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Research Article

LncRNA U731166 Increases the Accumulation of TGFBR1 by Sponging miR-3607-3p in Esophageal Squamous-Cell Carcinomas (ESCC) to Promote Tumor Metastasis

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Background: Long non-coding RNA (lncRNA) U731166 and microRNA (miR)-3607-3p are two ncRNAs with critical roles in cancer biology, while their involvement in esophageal squamous-cell carcinomas (ESCC) is unclear. We predicted that U731166 and miR-3607-3p might interact with each other. This study aimed to investigate their role and interaction in ESCC.

Objectives: This study was therefore conducted to explore the involvement of U731166 and miR-3607-3p in ESCC, with a focus on the interaction between them.

Materials and Methods: Paired ESCC and non-tumor tissue samples were recruited from 72 ESCC patients. By RT-Qpcr, level of U731166 and miR-3607-3p in paired tissues was measured. By RNA-RNA pulldown assay, the direct interaction between U731166 and miR-3607-3p was detected. U731166 overexpression or miR-3607-3p overexpression was performed to investigate their role in regulating the expression of each other. By RT-qPCR and Western blot analysis, the role of U731166 and miR-3607-3p in regulating the level of TGFBR1 was assessed. By Transwell assays, cell invasion and migration were analyzed.

Results: Compared to non-tumor tissues, U731166 was highly upregulated in ESCC, while miR-3607-3p was downregulated in ESCC. U731166 and miR-3607-3p directly interacted with each other, but they are not closely correlated and did not regulate the level of each other. Moreover, U731166 reversed the role of miR-3607-3p in downregulating TGFBR1 and inhibiting cancer cell invasion and migration. U731166 and miR-3607-3p were closely associated with patients' tumor metastasis but not tumor size.

Conclusion: U731166 may upregulate TGFBR1 by sponging miR-3607-3p in ESCC cells to promote tumor metastasis.

Keywords: Esophageal squamous-cell carcinomas; miR-3607-3p; TGFBR1, U731166

1. Background

As a common type of malignant and solid tumor, esophageal squamous-cell carcinomas (ESCC) originate from the flat and thin epithelial cells lining the esophagus (1-3). Prevalence of ESCC varies a lot across the world, with the highest incidence observed in east Asian countries such as China, with an estimated rise in incidence in the following decades (4). With proper treatment, such as endoscopic resection and esophagectomy, about half of ESCC patients diagnosed with localized tumors can survive more than 5 years (5). However, patients with tumor metastasis can only be treated with neoadjuvant or adjuvant therapy, resulting in a 5-year overall survival rate as low as 5% (6, 7). Therefore, more treatment approaches are needed to combat the increasing incidence of ESCC.

Elucidation of the molecular mechanisms of ESCC has revealed a large number of molecular players involved in the pathological changes in this cancer (8). In effect, the expression of some molecular factors, such as VEGFR1 and VEGFR2, can be regulated by targeted therapy to affect tumor growth and metastasis, thereby suppressing ESCC progression (9, 10). However, targeted therapy for ESCC is still limited. Long non-coding RNAs (lncRNAs) do not have coding capacity but can interact with functional DNAs, proteins and miRNAs to regulate cancers (11, 12). The regulation of lncRNAs have been suggested to have great potentials in the treatment of ESCC (13, 14). U731166 has been recently characterized as a cancer-related lncRNA in melanoma (15), while its involvement in ESCC is unclear. It was predicted that U731166 may interact with microRNA (miR)-3607-3p, which targeted TGFBR1 to suppress cancer progression (16).

2. Objectives

This study was carried out to investigate the role of U731166, miR-3607-3p and TGFBR1 in ESCC.

3. Materials and Methods

3.1. ESCC Patients and Tissue Samples

ESCC and paired non-tumor tissues were prepared by dissecting the resected tumors (for patients received surgical section) and biopsy (for patients not appropriate for surgery) from 72 ESCC patients admitted to the Fourth Hospital of Hebei Medical University Shijiazhuang City. The Ethics Committee of this hospital approved this study (Ethical approval no. 5231632). All patients were newly diagnosed ESCC cases and patients with recurrent tumors or initiated therapy were excluded. All patients signed the informed consent.

