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The diagnostic value and molecular mechanism of exosomal lncGIHCG in prostate cancer

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ABSTRACT

The biological mechanism of exosomal lncGIHCG in prostate cancer (PCa) is unclear. In this study, qRT-PCR detection found that lncGIHCG was highly expressed in cancer tissues, serum exosomes of PCa patients and various human PCa cell lines. Functional experiments showed that knockdown of lncGIHCG could inhibit the proliferation and migration of PCa cells and promote apoptosis, while overexpression of lncGIHCG produced the opposite result. In addition, lncGIHCG enriched in exosomes also had the capacity to modulate the proliferative activity, migratory behavior, and apoptotic rates of PCa cells. Mechanism studies found that lncGIHCG acted as a competitive endogenous RNA (ceRNA) to adsorb miR-181a-5p, relieving its inhibitory effect on the downstream target gene RALBP1, thereby promoting tumor progression. In conclusion, the serum exosomal lncGIHCG may be a potential therapeutic target for prostate cancer. Regulating the lncGIHCG / miR-181a-5p / RALBP1 axis may offer a novel approach for treating prostate cancer. This article studied the expression of exosomal lncGIHCG in prostate cancer (PCa), explored its potential as a biomarker of prostate cancer, the molecular mechanism underlying the role of lncGIHCG in the progression of prostate cancer was analyzed. qRT-PCR was used to detect the presence of GIHCG in cancer tissues and serum exosomes derived from PCa patients, as well as in various human PCa cell lines. GIHCG siRNA and overexpression plasmids were constructed and transfected into different PCa cells to investigate how GIHCG influenced PCa cell proliferation, migratory capacity, and apoptotic rate. PCa cell exosomes were extracted and identified, and the effect of lncGIHCG enriched in exosomes on cell proliferation, migration, and apoptosis was detected. The miRNA that binds to lncGIHCG and its regulatory target gene were predicted through bioinformatics methods, and verified through luciferase reporter gene detection. In the above interaction network, qRT-PCR and Western blot assay were employed to assess the levels of miRNA and its regulatory target gene. LncGIHCG exhibited elevated expression levels in cancer tissues and serum exosomes of PCa patients. By knocking down GIHCG, the formation of cell clones and migration were suppressed, while apoptosis was enhanced. Overexpression of GIHCG produced the opposite result. LncGIHCG enriched in exosomes had the capacity to modulate the proliferative activity, migratory behavior, and apoptotic rates of PCa cells. A binding site existed between lncGIHCG and miR-181a-5p. MiR-181a-5p was low expressed in prostate cancer, and miR-181a-5p mimic could play an anti-cancer role. RALBP1 served as a target gene regulated by miR-181a-5p. Knockdown of RALBP1 could inhibit cell cloning, migration, and promote apoptosis. In addition, knocking down RALBP1 reversed the promotion effect of lncGIHCG overexpression on prostate cancer, demonstrating that lncGIHCG plays a pro-cancer role through the miR-181a-5p/RALBP1 axis. The serum exosomal lncGIHCG may be a potential therapeutic target for prostate cancer. Regulating the lncGIHCG/miR-181a-5p/RALBP1 axis may offer a novel approach for treating prostate cancer.

KEYWORDS

Prostate cancer; Exosome; LncGIHCG; Molecular marker; MiR-181a-5p; RALBP1

1 Introduction

Globally, prostate cancer (PCa) ranks as the second most prevalent malignancy among males. Its poor prognosis, high metastasis and drug resistance remain major challenges for treating prostate cancer [1]. Castration therapy for prostate cancer is not effective and a large number of high-risk prostate cancer patients are at risk of metastasis progression after initial treatment. Finding efficient diagnosis and treatment targets and clarifying the molecular mechanisms of malignant progression of prostate cancer are crucial for relevant diagnosis and treatment [2, 3].

Exosomes are extracellular vesicles secreted by various tissues in organisms, which mediate intercellular communication in the way of autocrine, paracrine or endocrine, and participate in tumorigenesis, development, invasion and other processes [4]. In cancer research, exosomes can not only serve as markers, but also affect tumor formation, growth, metastasis and treatment resistance. During tumor metastasis, exosomes are thought to mediate the "seed-soil" interaction. For example, miR-125b, miR-130, etc. in prostate cancer cell exosomes are involved in tumor reprogramming and tumor formation of adipose stem cells [5, 6].

