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Inhibition of Glycogen Synthase Kinase 3 beta (GSK3 β) Suppresses the Progression of Esophageal Squamous Cell Carcinoma by Modifying STAT3 Activity

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Abstract

While GSK3 β has been reported to have contrasting effects on the progression of different tumors, its possible functions in esophageal squamous cell carcinoma (ESCC) and the related molecular mechanisms remain unknown. Here, we investigated the expression, function, and molecular mechanism of GSK3 β in the development of ESCC *in vitro* and *in vivo*. Though the expression of total GSK3 β was significantly increased, the phosphorylated (inactivated) form of GSK3 β (Ser9) was concurrently decreased in the cancerous tissues of patients with ESCC compared with controls, suggesting that GSK3 β activity was enhanced in cancerous tissues. Further pathological data analysis revealed that higher GSK3 β expression was associated with poorer differentiation, higher metastasis rates, and worse prognosis of ESCC. These results were confirmed in different ESCC cell lines using a pharmacological inhibitor and specific siRNA to block GSK3 β . Using a cancer phospho-antibody array, we found that STAT3 is a target of GSK3 β . GSK3 β inhibition reduced STAT3 phosphorylation, and overexpression of constitutively active GSK3 β had the opposite effect. Moreover, STAT3 inhibition mimicked the effects of GSK3 β inhibition on ESCC cell migration and viability, while overexpression of a plasmid encoding mutant STAT3 (Y705F) abrogated these effects, and these results were further substantiated by clinicopathological data. In

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Availability of data and materials: The authors declare that data supporting the findings of this study are available within the article and its supplementary information files.

Authors' contributions: SG, XF and HW conceived of the project and contributed to the study design. SG, SL, and XD performed immunohistochemistry and Western blot analyses and collected all related data. ZM and XY collected the real-time PCR and phospho-antibody array data, respectively. XD and ZG collected the data regarding wound healing and cell viability. SL, ZM, and XY collected the data from the tumor-bearing xenograft model. SG, XF and HW analyzed and interpreted the data. HW wrote the manuscript with input from all authors. All authors read and approved the final version of the manuscript.

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addition, a GSK3 inhibitor (LiCl) and/or STAT3 inhibitor (WP-1066) efficiently suppressed the growth of ESCC cells in a xenograft tumor model. Altogether, these results reveal that higher GSK3 β expression promotes ESCC progression through STAT3 *in vitro* and *in vivo*, and GSK3 β -STAT3 signaling could be a potential therapeutic target for ESCC treatment.

Keywords

Esophageal Cancer; GSK3 β ; PI3-Kinase; STAT3

Introduction

As the eighth most common cancer and the sixth leading cause of cancer-related deaths worldwide, esophageal cancer primarily occurs in certain regions of developing countries such as Northwest China, Southeastern Africa, Iraq, and South America ¹. Recent studies have shown that the frequency of new cases of esophageal cancer has been increasing by 10% annually in Western Europe and the USA ^{2,3}. Despite substantial improvements in the screening, diagnosis, and treatment of esophageal cancer, the incidence of this disease is still very high, and the prognosis remains unsatisfactory ^{4,5}. The annual incidence rate of esophageal cancer is 3/10⁵ person-years in the U.S. white population ⁶⁻⁸ and reaches 1000/10⁵ person-years in some areas of China ^{8,9}. The median survival time of patients with inoperable cancer is estimated to be only 13 to 29 months, and the five-year survival rate of EC patients is only 10%-20% after radiotherapy or esophagotomy ^{4,8,10}. These epidemiologic results reflect the low efficiency of current treatments, underscoring the need for a deeper understanding of the molecular mechanisms underlying this disease as well as for new therapeutic strategies for its treatment.

As the key protein kinase-mediated signaling events in cancer development have been identified and characterized, strategies aimed at manipulating the activity of protein kinases have been investigated for use in the treatment of a wide range of cancers. Noteworthy examples include the treatment of breast cancer with lapatinib (TYKEB, EGFR/Her-2 inhibitor) ¹¹, kidney cancer with temsirolimus (TORISEL, mTOR inhibitor) ¹², T-cell lymphoma with vorinostat (Zolinza, histone deacetylase inhibitor) ¹³, and skin cancer with vismodegib (Erivedge, Hedgehog blocker) ¹⁴. Moreover, more than 30 agents are currently being tested in clinical trials, suggesting a promising future for the application of kinase inhibitors ¹⁵. Therefore, further exploration into the molecular events involved in the development of ESCC may facilitate the identification of diagnostic markers, therapeutic targets or prognostic indicators for this disease.

Glycogen synthase kinase 3 is a serine/threonine kinase downstream of the PI3K pathway. Two major isoforms, GSK3 α and GSK3 β , have been identified, and both are widely expressed in most mammalian cells ^{16,17}. A distinct feature of GSK3 β is its high activity under basal conditions. Phosphorylation at Serine 9 of GSK3 β leads to a structural change and decreases its activity ¹⁶. PI3K activation results in Akt phosphorylation and the subsequent phosphorylation/inactivation of GSK3 β , which has been demonstrated to suppress the production of pro-inflammatory cytokines including IL-12, TNF α , IL-1 β , IL-6,

IFN γ , and IL-17, while it concurrently promotes the production of IL-10, IL-1Ra, and IFN β by immune cells^{10,18}. Moreover, GSK3 is also involved in Wnt signaling pathways. GSK3 β inhibition leads to the accumulation of β -catenin in the nucleus, which enhances the progression of most cancers^{19,20}. Since some inflammatory cytokines (e.g., IL-6) and β -catenin are considered to be protumorigenesis factors that induce the initiation and progression of cancer, GSK3 β was assumed to play a functional role in cancer biology. In this regard, opposing roles of GSK3 β have been reported in the development of different cancers²¹⁻²³. Aberrant expression of GSK3 β has been shown to promote cell growth in some cancers and to suppress it in others. Several studies have reported the abnormal expression of GSK3 β in esophageal cancer cell lines^{24,25}. However, the expression level and activity of GSK3 β in the tissue of esophageal patients remains essentially unknown, let alone the molecular mechanism by which GSK3 β functions in ESCC.

