LncRNA NCK1-AS1 Was Overexpressed in Esophageal Squamous Cell Carcinoma and Predicts Survival

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Abstract

Backgrounds: NCK1-AS1 promotes cervical cancer, while its involvement in esophageal cancer is hardly known. We therefore explored the involvement of NCK1-AS1 in esophageal squamous cell carcinoma (ESCC) and analyzed the possible interaction between NCK1-AS1 and TGF-β signaling.

Methods: Our study selected 52 cases (30 males and 22 females, 46 to 70 years, 56.4 ±6.6 years) to be used as the research subjects in this study. RT-qPCR and western blot were used for gene expression analysis. Transient transfections were used to analyze gene interaction. Transwell assays were performed to analyze cell invasion and invasion.

Results: Our data showed that NCK1-AS1 was overexpressed in ESCC patients. NCK1-AS1 in plasma was positively correlated with the NCK1-AS1 in tumor but not in non-tumor tissues. High plasma levels of NCK1-AS1 were accompanied by poor survival. TGF-β1 expression level was also increased in tumor tissues compared to tumor adjacent normal tissues. TGF-β1 was positively correlated with NCK1-AS1 in tumor tissues. TGF-β1 overexpression did not affect NCK1-AS1 expression, while NCK1-AS1 upregulated TGF-β1 in ESCC cells. TGF-β1 and NCK1-AS1 increased ESCC cell migration and invasion, TGF-β inhibitor reduced the effects of NCK1-AS1 overexpression.

Conclusion: Therefore, NCK1-AS1 may promote ESCC by upregulating TGF-β1.

Background

Incidence of esophageal cancer ranks the 8th place among all malignancies (1). Due to its extreme malignant nature, esophageal cancer is also the 6th leading cause of deaths among cancer patients (1). Esophageal squamous cell carcinoma (ESCC) is one of the two major subtypes based on histological findings (2). ESCC accounts for more than 90% of esophageal cancer in Asian countries, such as China (3). ESCC now is considered as a major burden of public health in China. In effect, more than 50% of newly diagnosed ESCC are in China (4). Early diagnosis of ESCC is difficult due to the lack of classical symptoms. Therefore, most ESCC patients are diagnosed with the existence of regional lymph node metastasis, local invasion or even distant invasion by the time of initial diagnosis (5), leading to poor prognosis (6).

Although environmental factors have certain effect on ESCC, it is generally believed that genetic factors are the major players in ESCC (7). Long (> 200 nt) non-coding RNAs (lncRNAs) are RNA transcripts without protein-coding capacity but have important functions in cancer biology through the regulation of downstream oncogene or tumor suppressors (8, 9). LncRNAs promote or inhibit cancer progression by regulating cancer-related signaling pathways, such as TGF-β signaling (10). It has been reported that NCK1-AS1 promotes cervical cancer (11). In cervical cancer NCK1-AS1 is overexpressed and its inhibition led to the inhibited expression of miR-134, thereby suppressing cell proliferation and migration [11]. However, its involvement in esophageal cancer is hardly known. By analyzing TCGA dataset we observed the upregulation of NCK1-AS1 in esophageal cancer than in non-tumor tissues (3.86 vs. 1.64). The

present study was carried out to explore the involvement of NCK1-AS1 in ESCC and to explore the possible interaction between NCK1-AS1 and TGF-β signaling.

Methods

Research subjects

From May 2009 to May 2013, Henan Key Laboratory for Esophageal Cancer Research of the First Affiliated Hospital admitted 98 patients with ESCC. From those patients, our study selected 52 cases (30 males and 22 females, 46 to 70 years, 56.4 ± 6.6 years) to be used as the research subjects in this study. Inclusion criteria: 1) no therapies received before treatment; 2) patients willing to join follow-up study (5-year). Exclusion criteria: 1) other medical conditions were observed; 2) any treatment received before admission; 3) history of previous malignancy. According to the staging criteria proposed by AJCC, patients were classified into stage I (n = 12), II (n = 16), III (n = 14) and IV (n = 10), respectively. Different treatments, such as esophagectomy, radio therapies, chemotherapies and the combination of them, were performed. Patients signed informed consent before admission. This study was approved by the aforementioned hospital Ethics Committee.