3.2. Cell Culture

Gene function analysis was performed through *in vitro* cell experiments with TE8 and KYSE-450 human ESCC cell lines (Chinese Type Culture Collection, Chinese Academy of Sciences, Shanghai, China). RPMI-1640 (10% FBS and 1% penicillin/streptomycin) was used to cultivate cells in 6-cm plates, which were monitored every day and plates with observed contamination were discarded immediately.

3.3. Cell Transfection

Empty pcDNA3.1 vector, pcDNA3.1-U731166 vector, pcDNA3.1-TGFBR1 vector, NC miRNA and miR-3607-3p mimic (GenePharma, Shanghai) were transfected into TE8 and KYSE-450 ESCC cells using lipofectamine 2000 (Invitrogen). Briefly, 1 x 10^6 cells in a 12-cm cell plate were transfected with 50 mM miRNA and/or 10 mM vector. Lipofectamine 2000 was then mixed with miRNA and/or vector to prepare transfection mixture. Then the transfection mixture was incubated with cells at 37 °C for 6 h. Finally, transfected cells were washed with fresh medium to reduce cytotoxicity.

3.4. RNA Preparation

In this study, total RNAs were extracted from clinical samples using High Pure RNA Isolation Kit (Product No. 11828665001, Roche Life Science). In brief, Lysis/Binding Buffer was mixed with clinical samples (10:1 ratio; volume/volume) to prepare lysate. After that, RNA binding was performed through centrifugation using RNA binding column, followed by gDNA removal by adding DNase I incubation buffer containing DNase I. After that, DNase I was removed through centrifugation, followed by washing using Wash Buffer I and II. Finally, elution buffer was added to elute RNAs into collection tubes through centrifugation. PureLink™ miRNA Isolation Kit (Thermo Fisher Scientific) was applied for miRNA isolation. RNA samples were analyzed using 2100 Bioanalyzer and the results showed that all RNA samples were with high purity and RNA integrity (RNA integrity number higher than 8.0). RNA samples were kept in liquid nitrogen prior to the subsequent assays.

3.5. RT-qPCR

RNA samples were reverse transcribed into cDNA samples through reverse transcription (RT) using M-MLV Reverse Transcriptase (Sigma-Aldrich). To measure the accumulation levels of U731166 and TGFBR1 mRNA and miR-3607-3p in clinical samples, qPCR was conducted using ChamQ Universal SYBR qPCR Master Mix (Vazyme) with 18S rRNA as an internal control. Stem-loop qRT-PCT was applied to determine the expression of miR-3607-3p with U6 as an internal control. Each qPCR was repeated three times and average values were presented. Values were compared by t test. MiR-3607-3p RT primer sequence was: 5'-GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACCATCA-3'. U6 RT primer sequence was: 5'-GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACAAAATATGGAA-3'. The $2^{-\Delta\Delta Ct}$ method was used to process Ct values. Primer sequences used in qPCR were: miR-3607-3p:5›-ATGACTGTAAACGCTTTCTG-3› (forward) and 5›-GTGCAGGGTCCGAGGT-3' (reverse); U6: 5'-TGCGGGTGCTCGCTTCGGCAGC -3' (forward) and 5'-GTGCAGGGTCCGAGGT-3' (reverse); U73166: 5'- GCGGTCCTCATCTCTACCAT-3' (forward) and 5'-GTAATTCCAGACCCCTGTGG-3' (reverse); TGFBR1: 5'-GTTCCGTGAGGCAGAGATTT-3'(forward) and 5'-CCGTGGACAGAGCAAGTTT-3'(reverse); 18S rRNA: 5'-GTTTCCCATCCTACGCTTCC-3' (forward) and 5'-AGACAAATCGCTCCACCAAC-3' (reverse). All qPCR reactions were carried out on a CFX Opus 96 Real-Time PCR System (Bio-Rad, Shanghai, China).