Signal transducer and activator of transcription-3, also known as STAT3, is a transcription factor required for normal cell growth. STAT3 is quiescent in the cytoplasm. Phosphorylation at Serine 727 and Tyrosine 705 leads to its dimerization and translocation into the nucleus, where it activates the transcription of target genes²⁶. STAT3 has been reported to be constitutively activated in various cancer tissues, and increased STAT3 activation is considered to be an important indicator of poor prognosis²⁷⁻²⁹. Recent studies have shown that STAT3 inhibition suppresses cell growth or enhances apoptosis in multiple cancer cell lines^{30,31}. Conversely, constitutively activated STAT3 signaling has been found in tumor cells and has been demonstrated to promote cell cycle progression and prevent apoptosis by modulating cell cycle-associated and apoptosis-associated proteins such as cyclin D1 and Bcl-2²⁷⁻²⁹. In squamous cell carcinomas, increased STAT3 activation has been found to enhance cell proliferation and migration, while siRNA-mediated *stat3* gene silencing efficiently inhibited STAT3 activation, increased tumor cell apoptosis, and dramatically decreased tumor growth *in vitro* and *in vivo*^{32,33}. While STAT3 is a potent therapeutic target involved in the progression of multiple cancers, the activation of STAT3 and the upstream molecular events in esophageal tumor cells have not been well characterized.

In the present study, we found that the expression of total GSK3 β is higher, but phosphorylated GSK3 β is lower, in cancerous tissues from ESCC patients. GSK3 β inhibition suppresses the growth of ESCC cells *in vitro* and *in vivo* by modifying the activity of STAT3. The expression levels of GSK3 β and STAT3 in cancerous tissues are associated with the differentiation, metastasis, and prognosis of ESCC. These results suggest that GSK3 β and the downstream molecule STAT3 could be diagnostic biomarkers and/or potential therapeutic targets in the treatment of ESCC.

Material and Methods

The study was approved by the Institutional Review Board of Henan University of Science and Technology (HUST).

Patients and human tissue samples

Fifty patients with ESCC who underwent esophagectomy surgery from 2010 to 2015 at the First Affiliated Hospital of HUST and Anyang People's Hospital were enrolled in this study. Adjacent tissue samples were obtained 3 cm away from the cancerous tissue. Fifty additional specimens were randomly selected from biopsies obtained during endoscopic examinations and histologically confirmed as normal esophagus mucosa. Demographics (sex and age) and clinicopathological features (differentiation status, lymphatic invasion, lymph node metastasis, and TNM stage) were obtained from medical records. Overall survival rates were determined over 48 months.

Immunohistochemistry

Tissues were fixed in formalin and then embedded in paraffin. Serial sections of 4 μ m thickness were prepared and deparaffinized by submersion in three separate concentrations of ethanol (100%, 95%, and 70%), followed by rinsing continuously in distilled water for 5 min. Antigen retrieval was performed by incubating slides in Antigen Retrieval Citra Plus Solution (BioGenex, San Ramon, USA) according to the manufacturer's instructions. The slides were blocked in 1.5% normal goat serum (Vector Laboratories, Burlingame, USA) for 30 min. Pre-immune rabbit IgG was used as a negative control. Primary antibodies were incubated with tissue sections for 12 h at 4°C, followed by a biotin-conjugated secondary antibody for one hour at room temperature, streptavidin-peroxidase for 30 min at room temperature, and enzyme substrate (3,3'-diaminobenzidine, Dako, Denmark). As an additional control, sections were incubated with phosphate-buffered saline (PBS) alone, followed by incubation with the biotin-conjugated secondary antibody, streptavidin-peroxidase, and enzyme substrate. PBS washes ($\times 3$, 5 min each) were performed after each incubation step. The sections were counterstained with methyl green and visualized by light microscopy (Eclipse 80i, Nikon, Japan). Each tissue section was evaluated by two pathologists (Drs. Mi and Zhang). The kappa statistic was used to assess inter-observer variability, with a score of >0.75 indicating excellent agreement. The staining intensity was classified using a numerical scale consisting of grade 0 (none, 0-10% staining), grade 1 (weak, 10-30%), grade 2 (moderate, 30-60%), and grade 3 (strong, over 60%), with a score of ≥ 2 considered to be positive.

Cell lines, reagents and antibodies—Esophageal squamous cell lines (KYSE-30, KYSE-70, SHEE, and EC-9706) were gifted by Dr. Zhan QiMin (State Key Laboratory of Molecular Oncology at the Chinese Academy of Medical Sciences (Beijing, China)). The cell lines were routinely tested to confirm the absence of mycoplasma, and all experiments were performed with cells at 60% to 80% confluence. KYSE-30 and KYSE-70 cells were maintained and propagated in DMEM and RPMI1640 medium, respectively, supplemented with 10% FBS and 0.1% gentamicin sulfate (Gemini Bio-Products). Transfections were performed in DMEM containing only 5% FBS and no antibiotics. The GSK3 inhibitor LiCl and the STAT3 inhibitor WP-1066 were purchased from Sigma-Aldrich (St. Louis, MO), and SB216763 was purchased from Torcic (Bristol, United Kingdom). Specific GSK3 β small interfering RNAs (siRNA) and the control scramble siRNA were obtained from Dharmacon (Lafayette, CO). The plasmids pcDNA3-GSK3 β (S9A) with HA tag and pcDNA3-STAT3 (Y705F) with FLAG tag were obtained from Addgene (plasmid numbers

14754 and 46933) and originally created by Drs. Jim Woodgett and Afshin Dowlati, respectively. The control plasmid pcDNA3 with HA tag was obtained from Invitrogen (Carlsbad, CA). Antibodies against phosphorylated and total GSK3 β , phosphorylated and total STAT3, HA, FLAG, and tubulin and the horseradish peroxidase (HRP)-conjugated secondary antibody were obtained from Cell Signaling Technology.