Specimen collection and cell lines

Fine needle aspiration was performed to collect ESCC and paired non-tumor tissue from all patients prior to therapies. Storage of tissue samples was performed at – 80 °C. Prior to therapy, blood (5 ml) was extracted after patients were fasted for 12 h. Plasma samples were collected after centrifuging (1200 g) blood samples in EDTA tubes for 15 min.

EC109 and KYSE150 cell lines were used in this study. EC109 and KYSE150 cells were purchased from ATCC (USA). FBS was added into RPMI-1640 medium to reach 10%, and the mixture was used as cell culture medium. Cells culture was performed at 37 °C with 5% CO_2.

Follow-up

Patients were monitored by performing a follow-up study for 5-year. Through a monthly manner, patients were visited through telephone. All patients were excluded from deaths caused by factors unrelated to ESCC.

RT-qPCR

Following RNA isolations using RNAzol reagent, cDNAs were synthesized through reverse transcriptions using SS-IV-RT (Invitrogen). SYBR ® Green Master Mix (Toyobo, Japan) was used to perform qPCRs. The

internal control of NCK1-AS1 and TGF-β1 mRNA was 18S rRNA. This expression was repeated 3 times and $2^{-\Delta\Delta CT}$ method was used for data normalizations.

Cell transient transfection

NCK1-AS1 or TGF-β1 expression vector was constructed by Sangon (Shanghai, China). Nucleofector™ Technology was used to achieve transient cell transfections with 10 nM vectors. Cells without transfection (control) and empty vector transfection (negative control) were included to serve as 2 controls. Subsequent experiments were performed at 24 h after transfections (for TGF-β inhibitor treatment cells were cultivated with medium containing TGF-β inhibitor SB431542 (SB, 10 nM, Sigma-Aldrich)).

Transwell assays

EC109 and KYSE150 cells were collected and 3×10^3 cells in 0.1 ml serum-free medium were transferred to upper chamber, while in lower chamber 20% FBS was added into medium to induce the movement of cells. Membranes were coated with Matrigel at 37 °C for 12 h before invasion assays, while migration assays were carried out using uncoated membranes. At 37 °C, cells were cultivated for 12 h, followed by 0.5% crystal violet (Sigma-Aldrich) staining for 15 min in dark. An optical microscope (Olympus, Japan) was used to count cells.

Western-blotting

RIPA (Invitrogen) was used to extrat total protein from EC109 and KYSE150 cells. After denaturing, 10% SDS-PAGE gel was used to separate proteins. After that, gel transfer to PVDF membranes. Blocking at room temperature in 5% non-fat milk was performed for 2 h. Membranes were then incubated with GAPDH (ab9485, 1: 1400, Abcam) and TGF-β1 (ab9758, 1:1600, Abcam) primary antibodies and goat anti-rabbit IgG-HRPs secondary antibody (1:1000, MBS435036, MyBioSource). ECL (Sigma-Aldrich, USA) was used for signal production and signals were processed using Image J v.1.46 software.

Statistical analysis

Gene expression levels were expressed by average values of three technical replicates, and paired t test was used for data comparison. ANOVA Tukey's test was used to compare data of three independent replicates of multiple transfection groups, and data were expressed as mean+/- SD. Linear regression was used for correlation analyses. Patients were grouped into low (n = 28) and high (n = 24) plasma NCK1-AS1 level groups based on Youden's index (cutoff value = 4.17). Survival curves were plotted and log-rank test was performed for survival curve comparison. Differences with $p < 0.05$ were statistically significant

