

# Interplay between the NF- $\kappa$ B and hedgehog signaling pathways predicts prognosis in esophageal squamous cell carcinoma following neoadjuvant chemoradiotherapy

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**Abstract.** Tumor recurrence and metastasis in esophageal squamous cell carcinoma (ESCC) are primary causes of patient mortality. The nuclear factor (NF)- $\kappa$ B signaling pathway and hedgehog signaling pathway were previously reported to contribute to cell growth and metastasis in ESCC. The present study therefore investigated the roles of the NF- $\kappa$ B and hedgehog pathways in ESCC tumors following neoadjuvant chemoradiotherapy (NCRT). By immunohistochemistry staining, it was observed that NF- $\kappa$ B and glioma-associated oncogene homolog 1 (Gli1), key components of the NF- $\kappa$ B and hedgehog pathways, respectively, were decreased following NCRT, which was further confirmed by western blotting and reverse transcription-quantitative polymerase chain reaction analysis. In addition, survival analysis suggested that high expression levels of either NF- $\kappa$ B or Gli1 were associated with poor overall survival (OS) of patients. In the esophageal cell line TE-8, NF- $\kappa$ B and Gli1 formed a positive feedback loop, and inhibition of either NF- $\kappa$ B or Gli1 may inhibit cell migration, invasion and proliferation. The results of the present study demonstrated that activation of the NF- $\kappa$ B and hedgehog signaling pathways limited the OS of patients with ESCC following NCRT, and may therefore be suitable targets for ESCC treatment.

## Introduction

Esophageal cancer is a lethal malignancy with >440,000 new cases arising around the world every year (1). In Asia, squamous cell carcinoma is the primary type among a variety of esophageal cancers (2). Curative surgery for esophageal squamous cell carcinoma (ESCC) was believed to be promising and may provide an increased chance of survival for patients with ESCC. However, with a 5-year survival rate of 20-50%, the prognosis of these patients is unsatisfactory (3,4). Distant metastases following surgery is a major cause of mortality for these patients. Although early studies indicated that neoadjuvant chemoradiotherapy (NCRT) may effectively reduce lymph node metastasis and offer a good opportunity for margin-negative resection, it remains controversial whether NCRT improves treatment outcomes in patients with resectable ESCC (5,6).

The nuclear factor (NF)- $\kappa$ B pathway contains a number of transcription factors (RelA/p65, c-Rel, RelB, p50 and p52), which may form  $\geq 12$  kinds of homodimers or heterodimers. NF- $\kappa$ B p65 is the most well-studied transcription factor of the NF- $\kappa$ B signaling pathway. Activation of the NF- $\kappa$ B pathway releases p65 from its inhibitor, and promotes the translocation of p65 into the nucleus to drive the transcription of various key genes (7). The phosphorylation of p65 induces a conformational change to enhance its binding to DNA (8). Incorrect regulation of NF- $\kappa$ B has been implicated in a number of types of disease, including cancer (9). Research in ESCC cell lines and ESCC tissues has indicated that the NF- $\kappa$ B pathway is constitutively activated in ESCC, and targeting NF- $\kappa$ B may effectively block fast cell growth and inhibit the strong metastatic ability of ESCC cells (10). In patients with ESCC, activation of the NF- $\kappa$ B pathway was closely associated with a poor prognostic outcome, and it was deemed likely that patients with a complete pathological response may benefit from NCRT (11).

The hedgehog signaling pathway is one of the key mediators of development in humans. It is involved in embryonic formation, tissue homeostasis, tumor initiation and tumor development (12,13). Due to its central role in stem cell regeneration, the hedgehog pathway is crucial for the

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maintenance of cancer cell stemness and thus contributes to cancer cell metastasis (14). The glioma-associated oncogene homolog 1 (Gli1), a zinc finger transcription factor, is the key mediator of the hedgehog pathway that regulates a number of genes important for tumor occurrence and progression (15). Hyper-activation of Gli1 has been implicated in a number of cancer types. In ESCC, the hedgehog pathway was activated upon epidermal growth factor stimulation and cooperated with the phosphatidylinositol 3-kinase/RAC- $\alpha$  serine/threonine-protein kinase pathway and mitogen-activated protein kinase pathway to promote cancer cell survival and growth (16). Overexpression of Gli1 was observed in ESCC tissues, particularly in ESCC cells with strong invasive and metastatic capabilities (17). Gli1 was positively regulated by the NF- $\kappa$ B pathway in claudin-low breast cancer (18). However, the association between the hedgehog pathway and the NF- $\kappa$ B pathway, and their status in response to NCRT, were largely unknown.