Transfection, Western blot, qRT-PCR and Immunoprecipitation—KYSE-30 and KYSE-70 cells were transfected by electroporation using a Nucleofector device (Amaxa, Germany) according to the manufacturer's protocol. Briefly, purified cells (4×10^6) were re-suspended in 100 μ l of Nucleofector solution (epithelial cell Nucleofector kit; Amaxa) along with 2 μ g of a green fluorescent protein (GFP)-encoding plasmid (pCMV-GFP) and 2 μ g of siRNA duplexes or ectopic plasmids for each target. Immediately after electroporation, 400 μ l of pre-warmed M-199 containing 10% FCS was added to the cells, which were then transferred into culture plates containing pre-warmed M-199 with 10% FCS. At 48 h post-transfection, the cells were exposed to a GSK3 or STAT3 inhibitor. Cell lysates were prepared as previously described³⁴. Images were acquired using the ImageQuant LAS 4100 imaging system (GE Healthcare Life Sciences, Pittsburgh, PA). The levels of total GSK3, STAT3, and tubulin were assessed by Western blot at 48 h post-transfection to evaluate transfection efficiency. For experiments using inhibitors, control cells were pre-treated for 2 h with 0.01% DMSO (organic solvent control). Relative *gsk3* gene expression was detected using quantitative RT-PCR (qRT-PCR), which was performed on an ABI 7500 Sequence Detection System (Applied Biosystems) using a SensiMix SYBR Low-ROX Kit (Bioline, USA). Relative quantification of gene expression was performed using the $\Delta\Delta$ CT method. The primer sequences for GSK3 β were as follows: forward primer, 5'-ATGCCACAGCAGCGTCAG-3'; and reverse primer, 5'-TGGTCTGTCCACGGTCTCC-3'. The Dynabeads Co-Immunoprecipitation Kit (ThermoFisher) was used for immunoprecipitation according to the manufacturer's protocol. A rabbit isotype control IgG Ab (Cell Signaling Technology) was used to ensure that the immunoprecipitation of GSK3 β and subsequent immunodetection of STAT3 were not due to nonspecific interference.

Cell viability and migration assays—MTT assays were performed to determine the viability of KYSE-30 and KYSE-70 cells. First, 1×10^4 cells per well were seeded and cultured in a 96-well plate, and 10 μ l of 12 mM MTT stock solution was added to each well and incubated at 37°C for 4 h. Then, 100 μ l of SDS-HCl solution was added to each well and mixed thoroughly by pipetting, followed by an additional 4 h of incubation at 37°C. Each sample was then mixed again using a pipette, and the absorbance was measured at 570 nm. The absorbance values were normalized by assigning the value of the control group in the medium without any inhibitor to 1.0, which is equal to 100% cells are viable, and the value of the no-cell control to 0. For migration assays, KYSE-30 and KYSE-70 cells were pre-treated with LiCl (25mM) or WP-1066 (20 μ M) or transiently transfected with GSK3 β siRNA or a constitutively active mutant of GSK3 β or STAT3 mutant, STAT3 (Y705F). Then, a scratch wound was made using a pipette tip. The medium was replaced, and the wounds were photographed at 0, 24 and 48 h. ImageJ software was used to measure wound closure over time by averaging six individual wound size measurements for each wound at each time

point. The results from three independent experiments with three replicates per experiment were pooled.

Microarray analysis of cancer signaling antibodies—Cancer signaling antibody microarray analysis was performed by Full Moon BioSystems, Inc., as described previously³⁵. Briefly, KYSE-70 cells were treated with LiCl (25 mM) or SB216763 (25 μ M) for 2 h or 4 h, and whole-cell lysates were then harvested to analyze the phosphorylation of cancer signaling molecules. The total cell lysates (1 mg) were hybridized with antibodies on the microarray according to the manufacturer's protocol, and signals were detected and analyzed by the customized service of Full Moon BioSystems, Inc. The results were normalized using the quantile normalization method with the Linear Models for Microarray Data package. Cluster analysis was performed using the Cluster and TreeView software programs.

Xenograft studies—Mice were handled in accordance with the HUST Animal Care and Use Committee protocols and regulations. A total of 10×10^6 KYSE-70 ESCC cells were inoculated subcutaneously into female severe combined immunodeficiency (SCID) mice (20–25 grams; 6 to 8 weeks old; $n=12$). LiCl (100 mg/kg), WP-1066 (20 mg/kg), LiCl and WP-1066 together, or the respective solvent control(s) were injected peritoneally beginning on day 10, when the inoculated KYSE-70 cells had developed into a tumor of appreciable size (approximately 60–80 mm³). The injections were administered every two days until day 25. In total, the mice were treated with these inhibitors 7 times. Tumor specimens were dissected from SCID mice xenografted with KYSE-70 cells at day 40. Tumor volume was measured using calipers and calculated as (length $\times$ width $\times$ width). Tumor growth inhibition was determined as the change in tumor volume of treated over control animals at the end of the study.

Statistical analysis—The Kaplan-Meier method was used to generate time-to-progression and survival curves. The statistical analysis was performed with SPSS, and statistical significance was set at 0.05. For analyses of categorical data (tumor stage, tumor grade, and residual tumor size), Fisher's exact test was used to calculate *P* values. Results were presented as mean $\pm$ standard error of the mean when applicable. Tumor volume, weight, and tumor cell viability were analyzed using analysis of variance (ANOVA) followed by Dunnett test for comparison of treatment versus control groups. Statistical analysis was performed using GraphPad Prism 5 (GraphPad Software, Inc., San Diego, CA, USA). Differences were considered significance at $p < 0.05$.

Results

Expression of total and phosphorylated GSK3 β in the cancerous tissue of esophageal cancer patients

To investigate the functions of GSK3 β in the development of esophageal cancer, we first examined the expression of total GSK3 β in esophageal tissues from ESCC patients and age- and gender-matched controls. As shown in Figure 1A, the expression of GSK3 β was substantially higher in the cancerous esophageal tissues. Further statistical analysis of the

staining results demonstrated that 64% of the cancerous tissue samples exhibited positive GSK3 β expression, which was significantly higher than that of the adjacent control tissues (14%) ($P<0.001$; $\chi^2=26.272$) (Table 1). Moreover, GSK3 β expression was primarily immunolocalized in the cytoplasm and occasionally present in nuclei, which is consistent with previous reports in other cancerous tissues³⁶⁻³⁸. We next used qRT-PCR to examine the expression of GSK3 β transcripts and observed a similar trend, as shown in Figure 1B, with higher expression and transcript levels of GSK3 β observed in ESCC cancerous tissues compared with adjacent control tissues (Fig. 1B). The average fold increase in GSK3 β mRNA in cancerous tissue was significantly higher than that in control tissues (Fig. 1D). Since GSK3 β is constitutively active in resting cells and can be inactivated by phosphorylation at an N-terminal serine residue (Serine 9), we next examined the level of phosphorylated GSK3 β in esophageal tissue sections from ESCC patients. Due to the lack of an available high-quality phospho-GSK3 β antibody for IHC, we used Western blot analysis to examine phospho-GSK3 β expression and surprisingly observed that the expression level of phospho-GSK3 β (Ser9), unlike that of total GSK3 β , was significantly decreased in the cancerous tissues compared with adjacent control tissues (Fig. 1C, E). These results show for the first time the expression profile of total and phospho-GSK3 β and indicate that GSK3 β activity is increased in the cancerous tissue of ESCC patients.

