

# lncRNA Erbb4-IR is downregulated in prostate carcinoma and predicts prognosis

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**Abstract.** Long non-coding (lnc) RNA Erbb4-IR has been associated with diabetic renal injury; however, its roles in other diseases remain unknown. Therefore, the present study investigated the involvement of Erbb4-IR in prostate carcinoma. Reverse transcription-quantitative PCR was used to analyze gene expression in tissue samples collected from patients with prostate carcinoma. Overexpression experiments via cell transfection were performed to determine the association between Erbb4-IR and microRNA (miR)-21. Furthermore, Cell Counting Kit-8 and cell apoptosis assays were performed to assess cell proliferation and apoptotic rate, respectively. The results revealed that Erbb4-IR was downregulated in prostate carcinoma tissues compared with adjacent non-cancerous tissues, and that low expression of Erbb4-IR in tumor tissues was closely associated with poor survival. Furthermore, miR-21 was upregulated in prostate carcinoma tissues compared with adjacent non-cancerous tissues and was inversely associated with Erbb4-IR expression in tumor tissues. *In vitro* cell experiments revealed that Erbb4-IR overexpression resulted in the downregulation of miR-21, while miR-21 overexpression did not significantly affect the expression of Erbb4-IR. Moreover, Erbb4-IR overexpression increased apoptosis and inhibited the proliferation of prostate carcinoma cells. miR-21 overexpression resulted in the opposite effect and attenuated the effects of Erbb4-IR overexpression. Therefore, the results of the present study suggested that lncRNA Erbb4-IR is downregulated in prostate carcinoma and may inhibit cancer development by downregulating miR-21.

## Introduction

Prostate carcinoma is the second most common malignancy in males and accounts for ~15% of all cancer cases in males (1). Due to its high prevalence and aggressive nature, prostate carcinoma is considered as one of the leading causes of cancer-related deaths, particularly in developed countries (2). The majority of patients with prostate carcinoma are diagnosed at advanced stages due to the low specificity of prostate specific antigen (PSA) testing and the lack of identifiable symptoms at the early stages (3,4). Therefore, the early and accurate diagnosis of the disease may improve the survival time of patients. However, this remains a challenge due to the complex mechanisms underlying prostate carcinoma (5).

The identification of oncogenes and tumor suppressors indicated that genetic factors play important roles in the pathogenesis of prostate carcinoma (6). However, the limited number of oncogenes and tumor suppressors involved in prostate carcinoma may not be able to explain the complexity of the molecular pathways associated with the disease. In recent years, long non-coding RNAs (lncRNAs) have been identified as critical determinants in cancer biology due to their functions in regulating cancer cell behaviors (7,8). Therefore, studies on the involvement of lncRNAs in cancer biology are required to improve the understanding of cancer development and progression. Erbb4-IR is a recently identified lncRNA with important functions in diabetic kidney injury (9). The deep sequencing data obtained in the present study showed that Erbb4-IR was downregulated in prostate carcinoma and was inversely associated with microRNA (miR/miRNA)-21, a known oncogenic miRNA in prostate carcinoma (10). Therefore, the present study investigated the interactions between Erbb4-IR and miR-21 in prostate carcinoma.

## Materials and methods

**Patient admission and follow-up.** A total of 60 male patients (range, 42-67 years; mean  $\pm$  standard deviation, 55.2 $\pm$ 6.7 years) with prostate carcinoma were enrolled at The 940 Hospital of the Joint Logistics Support Force of the Chinese People's Liberation Army between January 2010 and February 2013. The inclusion criteria were as follows:

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i) Histopathologically-confirmed first diagnosis assessed by at least 3 experienced pathologists; and ii) no therapies received prior to admission. The exclusion criteria were as follows: i) Co-morbidities, including chronic diseases; and ii) treatment received up to 3 months prior to admission. There were 12, 13, 20 and 15 cases at American Joint Committee on Cancer stage I, II, III and IV (11), respectively. The present study was approved by The 940 Hospital of the Joint Logistics Support Force of the Chinese People's Liberation Army Ethics Committee and written informed consent was obtained from all patients prior to the study start. All patients were followed up for 60 months after admission through outpatient visits or phone calls. Patients lost to follow-up or who succumbed as a result of other clinical conditions or accidents were excluded. A total of 54 patients completed the follow-up period.