In the present study, it was demonstrated that the NF- $\kappa$ B pathway and the hedgehog pathway were hyperactivated in patients with ESCC following NCRT. Low expression of either NF- $\kappa$ B or Gli1 was associated with better overall survival (OS). In addition, there was a strong association between NF- $\kappa$ B p65 and Gli1 in ESCC patient samples. In the ESCC cell line TE-8, there was a decrease in cell proliferation and cellular metastasis following inhibition of the NF- $\kappa$ B pathway or hedgehog pathway by small molecules. Notably, inhibition of the NF- $\kappa$ B pathway induced a sharp decrease in Gli1, whereas inhibition of the hedgehog pathway inactivated the NF- $\kappa$ B pathway. The data suggested that overactivation of and interplay between the NF- $\kappa$ B pathway and the hedgehog pathway were involved in poor prognosis in patients with ESCC who underwent NCRT.

## Materials and methods

**Patients.** Between July 2006 and September 2010, tumor samples from 54 patients with ESCC who underwent NCRT prior to surgery at the Nanjing General Hospital of Nanjing Command (Nanjing, China) were collected following surgical resection. Tissue samples were immediately stored at -80°C until use. The tumors of patients were staged according to the American Joint Committee on Cancer (Edition 7) (19). The patients included 41 men and 13 women, aged between 40 and 78 years. A total of 11 patients were classified as stage II, 30 patients were classified as stage III, and 13 patients were classified as stage IV. The study was approved by the Ethics Committee of Nanjing General Hospital of Nanjing Command and written consent from each patient was obtained.

**Neoadjuvant chemoradiotherapy and surgery.** All 54 patients received NCRT prior to surgery. Chemotherapy included 5-fluorouracil (5-FU) in combination with cisplatin (CDDP). 5-FU was administrated at 500 mg/m<sup>2</sup> per day by a 5 h continuous intravenous (i.v.) infusion starting on day 1, and CDDP was administered at 25 mg/m<sup>2</sup> at a 2-h i.v. infusion on days 1-5. During radiation therapy, patients received five fractions of 2 Gy radiation per week over 4 weeks, at a total dose of 30-40 Gy. In the first week, radiation therapy was conducted in combination with chemotherapy, whereas radiation therapy alone was performed for the next 3 weeks. After 4 weeks

of NCRT, total thoracic esophagectomy was performed and tumor tissues were used for following experiments.

**Immunohistochemistry (IHC) staining.** Primary antibodies against NF- $\kappa$ B p65 (cat. no. 8242S) and Gli1 (cat. no. 3538S) were obtained from Cell Signaling Technology, Inc. (Danvers, MA, USA). Horseradish peroxidase (HRP)-conjugated goat anti-rabbit secondary antibody (cat. no. SA00001-2) was purchased from ProteinTech Group, Inc. (Chicago, IL, USA). ESCC tumor samples were fixed with 4% formalin for 2 h at room temperature and then processed using the Max Vision™ kit (Fuzhou Maixin Biotech Co., Ltd., Fuzhou, China) by following the manufacturer's protocol. Briefly, all samples were subjected to antigen retrieval by heating at a high temperature of 95°C in 0.01 M sodium citrate buffer (pH 6.0) for 20 min. Tissues were embedded in paraffin. Subsequently, 3% H<sub>2</sub>O<sub>2</sub> was added to the slices to block the activity of endogenous peroxidase at room temperature for 15 min. The sections were then incubated with anti-NF- $\kappa$ B (1:500) or anti-Gli1 (1:300) antibodies at 37°C for 1 h. The slices were washed with PBS and incubated with secondary antibody (1:2,000) for 30 min at room temperature. The signal was developed with DAB solution for 5 min at room temperature. Hematoxylin was used for nuclei visualization for 30 sec at room temperature. For semi-quantification of NF- $\kappa$ B and Gli1 expression, images from NF- $\kappa$ B- or Gli1-stained slides were captured at x40 magnification under a standard light microscope (five fields per slide). The threshold values used for NF- $\kappa$ B and Gli1 were positive if  $\geq 10\%$  cells exhibited clear positive staining with the antibodies.

**Cell culture and reagents.** The ESCC cell line, TE-8, was purchased from RIKEN BioResource Center (Tsukuba, Japan). The cells were cultured in RPMI-1640 medium (Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (HyClone; GE Healthcare, Chicago, IL, USA) and 1% penicillin-streptomycin (Thermo Fisher Scientific, Inc.), in a 37°C incubator supplemented with 5% CO<sub>2</sub>. GANT61 and Bay 11-7082 were purchased from Selleck Chemicals (Houston, TX, USA).

