

LncRNA TP73-AS1 Activates TGF- β 1 to Promote the Migration and Invasion of Colorectal Cancer Cell

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Purpose: LncRNA TP73-AS1 has been demonstrated to promote the developments of several types of human cancer. However, its role in colorectal cancer (CRC) is unknown.

Methods: All CRC patients (n=70, 40 males and 30 females, 38 to 66 years' old, 52.1 \pm 5.3 years' old) in this study were enrolled in the Affiliated Hospital of Southwest Medical University from July 2012 to January 2014. Cells, vectors, and transient transfections, RT-qPCR, western-blotting, as well as measurements of cell migration and invasion abilities were carried out during the research.

Results: In the present study, we found that TP73-AS1 was upregulated in CRC tissues compared with adjacent non-CRC tissues in CRC patients. Upregulation of TP73-AS1 was closely correlated with poor prognosis. TGF- β 1 was also upregulated in CRC tissues and positive correlated with TP73-AS1. TP73-AS1 overexpression caused upregulated TGF- β 1 in CRC cells, while TGF- β 1 overexpression showed no significant effect on TP73-AS1. TP73-AS1 and TGF- β 1 overexpressions caused enhanced migration and invasion of CRC cells. TGF- β inhibitor treatment caused suppressed migration and invasion of CRC cells and attenuated effects of TP73-AS1 and TGF- β 1 overexpression.

Conclusion: Therefore, TP73-AS1 may inactivate TGF- β 1 to inhibit the migration and invasion of CRC cells.

Keywords: colorectal cancer, lncRNA TP73-AS1, TGF- β 1, prognosis

Introduction

The early diagnosis of many types of human cancers is quite technically challenging. As a result, many cancer patients are diagnosed at inoperable conditions and their survival rate is quite poor.¹ Therefore, accurate prediction of the prognosis of cancer patients at advanced stages is necessary to assist the development of individualized treatment strategies.² Colorectal cancer (CRC) is considered as a major cause of deaths in cancer patients, with aggressive nature and unacceptably high incidence rate of malignancy.³ Treatment of CRC patients mainly relies on chemotherapeutic and radiation therapies, which may induce severe side effects.^{4,5} In addition, many CRC patients experience recurrence even after curative resection.⁶ Therefore, novel biomarkers are urgently needed to guide the treatment of CRC.

Recently, researchers have characterized a considerable number of genetic alterations on the pathogenesis of CRC, which involved in the occurrence and development of CRC.⁷ Besides, it was also well established that non-coding RNAs, such as long non-coding RNAs (lncRNAs, >200nt) have also been proved to be critical factors in cancer biology.⁸ LncRNAs regulate cancer development mainly by regulating cancer-related genes.⁹ Therefore, the regulation of lncRNA expression may have

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potential therapeutic value for cancer treatment.¹⁰ Although LncRNA TP73-AS1 has been proved to promote several types of cancer including CRC,^{11–14} two recent studies reported opposite expression patterns and functionality of TP73-AS1 in CRC. Our study was therefore performed to further explore the role of TP73-AS1 in CRC.

Materials and Methods

Research Subjects and Specimens

All CRC patients (n=70, 40 males and 30 females, 38 to 66 years' old, 52.1 ± 5.3 years' old) included in this study were enrolled in the Affiliated Hospital of Southwest Medical University from July 2012 to January 2014. All patients were initially diagnosed as CRC by X-ray imaging and confirmed by histopathological examinations. Patients with other clinical disorders, with a history of malignancies, or received any therapies before admission were excluded from this study. Based on the staging criteria proposed by AJCC, 14, 17, 22 and 17 patients were classified into stages I–IV, respectively. The Affiliated Hospital of Southwest Medical University Ethics Committee approved this study before admission of patients. All patients signed informed consent.

During the diagnosis, a biopsy was performed on all patients to obtain cancer (CRC) tissues as well as non-cancer tissues. All tissue specimens were confirmed by 3 experienced pathologists. Specimens were stored in liquid nitrogen at the specimen library of the Affiliated Hospital of Southwest Medical University before use.