*Specimens and cell lines.* Patients were subjected to prostate biopsies to obtain prostate carcinoma and adjacent non-cancerous (within 2 cm of the tumor margin) tissues prior to receiving treatment.

The 22Rv1 and DU145 prostate carcinoma cell lines (American Type Culture Collection) were investigated in the present study. The cells were cultured in Eagle's minimum essential medium (Sigma-Aldrich; Merck KGaA) supplemented with 10% FBS (Sigma-Aldrich; Merck KGaA) at 37°C in 5% CO<sub>2</sub>.

*Reverse transcription-quantitative (RT-q)PCR.* Total RNA was extracted from tissue specimens and 22Rv1 and DU145 cells using TRIzol reagent (Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. RNAs were precipitated using 80% ethanol to retain the miRNAs. Reverse transcription was performed using AMV reverse transcriptase kit (Sangon Biotech Co., Ltd.), under the following conditions: 25°C for 10 min, 55°C for 20 min and 80°C for 10 min. qPCR was subsequently performed using the QuantiTect SYBR Green PCR kit (Qiagen, Inc.). Erbb4-IR expression was normalized to the expression of endogenous 18S rRNA. miRNA reverse transcription was performed using the TaqMan MicroRNA Reverse Transcription kit (Thermo Fisher Scientific, Inc.). qPCR was performed using MystiCq® microRNA® SYBR® Green qPCR ReadyMix™ (Sigma-Aldrich; Merck KGaA). miR-21 expression was normalized to the expression of endogenous U6. The following primer sequences were used for PCR: Erbb4-IR forward, 5'-ACTCGCCACAGAAATCCAC-3' and reverse, 5'-ACAACCCCAAACAAGCTGT-3'; 18S rRNA forward, 5'-CTACCACATCCAAGGAAGC-3' and reverse, 5'-TTTTCGTCACTACCTCCCCG-3'; miR-21 forward, 5'-TAGCTTATCAGACTGATGTT-3'. The miR-21 reverse primer and the forward and reverse U6 primers were from the MystiCq® microRNA® SYBR® Green qPCR ReadyMix™ kit (Sigma-Aldrich; Merck KGaA). The following thermocycling conditions were used for PCR: Initial denaturation at 95°C for 1 min; 40 cycles of 95°C for 10 sec and 60°C for 45 sec. RT-qPCR was performed in triplicate and expression levels were quantified using the 2<sup>-ΔΔCq</sup> method (12).

*Library preparation and deep sequencing.* The biopsies of 10 patients (range, 43-65 years; mean ± standard deviation,

54.3±6.8 years) were subjected to deep sequencing analysis. Following RNA extraction and reverse transcription as aforementioned, the NEBNext® Ultra™ Directional RNA Library Prep kit for Illumina® (cat. no. 7420S; New England Biolabs) was used to prepare libraries and High-seq (Illumina, Inc.) was used to sequence the libraries. Data were analyzed using a Python-based pipeline (version 3.8, <https://www.python.org/downloads/release/python-380>).

*Transient transfection.* Lipofectamine® 2000 reagent (Thermo Fisher Scientific, Inc.) was used to perform all transient transfections, according to the manufacturer's protocol. A miR-21 mimic and negative control miRNA were purchased from Sigma-Aldrich (Merck KGaA). A pcDNA3.1 vector delivering Erbb4-IR was constructed by Sangon Biotech Co., Ltd. Two control groups, including a negative control (NC; empty vector or NC miRNA transfection) or control (C; no transfection) were included. A total of 10 and 40 nM of the vectors and miRNAs, respectively, were transfected into the cells. Subsequent experiments were performed 24 h after transfection.

*Cell Counting Kit-8 (CCK-8) assay.* 22Rv1 and DU145 cells were harvested 24 h post-transfection and 4x10<sup>5</sup> cells were suspended in 10 ml Eagle's minimum essential medium supplemented with 10% FBS to create a cell suspension with a cell density of 4x10<sup>4</sup> cells/ml. The cell suspension was subsequently added to a 96-well plate (100 μl/well). Cells were cultured at 37°C and 5% CO<sub>2</sub> and 10 μl CCK-8 solution (Sigma-Aldrich; Merck KGaA) was added into each well every 24 h for a total of 96 h. The cells were cultured for an additional 4 h, and 10 μl DMSO were added to each well. The optical density values were measured at a wavelength of 450 nm.