Long non-coding ribonucleic acid (LncRNA) is a type of RNA molecule approximately 200 bp in length. LncRNAs with different subcellular localization can act as competing endogenous RNAs (ceRNAs) for mRNAs by competitively binding to target microRNAs (miRNAs) through the ceRNA mechanism, or they can influence various biological functions through direct protein-anchoring mechanisms or mechanisms involved in short peptide coding [7]. Research has revealed that

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lncRNA plays a crucial part in epigenetic regulation, dose compensation effects, post-transcriptional regulation and other activities [8]. In the past few years, the role of lncRNA GIHCG in malignant tumors has attracted the attention of many scholars. For example, Ma et al. found that the expression level of lncRNA GIHCG was elevated in tongue squamous cell carcinoma tissues; He et al. found that lncRNA GIHCG exhibited heightened expression in renal cell carcinoma tissues, and knockdown of GIHCG could inhibit the growth of renal cell carcinoma [9, 10]. However, the expression of lncRNA GIHCG in prostate cancer remains unknown.

The aim of this research was to explore the presence of exosomal lncGIHCG in prostate cancer and analyze its feasibility as a potential diagnostic marker. And further explore its molecular mechanism in the progression of prostate cancer, providing a new target for clinical prostate cancer treatment.

2 Materials and methods

2.1 Clinical samples

The tissues source were cancer tissue and corresponding adjacent normal tissue from 38 prostate cancer surgery patients in Shandong Provincial Hospital Affiliated to Shandong First Medical University from January to December 2022. Serum samples of these 38 patients were collected before surgery, and serum samples from 12 healthy physical examiners at the same time were collected as controls.

2.2 Cell lines

Human prostate epithelial cell PrEC LH, and human prostate cancer cells including DU 145, NCI-H660, LNCaP clone FGC, PC-3, VCaP, 22RV1, and MDA-PCA-2B, were all purchased from Nanjing Cobioer Biosciences Co., Ltd.

2.3 Cell culture and transfection

The cells were maintained in complete RPMI 1640 medium (Gibco, USA), which consisted of 100 mL/L FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin in a 5% CO_2 incubator at 37℃. Shanghai GenePharma Co., Ltd designed and synthesized two specific siRNAs of lncGIHCG and its full-length overexpression plasmid, miR-181a-5p overexpression plasmid and two specific siRNAs of RALBP1. When the cells were grown to a suitable confluence in the 6-well plate, Lipofectamine 2000 (Invitrogen, USA) was used to transfect siRNA and overexpression plasmid into PCa cells for subsequent experiments according to the instructions.

2.4 qRT-PCR

RNA was isolated from tissues and cells employing TRIzol reagent (TaKaRa, Japan), and then the RNA underwent reverse transcription into cDNA using a reverse transcription kit (Vazyme, China), and qRT-PCR assay was used to amplify the target gene.

2.5 Clone formation assay

After trypsin digestion, 500-1000 cells were planted in a 3.5 cm culture dish and placed in a cell incubator for culture. When the cells grew to the cell colony, the culture was stopped, and the cells were cultured for about 10 days. After fixation, staining, and washing, the samples were photographed and saved.

2.6 Wound healing assay

Cells undergoing logarithmic growth were harvested, digested and centrifuged, and inoculated into 6-well plates. When the cells grew to confluence, a scratch was made and the cells were then further cultured. At 0 and 24 hours of incubation, the scratched areas were observed and photographed through an inverted microscope, and the relative mobility was detected using Image J software and statistically analyzed.

2.7 Flow apoptosis assay

The supernatant of the treated cells in the 6-well plate was transferred to the centrifuge tube, EDTA-free trypsin was used to digest the cells, and the supernatant was removed after centrifugation. The supernatant was removed, while the cells underwent two washing procedures using pre-cooled PBS. Each tube was prepared with 100 μL of binding buffer to form a cell suspension, subsequently supplemented with 5 μL of both Annexin V-FITC and PI dye (Yeasen, China),

respectively. Protected from light, the tubes were incubated for 10 min at room temperature, followed by the addition of 400 μL of binding buffer to each. At this point, flow cytometry detection could be performed.