3.6. Biotin RNA-RNA Pull-Down

In vitro transcriptions were performed to prepare the transcripts of both NC and U731166, followed by labeling with Biotin. The labeled RNAs were named Bio-NC and Bio-U731166, respectively. Then the labeled RNAs were transfected into cells, then cell culture for 48 h. Cell lysates were then prepared and incubated with Dynabeads. Then RNA pulldown was performed, and after RNA purification, accumulation of miR-3607-3p was detected by RT-PCR.

3.7. Western Blot Analysis

Cell lysate was made by mixing RIPA (Sigma-Aldrich) and cells to prepare protein samples, which were then quantified using BCA assay (Sigma-Aldrich). The composition of RIPA solution was 150 mM NaCL, 1% Nonidet P-40, 0.5% DOC, 0.1% SDS, and 50 mM Tris (pH 7.4). Protein samples were subjected to electrophoresis using 10% sodium dodecyl sulfate-polyacrylamide gel. After gel transfer and blocking, samples were incubated with primary rabbit polyclonal antibody to TGFBR1 (1:1,000, A90567, Abcam) and goat anti-rabbit IgG H&L (HRP) secondary antibody (ab6721, Abcam). Signals were developed with ECL (Sigma-Aldrich). MYECL imager (Thermo Fisher Scientific) was used to capture gray values. All other groups were normalized to the control group, and the the control group value was set to "1".

3.8. Transwell Assay

Cells were collected and count to analyze their invasion

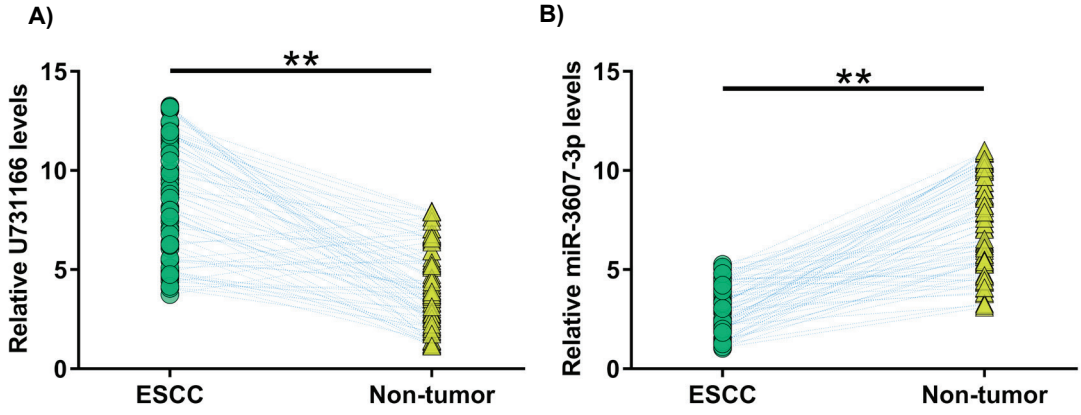

Figure 1. **Differential expression of U731166 (A), and miR-3607-3p (B) in paired ESCC and non-tumor samples, collected form 72 ESCC patients, determined by RT-qPCR**. ** $p< 0.01$.

and migration ability using Transwell inserts (Corning, USA). To mimic invasion condition, inserts were coated with serum-free RPMI-1640 medium containing Matrigel (Corning, USA) for overnight. Migration assay was performed using uncoated inserts. Cells were seeded to the upper chamber filled with non-serum medium, and complete medium was added to the low chamber. Cells were cultivated for 12 h. Cells were then collected, and membranes were fixed with crystal violet staining following 4% paraformaldehyde. A microscope (Olympus, Tokyo, Japan) was then used to count cells.