*Cell apoptosis assay.* 22Rv1 and DU145 cells were harvested 24 h post-transfection and 1x10<sup>6</sup> cells were washed with PBS and mixed with 500 μl binding buffer, prior to incubation with FITC labeled Annexin-V (5 μl) and PI solution (5 μl) for 10 min in the dark. These steps were performed using the FITC Annexin V Apoptosis Detection kit with PI (BioLegend, Inc.). Early apoptotic cells were detected using a flow cytometer.

*Statistical analysis.* GraphPad Prism (version 6.0; GraphPad Software, Inc.) was used for data analysis. Differences between prostate carcinoma and adjacent non-cancerous tissues were analyzed using a paired Student's t-test. Differences among multiple cell treatment groups were analyzed using a one-way ANOVA followed by a Tukey post hoc test. Linear regression was used for association analysis. The 54 patients who completed follow-up were divided into high (n=25) and low (n=29) Erbb4-IR expression groups, based on Youden's index. Based on follow-up data, survival curves were plotted and compared using the Kaplan-Meier method and the χ<sup>2</sup> test. All experiments were performed in triplicate. P<0.05 was considered to indicate a statistically significant difference.

## Results

*Erbb4-IR is downregulated in prostate carcinoma.* Deep sequencing analysis showed that the expression levels

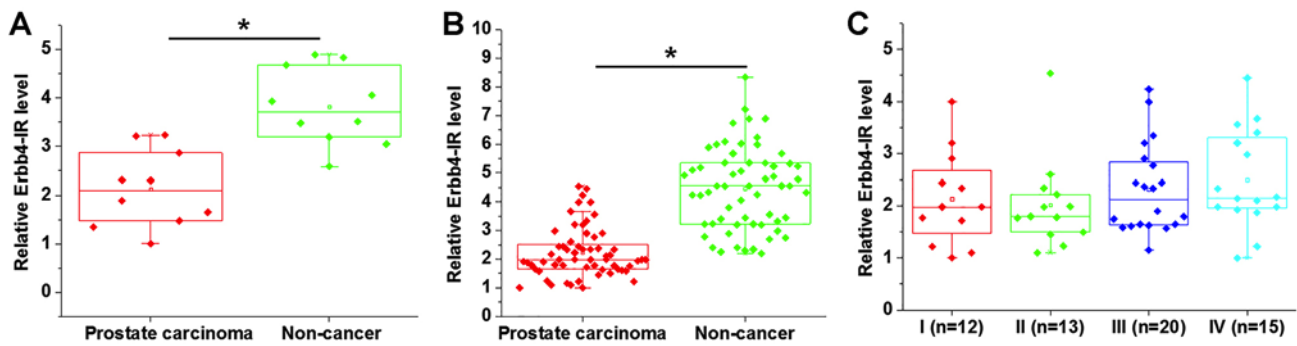


Figure 1. Erbb4-IR expression is upregulated in prostate carcinoma tissues compared with adjacent non-cancerous tissues. (A) Deep sequencing analysis and (B) reverse transcription-quantitative PCR data demonstrated that the expression level of Erbb4-IR was significantly lower in prostate tissues compared with adjacent non-cancerous tissues. (C) Erbb4-IR expression levels in prostate carcinoma tissues from patients with different clinical stages were not significantly different. \* $P < 0.05$ .

of Erbb4-IR were significantly lower in prostate tissues compared with adjacent non-cancerous tissues (Fig. 1A;  $P < 0.05$ ). Erbb4-IR expression in prostate carcinoma and adjacent non-cancerous tissues was subsequently detected by RT-qPCR. The data were analyzed using the paired Student's t-test, and the results showed that Erbb4-IR was significantly upregulated in prostate carcinoma tissues compared with adjacent non-cancerous tissues (Fig. 1B;  $P < 0.05$ ). In addition, no significant differences in the expression levels of Erbb4-IR were observed in prostate carcinoma tissues obtained from patients with different clinical stages (Fig. 1C;  $P > 0.05$ ).