A 5-Year's Follow-Up

All the 70 CRC patients were followed up for 5 years. Follow-up was performed through telephone or by an out-patient visit in some cases to monitor their survival. Patients who were lost during follow-up were not included. Patients died of other clinical disorders or accidents were also excluded.

Cells, Vectors and Transient Transfections

Human CRC cell lines CR4 (Sigma-Aldrich, USA) and RKO (ATCC, USA), as well as normal colon cell line CCD-18Co, were used in this study. Eagle's Minimum Essential Medium (10% FBS) was used as cell culture medium. Cell culture conditions were 5% CO₂ and 37°C.

TP73-AS1 and TGF-β1 expression vectors were constructed by inserting full-length TP73-AS1 or TGF-β1 cDNAs into pcDNA3.1 vectors (Sangon, Shanghai, China). CR4 and RKO cells were cultivated overnight to confluence

of 70–80%, followed by transient transfections performed using lipofectamine™ 2000 (Thermo Fisher Scientific) with vector at dose of 10 nM. Subsequent experiments were performed at 24 hrs after transfections. Cells without transfections (control) and cells transfected with empty vectors (negative control) were included to serve as two controls.

Total RNA Extraction and RT-qPCR

Total RNA extractions from CR4, RKO and CCD-18Co cells, as well as tissues, were performed using Ribozol reagent (Sigma-Aldrich, USA). Following cDNA synthesis using AMV Reverse Transcriptase (Promega Corporation, USA), qPCR reaction systems were prepared using SYBR® Green master mix (Bio-Rad, USA) with 18S rRNA or GAPDH as endogenous controls to detect the expression of TP73-AS1 and TGF-β1, respectively. PCR reactions were repeated 3 times. All data were analyzed using 2^{-ΔΔCT} method.

Western Blotting

Total protein extractions from CR4 and RKO cells (collected at 24 hrs after transfection) were performed using ReadyPrep™ Protein Extraction Kit (Bio-Rad). After denaturing, protein samples were subjected to gel electrophoresis using 10% SDS-PAGE. Following gel transfer to PVDF membranes, blocking was performed in 5% non-fat milk for 2 hrs at room temperature. After that, membranes were first incubated with rabbit polyclonal TGF-β1 (ab92486, 1:1200; Abcam) and GAPDH (ab9485, 1:1200, Abcam) primary antibodies, followed by incubation with an anti-rabbit IgG-HRP secondary antibody (1:1200, MBS435036, MyBioSource). Signals were developed using Pierce ECL Western Blotting Substrate (Thermo Fisher Scientific). Image J v.1.46 software was used to process all data.

Measurement of Cell Migration and Invasion Abilities

CR4 and RKO cells (collected at 24 hrs after transfection) were used to prepare single-cell suspensions using Eagle's Minimum Essential Medium containing 1% FBS. In cases of TGF-β inhibitor treatment, SD 208, a potent ATP-competitive TGF-β receptor inhibitor (RI) inhibitor (10 ng/mL, R&D Systems, Inc., Minneapolis, MN, USA) was used to treat cells at 37°C for 24 hrs before use. Cell suspensions were transferred to upper Transwell chamber with 0.1 mL per well, and Eagle's Minimum Essential Medium (20% FBS) was added into the lower Transwell chamber. It is worth noting that Matrigel (356234, Millipore, USA) was used to coat the

upper chamber membrane before invasion assay to mimic *in vivo* cancer cell invasion. After cell culture for 2 hrs, 0.5% crystal violet (Sigma-Aldrich, USA) was used to stain the upper chamber membrane and stained cells were observed under an optical microscope.