3.9. Statistical Analysis

Data analysis and data visualization were performed using GraphPad Prism 7.0. Two groups were compared by Student's *t* test and multiple groups were compared by ANOVA Tukey's test. Paired tissues were compared by paired *t* test. The 72 patients were divided into high and low U731166 or miR-3607-3p level groups (cutoff = median expression level). Associations between patients' clinical data and the expression of U731166 or miR-3607-3p in ESCC tumors were analyzed by performing Chi-squared test. All in vitro experiments were performed in three biological replicates and mean +/- SD values were presented and compared. $P < 0.05$ indicated a difference with statistical significance.

4. Results

4.1. Differential Expression of U731166 and miR-3607-3p in ESCC

To explore the involvement of U731166 and miR-3607-3p in ESCC, their expression in ESCC and paired nontumor samples collected form the 72 ESCC patients was determined by performing RT-qPCR. Compared to non-tumor samples, U731166 was highly upregulated in ESCC samples (**Fig. 1A**, $p < 0.01$), while miR-3607-3p was significantly downregulated in ESCC samples (**Fig. 1B**, $p < 0.01$). These results suggested the participation of U731166 and miR-3607-3p in ESCC.

4.2. U731166 and miR-3607-3p Bind to Each Other But did not Regulate the Expression of Each Other

The directing binding of miR-3607-3p to U731166 was performed using IntaRNA 2.0 (**Fig. 2A**), and it showed that miR-3607-3p might bind to U731166. The direct interaction of U731166 and miR-3607-3p in TE8 and KYSE-450 cells was then analyzed by RNA-RNA binding assay using biotin (Bio-) labeled RNAs. Compared with Bio-NC group, significantly overexpressed miR-3607-3p (**Fig. 2B**, $p < 0.01$) was observed in Bio-U731166 group, suggesting that miR-3607-3p direct bind to U731166. To further confirm the interaction, miR-3607-3p or U731166 was overexpressed in TE8 and KYSE-450 cells (**Fig. 2C**, $p < 0.01$). By RT-Qpcr, their role in regulating the level of each other was analyzed. It showed that they did not affect the expression of each other (**Fig. 2D**). Therefore, U731166 is unlikely a target of miR-3607-3p. To explore the possible interaction between U731166 and miR-3607-3p *in vivo*, their correlation across ESCC and non-tumor tissue samples was analyzed by Pearson's correlation coefficient. U731166 showed no close correlation to miR-3607-3p across ESCC (**Fig. 2E**) and non-tumor (**Fig. 2F**) groups.

4.3. The Role of U731166 and miR-3607-3p in Regulating the Level of TGFBR1

Data presented above indicated that U731166 is unlikely a direct target of miR-3607-3p, while they could bind to each other. Therefore, U731166 may serve as a sponge (endogenous competing RNA) for miR-3607-3p. To explore this possibility, the role of U731166 and miR-3607-3p in regulating the level of TGFBR1, which is a direct target of miR-3607-3p (16), was evaluated by RT-qPCR (**Fig. 3A**) and Western blot analysis (**Fig. 3B**). U731166 increased the expression levels of TGFBR1 at both mRNA and protein levels. In contrast, miR-3607-3p decreased the expression levels of TGFBR1 at both mRNA and protein levels. Moreover, U731166 suppressed the role of miR-3607-3p in downregulating TGFBR1 ($p < 0.01$). Original images of Western blots were presented in **Supplementary data 1**.

4.4. Associations Between the Level of U731166 and miR-3607-3p with Patients' Clinical Data in ESCC Tissues

The 72 patients were divided, which was named as high and low U731166 or miR-3607-3p level groups (cutoff = median expression level). By Chi-squared test, associations between the level of U731166 or miR-3607-3p with patients' clinical data in ESCC tumors were analyzed. U731166 and miR-3607-3p were only closely associated with patients' tumor metastasis, but not tumor size and other factors (**Table 1**), indicating the potential involvement of U731166 and miR-3607-3p in tumor metastasis.