GSK3 β expression is associated with the progression and prognosis of esophageal cancer

Because high expression of the active form of GSK3 β was more frequently detected in cancerous tissues, we next analyzed the possible correlation between GSK3 β expression and clinicopathological factors including differentiation, depth of invasion, metastasis, TNM, and survival rate in ESCC patients (Table 1). Twelve out of 13 (92.3%) cases with lymphatic or distant organ metastases at diagnosis showed positive GSK3 β staining compared with 20 out of 37 (54.1%) cases without metastases ($P<0.05$; $\chi^2=6.11$). Similar trends were observed for ESCC differentiation and invasion, with higher GSK3 β expression being closely associated with poorer differentiation and severe invasion. Importantly, positive GSK3 β staining was observed in 30 of the 42 cases (71.4%) of advanced ESCC (TII or TIII), whereas early ESCC limited to the muscularis (<TII) exhibited lower positive GSK3 β staining, with only 2 of 8 cases showing positive staining (25%; $P<0.05$; $\chi^2=6.29$), suggesting that higher GSK3 β expression is indicative of late-stage ESCC. Moreover, the overall survival time of the patients with positive GSK3 β staining was only 26.446 months, which was significantly lower ($P=0.027$) than that of patients with negative GSK3 expression, who exhibited a survival time of 36.845 months (Fig. 2A, Table 2). Collectively, these results suggest that GSK3 β expression is closely related to ESCC development and that higher GSK3 β activity is an indicator of poorer differentiation, higher metastasis rates, and worse prognosis.

GSK3 β inhibition suppresses the viability and migration of ESCC cell lines

To further investigate the association between GSK3 β expression and ESCC progression and to select the proper cell lines for our analyses, we first examined the basal expression levels of GSK3 β in different ESCC cell lines including KYSE-30 (well-differentiated ESCC cells), KYSE-70 (poorly differentiated ESCC cells), EC9706, and an immortalized esophageal epithelial cell line (SHEE). KYSE-30 and KYSE-70 cells exhibited higher expression levels

of GSK3 β than EC-9706 and SHEE cells (Fig. 3A). Next, we used a (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay to examine the effect of GSK3 β inhibition on the viability of KYSE-30 and KYSE-70 cells. As shown in Figure 3B and C, treatment with a pharmacological inhibitor of GSK3 β either LiCl (Fig. 3B) or SB216763 (Fig. 3C), significantly reduced the viability of KYSE-30 and KYSE-70 cells after 48 h. To exclude the possibility of off-target inhibitor effects, we used a specific siRNA to knock down GSK3 β and found that siRNA-mediated gene silencing of GSK3 β (Fig. 3D) resulted in a similar suppression of the viability of both KYSE-30 and KYSE-70 cells compared with cells transfected with a control siRNA (Fig. 3E). We further determined the impact of GSK3 β inhibition on the migration of these cancer cells using a wound healing assay. Inhibiting GSK3 β with either a pharmacological inhibitor or a specific siRNA significantly reduced the migration of KYSE-30 (Fig. 3F to I) and KYSE-70 (Fig. 3J to M) cells in the wound healing assay at the indicated time points compared with the control cells. Collectively, these results demonstrated that GSK3 β inhibition suppresses the viability and migration of ESCC cells.

Cancer signaling phospho-antibody array shows that GSK3 β inhibition suppresses STAT3 activity

To further explore the molecular mechanisms by which GSK3 β affects the behavior of ESCC cells, a cancer signaling-specific phospho-antibody microarray was used to comprehensively analyze the site-specific phosphorylation of 269 kinases involved in cancer cell signaling pathways. Since KYSE-30 and KYSE-70 cells exhibited similar trends in viability and migration upon GSK3 β inhibition, we used KYSE-70 as the representative cell line in the antibody array analysis and subsequent experiments. As shown in Figure 4A, the phosphorylation level of numerous molecules, such as STAT3, Bcl-XL, p53, c-Kit, p27Kip1 and integrin- β varied >25% in KYSE-70 cells treated with LiCl for 2 h and 4 h compared with untreated cells (Fig. 4A, Table 3). Due to the obvious change in STAT3 phosphorylation and to previous studies showing that GSK3 β inhibition suppressed the levels of phosphorylated STAT3^{39,40}, we next used Western blotting to confirm the effect of GSK3 β inhibition on STAT3 phosphorylation in ESCC cells. As shown in Figure 4B and C, a significant decrease was observed in STAT3 phosphorylation at both Tyrosine 705 and Serine 727 in KYSE-70 cells when GSK3 β was inhibited by LiCl or SB216763 (Fig. 3B, C). Collectively, these data indicated that GSK3 β inhibition leads to decreased STAT3 phosphorylation in ESCC cells.

STAT3 overexpression in ESCC patient tissues

The effect of GSK3 β inhibition on STAT3 phosphorylation raises the question of whether STAT3 plays a role in ESCC progression. Thus, we next investigated the expression of STAT3 in the cancerous tissues of ESCC patients and its association with ESCC development. As shown in Figure 5, STAT3 expression was noticeably higher in the cancerous tissues of ESCC patients than in the surrounding tissues. Moreover, statistical analysis of the pathological data demonstrated that the STAT3 expression level was significantly higher (Table 4, $P=0.003$) in cancerous tissues of ESCC patients than in the adjacent tissues, similar to the expression of total GSK3 β . Moreover, STAT3 expression was correlated with differentiation, metastasis, invasion, overall survival rate, and TNM stage in

ESCC patients (Table 4). Positive STAT3 expression was observed more frequently in late-stage ESCC ($P<0.05$; $\chi^2=12.743$) and indicated poorer differentiation ($P<0.05$; $\chi^2=5.348$) and a higher likelihood of lymph node invasion ($P<0.001$; $\chi^2=11.611$). Importantly, the overall survival period of the patients with positive STAT3 expression (26.705 months) was significantly lower ($P<0.05$) than that of patients with negative STAT3 expression (36.460 months) (Fig. 2B; Table 2), which is also similar to the results observed for GSK3 β expression. These results are consistent with previous studies^{28,30,41,42} on the functional roles of STAT3 in the development of other cancers, indicating that the GSK3-STAT3 signaling axis may serve as a potential prognosis marker for ESCC patients.