*Low expression of Erbb4-IR in prostate carcinoma tissues is closely associated with poor survival.* The 54 patients who completed the follow-up period were divided into high ( $n=25$ ) and low ( $n=29$ ) Erbb4-IR expression groups based on Youden's index. Based on follow-up data, the survival curves revealed that patients with a low expression level of Erbb4-IR had a significantly lower overall survival rate compared with patients with a high expression level (Fig. 2).

*miR-21 is upregulated in prostate carcinoma tissues and is inversely associated with Erbb4-IR expression.* miR-21 expression in prostate carcinoma and adjacent non-cancerous tissues was detected by RT-qPCR. The results revealed that miR-21 was significantly downregulated in prostate carcinoma tissues compared with the adjacent non-cancerous tissues (Fig. 3A;  $P < 0.05$ ). Linear regression analysis was performed to explore the association between miR-21 and Erbb4-IR and revealed that miR-21 and Erbb4-IR were significantly and inversely associated in prostate carcinoma tissues (Fig. 3B), but not in adjacent non-cancerous tissues (Fig. 3C).

*Erbb4-IR overexpression downregulates miR-21 expression.* An Erbb4-IR-overexpression vector and miR-21 mimic were transfected into 22Rv1 and DU145 cells. Compared with the two control groups (C and NC), Erbb4-IR and miR-21 were significantly upregulated in the overexpression group (Fig. 4A;  $P < 0.05$ ). Furthermore, Erbb4-IR overexpression resulted in the downregulation of miR-21 (Fig. 4B;  $P < 0.05$ ), while miR-21 overexpression did not significantly affect Erbb4-IR expression (Fig. 4C).

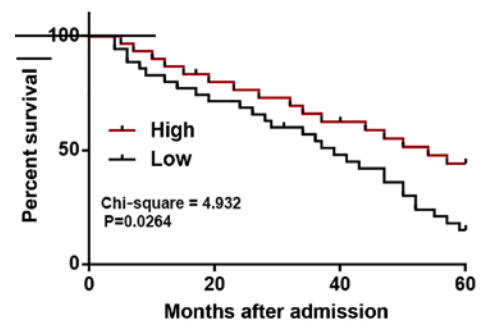


Figure 2. Low Erbb4-IR expression in prostate carcinoma tissues is closely associated with poor survival. The 54 patients who completed follow-up were divided into high ( $n=25$ ) and low ( $n=29$ ) Erbb4-IR expression groups, based on Youden's index. Survival curve analysis demonstrated that low Erbb4-IR expression in prostate carcinoma tissues was closely associated with poor survival.

*Erbb4-IR regulates cancer cell proliferation and apoptosis through miR-21.* Compared with the two control groups (C and NC), Erbb4-IR overexpression promoted apoptosis (Fig. 5A) and inhibited the proliferation (Fig. 5B) of prostate carcinoma cells ( $P < 0.05$ ). miR-21 overexpression resulted in an opposite effect and attenuated the effects of Erbb4-IR overexpression ( $P < 0.05$ ).

## Discussion

The function of lncRNA Erbb4-IR has only been characterized in diabetic kidney injury (9), and its involvement in cancer biology is unknown. To the best of our knowledge, the present study was the first to show that Erbb4-IR was downregulated in prostate carcinoma tissues. Furthermore, *in vitro* results revealed that the overexpression of Erbb4-IR downregulated miR-21, a miRNA that has been previously shown to promote prostate carcinoma (10).

Although the exact mechanism of Erbb4-IR in cancer biology has not been elucidated, Erbb4-IR interacts with TGF- $\beta$  signaling (13), which has a critical role in the development of most, if not all, types of cancer (14). Therefore, Erbb4-IR may be involved in carcinogenesis. The preliminary deep sequencing data obtained in the present study revealed that Erbb4-IR was downregulated in prostate

