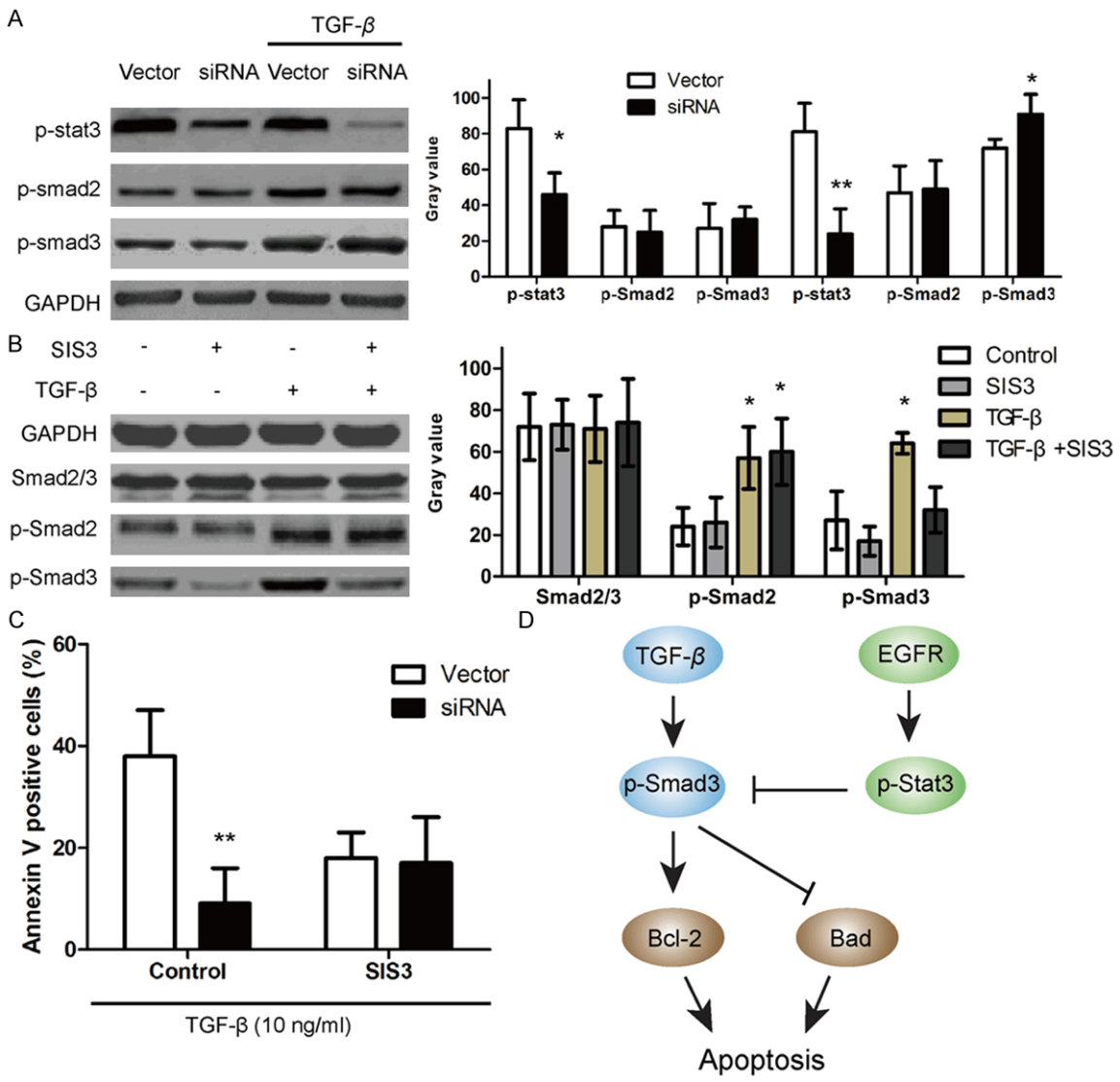

Figure 4. NPC cell apoptosis is mediated by the EGFR/Stat3/Smad3 Signaling. EGFR siRNA-transfected (siRNA) and vector-transfected (Vector) CNE1 cells were cultured with the 10 ng/ml TGF-β for 48 hs, respectively. A. The amount of p-Smad2, p-Smad3 and p-Stat3 proteins in lysates from CNE1 was analyzed by western blot. B. CNE1 cells were cultured with 3 μM SIS3 for 4 hs, and then washed by cold PBS and followed by the 10 ng/ml TGF-β treatments for 48 hs, respectively. C. EGFR siRNA-transfected (siRNA) and vector-transfected (Vector) CNE1 cells were cultured with 3 μM SIS3 for 4 hs, and then washed by cold PBS and followed by the 10 ng/ml TGF-β treatment for 48 hs, respectively. CNE1 were treated with the same volume of DMSO as control. Annexin V-positive cells were analyzed by Annexin V/PI staining after 48 hs treatment. D. The schematic chart of human NPC cells apoptosis mediated by the cooperation of EGFR/Stat3/Smad3 signaling. All the quantification data were from three independent experiments. Results are showed as the mean ± SEM., $^{*}P<0.05$, $^{**}P<0.01$; compared with the control.

induced a much more significant apoptotic changes on CNE1 cells than TGF-β treatment alone. It indicates that AEE788 pose a synergistic effect on TGF-β-induced CNE1 cells apoptosis. To further verify the role of EGFR inhibition in TGF-β-induced CNE1 cells apoptosis, EGFR siRNA was transfected into CNE1 cells to inhibit the expression of EGFR (**Figure 3C**). EGFR knockdown led to a higher percentage of Annexin V positive cells in CNE1 (**Figure 3D**). These data demonstrate EGFR inhibition synergistically promotes TGF-β-induced NPC cells apoptosis.