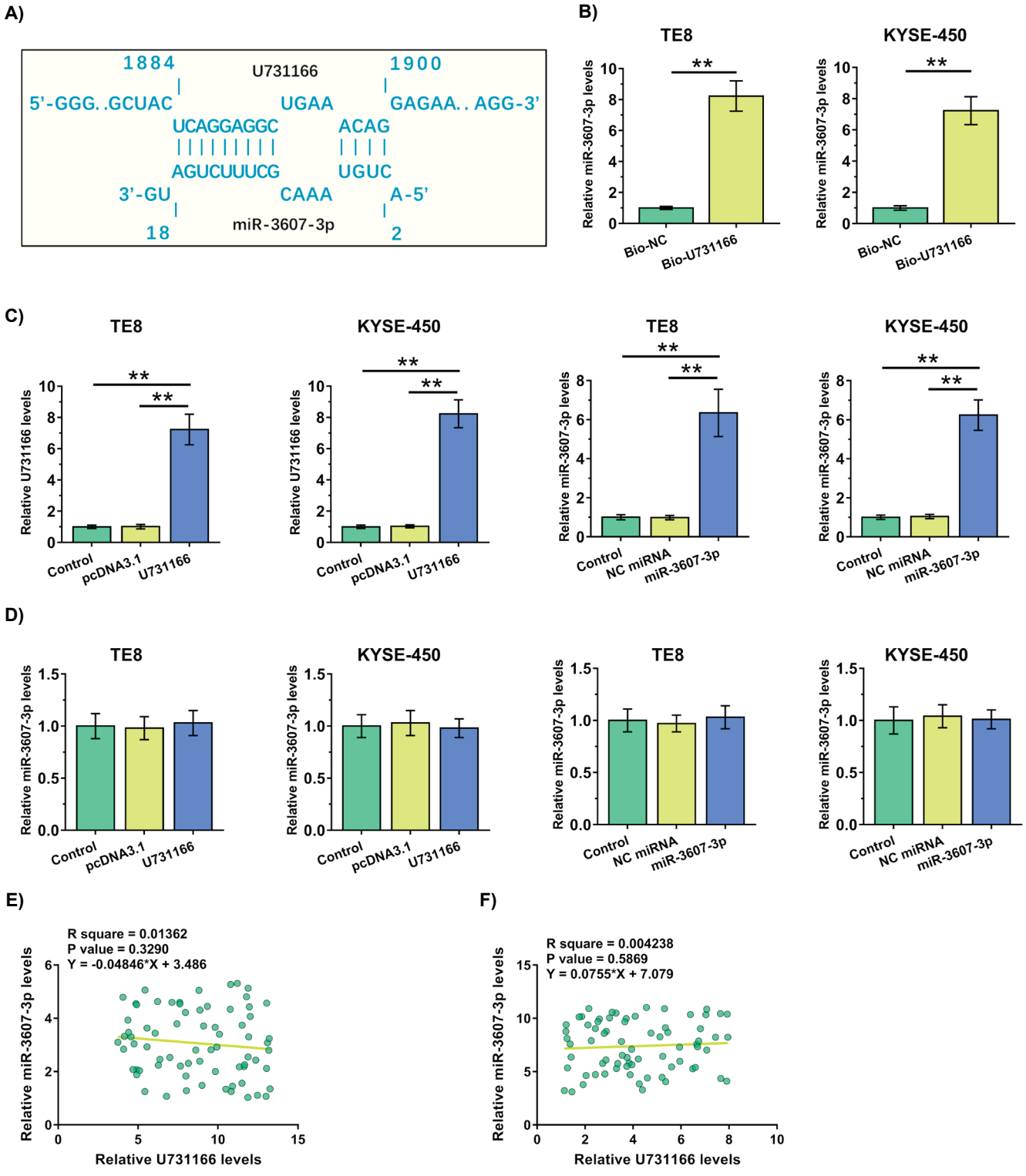

Figure 2. Investigation of binding and cross-regulation of U731166 and miR-3607-3p. Prediction of binting of U731166 and miR-3607-3p (A), Analysis of direct interaction between U731166 and miR-3607-3p in TE8 and KYSE-450 cells **(B)**, Over expression of 731166 and miR-3607-3p in TE8 and KYSE-450 cells **(C)**, Analysis of the regulatory