Results

NCK1-AS1 was upregulated in ESCC and positive correlated with its plasma level

NCK1-AS1 expression in ESCC and non-cancer tissues was analyzed by RT-qPCR. Expression data were analyzed by paired t test. It was observed that NCK1-AS1 was significantly upregulated in ESCC tissues compared to non-cancer tissues (Fig. 1A, $p < 0.05$). Plasma levels of NCK1-AS1 were also measured by RT-qPCR. Linear regression was carried out to analyze the correlation between NCK1-AS1 expression in plasma and NCK1-AS1 expression in tissues. NCK1-AS1 expression levels in plasma were positively and significantly correlated with levels of NCK1-AS1 in ESCC tissues (Fig. 1B), but not in adjacent non-cancer tissues (Fig. 1C).

High levels of plasma NCK1-AS1 in were accompanied by poor survival

No significant differences in levels of plasma NCK1-AS1 were found among different clinical stages. Therefore, all patients were grouped into high (n = 24) and low (n = 28) plasma NCK1-AS1 level groups based on Youden's index. Survival curve analysis showed that the overall condition of patients with high level of NCK1-AS1 was significantly worse than that of patients with low levels of NCK1-AS1 (Fig. 2).

TGF-β1 mRNA was positively correlated with NCK1-AS1 in ESCC

TGF-β1 expression was also analyzed by RT-qPCR. TGF-β1 was significantly overexpressed in ESCC tissues in comparison to non-cancer tissues at mRNA level (Fig. 3A, $p < 0.05$). Correlation analysis showed that TGF-β1 and NCK1-AS1 were significantly and positively correlated across ESCC tissues (Fig. 3B), but not in adjacent non-cancer tissues (Fig. 3C).

Overexpression of NCK1-AS1 stimulated TGF-β1 expression

Vectors expressing TGF-β1 and NCK1-AS1 were transfected into EC109 and KYSE150 cells. Overexpression of TGF-β1 mRNA and NCK1-AS1 was confirmed at 24 h after transient transfections (Fig. 4A, $p < 0.05$). Overexpression of TGF-β1 did not significantly affect NCK1-AS1 expression (Fig. 4B), while NCK1-AS1 overexpression was followed by the upregulated TGF-β1 expression in cells of ESCC cell lines at both mRNA and protein levels (Fig. 4C, $p < 0.05$).

NCK1-AS1 stimulated ESCC cell invasion and migration through TGF-β1

Comparing to two controls (control, C and negative control, NC), TGF-β1 and NCK1-AS1 overexpression was followed by the increased migration (Fig. 5A) and invasion (Fig. 5B) rates of ESCC cells (p < 0.05). In addition, TGF-β inhibitor SB431542 (SB, 10 nM, Sigma-Aldrich) incubation for 24 h reduced the effects of NCK1-AS1 overexpression (p < 0.05).

Discussion

It has been reported that NCK1-AS1is an oncogenic lncRNA in cervical cancer (11). We reported the involvement of NCK1-AS1 in ESCC and explored its prognostic values. We concluded that NCK1-AS1 could upregulate TGF-β1 to promote ESCC.

ESCC is accompanied by the altered expression of a huge number of lncRNAs (12, 13). Some altered lncRNAs participate in ESCC by affecting cancer cell behaviors and chemo-sensitivity of cancer cells to chemotherapies (12, 13). Our study first showed that NCK1-AS1 was upregulated in ESCC and overexpression of NCK1-AS1 resulted in promoted invasion and migration of ESCC cells. Therefore, NCK1-AS1 is also likely an oncogenic lncRNA in ESCC.

LncRNAs are usually specifically expressed during specific developmental or pathological stages to regulate downstream gene expression (14). However, lncRNAs may enter blood to traffic systemically, thereby regulating systemic gene expression (15). We detected NCK1-AS1 in plasma of all ESCC patients. In addition, plasma NCK1-AS1 reflects its expression levels in ESCC tissues. Therefore, our speculation is that NCK1-AS1 synthesized in ESCC tissues can be released into blood and plasma NCK1-AS1 level can reflect it level in cancer tissue. Comparing to detection of gene expression in tissue, detection of plasma biomarker as a non-invasive approach may be accepted by more patients for disease diagnosis and prognosis. In effect our study proved that high plasma levels of NCK1-AS1 were accompanied by poor survival of ESCC patients. Therefore, plasma NCK1-AS1 may serve as a prognostic marker for ESCC. It is worth noting that NCK1-AS1 expression was not significantly affected by clinical stages, which were closely correlation with patients' survival. Therefore, NCK1-AS1 may be an independent prognostic marker for ESCC.