Statistical Analysis

Experiments were repeated 3 times to obtain solid data. Differences between cancer and non-cancer tissues were analyzed by performed paired *t*-test. Differences among different cell transfection groups or among different clinical stages were analyzed by performed one-way ANOVA and Tukey's test. Linear regression was used to analyze the correlation between TP73-AS1 and TGF- β 1. For survival analysis, the 70 patients were first grouped into low (n=37) and high (n=33) TP73-AS1 groups (Youden's index) using TP73-AS1 expression data in CRC tissues, followed by performing K-M method and log-rank test to plot and compare survival curves. Differences were statistically significant when $p < 0.05$.

Results

TP73-AS1 Was Upregulated in CRC

TP73-AS1 expression was detected by performing RT-qPCR. TP73-AS1 expression in CRC and non-cancer tissues were compared by performing a paired *t*-test. It was found that expression levels of TP73-AS1 were significantly higher in CRC tissues comparing to non-cancer tissues (Figure 1A, $p < 0.05$). In addition, expression levels of TP73-AS1 were

also higher in cells CRC cell line CR4 and RKO than in cells of normal colon cell line CCD-18Co (Figure 1B, $p < 0.05$).

TP73-AS1 Is Correlated with the Survival of CRC Patients

Before survival analysis, TP73-AS1 expression in CRC tissues was first compared by performing ANOVA (one-way) and Tukey's test. It was observed that TP73-AS1 expression levels were not significantly different among patients with different clinical stages (Figure 2A). Seventy patients were first grouped into low (n=37) and high (n=33) TP73-AS1 groups (Youden's index) using TP73-AS1 expression data in CRC tissues, followed by performing K-M method and log-rank test to plot and compare survival curves. The results showed that patients with high levels of TP73-AS1 had significantly worse survival conditions (Figure 2B).

TP73-AS1 Promoted TGF- β 1 Expression

TGF- β 1 mRNA was also detected by performing RT-qPCR. TGF- β 1 mRNA expression in CRC and non-cancer tissues were compared by performing a paired *t*-test. It was found that expression levels of TGF- β 1 mRNA were significantly higher in CRC tissues comparing to non-cancer tissues (Figure 3A, $p < 0.05$). Linear regression was used to analyze the correlation between TP73-AS1 and TGF- β 1. It was found that TP73-AS1 and TGF- β 1 mRNA were significantly and positively correlated in CRC tissues (Figure 3B), but not in non-cancer tissues (Figure 3C). To further investigate the relationship between TP73-AS1 and TGF- β 1, TP73-AS1 and TGF- β 1