**Western blotting.** The 5- $\mu$ m frozen tissue samples at -80°C were thawed on ice, and lysates were prepared with radio-immunoprecipitation assay lysis buffer (Beyotime Institute of Biotechnology, Haimen, China). The antibody against phospho-NF- $\kappa$ B p65 (Ser536) (cat. no. 3033S; 1:1,000) was purchased from Cell Signaling Technology, Inc. and the anti-GAPDH antibody (cat. no. G8795; 1:8,000) was purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). The HRP-conjugated goat anti-mouse antibody (cat. no. SA00001-1; 1:10,000) was purchased from ProteinTech Group, Inc.

The concentration of protein lysates was determined using a Bicinchoninic Acid kit for Protein Determination (Sigma-Aldrich; Merck KGaA). Protein lysates (30  $\mu$ g each) were loaded onto an 8% SDS-PAGE gel, and subsequently transferred to a polyvinylidene difluoride membrane. The membrane was blocked with 5% non-fat milk at room temperature for 1 h, and incubated with the indicated primary antibodies at room temperature for 1 h, and followed by incubation with

secondary antibodies at room temperature for a further 1 h. Blots were developed with SuperSignal West Femto Maximum Sensitivity substrate (Pierce; Thermo Fisher Scientific, Inc.) and the images were obtained by using ImageQuant LAS 4000 (GE Healthcare) using ImageQuant TL 8.0.

**Reverse transcription-quantitative-polymerase chain reaction (RT-qPCR).** For RT-qPCR, total RNA from tumor samples was prepared using an RNeasy kit (Qiagen GmbH, Hilden, Germany), according to the manufacturer's protocol. Reverse transcription of RNA was performed with a PrimeScript RT reagent kit (Takara Biotechnology Co., Ltd., Dalian, China). PCR was performed using SYBR Premix Ex Taq kit (Takara Biotechnology Co., Ltd.) on a Bio-Rad CFX96 Real-Time PCR system (Bio-Rad Laboratories, Inc., Hercules, CA, USA) and normalized to the internal control GAPDH. The qPCR program was as follows: Stage 1, 95°C for 30 sec; stage 2, 95°C for 5 sec and 60°C for 30 sec (35 repeats). The relative expression of genes was calculated using the  $2^{-\Delta\Delta C_q}$  method (20). The primer sequences were as follows: GAPDH forward, AAT CCCATCACCATCTTCCA; GAPDH reverse, TGGACTCCA CGACGTACTCA; NF- $\kappa$ B p65 forward, ATGGCAGACGAT GATCCCTAC; NF- $\kappa$ B p65 reverse, CGGAATCGAAATCCC CTCTGTT; Gli1 forward, GTGCAAGTCAAGCCAGAACA; and Gli1 reverse, ATAGGGGCCTGACTGGAGAT.

**Cell invasion assay.** Cell invasion assays were performed using Transwell permeable supports (Corning Incorporated, Corning, NY, USA), according to manufacturer's protocol. Cells of 90% confluence were treated and incubated with dimethyl sulfoxide (DMSO; 0.02%), GANT61 (2  $\mu$ M) or Bay 11-7082 (1  $\mu$ M) in a 37°C incubator for 24 h, then plated onto a Matrigel-coated membrane in the upper chamber of a 24-well insert containing serum-free RPMI-1640 medium. The bottom chamber contained RPMI-1640 medium supplemented with 10% FBS. The cells were subsequently cultured with DMSO (0.02%), GANT61 (2  $\mu$ M) or Bay 11-7082 (1  $\mu$ M) in a 37°C incubator for 48 h. Subsequently, the bottom of the chamber insert was collected and fixed with 10% methanol for 5 min, and stained with crystal violet for 5 min at room temperature. Cells that remained in the upper chamber were removed with a cotton swab. Images of cells that invaded into the bottom area were captured with an inverted microscope (five fields per well) and the cell number was calculated with ImageJ software (version 1.50) (National Institutes of Health, Bethesda, MD, USA). Each experiment was performed in triplicate.