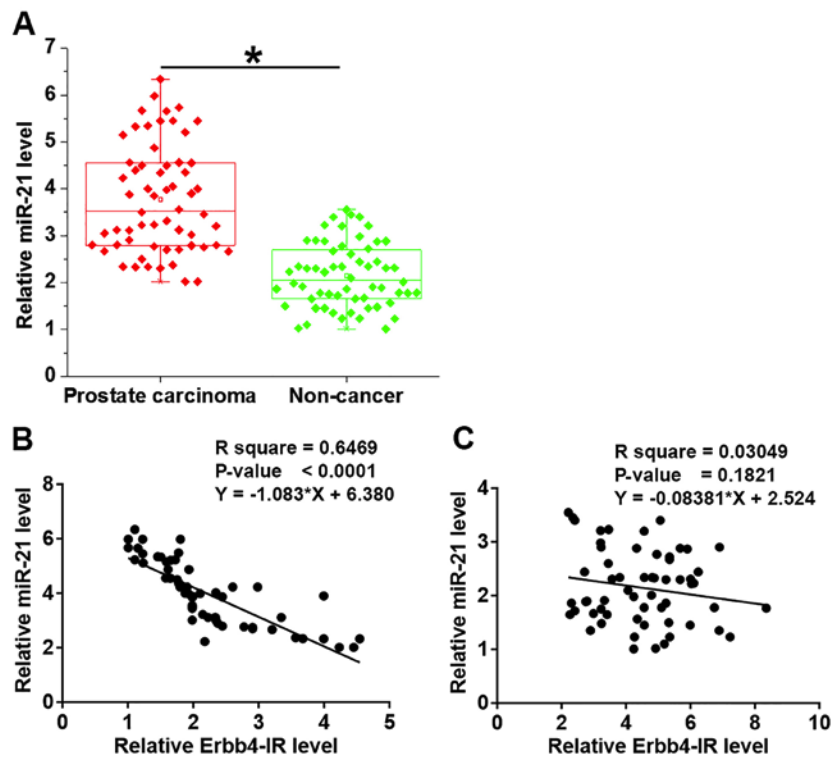


Figure 3. miR-21 is upregulated in prostate carcinoma tissues and inversely associated with Erbb4-IR. (A) Reverse transcription-quantitative PCR results demonstrated that miR-21 was upregulated in tumor tissues compared with adjacent non-cancerous tissues. miR-21 was inversely associated with Erbb4-IR in (B) prostate carcinoma tissues, but not in (C) adjacent non-cancerous tissues. \* $P < 0.05$ . miR-21, microRNA-21.