GSK3 inhibition-mediated suppression of cancer cell viability is dependent on STAT3 activation

Since we demonstrated that GSK3 β inhibition suppresses STAT3 phosphorylation, we next investigated if the suppressive effect of GSK3 β inhibition on the viability and migration of ESCC cells is mediated by STAT3 modifications. To this end, we first confirmed the effect of GSK3 β on STAT3 phosphorylation using a GSK3 β -specific siRNA and a constitutively active mutant of GSK3 β GSK3 β (S9A). Specific GSK3 β siRNA led to a substantial reduction of GSK3 β expression (Fig. 6A) and a considerable decrease of the STAT3 phosphorylation in KYSE-70 cells compared with the control cells (Fig. 6A, B), while constitutively active GSK3 β considerably increased STAT3 phosphorylation (Fig. 6C, D), suggesting that GSK3 β inhibition indeed reduces STAT3 phosphorylation in ESCC cells. We next used MTT and wound healing assays to examine the role of STAT3 in the GSK3 β -mediated suppression of KYSE-70 cell viability and migration. As shown in Figure 6, transfection with STAT3 (Y705F), a mutant plasmid that has been demonstrated to enhance the activity of STAT3⁴³, significantly increased the total expression level of STAT3 (Fig. 6E, F) and the viability of ESCC cells and abrogated the anti-proliferative effect of GSK3 inhibition on KYSE-70 cells (Fig. 6H). By contrast, a STAT3 inhibitor, WP-1066, enhanced the suppressive effect of the GSK3 inhibitor on the viability of KYSE-70 cells (Fig. 6I). These results are consistent with previous studies indicating that STAT3 inhibition suppressed the growth of ESCC cells, while STAT3 activation had the opposite effect⁴⁴⁻⁴⁶. Similar effects were observed in the wound healing assay. Active STAT3 enhanced cell migration and offset the suppressive effect of LiCl on the migration of KYSE-70 cells (Fig. 6J, L). By contrast, the STAT3 inhibitor (WP-1066) substantially enhanced the suppressive effect of LiCl (Fig. 6K, M). Collectively, these results demonstrated that the ability of GSK3 inhibition to suppress the viability of ESCC cells is dependent at least in part on the activity of STAT3. Notably, the direct association of GSK3 β and STAT3 was not observed when we immunoprecipitated GSK3 β and tested the expression of total STAT3 in the presence or absence of LiCl treatments (Fig. 6G), which indicated other molecules may be involved in the regulatory effects of GSK3 β on STAT3 activation in tumor cells.

Inhibition of GSK β -STAT3 signaling suppresses the progression of ESCC in SCID tumor-bearing mice

To evaluate the effects of GSK3 β -STAT3 signaling activity on the growth of esophageal squamous cancer *in vivo*, KYSE-70 cells were inoculated under the skin of SCID mice to generate a tumor-bearing mouse model. As shown in Figure 7A, all tumor specimens were

dissected from SCID mice at the end of the experiment. The average tumor volume was considerably reduced in mice pre-treated with LiCl, WP-1066, or both compared with mice treated with a solvent control (Fig. 7C). Moreover, the mean tumor weights of LiCl-, WP-1066-, or LiCl- and WP-1066-treated mice were significantly decreased compared with those of solvent control-treated mice (Fig. 7B, $P < 0.05$). The differences in tumor volume between the control and inhibitor(s)-treated mice were statistically significant after 22 days for WP-1066 and LiCl, 22 days for WP-1066, and 26 days for LiCl (Fig. 7C), demonstrating that inhibition of the GSK3-STAT3 signaling axis blocked tumor growth in the esophageal squamous cancer xenograft model. Moreover, immunohistochemical staining showed that the expression of phospho-STAT3 was decreased in the cancer tissues of LiCl-treated mice compared with the control mice (Fig. 7D), indicating the *in vivo* inhibitory effect of LiCl on STAT3 phosphorylation. Importantly, compared with a single dose of either inhibitor, the combined use of LiCl and WP-1066 showed enhanced suppressive effects although there is no significant synergistic effects were observed, on the growth of ESCC cells in this model, suggesting that both GSK3 β and STAT3 contribute to the development of ESCC and may serve as potent therapeutic targets for the control of esophageal cancers.

Discussion

The expression pattern and possible functions of GSK3 β in ESCC patients, along with the related molecular mechanisms through which GSK3 β is involved in ESCC, remained unknown. Our results demonstrated for the first time that expression of total GSK3 β is significantly higher in the cancer tissues of ESCC patients than in adjacent control tissues, and, interestingly, phosphorylated GSK3 β is concurrently decreased in cancer tissues. The inverse expression patterns of total and phospho-GSK3 β indicated that GSK3 β activity is higher in the cancerous tissues of patients with ESCC than in normal tissues, which was substantiated by the result that GSK3 β inhibition leads to the suppression of ESCC cell viability and migration. Moreover, statistical analysis of clinical data revealed that higher GSK3 β expression was also associated with poorer differentiation, higher rates of metastasis and worse prognosis of ESCC. Further antibody array screening and multiple gain- and loss-of-function studies demonstrated that GSK3 β affects the development of ESCC by modifying the activity of STAT3 (Figure 8). Additionally, our *in vivo* data also demonstrated the suppressive effect of a GSK3 β inhibitor on the viability of ESCC in SCID mice bearing esophageal cancer cell xenograft tumors. These findings established the pro-tumorigenesis role of GSK3 β in the development of ESCC *in vitro* and *in vivo*, suggesting that a GSK3 β inhibitor could be a potential molecular therapeutic in the control of esophageal cancer.