**Cell proliferation assay.** The cell proliferation assay was conducted with a Cell Counting Kit-8 (CCK-8) assay (Dojindo Molecular Technologies, Inc., Kumamoto, Japan). Briefly, cells were seeded onto a 96 well plate at 50% confluence, and the next day, the medium was replaced with medium containing DMSO (0.02%), GANT61 (2  $\mu$ M) or Bay 11-7082 (1  $\mu$ M) and incubated at 37°C for 72 h. A total of 10  $\mu$ l CCK-8 solution was added into each well and incubated at 37°C for 2 h, and absorbance was measured at a wavelength of 450 nm. The cell proliferation curves were generated using GraphPad Prism version 6.0 software (GraphPad Software Inc., La Jolla, CA, USA).

**Statistical analysis.** All data were analyzed using GraphPad Prism version 6.0 software (GraphPad Software Inc.). The values were expressed as the mean  $\pm$  standard deviation. Student's t-test was applied to compare continuous variables and Pearson's Chi-squared test was employed to compare dichotomous variables.  $P < 0.05$  was considered to indicate a statistically significant difference.

OS was defined as the length of time from the date of ESCC diagnosis to either mortality of the patient or the date of the last available information on vital status. Distant metastasis-free survival (DMFS) was defined as the period from the date of ESCC diagnosis to the date of metastasis detection. Comparison between OS and DMFS between patients with negative and positive IHC staining was achieved using the Kaplan-Meier method.

## Results

**Hyperactivation of the NF- $\kappa$ B and hedgehog signaling pathways in tumors from patients with ESCC following NCRT.** To investigate the status of the NF- $\kappa$ B and hedgehog signaling pathways following NCRT, IHC was performed to evaluate the expression levels of NF- $\kappa$ B p65 and Gli1 in tissue samples from patients who underwent NCRT prior to surgery. A total of 32 out of 54 cases were positive for NF- $\kappa$ B p65, 28 out of 54 cases were positive for Gli1 and 20 cases were positive for NF- $\kappa$ B p65 and Gli1 (Table I). Representative expression for positive and negative staining of NF- $\kappa$ B p65 and Gli1 in ESCC tissue samples was demonstrated in Fig. 1.

To further examine the association between the NF- $\kappa$ B pathway and the hedgehog pathway, RT-qPCR analysis and western blotting were performed to detect the mRNA and protein expression levels of NF- $\kappa$ B p65 and Gli1, respectively. At the transcriptional and translational level, NF- $\kappa$ B p65 was positively associated with Gli1 (Fig. 2A and B). Representative examples of western blotting and RT-qPCR are presented in Fig. 2C and D, respectively.

**High NF- $\kappa$ B p65 and Gli1 expression is associated with poor prognosis.** The NF- $\kappa$ B pathway and hedgehog pathway are involved in cancer initiation and development, and therefore, the present study analyzed OS between patients that exhibited positive and negative NF- $\kappa$ B p65 expression levels. The OS of NF- $\kappa$ B p65-positive patients was significantly lower compared with that of the NF- $\kappa$ B p65-negative patients (Fig. 3A). In addition, comparison of OS between Gli1-positive and -negative patients indicated that Gli1 positivity was associated with a poorer survival rate (Fig. 3B). This association was additionally observed when NF- $\kappa$ B p65 and Gli1 were positive (Fig. 3C). These data implied that NF- $\kappa$ B p65 and Gli1 were important for predicting patient survival, and that NCRT may improve patient treatment outcomes via inhibition of these two proteins.

**NF- $\kappa$ B and hedgehog signaling pathways are crucial for ESCC cell survival and invasion.** To examine the role of the NF- $\kappa$ B pathway and hedgehog pathway in ESCC cell behavior, cell proliferation was determined following inhibition of the NF- $\kappa$ B or hedgehog pathways with inhibitors. Inhibition of NF- $\kappa$ B with Bay 11-7082 resulted in a decrease in cell



Table I. Characteristics of patients.

Characteristic	NF- $\kappa$ B p65		$\chi^2$ test P-value	Gli1		$\chi^2$ test P-value	NF- $\kappa$ B p65 and Gli1		$\chi^2$ test P-value
	Positive cases (%)	Negative cases (%)		Positive cases (%)	Negative cases (%)		Positive cases (%)	Negative cases (%)	
Sex									
Male	24 (44)	17 (31)	1.00	23 (43)	18 (33)	0.35	16 (30)	25 (46)	0.75
Female	8 (15)	5 (9)		5 (9)	8 (15)		4 (7)	9 (17)	
Age (years)									
$\leq 50$	15 (28)	10 (19)	1.00	11 (20)	14 (26)	0.41	10 (19)	15 (28)	0.78
$> 50$	17 (31)	12 (22)		17 (31)	12 (22)		10 (19)	19 (35)	
Clinical stage									
II	3 (6)	8 (15)	0.04	2 (4)	9 (17)	0.01	2 (4)	9 (17)	0.01
III	19 (35)	11 (20)		16 (30)	14 (26)		9 (17)	21 (39)	
IV	10 (19)	3 (6)		10 (19)	3 (6)		9 (17)	4 (7)	
Total	32 (59)	22 (41)		28 (52)	26 (48)		20 (37)	34 (63)	

NF- $\kappa$ B, nuclear factor- $\kappa$ B; Gli1, glioma-associated oncogene homolog 1.