NPC cell apoptosis is mediated by the EGFR/Stat3/Smad3 signaling

The EGFR/Stat3 signaling is involved in various types of cell apoptosis [14]. Next, we explored

whether EGFR inhibition synergistically induce TGF-β-mediated NPC cells apoptosis through Stat3 regulation. We found that the expressions of p-Stat3 were suppressed in EGFR siRNA-transfected CNE1 cells. There is no significant difference on the expressions of p-Smad3 and p-Smad2 between EGFR siRNA-transfected and vector-transfected CNE1 cells. Consistent with the results that TGF-β treatment led increased expressions of p-Smad3 and p-Smad2 in CNE1 cells in **Figure 2A**, the expressions of p-Smad3 and p-Smad2 were also upregulated in EGFR siRNA-transfected CNE1 cells after TGF-β treatment (**Figure 4A**). We also found that the expressions of TGF-β-mediated p-Smad3 upregulation in EGFR siRNA-transfected CNE1 cells are more abundant than that in vector-transfected cells. But, the expressions of p-Smad2 are similar in these two cells. It indicates that EGFR inhibition upregulate TGF-β-induced human NPC CNE1 cells apoptosis specifically through promoting Smad3 phosphorylation. To further confirm it, the Smad3 specific inhibitor SIS3 was utilized to inhibit Smad3 activation. It showed that SIS3 could remarkably decrease TGF-β-induced Smad3 phosphorylation, but minimal effects on Smad2 phosphorylation (**Figure 4B**). Next, the TGF-β-induced apoptotic changes on EGFR siRNA-transfected CNE1 cells and the vector ones were detected after p-Smad3 inhibition by SIS3 treatment, respectively. It showed that the level of TGF-β-induced apoptotic changes on EGFR siRNA-transfected CNE1 cells and the vector ones are similar after p-Smad3 inhibition (**Figure 4C**). It indicates that EGFR inhibition synergistically promotes TGF-β-induced NPC cells apoptosis through the cooperation of EGFR/Stat3/Smad3 signaling (**Figure 4D**).

Discussion

The current study showed that activation of TGF-β signaling induced human nasopharyngeal carcinoma cells apoptosis *in vitro*. The pro apoptotic effects of TGF-β signaling were also reported in others cancer cells types [6, 7]. Furthermore, we found EGFR inhibition by antagonist reinforce the tumor suppression effects of TGF-β signaling activation on human NPC cells through the crosstalk of EGFR/Stat3/Smad3 signaling. These data gives us insights that precise regulation and proper operation of TGF-β and EGFR signaling could virtually enhance the anti-cancer efficiency in human nasopharyngeal carcinoma treatment.

TGF-β receptor engaged by extracellular ligands initiates TGF-β/Smad2/3 signaling activation led to cytoplasmic Smad2 and Smad3 phosphorylation [15]. Phosphorylated Smad proteins were transported into nucleus to regulate various genes transcriptions that involved in a wide range of cellular processes including cell proliferation, apoptosis, differentiation and migration [16, 17]. The regulation of TGF-β/Smad2/3 on tumor cells is two-fold. On the one hand, it exerts the tumor promotion effect in glioblastoma [4]. One the other hand, it has tumor suppression effects in endometrial cancer [6], which is consistent with current results that TGF-β treatment induced NPC cells apoptosis through Smad2/3 phosphorylation (**Figure 2**). This phenomenon occurs mainly because some tumor cells may escape the inhibitory effects of the canonical TGF-β/Smad2/3 signaling through mutations that can provide a growth advantage suppression effect [7].

It has been reported that epidermal growth factor receptor (EGFR) is robustly abundant in most NPC patients [10, 18]. Its abnormal expressions are related to human cancers pathogenesis. EGFR overexpression is also associated with shorter survival and metastasis in locoregionally advanced NPC [19]. In current study, we found that EGFR signaling inhibition had minimal effects on NPC cells apoptosis *in vitro*, but synergistically induced TGF-β-mediated NPC cells apoptosis (**Figure 3**), suggesting s potential crosstalk between TGF-β and EGFR signaling. It widely reported that EGFR/Stat3 participate in the regulation of different cancer cells proliferation and apoptosis [20-22]. In that case, EGFR inhibition dephosphorylated Stat3 at the site of Tyr705 or Ser727 by the direct interaction, leading to cells apoptosis by stat3 inactivation [23]. We found that EGFR inhibition reduced Stat3 phosphorylation. Down-expression of p-Stat3 attenuated the inhibitory effects on p-Smad3 leading to a higher expression of p-Smad3, which results in over expression of TGF-β-activated Smad3 phosphorylation (**Figure 4D**). It subsequently produced synergistic effects on NPC cells apoptosis. These finding was also supported by the report that the STAT3-Smad3 interaction contributes to STAT3-mediated inhibition of TGF-β signaling in human keratinocyte cell line HaCaT cells [24].

In conclusion, our studies showed that agonist-mediated TGF-β activation induced human NPC

cells apoptosis *in vitro*. Antagonist-mediated EGFR inhibition poses synergistic apoptosis-inducing effects of TGF-β activation on human NPC cells. The synergistic apoptosis-inducing effects were mainly contributed by the interaction of EGFR/Stat3/Smad3 axis. Thoroughly understanding the novel pro apoptotic molecular mechanisms will enrich our insights to develop the related treatment strategies for nasopharyngeal carcinoma.

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Yulin Zhao, Department of Rhinology, The First Affiliated Hospital of Zhengzhou University, NO. 1 Eastern Jianshe Road, Zhengzhou 450052, Henan, China. Tel: +86 0371 6796 7276; Fax: +86 0371 6796 7276; E-mail: yulinzhao@outlook.com

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