functions of U731166 and miR-3607-3p **(D)**, Correlation between U731166 and miR-3607-3p across ESCC **(E)**, and non-tumor **(F)** tissue samples . **, $p < 0.01$.

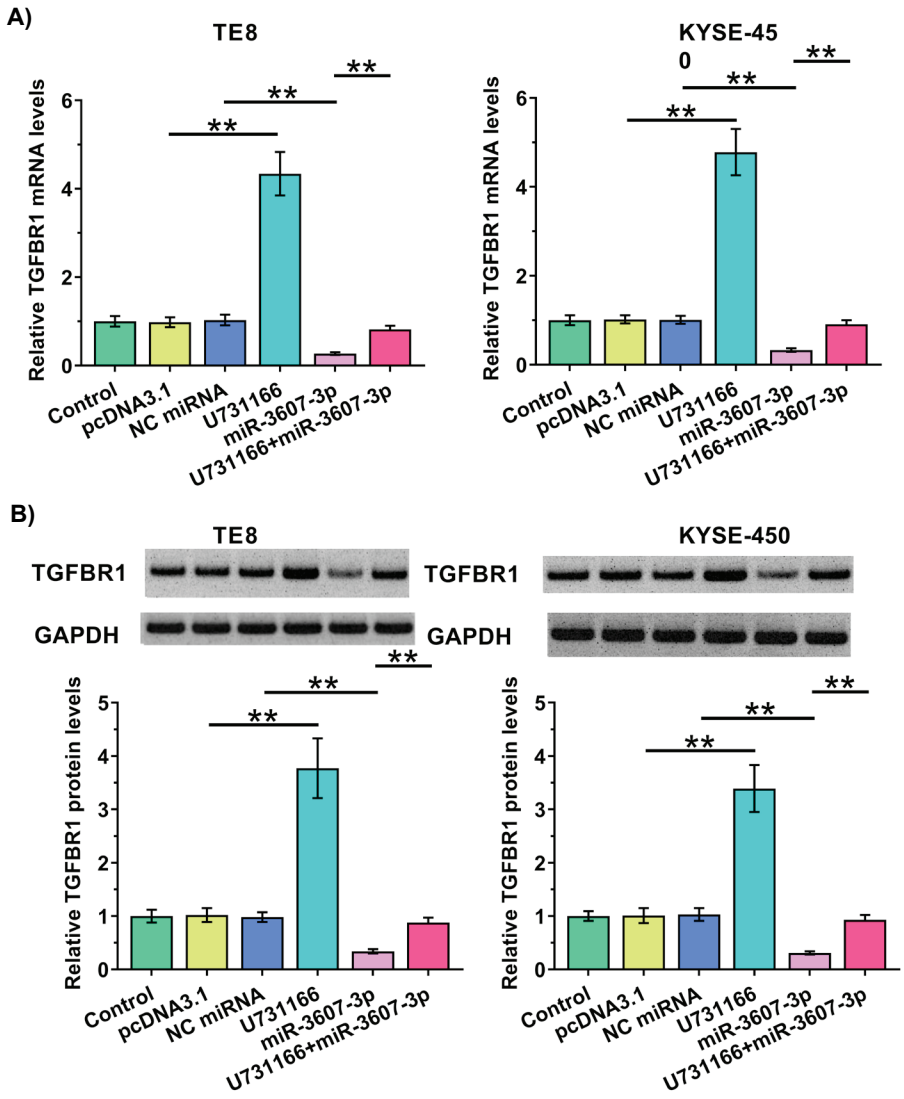

Figure 3. Analysis of the regulatory functions of U731166 and miR-3607-3p on the TGFBR1 expression, based on RT-qPCR (A), and Western blotting (B). **, $p < 0.01$.