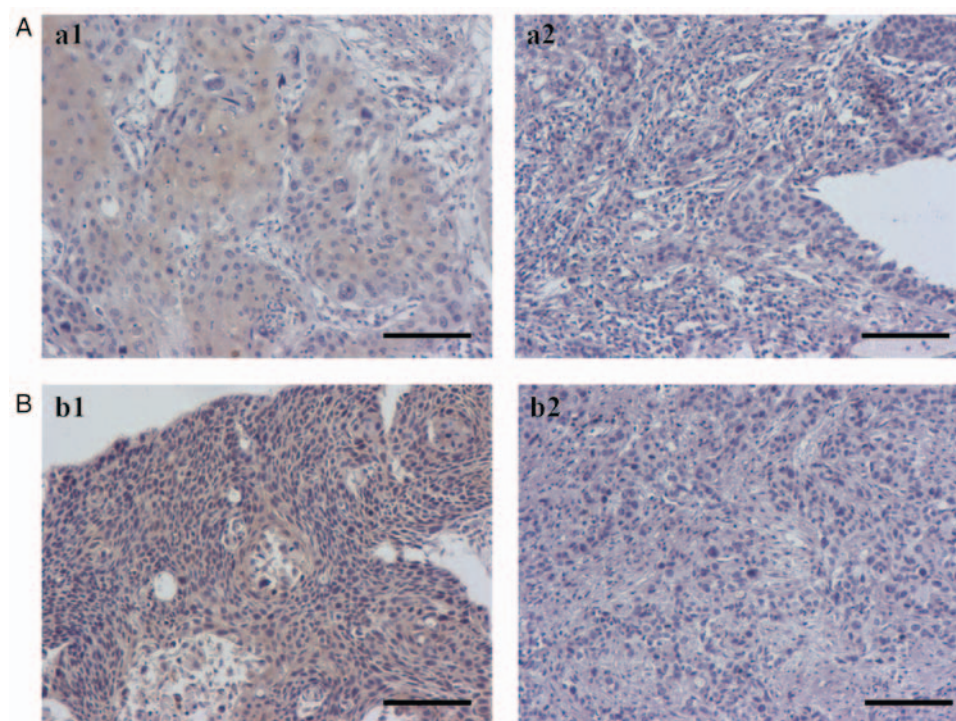


Figure 1. Immunohistochemical detection of NF- $\kappa$ B p65 and Gli1 in esophageal squamous cell carcinoma following neoadjuvant chemoradiotherapy. (A) Representative (a1) positive and (a2) negative staining of NF- $\kappa$ B p65 in tissue samples. (B) Representative (b1) positive and (b2) negative staining of Gli1 in tissue samples. Scale bars, 100  $\mu$ m. NF- $\kappa$ B, nuclear factor  $\kappa$ B; Gli1, glioma-associated oncogene homolog 1.

growth in TE-8 cells, an ESCC cell line (Fig. 4A). In addition, inhibition of Gli1 with GANT61 resulted in a decrease in proliferation rate in TE-8 cells (Fig. 4B). Blocking either the NF- $\kappa$ B pathway or hedgehog pathway reduced the number of cells that invaded through the Matrigel membrane in the chambers, suggesting their importance in promoting ESCC cell metastasis (Fig. 4C and D).

*NF- $\kappa$ B pathway and hedgehog pathway form a positive loop in ESCC cells.* As the NF- $\kappa$ B and hedgehog signaling pathways are crucial for ESCC development, the present study determined their association in ESCC. Inhibition of the NF- $\kappa$ B pathway resulted in a decrease in Gli1 at the mRNA and protein levels (Fig. 5A and B). Treatment with the hedgehog pathway inhibitor reduced the phosphorylation of NF- $\kappa$ B p65 (Fig. 5C).