2.8 Western blot

The treated cells were fully ground with a mixture of protease inhibitor and lysis buffer pre-mixed at a ratio of 1:100. Subsequently, they were centrifuged at 12000 r/min at low temperature for 10 min. An equivalent amount of loading buffer were added, and the mixture was boiled for 10 min. Western blot detection was performed: Following electrophoresis, membrane transfer, and blocking procedures, the membrane underwent incubation with the primary antibody overnight at 4°C. After TBST washes, it was incubated with the secondary antibody for 1 hour. Subsequently, it underwent more TBST washes, followed by the addition of ECL luminescent solution (ABclonal, China) to facilitate visualization under a gel imaging system (Bio-Rad, USA).

2.9 Extraction and identification of exosomes

After the serum samples were defrosted, exosome extraction reagent (Umibio, China) was added and placed at 4℃ for 30 min. After being centrifuged at 4℃ for 30 min at a speed of 1500 *g*, the supernatant was then discarded. The samples were centrifuged again for 5 min and the supernatant was discarded. Then, 200 μL of PBS was added, and the mixture was vortexed to obtain serum-derived exosomes. Exosomes derived from cells were extracted by ultracentrifuge method. The obtained exosomes were then identified by transmission electron microscopy (TEM), analyzed for particle size using nanoparticle tracking analysis (NTA), and detected for marker proteins through Western blot.

2.10 Nuclear-cytoplasmic separation assay

The cells from each group underwent trypsin digestion to form a cell suspension. Following centrifugation, the supernatant was removed. The cells, after undergoing two rinses with PBS, were moved into a centrifuge tube. Subsequently, 200 μL of Lysis buffer was introduced to the tube, and the mixture was placed on ice for 5 min. Subsequently, the cytoplasm (supernatant) and nucleus (precipitate) were separated by centrifugation at 5000 r/min at 4°C for 5 min.

2.11 RNA FISH

The cells were inoculated on a slide, fixed using 4% paraformaldehyde for 10 min, followed by rinsing with PBS. Next, the cells were placed in hybridization buffer containing 20 μL of FISH probe and incubated overnight at 37°C. The next day, the slides underwent rinsing with PBS and were subsequently counterstained with DAPI. Finally, the samples were observed under a confocal fluorescence microscope (Olympus, Japan). The lncGIHCG probe was synthesized by Guangzhou RiboBio Co., Ltd.

2.12 Dual luciferase report analysis

The vectors required for the dual luciferase reporter gene experiment were constructed. Well-grown 293T cells were digested and inoculated onto a 12-well plate, then further cultured in a cell incubator. When the cell density reached more than 80%, the prepared plasmids, mixed with Lipofectamine 2000 transfection reagent based on the experimental grouping, were introduced into the 293T cells and incubated for an additional 48 hours. Afterwards, the cell supernatant was harvested and the luciferase activity was subsequently measured (Promega, China).

2.13 Statistical analysis

The statistical analysis was carried out using GraphPad Prism 8.0 software. For comparing two groups, independent sample *t*-test was employed. When comparing multiple groups, variance (ANOVA) was conducted, followed by Bonferroni's correction for multiple pairwise comparisons. Statistical significance was determined when P was less than 0.05.

3 Results

3.1 LncGIHCG serves as a diagnostic marker for prostate cancer

The level of GIHCG expression in PCa tissues was notably elevated compared to that in paracancer control tissues

(Figure 1A). The extraction of exosomes from patient serum, upon identification, showed that the exosomes met the criteria (Figure 1B-D). Compared with healthy physical examiners, the serum exosomes of PCa patients exhibited a marked elevation in GIHCG expression levels (Figure 1E). Prostate-specific antigen (PSA) is a specific marker of prostate cancer. When lncGIHCG and PSA were jointly diagnosed, the AUC area increased, improving the accuracy and efficiency of the diagnosis of PCa (Figure 1F).