GSK3 β has been shown to suppress the Wnt/ β -catenin pathway by phosphorylating β -catenin, resulting in the ubiquitin/proteasome-dependent degradation of β -catenin⁴⁷. Since the accumulation of β -catenin has been demonstrated to promote the development of multiple cancers^{48,49}, GSK3 β was thought to act as a tumor suppressor by controlling the level of β -catenin. Several studies have reported the anti-tumorigenesis role of GSK3 β using gain- and loss-of-function approaches in different tumors such as breast and skin tumors^{50,51}. Moreover, GSK3 β overexpression has been reported to affect the viability of cancer cells by enhancing the degradation of pro-tumorigenesis factors such as Cdc25A and c-Myc^{24,52}. On the other hand, the pro-tumorigenesis role of GSK3 β has also been

observed in various tumor types including colon, liver, ovarian, and pancreatic cancer with underlying molecular mechanisms of controlling the expression of cyclin proteins or modifying the activity of telomerase⁵³⁻⁵⁵. Moreover, GSK3 β inhibition has been shown to enhance lysosomal acidification and thus increase the degradation of the epidermal growth factor receptor⁵⁶, indicating an alternative pro-tumorigenesis mechanism of GSK3 β . Here, we provided direct evidence of higher expression of the active GSK3 β form in the cancerous tissues of ESCC patients than in normal tissues and elucidate a novel molecular mechanism by which GSK3 β promotes the viability and migration of ESCC cells *in vitro* and *in vivo* that involves regulating STAT3 phosphorylation (Figure 8). Inhibition of GSK3 β , STAT3 or both significantly suppressed ESCC development, indicating that GSK3 β -mediated STAT3 signaling is a promising therapeutic target for ESCC. Since LiCl has been used in the clinic to treat patients with bipolar disorder, this inhibitor carries an additional advantage as a therapeutic candidate for ESCC. Moreover, our data showed that differential expression of GSK3 β was closely correlated with the viability status and lymph node metastasis of ESCC, which suggests that the ratio of phospho- to total GSK3 β could be developed as a valuable prognostic biomarker. The status of the GSK3-STAT3 pathway may have the potential to guide personalized therapies in the future by directing ESCC patients to therapies modifying the activity of GSK3 β , STAT3 or both.

STAT3 is constitutively activated in many types of human cancers and plays a key role in regulating the proliferation and migration of cancer cells^{57,58}. Dysregulation of STAT3 has been reported in several epithelial cancers, such as lung, head and neck, stomach, and esophageal cancer^{59,60}. In our study, we found that STAT3 expression was significantly higher in the cancerous tissues of ESCC patients compared with adjacent normal tissues, a result consistent with previous findings on the influence of STAT3 on the progression of other cancers^{42,61-63}. Even though we found that higher GSK3 β activity leads to increased STAT3 expression in the cancerous tissues of ESCC patients, the extremely high expression of STAT3 suggests that there might be other regulatory mechanisms involved in the control of STAT3 in addition to the direct influence of GSK3 β . As the activity of STAT3 is controlled by phosphorylation of tyrosine and serine residues^{27,39}, our data showing that GSK3 inhibition reduced the phosphorylation of both Serine 727 and Tyrosine 705 indicate that GSK3 may be one of the major factors affecting STAT3 activity. On the other hand, our cancer signaling array data revealed that GSK3 affects numerous other molecules, such as β -catenin, Jun protein, cyclin, NF- κ B, and p53, in cancer cells, suggesting that STAT3 is not the only target of GSK3 β that controls the viability and migration of cancer cells. These results suggest the existence of a complex signaling network that comprehensively controls cancer cell behavior. The aberrant signaling in cancer cells is likely a result of “multiples to multiples” effects and not straightforward “individual to individual” effects, emphasizing the necessity of high-throughput signaling research in cancer development.

In the present study, we found that inhibition of either GSK3 β or STAT3 alone resulted in a significant decrease in ESCC cell viability and migration both *in vitro* and *in vivo*, indicating that dysregulation of either GSK3 β or STAT3 contributes to the malignant manifestation of ESCC cells. Since our data show that GSK3 β inhibition suppressed STAT3 phosphorylation and in turn reduced the viability and migration of ESCC cells, GSK3 β might directly control STAT3 activation and may thus be a critical factor in ESCC tumorigenesis. However, we did

not observe the direct interaction between GSK3 β and STAT3 in our immunoprecipitation experiments in KYSE-70 cells (Fig. 6G), indicating that other molecules might be involved in the GSK3 β -mediated regulation of STAT3. Considering that GSK3 β can modify multiple other transcription factors that in turn control tumor progression, the activation of STAT3 by an alternative GSK3-mediated mechanism will highlight the central regulatory role and diverse influences of GSK3 β in ESCC tumorigenicity. One possible mechanism by which GSK3 β may activate STAT3 in ESCC cells is through the control of *de novo* cytokine expression and subsequent second signaling. Our previous findings demonstrated that GSK3 β regulates the production of inflammatory cytokines (such as IL-6) and is involved in cytokine signaling³⁴. Since STAT3 phosphorylation mediated by cytokines (such as IL-6) also plays a critical role in the progression of cancer, it is possible that GSK3 β regulates STAT3 phosphorylation by modifying the amount and subsequent signaling of certain cytokines, e.g., IL-17 and IL-6.

In summary, our current study identified the expression profile and functional role of GSK3 β and its mediation of STAT3 signaling in ESCC development *in vitro* and *in vivo*. Since esophageal cancer is well characterized as a group of heterogeneous diseases caused by many genomic and epigenetic alterations as well as various environmental stresses, further studies on the function of genomic mutation-mediated aberrant activity and expression of GSK3 β and STAT3 and the detailed signaling network that acts upstream of GSK3 β and downstream of STAT3 would be beneficial to the development of efficient interventional therapeutics for the control of ESCC.

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Figure 1. Differential expression of total and phospho-GSK3β in ESCC patient tissues
 (A) Representative images of the immunohistochemical detection of GSK3β in normal esophagus mucosa and cancerous and adjacent tissues of ESCC patients. (A1), (A2), and (A3) are representative images of GSK3β in well-differentiated (A1), moderately differentiated (A2), and poorly differentiated (A3) ESCC tissues (20× magnification; scale bar=50 μm; the insets in the left corners amplify the area indicated by red arrows; same as below). Pre-immune rabbit IgG was used as a control in serial tissue sections from the same paraffin-embedded tissue block of (A4) well-differentiated, (A5) moderately differentiated,

and (A6) poorly differentiated ESCC. (A7) Normal esophagus mucosa stained with an anti-GSK3 β antibody. (A8) and (A9) are representative negative/positive images of GSK3 β immunostaining in the adjacent cancerous tissues. (B, D) mRNA levels of total GSK3 β in the cancerous and adjacent tissues from the same ESCC patient (B), and the average fold increase in mRNA in all cancerous tissues and adjacent tissues from ESCC patients (D). (C) Western blot analysis of whole-cell lysates from cancerous (T) or adjacent tissues (N) of ESCC patients. The blots were probed with antibodies against phospho-GSK3 β , total GSK3 β and tubulin. (E) Densitometric quantification using the mean of the sum of the ratios of total and phospho-GSK3 β to tubulin for all samples.