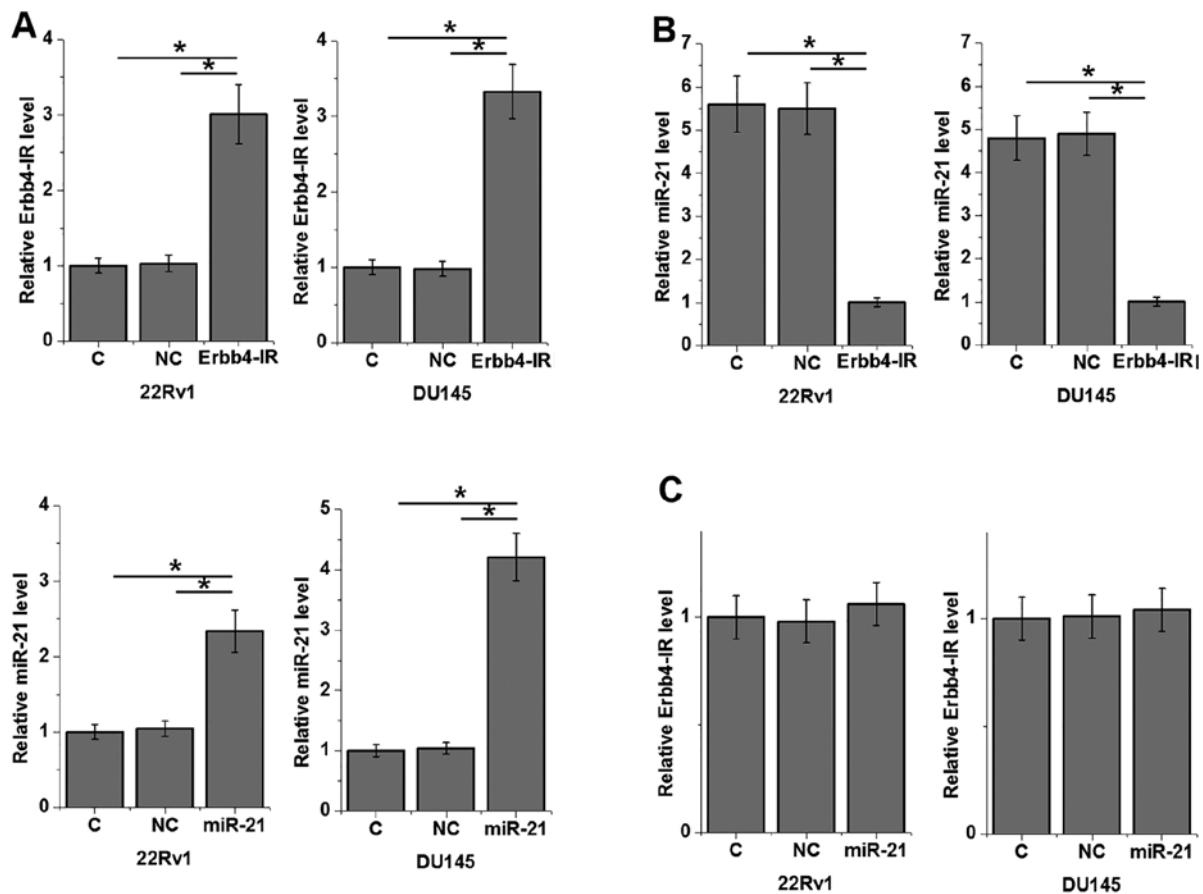


Figure 4. Erbb4-IR overexpression downregulates miR-21 expression in prostate carcinoma cells *in vitro*. (A) Erbb4-IR was significantly upregulated in the Erbb4-IR overexpression group compared with the C and NC groups. (B) Erbb4-IR overexpression resulted in the downregulation of miR-21, while (C) miR-21 overexpression was demonstrated to have no significant effect on Erbb4-IR expression. \* $P < 0.05$ . miR, microRNA; C, control; NC, negative control.