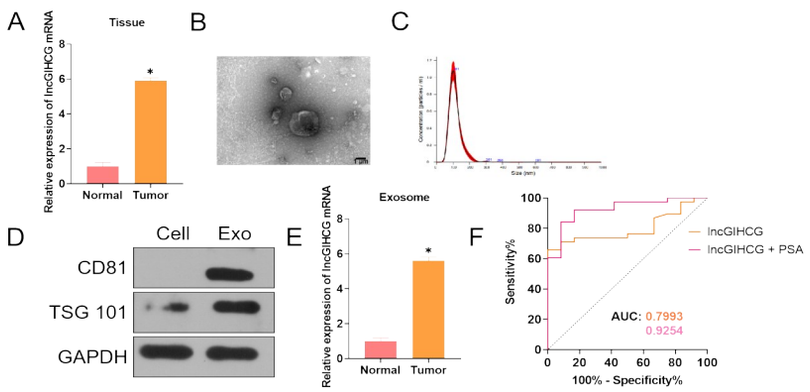

Figure 1 LncGIHCG serves as a diagnostic marker for prostate cancer. (A) The level of GIHCG expression in PCa tissues and paracancer control tissues. (B) Morphology of exosomes isolated from serum under TEM. (C) NTA analysis of exosomes isolated from serum. (D) Detection of protein markers in serum exosomes. (E) GIHCG expression levels in serum exosomes from PCa patients and healthy people. (F) The ROC curve for the combined diagnosis of lncGIHCG and PSA in serum exosomes of PCa patients.

3.2 LncGIHCG plays a pro-cancer role in prostate cancer

RNA was extracted from prostate epithelial cells (PrEC LH) and various PCa cell lines (DU 145, NCI-H660, LNCaP clone FGC, PC-3, VCaP, 22RV1 and MDA-PCA-2B) to detect the expression of lncGIHCG. The quantitative analysis revealed that all PCa cells exhibited a high level of GIHCG expression, with the highest relative expression in DU 145 and NCI-H660 cells, and the lowest relative expression in MDA-PCA-2B cells (Figure 2A). Two siRNAs were used to inhibit GIHCG in DU 145 and NCI-H660 cell lines in vitro, and plasmid vectors were constructed in vitro to transfect MDA-PCA-2B cells to overexpress lncGIHCG, and the knockdown and overexpression efficiency was detected respectively (Figure 2B). In the clone formation assay, after knocking down GIHCG, cell cloning ability was weakened (Figure 2C). In the wound healing assay, a decrease in migration capacity was observed following the knockdown of GIHCG (Figure 2D). In the apoptosis experiment, after knocking down GIHCG, the apoptosis proportion increased significantly (Figure 2E). Clone formation assay confirmed that the cells exhibited an improved cloning ability after overexpressing GIHCG (Figure 2F). Wound healing assay showed that cell migration ability was enhanced after GIHCG was overexpressed (Figure 2G).

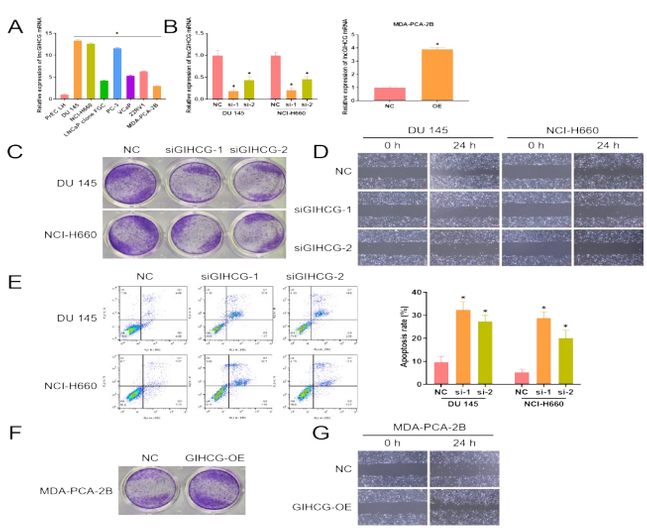

Figure 2 LncGIHCG plays a pro-cancer role in prostate cancer. (A) The level of lncGIHCG expression was observed in PrEC LH and prostate cancer cells. (B) Knockdown and overexpression efficiency of GIHCG. (C) Clone formation assay after knocking down GIHCG. (D) The impact of reducing GIHCG expression on cell migration. (E) The influence of suppressing GIHCG on apoptosis. (F) Cell cloning ability after GIHCG overexpression. (G) Effect of GIHCG overexpression on cell migration.

3.3 Enriched lncGIHCG in exosomes can regulate the proliferation, migration and apoptosis of prostate cancer cells

Exosomes from DU 145, NCI-H660 and MDA-PCA-2B cells were extracted, the morphology and size of the exosomes were detected by TEM (Figure 3A), and the particle size was analyzed by NTA (Figure 3B). The protein markers CD81 and TSG101 of the exosomes were detected (Figure 3C), which provided additional verification of the existence of exosomes. qRT-PCR detected the expression of lncGIHCG in three cell exosomes (Figure 3D). The results of clone formation assay and wound healing assay evidenced that the siGIHCG-Exo group exhibited decreased cellular proliferation and migration abilities (Figure 3E, F). The experiment on apoptosis revealed an upregulation in the apoptosis rate in the siGIHCG-Exo group (Figure 3G). GIHCG-OE-Exo could promote the proliferation and migration of MDA-PCA-2B cells; after co-culture of exosomes from DU 145 cells that highly express GIHCG with MDA-PCA-2B cells, it was found that exosomes derived from DU 145 cells could also promote the proliferation and migration of MDA-PCA-2B cells (Figure 3H, I).