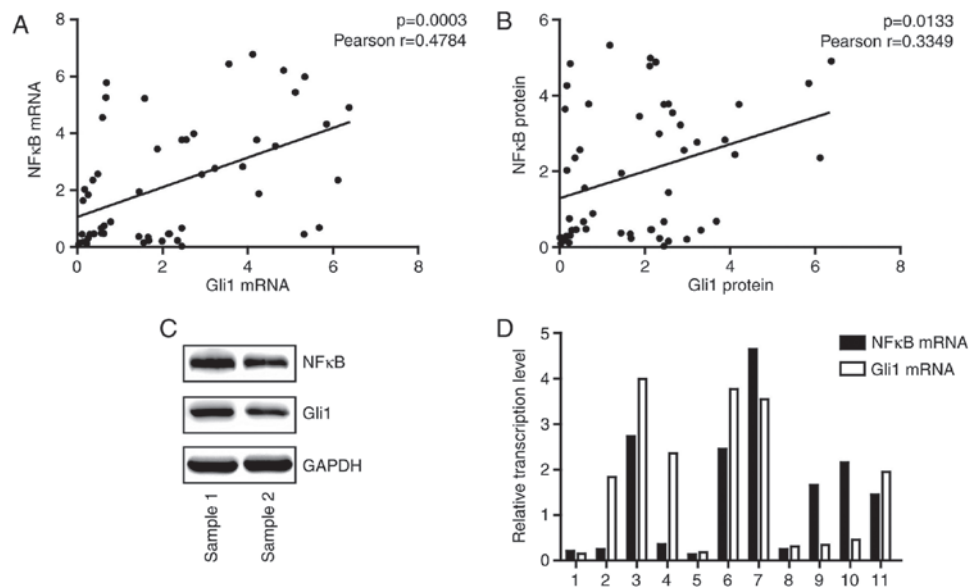


Figure 2. Positive association between the expression levels of NF- $\kappa$ B p65 and Gli1 in ESCC following NCRT. (A) Association between the mRNA expression levels of NF- $\kappa$ B p65 and Gli1 in specimens from patients with ESCC following NCRT. (B) Association between the protein expression levels of NF- $\kappa$ B p65 and Gli1 in specimens from patients with ESCC following NCRT. (C) Immunoblot detection of NF- $\kappa$ B p65 and Gli1 proteins in specimens from patients with ESCC following NCRT. (D) Reverse transcription-quantitative polymerase chain reaction detection of NF- $\kappa$ B p65 and Gli1 mRNA expression levels in specimens from patients with ESCC following NCRT. NF- $\kappa$ B, nuclear factor  $\kappa$ B; Gli1, glioma-associated oncogene homolog 1; ESCC, esophageal squamous cell carcinoma; NCRT, neoadjuvant chemoradiotherapy.

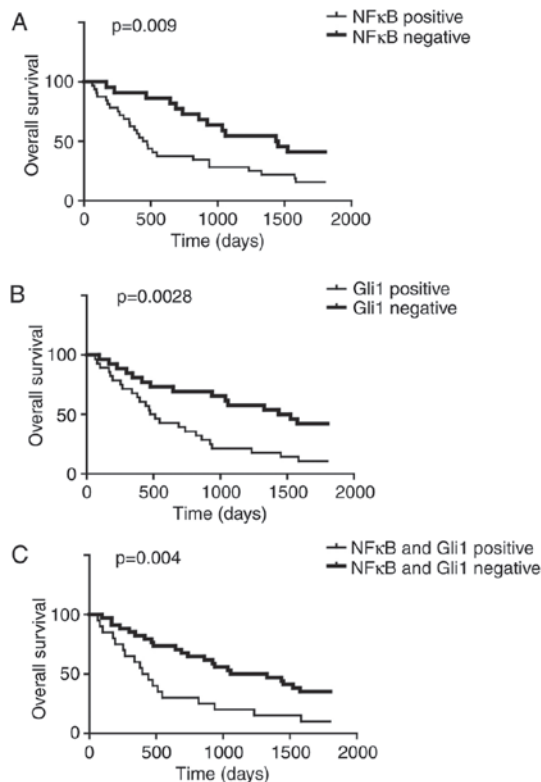


Figure 3. Kaplan-Meier analysis of OS according to immunohistochemical staining of NF- $\kappa$ B p65 and Gli1 in patients with ESCC. (A) OS differences between patients with ESCC undergoing NCRT with NF- $\kappa$ B p65 positive staining, and patients with NF- $\kappa$ B p65 negative staining. (B) OS differences between patients with ESCC undergoing NCRT with Gli1 positive staining, and patients with Gli1 negative staining. (C) OS differences between patients with ESCC undergoing NCRT with NF- $\kappa$ B p65 and Gli1 positive staining, and patients with NF- $\kappa$ B p65 and Gli1 negative staining. OS, overall survival; NF- $\kappa$ B, nuclear factor  $\kappa$ B; Gli1, glioma-associated oncogene homolog 1; ESCC, esophageal squamous cell carcinoma; NCRT, neoadjuvant chemoradiotherapy.