Our study proved that NCK1-AS1 can regulate TGF-β1 on its upstream. This is because: 1) NCK1-AS1 overexpression led to the upregulated TGF-β1, while TGF-β1 overexpression failed to affect NCK1-AS1; 2) TGF-β1 inhibition attenuated the effects of NCK1-AS1 overexpression on cell invasion and migration. TGF-β signaling can be inactivated or activated by certain lncRNAs (16, 17). Our study observed thatNCK1-AS1 overexpression resulted in upregulated TGF-β1 mRNA. LncRNAs regulate gene expression mainly at 3 levels, namely posttranscriptional level, translational level and epigenetic level (18). Therefore, NCK1-AS1 may affect the stability of TGF-β1 mRNA to regulate TGF-β1 expression.

Conclusion

NCK1-AS1 was upregulated in ESCC and overexpression of NCK1-AS1 may promote ESCC cell migration and invasion by activating TGF-β signaling.

List Of Abbreviations

esophageal squamous cell carcinoma (ESCC); Long non-coding RNAs (lncRNAs); polyvinylidene fluoride (PVDF).

Declarations

Ethics approval and consent to participate

Ethics Committee of the First Affiliated Hospital, Zhengzhou University approved this study. Written informed consent was obtained from all individual participants included in the study.

Consent for publication

Not applicable.

Availability of data and material

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

Funding

No funding was obtained for this study.

Authors' contributions

FKX, XF, FY, SG, YRQ and LDW have made substantial contributions to conception and design, acquisition of data, and analysis and interpretation of data. FKX was involved in drafting the manuscript or revising it critically for important intellectual content. FKX, XF, FY, SG, YRQ and LDW gave final approval of the

version to be published. Each author agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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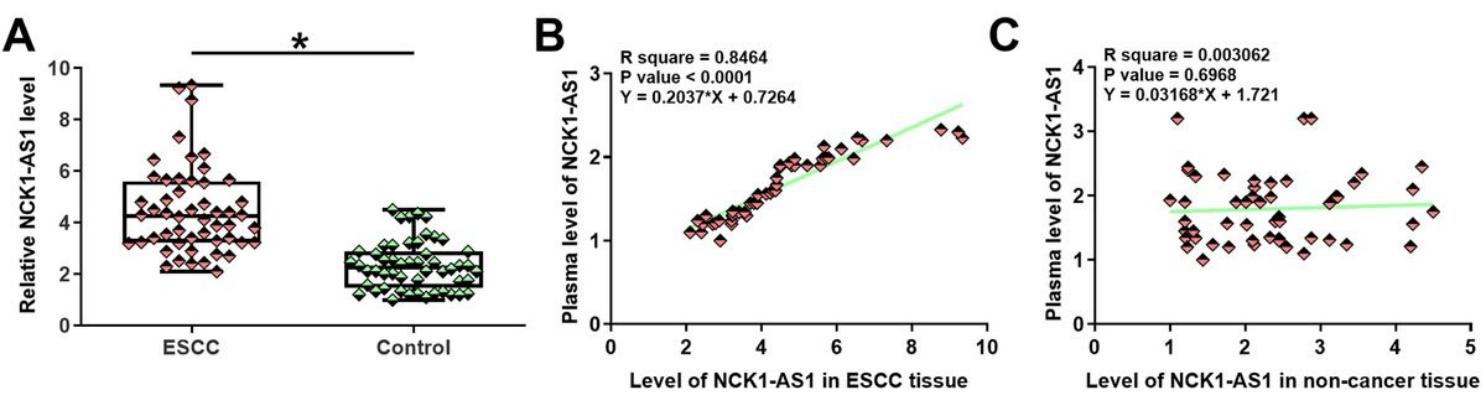
Figures