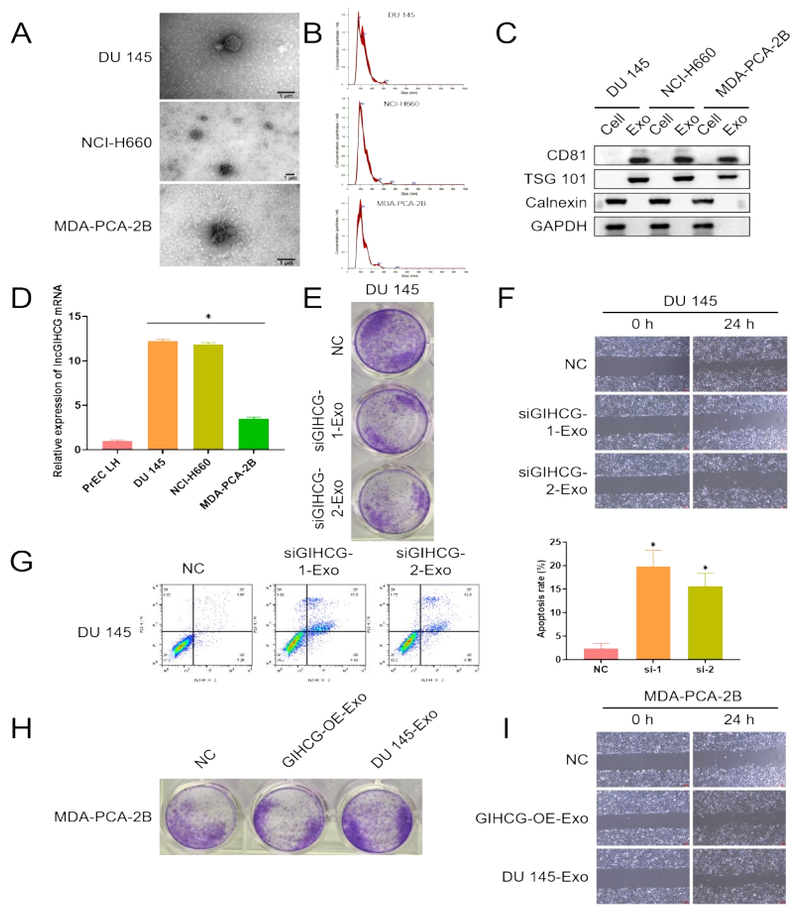

Figure 3 Enriched lncGIHCG in exosomes can regulate the proliferation, migration and apoptosis of prostate cancer cells. (A) Morphology of exosomes isolated from cell supernatant under TEM; (B) NTA analysis of exosomes isolated from cell supernatant; (C) Detection of protein markers in cell exosomes; (D) The expression of GIHCG in exosomes of three PCa cells was detected; (E) The cloning ability of cells in PCa cell exosomes after knocking down GIHCG; (F) Cell migration ability in PCa cell exosomes after knocking down GIHCG; (G) Apoptosis rate in PCa cell exosomes after downregulation of GIHCG; (H) The cloning ability of PCa cells after different treatments; (I) Migration ability of PCa cells after different treatments.

3.4 LncGIHCG plays a pro-cancer role by regulating miR-181a-5p

The qRT-PCR results indicated that the mainly localization of GIHCG in PCa cells was in the cytoplasm (Figure 4A), which was confirmed by the RNA FISH experimental results (Figure 4B). LncBase predicted that miR-181a-5p might bind to lncGIHCG (Figure 4C). MiR-181a-5p was downregulated in both Pca tissues and cells (Figure 4D). The dual luciferase reporter assay results demonstrated that miR-181a-5p could indeed bind to lncGIHCG (Figure 4E). After transfection with miR-181a-5p mimic, the cloning number of Pca cells decreased and their migration ability decreased (Figure 4F, G). Overexpression of miR-181a-5p could induce apoptosis in Pca cells (Figure 4H). After overexpressing GIHCG and miR-181a-5p in MDA-PCA-2B cells simultaneously, miR-181a-5p could reverse the proliferation and migration promotion effect of GIHCG overexpression on MDA-PCA-2B cells (Figure 4I, J).