This suggested that interplay between the NF- $\kappa$ B pathway and the hedgehog pathway may exist and that these two pathways may cooperate to promote ESCC development.

## Discussion

Although substantial advantages have been achieved in the screening, diagnosis and treatment of ESCC, the prognosis for patients with ESCC remains poor. Surgical resection was considered to provide a better survival for ESCC patients; however, numerous patients continued to succumb as a result of recurrence or distant metastasis (21). In a phase III randomized trial, compared with the surgical treatment alone group of patients, NCRT prior to surgery did not improve OS, which was 47.5% with trimodal therapy vs. 53% with surgery at 3 years, with a P-value of 0.94 (22). In a large randomized trial that included 366 patients, the OS of trimodal therapy was significantly improved compared with surgery alone, with a median OS of 49.4 months in the NCRT group and 24 months for the surgery group (23). The function of NCRT in ESCC has been debated for a number of years. The dysregulation of numerous proteins has been proven to predict recurrence and prognosis in patients with ESCC following NCRT (24-26). In this respect, inhibition of a number of key molecules was demonstrated to be effective in enhancing the sensitivity of ESCC cells towards chemotherapy or chemoradiotherapy (10,27,28). The present study demonstrated that overactivation of the NF- $\kappa$ B and hedgehog signaling pathways, and their interplay, was associated with poor prognosis post-NCRT.

In esophageal cancer, NF- $\kappa$ B activation prior to therapy was associated with chemotherapy resistance and contributed to metastasis, and eventually led to patient mortality (29). Another study on localized esophageal cancer demonstrated that activated NF- $\kappa$ B was associated with chemoradiation resistance,

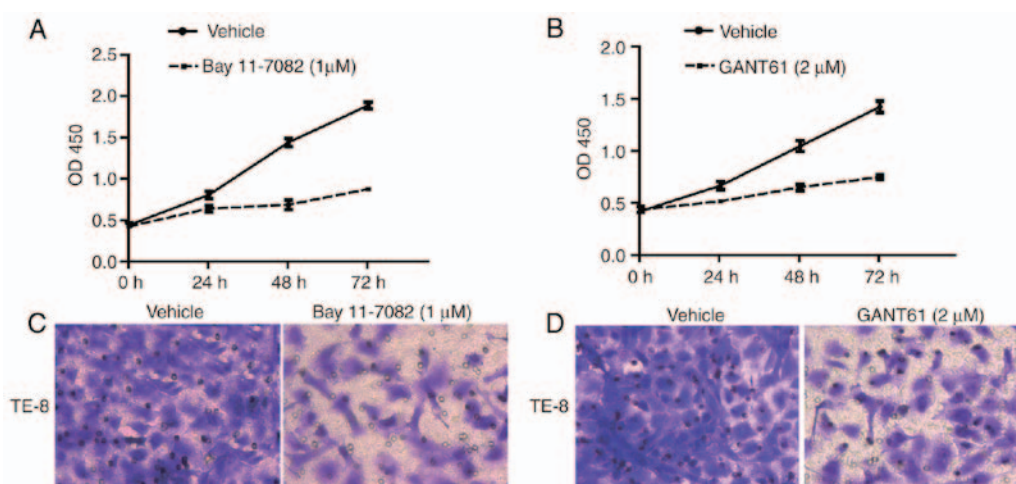


Figure 4. NF- $\kappa$ B and hedgehog signaling pathways contribute to cell proliferation and invasion in the TE-8 esophageal squamous cell carcinoma cell line. (A) Inhibition of the NF- $\kappa$ B pathway by 1  $\mu$ M Bay 11-7082 reduced cell proliferation in TE-8 cells. (B) Inhibition of the hedgehog pathway by 2  $\mu$ M GANT61 reduced cell proliferation in TE-8 cells. (C) Inhibition of the NF- $\kappa$ B pathway by 1  $\mu$ M Bay 11-7082 weakened cell invasion in TE-8 cells (magnification, x200). (D) Inhibition of the hedgehog pathway by 2  $\mu$ M GANT61 weakened cell invasion in TE-8 cells (magnification, x200). NF- $\kappa$ B, nuclear factor- $\kappa$ B.