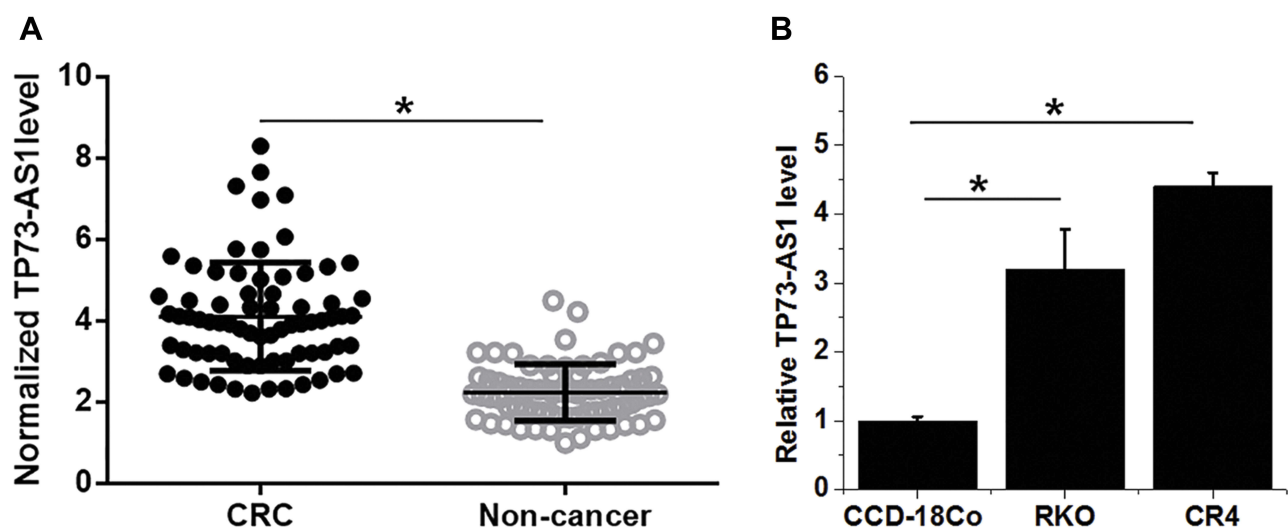


Figure 1 TP73-AS1 was upregulated in CRC tissues. Analysis of TP73-AS1 expression by paired *t*-test revealed that expression levels of TP73-AS1 were significantly higher in CRC tissues comparing to non-cancer tissues (A). In addition, ANOVA (one-way) and Tukey's test analysis showed that expression levels of TP73-AS1 were also higher in CRC cell line CR4 and RKO than in cells of normal colon cell line CCD-18Co (B) (* $p < 0.05$).

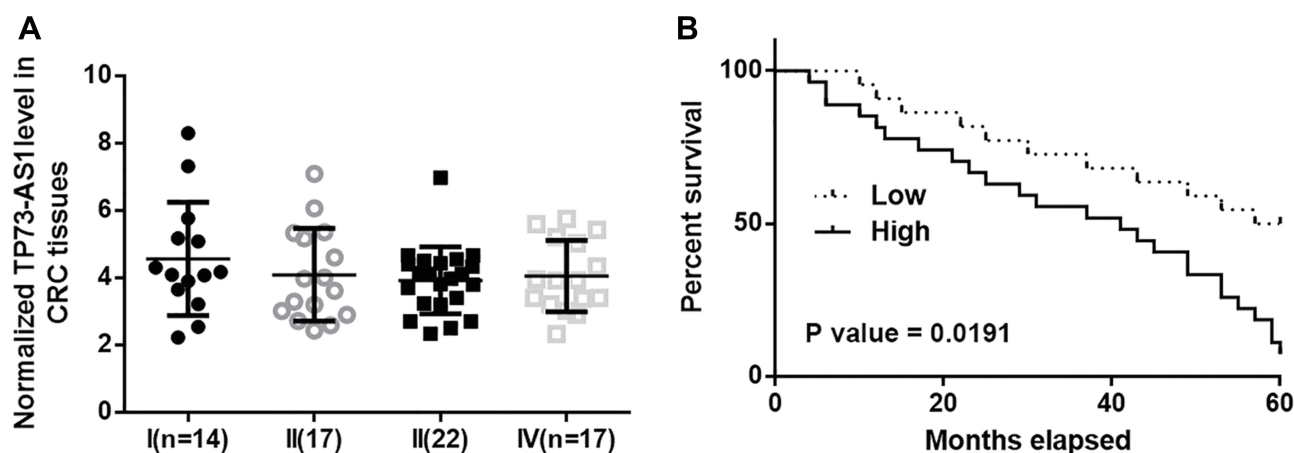


Figure 2 TP73-AS1 is correlated with the survival of CRC patients. Analysis of TP73-AS1 expression by performing ANOVA (one-way) and Tukey's test showed that TP73-AS1 expression levels were not significantly different among patients with different clinical stages (A). Survival curve analysis showed that patients with high levels of TP73-AS1 had significantly worse survival conditions (B).

expression vectors were transfected into CR4 and RKO cells. Expression levels of TP73-AS1 and TGF- β 1 mRNA were significantly increased at 24 hrs after transfections comparing to C and NC two controls (Figure 3D, $p < 0.05$). In addition, TP73-AS1 overexpression caused upregulated TGF- β 1 mRNA and protein in CRC cells (Figure 3E, $p < 0.05$), while TGF- β 1 overexpression showed no significant effect on TP73-AS1 (Figure 3F).