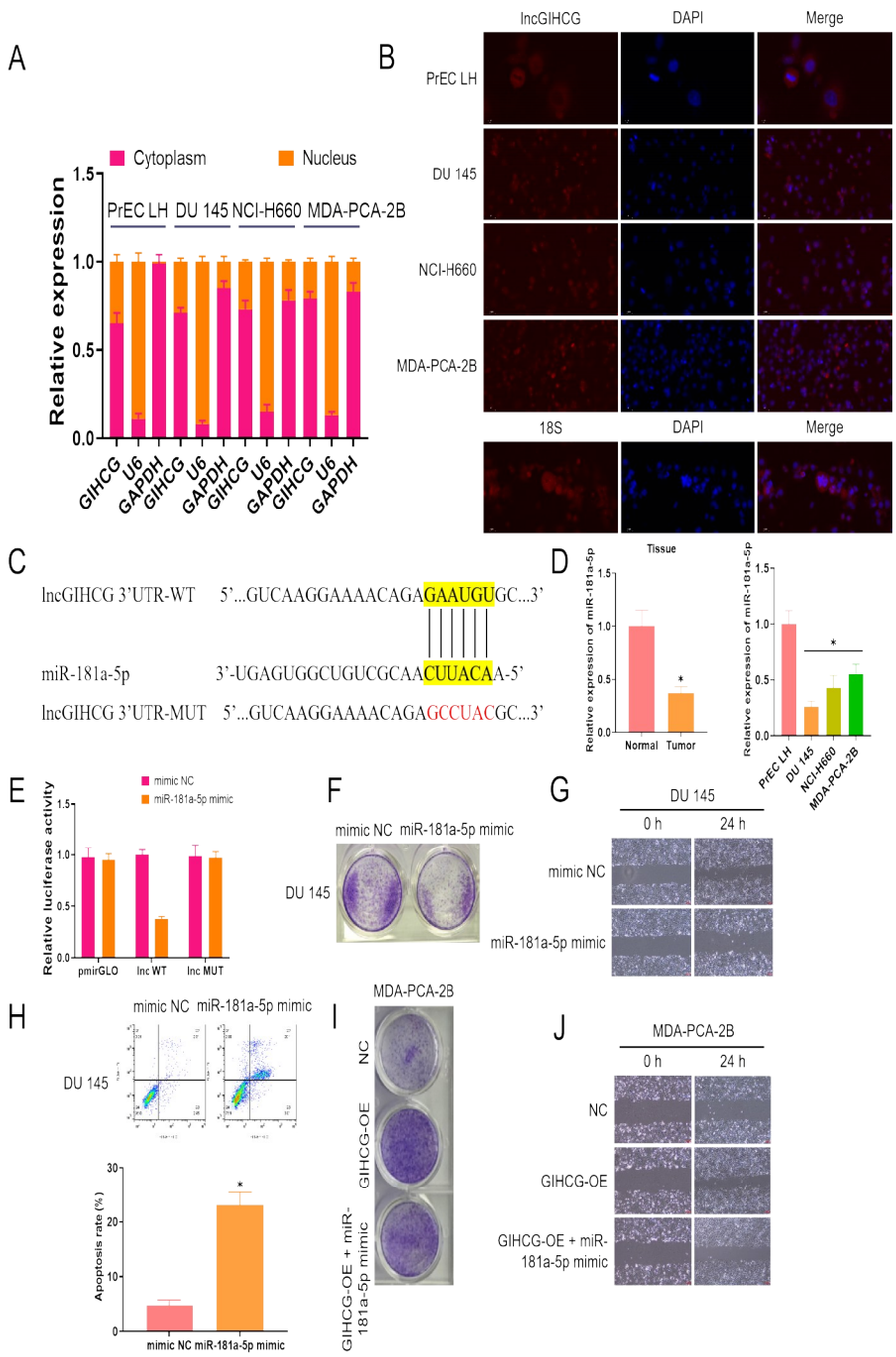

Figure 4 LncGIHCG plays a pro-cancer role by regulating miR-181a-5p. (A) qRT-PCR detection after nuclear-cytoplasmic isolation of Pca cells; (B) Subcellular localization of GIHCG in Pca cells; (C) Binding site of hsa-miR-181a-5p and lncGIHCG; (D) The level of miR-181a-5p expression in Pca tissues and cells was detected; (E) The binding of miR-181a-5p to lncGIHCG was confirmed; (F) Clone formation results of Pca cells after transfection with miR-181a-5p mimic; (G) Effect of transfection of miR-181a-5p mimic on migration ability of Pca cells; (H) Changes in apoptosis rate of Pca cells after transfection with miR-181a-5p mimic; (I) Clone formation results of MDA-PCA-2B cells after co-transfection with GIHCG and miR-181a-5p; (J) After co-transfection with GIHCG and miR-181a-5p, MDA-PCA-2B cell migration experiment was performed.

3.5 LncGIHCG promotes prostate cancer progression through the miR-181a-5p/RALBP1 axis

TargetScan predicted that the downstream target gene of miR-181a-5p might be RALBP1 (Figure 5A). After overexpression of miR-181a-5p, RALBP1 expression was downregulated in DU 145 and NCI-H660 cells (Figure 5B). Dual luciferase reporter assay demonstrated that miR-181a-5p could indeed bind to RALBP1 (Figure 5C). After knocking down RALBP1, the cloning number of PCa cells decreased, the migration ability was weakened (Figures 5D, E), and the apoptosis rate was upregulated (Figure 5F). After simultaneously knocking down RALBP1 and overexpressing GIHCG in MDA-PCA-2B cells, RALBP1 could reverse the proliferation and migration promotion effect of GIHCG overexpression on MDA-PCA-2B cells (Figure 5G, H).