Figure 1

NCK1-AS1 was upregulated in ESCC tissues and positive correlated with it plasma level Expression data analyzed by paired t test showed that NCK1-AS1 expression was significantly upregulated in ESCC (A) (*, p<0.05). Linear regression showed that levels of NCK1-AS1 in plasm were positively and significantly correlated with its levels in ESCC tissues (B), but not in adjacent non-cancer tissues (C).
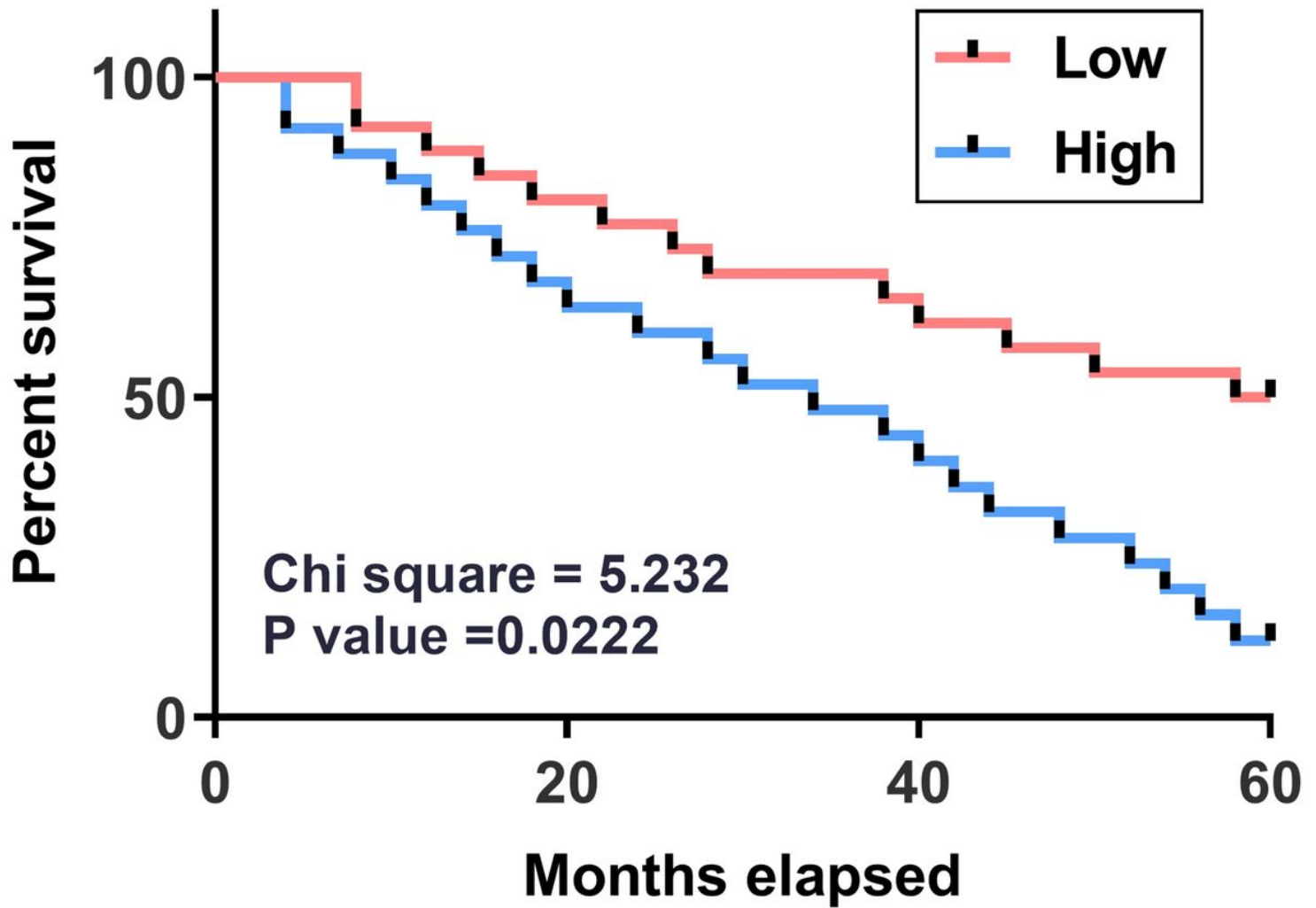
Figure 2