Upregulating of TGF- β 1 by TP73-AS1 Regulated CRC Cell Migration and Invasion

Analysis of Transwell migration and invasion data by one-way ANOVA and Tukey's test showed that TP73-AS1 and TGF- β 1 overexpression caused promoted migration and invasion of both CR4 (Figure 4A, $p < 0.05$) and RKO (Figure 4B, $p < 0.05$) cells. TGF- β inhibitor treatment caused inhibited migration and invasion of CRC cells and attenuated effects of TP73-AS1 and TGF- β 1 overexpression.

Discussion

The present study mainly investigated the role of TP73-AS1 in CRC. We found that TP73-AS1 has prognostic value for CRC and overexpression of TP73-AS1 may promote CRC cell migration and invasion through the upregulation of TGF- β 1.

Previous studies showed that TP73-AS1 was a key player in many types of cancer, such as osteosarcoma¹¹ and bladder,¹² indicating that TP73-AS1 may also participate in CRC. Interesting, in one study Jia et al showed that TP73-

AS1 is downregulated in CRC and inhibited cell proliferation by sponging miR-103 to regulate PTEN.¹³ In contrast, in another study, Cai et al reported that TP73-AS1 was overexpressed in CRC and promoted cancer cell proliferation, migration and invasion by upregulating TGF α .¹⁴ In this study we found TP73-AS1 was upregulated in CRC tissues but was not affected by clinical stages. Therefore, the TP73-AS1 was not further upregulated with the development of CRC. It is possible that TP73-AS1 was upregulated at the beginning of the formation of CRC tumors and participates in the whole procedure of cancer development. Interestingly, our follow-up study showed that high TP73-AS1 level was closely correlated with the poor survival of CRC patients. Therefore, detecting the expression of TP73-AS1 in cancer tissues may predict the survival of CRC patients.

TGF- β signaling is a critical player in cancer development.¹⁵ Although the role of TGF- β in regulating the proliferation of cancer cells is contradictory in different types of cancer, it is generally believed that the activation of TGF- β has enhancing effects on cancer cell migration and invasion.¹⁶ It has been reported TP73-AS1 interacts with epithelial-to-mesenchymal transition (EMT) pathway to promote cancer development,¹² while TGF- β signaling is a key player in EMT.^{17,18} In the present study, we showed that TP73-AS1 was likely an upstream activator of TGF- β signaling, and this interaction participates in the regulation of cancer cell migration and invasion. However, the mechanism of the interaction between these 2 factors is unknown. It has been reported that TGF- β in cancer can be regulated by miR-142,¹⁹ which can also interact with TP73-AS1.²⁰ Therefore, miR-142