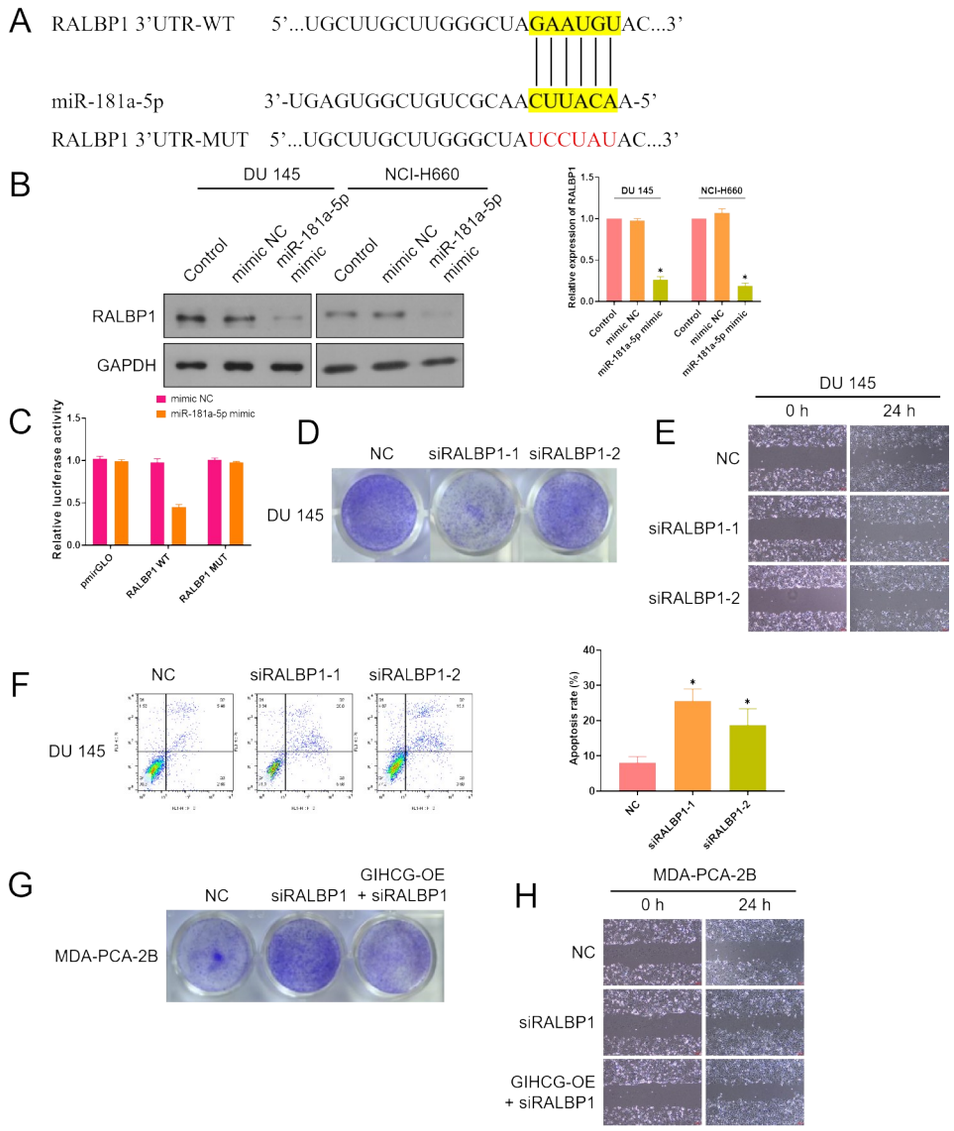

Figure 5 LncGIHCG promotes prostate cancer progression through the miR-181a-5p/RALBP1 axis. (A) Binding sites of miR-181a-5p and RALBP1; (B) Changes in RALBP1 protein expression in DU 145 and NCI-H660 cells after transfection with miR-181a-5p mimic; (C) The binding of miR-181a-5p to RALBP1 was confirmed; (D) Clone formation results of Pca cells after knocking down RALBP1; (E) Effect of RALBP1 knockdown on migration ability of Pca cells; (F) Changes in apoptosis rate of Pca cells after knocking down RALBP1; (G) After simultaneously knocking down RALBP1 and overexpressing GIHCG, the clone formation results of MDA-PCA-2B cells; (H) After simultaneously knocking down RALBP1 and overexpressing GIHCG, MDA-PCA-2B cell migration assay was performed.

4 Discussion

Prostate cancer belongs to a very malignant type of urinary tract tumor [11]. Due to the high heterogeneity and drug resistance of prostate cancer, there is no cure drug available. Clarifying the molecular mechanism of malignant progression of prostate cancer will be of great benefit to improving drug resistance of prostate cancer and improving patient prognosis [12]. The development of prostate cancer is inseparable from the tumor microenvironment. More and more studies have shown that tumor derived exosomes can reshape the tumor microenvironment [13, 14]. Studies have found that lncRNAs are highly tissue specific and participates in multiple biological processes in tumor cells. In addition, lncRNAs are involved in the initiation and progression of tumors through various means such as epigenetic modification, transcriptional regulation, and post-transcriptional regulation [15]. LncRNA GIHCG is a newly identified lncRNA which exhibits elevated expression levels in malignant tumors such as kidney cancer [16].