High plasma levels of NCK1-AS1 were correlated with poor survival Overall survival condition of patients in high level group was significantly worse in comparison to patients in low level group.
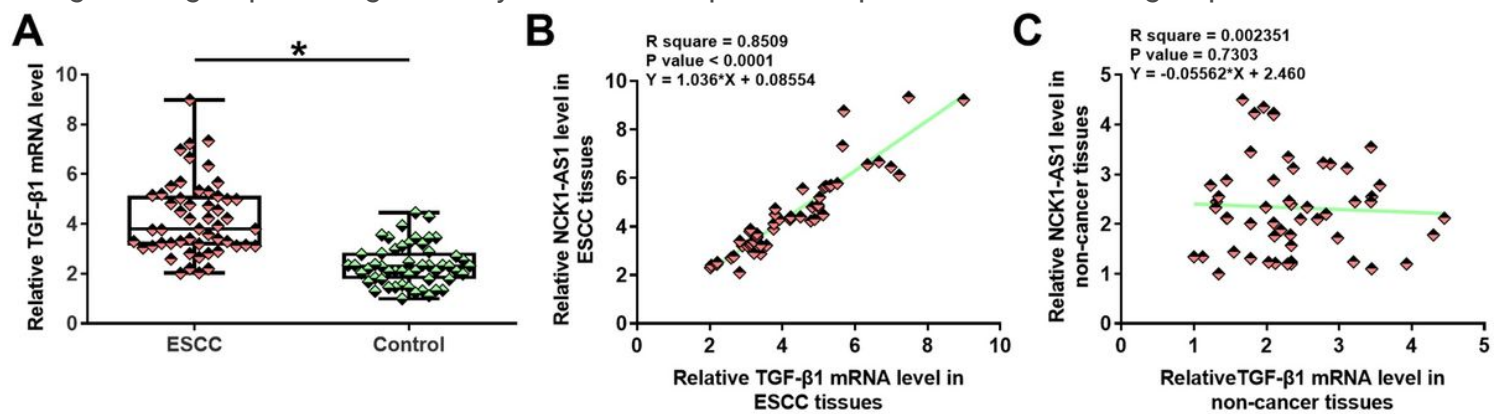

Figure 3

TGF-β1 mRNA expression was upregulated in ESCC tissue and positive correlated with NCK1-AS1 Expression data analyzed by paired t test showed that TGF-β1 mRNA expression was significantly