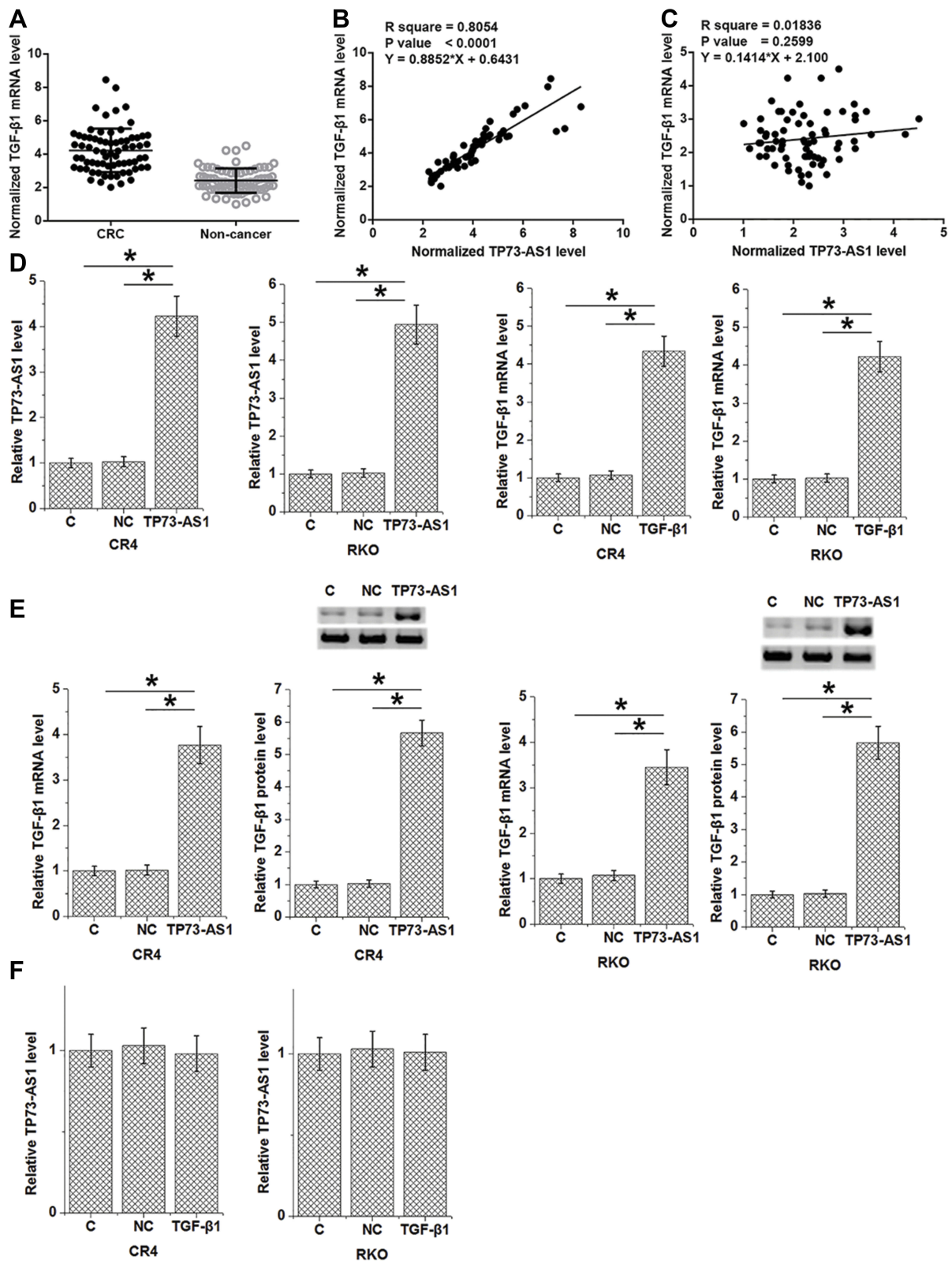


Figure 3 TP73-AS1 promoted TGF- β 1 expression. Paired t-test analysis showed that expression levels of TGF- β 1 mRNA were significantly higher in CRC tissues comparing to non-cancer tissues (**A**). Linear regression showed that TP73-AS1 and TGF- β 1 mRNA were significantly and positively correlated in CRC tissues (**B**), but not in non-cancer tissues (**C**). Expression levels of TP73-AS1 and TGF- β 1 mRNA were significantly increased at 24 hrs after transfections comparing to C and NC two controls (**D**). In addition, TP73-AS1 overexpression caused upregulated TGF- β 1 mRNA and protein in CRC cells (**E**), while TGF- β 1 overexpression showed no significant effect on TP73-AS1 (**F**) (* p <0.05).