When detected in serum exosomes from these prostate cancer patients, the trend of GIHCG was consistent with that in tissues. Overexpression of GIHCG resulted in the opposite result. When exosomes enriched in GIHCG were co-cultured with prostate cancer cells, it was further confirmed that lncGIHCG enriched in exosomes had the capacity to modulate the processes of proliferation, migration, and apoptotic activity in prostate cancer cells. Nuclear-cytoplasmic separation and RNA FISH experimental results confirmed that lncGIHCG was localized in the cytoplasm. Dual luciferase reporter assay

confirmed the presence of a binding site between lncGIHCG and miR-181a-5p. MiR-181a-5p, belonging to the miR-181a family, holds a significant position in the development of various diseases. The role of miR-181a-5p in different tumor cells is different and complex [17]. Our research results showed that miR-181a-5p plays an anti-cancer role in prostate cancer cells, and miR-181a-5p could inhibit the proliferation and migration of prostate cancer cells. The downstream target gene of miR-181a-5p was predicted, and the expression of its possible target gene-RALBP1 was detected. It was found that its expression was opposite to that of miR-181a-5p. Dual luciferase reporter assay also confirmed that RALBP1 could directly bind to miR-181a-5p. RALBP1 is essential for the metastasis of human cancer cells. RALBP1 is the earliest discovered RAL effector protein [18]. RALBP1 binds to the two Swtich regions of RAL through the RAL binding domain (RBD), responds to RAL signals, and plays a role in RAL dependent endocytosis, normal cell stress defense, and tumor cell drug resistance [19].

In conclusion, this study not only revealed the important role of exosomal lncGIHCG in prostate cancer, but also deeply explored the mechanism by which it affected the biological behavior of prostate cancer cells by regulating the miR-181a-5p/RALBP1 axis. These findings provide new ideas and methods for the diagnosis, treatment and prognosis of prostate cancer.

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