upregulated in ESCC tissues comparing to non-cancer tissues (A) (*, p<0.05). Linear regression showed that TGF-β1 and NCK1-AS1 were significantly and positively correlated in ESCC tissues (B), but not in adjacent non-cancer tissues (C).
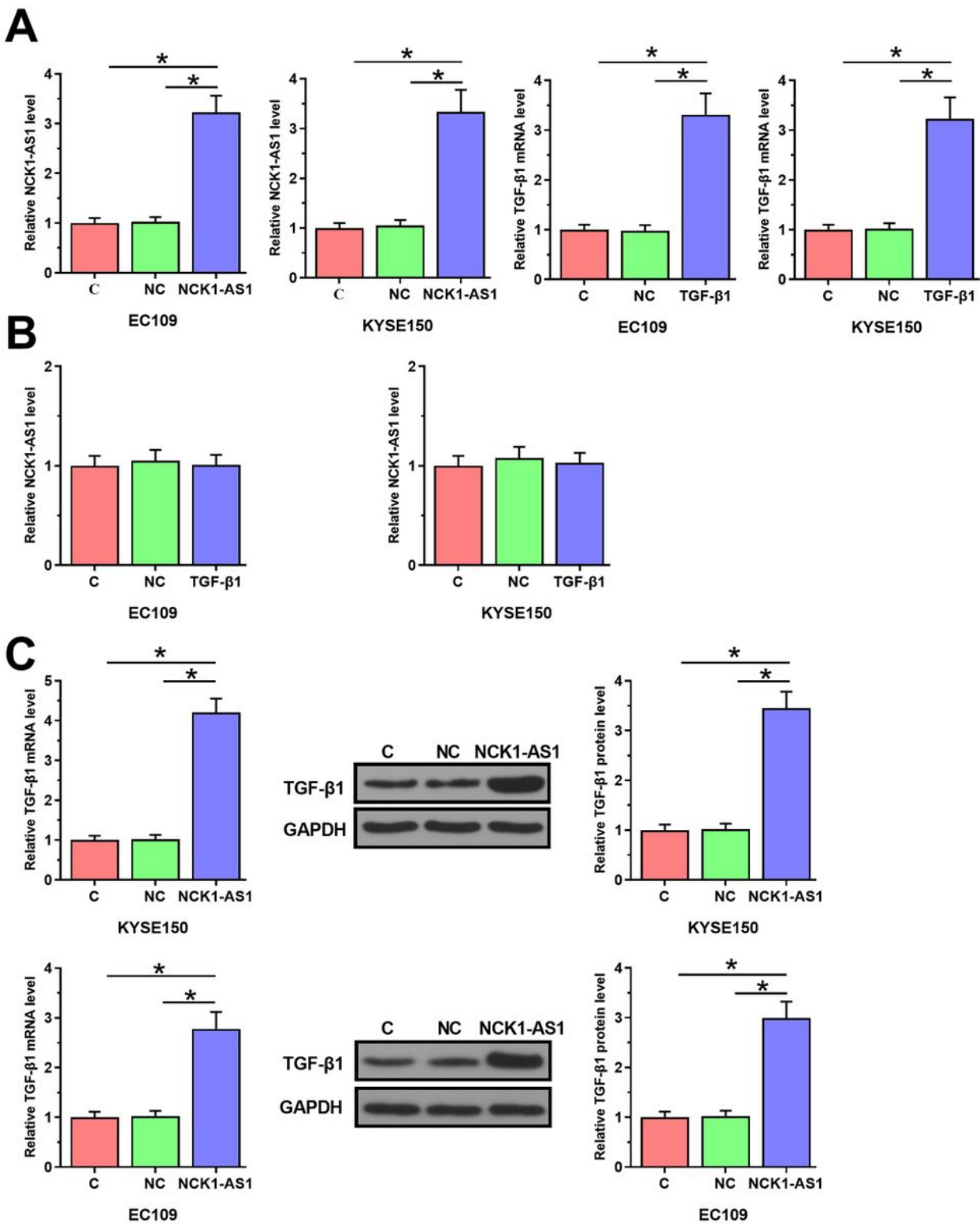

Figure 4

Overexpression of NCK1-AS1 stimulated TGF-β1 expression Overexpression of TGF-β1 and NCK1-AS1 was confirmed at 24h after transient transfections (A). TGF-β1 overexpression did not affect NCK1-AS1