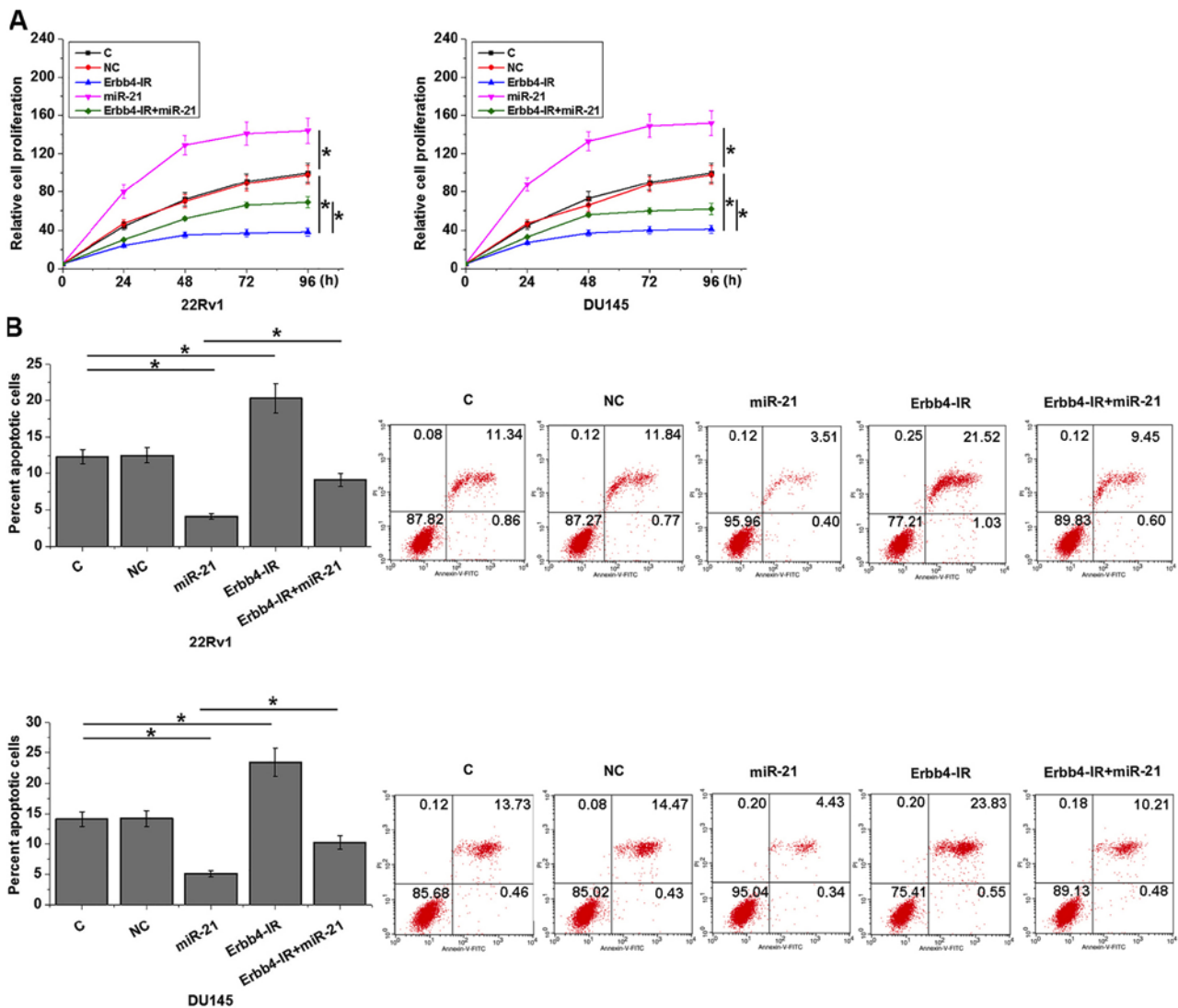


Figure 5. Erbb4-IR regulates cancer cell proliferation and apoptosis through miR-21-associated pathways. Cell proliferation and apoptosis assays revealed that Erbb4-IR overexpression (A) promoted apoptosis and (B) inhibited proliferation of prostate carcinoma cells. miR-21 overexpression served the opposite role and attenuated the effects of Erbb4-IR overexpression. \* $P < 0.05$ . miR, microRNA; C, control; NC, negative control.

carcinoma tissues compared with adjacent non-cancerous tissues. Furthermore, low expression of Erbb4-IR in prostate carcinoma tissues was significantly associated with poor survival rate. In addition, the overexpression of Erbb4-IR promoted apoptosis and inhibited the proliferation of prostate carcinoma cells *in vitro*. Therefore, Erbb4-IR is likely to serve as a tumor suppressor in prostate carcinoma, and the regulation of Erbb4-IR expression may be a useful therapeutic strategy to decrease cancer cell proliferation and increase apoptosis.

miR-21 is an oncogenic miRNA in several types of cancer (13-15). Overexpression of miR-21 in cancer cells not only regulates cell behavior, such as cell growth, migration and invasion, but also regulates the sensitivity of cancer cells to chemotherapy (15-17). The present study revealed that the overexpression of miR-21 in prostate carcinoma cells decreased apoptosis and increased proliferation. A previous study revealed that miR-21 participates in cancer biology primarily by downregulating the downstream tumor suppression pathway associated with reversion inducing cysteine rich

protein with kazal motifs (18). However, the upstream regulators of miR-21 remain largely unknown. The present study revealed that Erbb4-IR was likely an upstream inhibitor of miR-21, however, the underlying molecular mechanism was not investigated. It is known that miR-21 may interact with TGF- $\beta$  (19), which also interacts with Erbb4-IR (11). Therefore, TGF- $\beta$  may mediate the interaction between Erbb4-IR and miR-21.

Future studies investigating additional more cell lines are required to validate the results obtained in the present study. In addition, *in vivo* studies will allow more in-depth investigations.

In conclusion, the present study revealed that Erbb4-IR was upregulated in prostate carcinoma and that overexpression of Erbb4-IR may downregulate miR-21 to inhibit prostate carcinoma.

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## Availability of data and materials

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

## Authors' contributions

JZ, QS and HQ conceived and designed the study. JZ, QS, XL, HY, YW, LZ and SP analyzed and interpreted the data. JZ, XL and HY drafted the manuscript. YW and HQ revised the manuscript. All authors reviewed and approved the manuscript.

## Ethics approval and consent to participate

This study was approved by The 940 Hospital of the Joint Logistics Support Force of the Chinese People's Liberation Army Ethics Committee.

## Patient consent for publication

Not applicable.

## Competing interests

The authors declare that they have no competing interests.

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