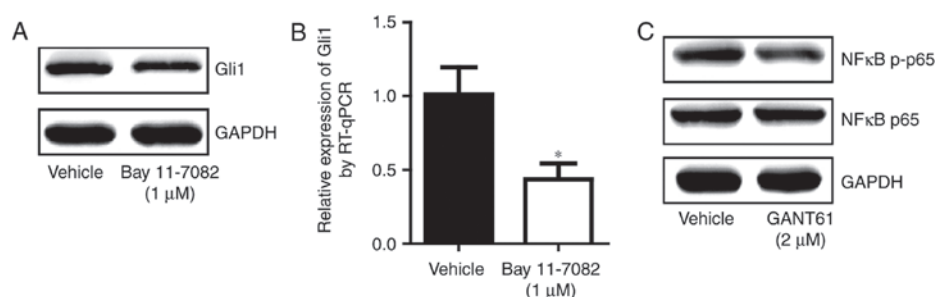


Figure 5. Crosstalk between the NF- $\kappa$ B pathway and hedgehog pathway in TE-8 cells. Inhibition of the NF- $\kappa$ B pathway by 1  $\mu$ M Bay 11-7082 induced a decrease in Gli1 (A) protein and (B) mRNA expression levels in TE-8 cells. (C) Treatment with 2  $\mu$ M Gli1 inhibitor GANT61 resulted in decreased phosphorylation of NF- $\kappa$ B p65 in TE-8 cells. \* $P$ <0.05. NF- $\kappa$ B, nuclear factor  $\kappa$ B; RT-qPCR, reverse transcription-quantitative polymerase chain reaction.

and inversely associated with metastatic potential and OS (30). However, the above studies were based on a cohort of patients including esophageal adenocarcinoma and squamous cell carcinoma. In ESCC, the present study demonstrated that the NF- $\kappa$ B signaling pathway was active in a majority of patients (32 of 54 cases) that received NCRT, and patients with NF- $\kappa$ B p65 positive IHC staining exhibited significantly shorter OS compared with patients with negative staining of NF- $\kappa$ B p65. This suggested that NF- $\kappa$ B was a predictor for poor prognosis in patients undergoing NCRT.

The hedgehog signaling pathway was essential for esophageal tumor formation, and associated with invasion and a poor prognosis. In patients with ESCC undergoing NCRT, Gli1 expression was observed to be a predictor for patients with poor treatment outcomes (31). Inhibitor of NF- $\kappa$ B, an inhibitor of NF- $\kappa$ B signaling, was discovered to serve a role in the phosphorylation of Gli1, and thus regulated its transcriptional activity in diffuse large B cell lymphoma (32). In pancreatic cancer, NF- $\kappa$ B activation induced the activation of the hedgehog pathway by targeting sonic hedgehog protein (33). In the present study, it was confirmed that Gli1 was activated in patients with ESCC following NCRT and was associated with clinical outcome. A total of 20 out of 54 patients exhibited overexpression of NF- $\kappa$ B p65 and Gli1. Additionally, the present

data revealed that the expression of NF- $\kappa$ B p65 was associated with Gli1 in the samples analyzed. In the ESCC cell line TE-8, inhibition of either NF- $\kappa$ B or Gli1 resulted in decreased cell proliferation and cell invasion ability, and treatment with an NF- $\kappa$ B inhibitor reduced the mRNA and protein expression levels of Gli1 in TE-8. A number of oncogenes, including epidermal growth factor receptors (ErbB), have been reported to contribute to carcinogenesis by activating the hedgehog pathway and the NF- $\kappa$ B pathway (34,35). As overexpression of ErbB was frequently observed in ESCC, ErbB may activate the hedgehog and NF- $\kappa$ B pathways to promote ESCC progression. A study in refractory acute myeloid leukemia cells reported that inhibition of smoothened homolog, a transducer of the hedgehog pathway, was accompanied by a decrease in the expression of nuclear NF- $\kappa$ B p65 (36). Inhibition of Gli1 by an inhibitor additionally resulted in inactivation of the NF- $\kappa$ B pathway by reducing the phosphorylation levels of NF- $\kappa$ B p65 in TE-8 cells. Therefore, interplay between the NF- $\kappa$ B pathway and the hedgehog pathway may exist in patients with ESCC undergoing NCRT.

In conclusion, the present study suggested that crosstalk between the NF- $\kappa$ B pathway and hedgehog pathway may be a predictor for prognosis in patients with ESCC following NCRT and, thus, may be a putative therapeutic target.



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## Competing interests

The authors declare that they have no competing interests.

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