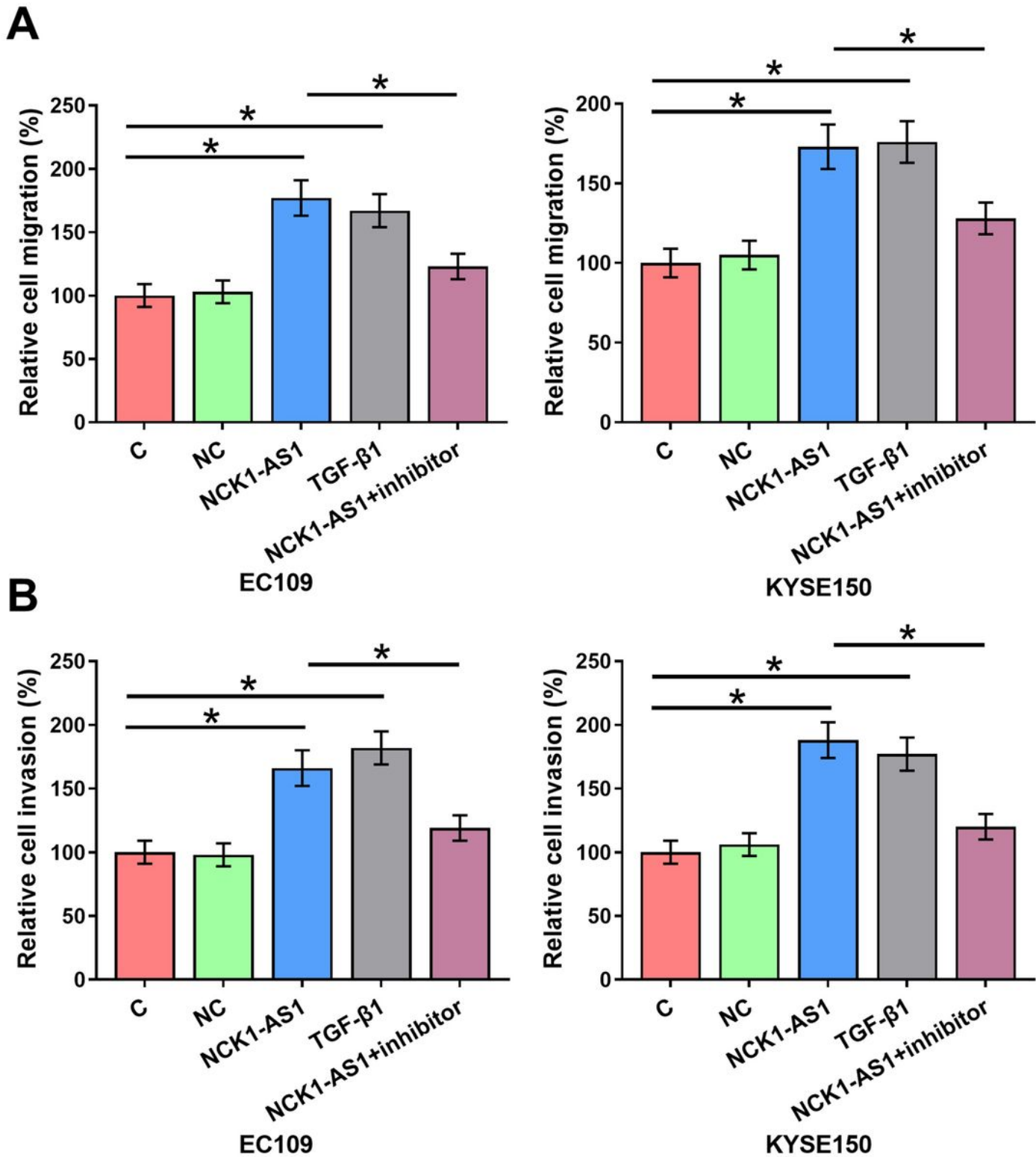(B), while NCK1-AS1 upregulated TGF-β1 expression in cells of ESCC cell lines (C) (*, p<0.05).

Figure 5

NCK1-AS1 stimulated ESCC cell migration and invasion through TGF-β1 TGF-β1 and NCK1-AS1 overexpression increased ESCC cell migration (A) and invasion (B). In addition, TGF-β inhibitor SB431542 attenuated reduced the effect of NCK1-AS1 overexpression (*, p<0.05).

Supplementary Files

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