Figure 2. Kaplan-Meier survival analysis of the association between the expression of GSK3 β or STAT3 and the overall survival rate of ESCC

Higher expression of either GSK3 β (A) or STAT3 (B) was positively correlated with a poorer overall survival rate of ESCC, with P values of 0.027 (A) and 0.047 (B), respectively.

Figure 3. GSK3β inhibition suppresses ESCC cell viability and migration *in vitro*

A. The mRNA expression of GSK3β was detected by qPCR in KYSE-30, KYSE-70, EC-9706 and SHEE cells. The viability of the ESCC cell lines KYSE-30 and KYSE-70 was assessed using a (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. A wound healing assay was used to study cell migration. Cells were pre-treated with various concentration of LiCl (25 mM) or SB216763 (25 μM) for 2 h, and the anti-proliferative effects of LiCl (B) and SB216763 (C) on KYSE-30 and KYSE-70 cells were assessed at 48 h. (D, E) After KYSE-30 and KYSE-70 cells were transfected with either a

GSK3 β -specific siRNA or a scramble control siRNA, MTT cell viability assays were performed at different time points on days 1 and 2. siRNA-mediated GSK3 β silencing (D) significantly decreased the viability of KYSE-30 and KYSE-70 cells (E). Using the same treatments as above, a wound healing assay was performed to examine the effects of LiCl (25 mM), SB216763 (25 μ M), and GSK3 β -specific siRNA on the migration of KYSE-30 (F-I) and KYSE-70 (J-M) cells. All results are the average of at least three independent experiments. Error bars represent standard deviation; $n=3$. * $P<0.05$; *** $P<0.001$, compared with control cells.

Figure 4. Cancer signaling phospho-antibody array shows that STAT3 is suppressed by GSK3 β inhibition

KYSE-70 cells were pre-treated with LiCl for 2 h and 4 h, and then whole-cell lysates were collected to detect the phosphorylation of cancer-related molecules using a cancer signaling phospho-antibody array. A. Cluster analysis of antibody array data. B to E, Western blot analysis of the whole-cell lysates from LiCl- or SB216763-treated KYSE-70 cells. The blots were probed with antibodies against phospho-STAT3, total STAT3 and tubulin (B). Densitometric quantification was performed by calculating the ratio of total and phospho-STAT3 to tubulin (C). The results of Western blots are the average of three independent

experiments. Error bars represent standard deviation of the mean of intensity ratios; “***” indicates $P<0.001$, compared with control group.

Figure 5. Expression of STAT3 in esophageal tissues from ESCC patients

Representative images of the immunohistochemical detection of STAT3 in normal esophagus mucosa and cancerous and adjacent tissues of ESCC patients. (A), (B), and (C) are representative images of STAT3 in well-differentiated (A), moderately differentiated (B), and poorly differentiated (C) ESCC tissues (20× magnification; scale bar=50 μm; the insets in the left corners amplify the area indicated by the red arrows; same as below). Pre-immune rabbit IgG was used as a control in serial tissue sections from the same paraffin-embedded tissue block of (D) well-differentiated, (E) moderately differentiated, and (F) poorly differentiated ESCC. (G) Normal esophagus mucosa stained with an anti-STAT3 antibody. (H) and (I) are representative negative/positive images of STAT3 immunostaining in the adjacent cancerous tissues.

Figure 6. GSK3β inhibition-mediated suppression of cancer cell viability and migration is dependent on STAT3 activation

KYSE-70 cells were transfected with a GSK3β-specific siRNA (A), a constitutively active mutant of GSK3β (S9A) (C) or a mutant plasmid STAT3 (Y705F) (E), or the relevant control including a scramble siRNA and empty control plasmids for 48 h, and whole-cell lysates were then collected and analyzed by immunoblot using antibodies against phospho-STAT3/total STAT3, HA tag, FLAG tag, and tubulin (A, C, and E). Densitometric quantification was performed by calculating the ratio of target proteins to tubulin (B, D and F). Direct association between GSK3β and STAT3 was determined by immunoprecipitation

of GSK3 β followed by Western blot for STAT3 and total GSK3 β in KYSE-70 cells(G). Cells transfected with a STAT3 (Y705F) mutant or the empty control vector (F) were pre-treated with LiCl, and the viability and migration of the cells were assessed by MTT and wound healing assays, respectively. (H, I) Viability analysis of cells transfected with overexpression of STAT3 mutant indicates that STAT3 enhances cell viability and abrogates the effects of GSK3 β inhibition on KYSE-70 cells (H). By contrast, a STAT3 inhibitor (WP-1066; 20 μ M) enhances the effect of LiCl (I). (J to M) In a wound healing assay, overexpression of active STAT3 enhances cell migration and offsets the suppressive effect of LiCl on the migration of KYSE-70 cells (J, L). By contrast, a STAT3 inhibitor (WP-1066) substantially enhances the suppressive effect of LiCl (K, M). All results are the average of at least three independent experiments. Error bars represent standard deviation; $n=3$. * $P<0.05$; *** $P<0.001$, compared with control cells.

Figure 7. Inhibition of GSK3 β -STAT3 signaling suppresses the initiation and progression of ESCC in SCID tumor-bearing mice

ESCC was induced in SCID mice by implanting KYSE-70 cells in the flanks of the mice ($n=12$) and allowing the tumors to develop to an appreciable size (approximately 60-80 mm³). Then, LiCl (100 mg/kg), WP-1066 (20 mg/kg), or both were injected intraperitoneally beginning on day 10 and were administered every two days for a total of 7 treatments. (A) Representative tumor specimens dissected from the SCID mice xenografted with KYSE-70 cells at the end of the study. The average tumor weight (B) and volume (C) were calculated at the time indicated. Both LiCl and WP-1066 suppressed tumor growth,

and the combined use of LiCl and WP-1066 decreased tumor growth more significantly than either treatment alone. However, no significantly synergistic effects were observed between the treatment by WP-1066 or LiCl only and their combination. The data represent the mean $\pm$ standard deviation of twelve mice per group. (D) Phospho-STAT3 expression in xenograft tissues from mice treated with different inhibitor(s) was detected by immunohistochemistry, and the representative images are shown here. “*” indicates $P<0.05$, as compared with controls.