4.5. The Role of U731166, miR-3607-3p and TGFBR1 in the Invasion and Migration of ESCC

To explore the role of U731166, miR-3607-3p and TGFBR1 in tumor metastasis of ESCC patients, TE8 and KYSE-450 cells were collected after transfection at 48 h, which was used for transwell invasion (**Fig. 4A**) and migration (**Fig. 4B**) assays. It showed that U731166 and TGFBR1 increased cell invasion and migration. In contrast, miR-3607-3p decreased cell invasion and migration. Moreover, U731166 and TGFBR1 reversed the inhibited role of miR-3607-3p in cell invasion and migration ($p < 0.01$).

5. Discussion

The involvement of U731166 in ESCC and its role in regulating the miR-3607-3p/TGFBR1 axis was explored in this study. We showed that U731166 was highly overexpressed in ESCC. We also observed that U731166 may increase the expression levels of TGFBR1 protein in ESCC patients by sponging miR-3607-3p.

In a recent study, lncRNA U731166 was found to be highly expressed in melanoma and was closely correlated with vemurafenib resistance and cell invasion and migration (15). However, the role of U731166 in

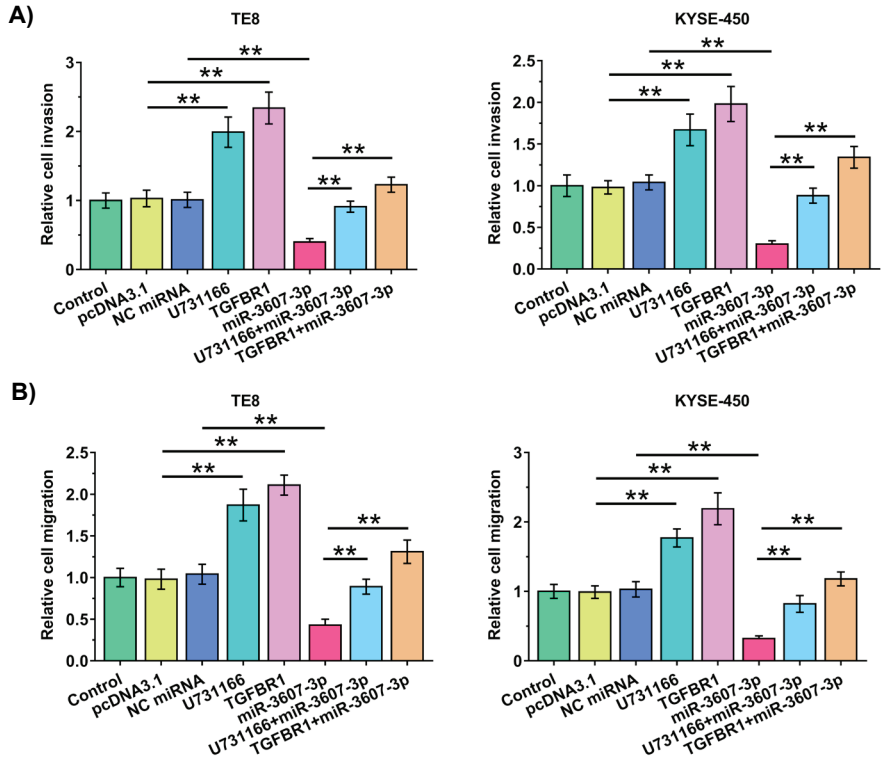

Figure 4. Evaluation of the role of U731166, miR-3607-3p and TGFBR1 in regulating invasion and migration of ESCC cells in TE8 and KYSE-450 cells at 48h post-transfection by performing Transwell invasion (A), and migration (B) assays. **, $p < 0.01$.