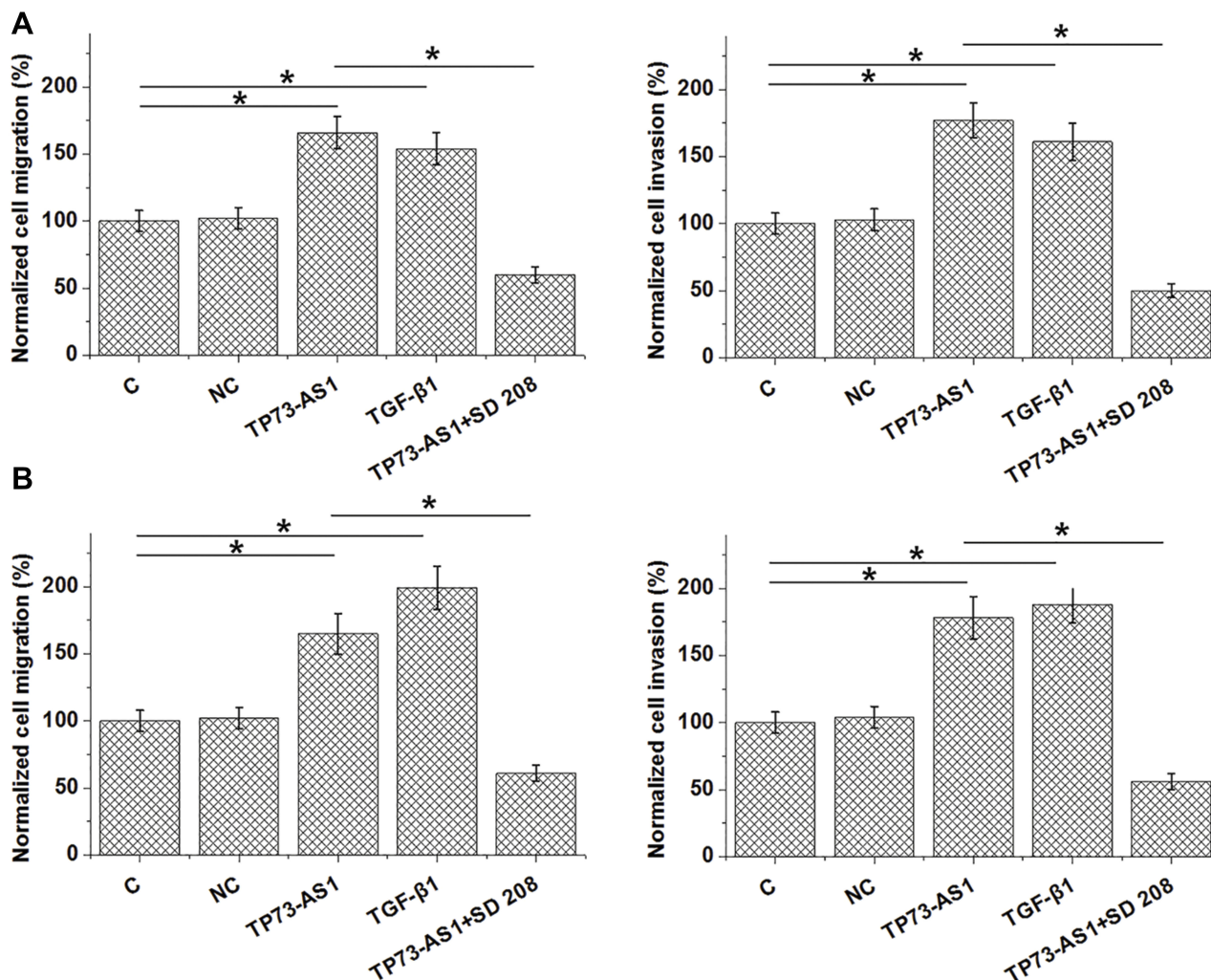


Figure 4 Upregulating of TGF- β 1 by TP73-AS1 regulated CRC cell migration and invasion. Transwell migration and invasion data showed that TP73-AS1 and TGF- β 1 overexpression caused promoted migration and invasion of both CR4 (A) and RKO (B) cells. TGF- β inhibitor treatment caused inhibited migration and invasion of CRC cells and attenuated effects of TP73-AS1 and TGF- β 1 overexpression (* p <0.05).

may be a mediator between TP73-AS1 and TGF- β . We will test this possibility and explore other mediators in our future studies.

Conclusion

In conclusion, TP73-AS1 was upregulated in CRC and overexpression of TP73-AS1 resulted in TGF- β upregulation, which in turn led to the promoted migration and invasion of CRC cells.

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Disclosure

The author reports no conflicts of interest in this work.

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