Figure 8. Model of how GSK3 β affects ESCC cell survival and cancer progression

Pro-tumorigenic factors led to the higher expression of total- and lower expression of phospho-GSK3 β in cancerous tissue of ESCC, which enhanced the activity of GSK3 β . Higher levels of GSK3 β activation increased the phosphorylation of STAT3 and by which promoted cancer cell survival and progression of ESCC (Green arrows). In contrast, inhibition of GSK3 β by LiCl or SB216763 reduced phosphorylation of STAT3 and consequently suppressed the growth of cancer cell (Pink arrows). Direct association between GSK3 β and STAT3 was not observed in this study. Other factors, i.e., IL-6, might be involved in this process. GSK3 β activation could enhance the production of IL-6, and through which facilitate the phosphorylation of STAT3 and promote the growth of ESCC cells.

Table 1
Expression of GSK3 β and its association with the clinicopathological factors of the patients with ESCC

Factors	Nucleus and Cytoplasm GSK3 β (%)		χ^2	<i>p</i> value
	Positive	Negative		
ESCC Cancerous Tissue	32(64.0)	18(36.0)	26.27	0.000
Adjacent Control Tissue	7(14.0)	43(86.0)		
Gender				
Male(n=31)	24(77.4)	7(22.6)	6.38	0.012
Female(n=19)	8(42)	11(58)		
Age(years)				
60(n=23)	16(69.6)	7(30.4)	0.57	0.449
>60(n=27)	16(59.3)	11(40.7)		
Differentiation				
Well(n=13)	6(46.2)	7(53.8)	2.43	0.119
Moderate(n=27)	17(62.9)	10(37.1)		
Poor(n=10)	9(90)	1(10)		
Lymph node metastasis				
Positive(n=13)	12(92.3)	1(7.7)	6.11	0.013
Negative (n=37)	20(54.1)	17(45.9)		
Depth of invasion				
Deep muscularis(n=19)	7(36.8)	12(63.2)	9.81	0.002
>Deep muscularis(n=31)	25(80.6)	6(19.4)		
TNM stage				
I(n=8)	2(25)	6(75)	6.29	0.012
II(n=29)	19(65.5)	10(34.5)		
III(n=13)	11 (84.6)	2(15.4)		

Table 2
Means and medians for the overall survival time of ESCC patients with positive/negative expression of GSK3 β and STAT3

Factors	Group	Mean 95% Confidence Interval			Median 95% Confidence Interval			p value	
		Est. *	Std.Error	Lower Bound	Upper Bound	Est. *	Std.Error		Lower Bound
GSK3 β	Positive	26.446	2.458	21.629	31.263	29.000	4.622	19.941	38.059
	Negative	36.845	1.989	32.946	40.743				
	Overall	30.534	1.888	26.833	34.235	37.000	3.209	30.711	43.289
STAT3	Positive	26.705	2.335	22.128	31.283	30.000	5.335	19.543	40.457
	Negative	36.460	2.195	32.157	40.763				
	Overall	30.534	1.888	26.833	34.235	37.000	3.209	30.711	43.289

*“Est.” is for “estimation”, which is limited to the largest survival time.

Table 3
The effects of GSK3 inhibition on the levels of phosphorylation of cancer related molecules (altered percentage more than 25% at either time point) in ESCC cells

Phosphorylation Sites	LiCl treated 2h	LiCl treated 4h	Average Percent Variation
Bcl-XL (S62)	0.73	0.77	-25%
C-Kit (Y721)	0.60	0.69	-35.5%
FAK (Y925)	0.86	0.67	-23.5%
HSP27 (S15)	0.82	0.72	-23%
I κ B α , (S32/36)	0.67	0.76	-28.5%
Integrin β 3 (Y773)	0.65	0.67	-34%
Jun B (S259)	0.71	0.84	-22.5%
Jun B (S79)	0.73	0.86	-20.5%
NF- κ B p100/p52 (S865)	0.65	0.97	-19%
P27Kip1 (T187)	0.74	0.73	-26.5%
P53 (S6)	0.49	0.48	-51.5%
PI3K P85 α (Y607)	1.33	1.21	+27%
Shc (Y349)	0.68	0.54	-39%
Smad (S425)	1.47	1.44	+45.5%
STAT3 (S727)	0.71	0.73	-28%
VEGFR2 (Y951)	0.71	0.79	-25%

The Cancer Phosphorylation Profiling (PAC-155) antibody microarray identified a list of phosphorylated proteins whose phosphorylation states decreased in the presence of LiCl sodium (25mM). The ratio of the phosphorylated proteins between LiCl-treated group and non-stimulated control group at indicated time point was shown. “-” and “+” represent “decrease” and “increase”, respectively.

Table 4
Expression of STAT3 and its association with the clinicopathological factors of the patients with ESCC

Factors	Nucleus and cytoplasm STAT3 (%)		χ^2	p value
	Positive	Negative		
ESCC Cancerous Tissue	33(66.0)	17(34.0)		
Adjacent Control Tissue	18(36.0)	32(64.0)	9.004	0.003
Gender				
Male(n=31)	21(67.7)	10(32.3)		
Female(n=19)	12(63.2)	7(36.8)	0.110	0.740
Age(years)				
60(n=23)	17(73.9)	6(26.1)		
>60(n=27)	16(59.3)	11(40.7)	1.188	0.276
Differentiation				
Well(n=13)	4(30.8)	9(69.2)		
Moderate(n=27)	20(74.1)	7(25.9)		
Poor(n=10)	9(90)	1(10)	5.348	0.021
Lymph node metastasis				
Positive(n=13)	12(92.3)	1(7.7)		
Negative (n=37)	21(56.8)	16(43.2)	3.950	0.047
Depth of invasion				
Deep muscularis(n=19)	7(36.8)	12(63.2)		
>Deep muscularis(n=31)	26(83.9)	5(16.1)	11.611	0.001
TNM stage				
I(n=8)	1(12.5)	7(87.5)		
II(n=29)	21(72.4)	8(27.6)		
III(n=13)	11 (84.6)	2(15.4)	12.743	0.002