Table 1. Associations between patients' clinical data and the accumulation of U731166 and miR-3607-3p in ESCC tissues. The clinical data recorded in this table, were obtained based of the analysis of the expression of U731166 and miR-3607-3p.

Clinical Parameters		Cases	U731166		P	miR-3607-3p		P
			Low	High		Low	High	
Age	<60y	40	18	22	>0.05	17	23	>0.05
	>60y	32	18	14		19	13	
Gender	Male	54	28	26	>0.05	28	26	>0.05
	Female	18	8	10		8	10	
Differentiation grade	Well/Moderate	62	30	32	>0.05	32	30	>0.05
	Poor	10	6	4		4	6	
Distant metastasis	No	58	24	34	0.02	35	23	0.0004
	Yes	14	12	2		1	13	
Lymphnode	Negative	42	9	33	< .00001	32	10	< .00001
	Positive	30	27	3		4	26	
Tumor size	>=7 cm	44	24	20	>0.05	19	25	>0.05
	<7cm	28	12	16		17	11	
Smoking	Yes	55	27	28	>0.05	26	29	>0.05
	No	17	9	8		10	7	
Drinking	Yes	56	29	27	>0.05	30	26	>0.05
	No	16	7	9		6	10	

other cancers is unknown (17). In this study we showed increased expression levels of U731166 in ESCC tissues. Additionally, increased expression levels of U731166 resulted in increased cancer cell invasion and migration in ESCC cells. Therefore, U731166 may also serve as an oncogenic lncRNA in ESCC by promoting tumor metastasis. This is consistent with our association analysis, which showed the close association between U731166 and tumor metastasis, but not other clinical factors. However, animal model experiments need be performed in future studies to further explore the role of U731166 in tumor metastasis. Moreover, this study did not confirm the role of U731166 in the development of chemo-resistance in ESCC cells. Future studies should explore the involvement of U731166 in this process.

Although U731166 is known to be involved in melanoma (15), its downstream targets in cancer biology are not mentioned before. This study predicted and confirmed the interaction between U731166 and miR-3607-3p, which is downregulated in non-small cell lung cancer and targets TGFBR1 to suppress the cancer progression (16, 18). In this study we also revealed the decreased the expression levels of miR-3607-3p in ESCC. In addition, miR-3607-3p also suppressed the level of TGFBR1 in ESCC cells and suppressed cell invasion and migration. Therefore, miR-3607-3p may also target TGFBR1 in ESCC cells to suppress tumor metastasis. Therefore, the same molecular pathway may participate in different cancers. Interestingly, the suppressed effect of miR-3607-3p in downregulating the expression of TGFBR1 and inhibiting cell migration and invasion was suppressed by U731166, while U731166 and miR-3607-3p did not regulate the level of each other. Therefore, the best explanation for our findings is that U731166 could sponge miR-3607-3p to upregulate TGFBR1, then promoting ESCC invasion and migration.

6. Conclusion

U731166 was highly upregulated in ESCC and it may sponge miR-3607-3p to upregulate TGFBR1, thereby promoting ESCC invasion and migration.

Acknowledgements

Not applicable

Conflict of interests

The authors declare that they have no conflict of interests.

Authors contribution

Mingbo Wang, Meng Wang, Ziqiang Tian, Chao Gao, Xingpeng Han concept, manuscript writing, editing and review

Chao Huang, Yonggang Zhu, Fan Zhang, Wenda Gao, Zhenhua Li, Liangbiao Peng data collection and analysis, manuscript preparation

All authors have read and approve the submission of the manuscript.

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Availability of data and materials

The analyzed data sets generated during the study are available from the corresponding author on reasonable request.

Ethical Approval and Consent to participate

All patients signed the written informed consent. All procedures were approved by The Fourth Hospital of Hebei Medical University and Tianjin Chest hospital Ethics Committee. Procedures operated in this research were completed in keeping with the standards set out in the Announcement of Helsinki and laboratory guidelines of research in China.

Consent for publication

Not applicable

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