Research Article

Murat Kaya, Ilknur Suer*, Abdulmelik Aytatli, Omer Faruk Karatas, Sukru Palanduz, Kivanc Cefle and Sukru Ozturk

CDR1as/miR-7-5p/*IGF1R* axis contributes to the suppression of cell viability in prostate cancer

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Abstract

Objectives: Prostate cancer is the most frequently diagnosed male cancer and the fifth highest cause of cancer mortality in men. CDR1as has played an essential role in the growth of several malignancies. However, its significance in the progression of prostate cancer has not been investigated. We aimed to investigate the role and mechanism of CDR1as in the development of prostate cancer and identify a new target for diagnostics and treatment.
Methods: CDR1as siRNA and miR-7-5p mimic were transfected into PC3 and DU145 PCa cell lines and their effects on cellular processes were investigated. Cell viability was measured by WST-8 assay. The role of CDR1as and/or miR-7-5p on PCa cell migration was detected using the scratch-wound assay. The apoptotic capacity of the cells was evaluated using the Caspase-3 kit. The potential targets of miR-7-5p were defined via in silico tools. mRNA and protein expression levels of *IGF1R* and *EIF4E* were detected by qRT-PCR and western blot assays, respectively. The matching between miR-7-5p and *IGF1R* was defined via luciferase reporter assay.
Results: Inhibiting CDR1as or restoring miR-7-5p reduced prostate cancer cell proliferation and migration while increasing apoptosis. Silencing CDR1as elevated the expression of miR-7-5p while decreasing IGF1R.
Conclusions: CDR1as functions as a miR-7-5p sponge, increasing *IGF1R* expression and promoting tumor development.

Keywords: CDR1as; miR-7-5p; *IGF1R*; prostate cancer; PC3; DU145

Introduction

Prostate cancer (PCa) is the fourth most frequently reported cancer worldwide and the most prevalent malignancy among men in Europe [1]. The incidence rate of PCa is also steadily increasing. It is known that various factors such as age, race, hormones and genetics are important in the development of prostate cancer. Treatments such as surgery and radiation therapy, which are routinely used in the treatment of prostate cancer, may be inadequate [2]. PCa mortality is highest in the majority of advanced, late stage and metastatic cases [3]. In addition, the absence of curative treatment choices for individuals with metastatic disease results in higher mortality and morbidity rates. The high metastatic rate of PCa and the inadequate curative therapy underline the critical requirement for new and more effective treatment approaches [4]. To create more effective therapy, it is required to uncover novel targets to prevent the invasive process, as well as novel markers for early detection and progression.

Murat Kaya and Ilknur Suer contributed equally to this work.

***Corresponding author: Dr. Ilknur Suer**, Associate Professor, Department of Medical Genetics, Istanbul Faculty of Medicine, Istanbul University, Istanbul, Türkiye, E-mail: ilknursuer@istanbul.edu.tr. https://orcid.org/0000-0003-1954-4190
Murat Kaya, **Sukru Palanduz**, **Kivanc Cefle and Sukru Ozturk**, Department of Internal Medicine, Division of Medical Genetics, Istanbul Faculty of Medicine, Istanbul University, Istanbul, Türkiye, E-mail: kmurat@istanbul.edu.tr (M. Kaya), spalanduz@istanbul.edu.tr (S. Palanduz), ceflek@istanbul.edu.tr (K. Cefle), sozturk@istanbul.edu.tr (S. Ozturk). https://orcid.org/0000-0003-2241-7088 (M. Kaya). https://orcid.org/0000-0002-9435-009X (S. Palanduz). https://orcid.org/0000-0002-9420-4543 (K. Cefle). https://orcid.org/0000-0002-8809-7462 (S. Ozturk)
Abdulmelik Aytatli and Omer Faruk Karatas, Department of Molecular Biology and Genetics, Erzurum Technical University, Erzurum, Türkiye; and Molecular Cancer Biology Laboratory, High Technology Application and Research Center, Erzurum Technical University, Erzurum, Türkiye, E-mail: abdulmelik.aytatli@erzurum.edu.tr (A. Aytatli), faruk.karatas@erzurum.edu.tr (O.F. Karatas). https://orcid.org/0000-0002-9204-1234 (A. Aytatli). https://orcid.org/0000-0002-0379-2088 (O.F. Karatas)

MicroRNAs (miRNAs) are non-coding RNAs that are brief of around 18–22 nucleotides in length [5]. miRNAs can exhibit either tumor-suppressive or oncogenic properties, depending on the specific genes that they target [6]. It has been known since the early 2000s that miRNAs can play critical roles in many cellular processes by regulating the expression of their target genes [7]. In recent years, a unique class of RNAs called circular RNAs (circRNAs) was revealed that influence gene expression via miRNAs [8]. CircRNAs are covalently packed single-strand RNAs produced by pre-mRNA backsplicing. This unique structure of circRNAs protects them from exonucleases and degradation, providing them more stability than linear RNA [8, 9]. Zhou et al. have shown that circROBO1 plays a role in the prostate cancer process by accelerating glycolysis [10]. Hsa_circRNA_100,146 has been shown to affect prostate cancer cell proliferation by up-regulating the expression of *TRIP13* via miR-615-5p [11]. Pappalysin 1 circRNA has been reported to trigger prostate cancer development via the miR-515-5p/*FKBP1A* axis [12].

CDR1as, also known as ciRS-7, is one of the critically important circRNAs in the cell. CDR1as expression is elevated in several kinds of cancers, including, laryngeal squamous cell carcinoma, ovarian cancer, and others, functioning as an oncogene [13, 14]. However, the association between prostate cancer and CDR1as is mostly uncertain. Aghajani et al. found that CDR1as level was substantially greater in PCa patient's tissue specimens than in BPH tissue specimens [15]. In another study, CDR1as was found to be nearly 200 times higher in PC3 cells compared to healthy epithelial prostate cells RWPE-1. However, the functional effects of CDR1as on prostate cancer cells remain unknown. Therefore, we investigated the functional impacts of the CDR1as/miR-7/*IGF1R* axis on prostate cancer cell lines.

Materials and methods

Selection of potential sponge miRNA of CDR1as

CircInteractome is an online tool for finding circRNAs, the protein molecules and miRNAs that interact with them [16]. The tool was used to investigate the potential sponge miRNAs of CDR1as.

Culture processes of cell lines

PC3 (prostate adenocarcinoma; Grade IV) and DU145 (prostate adenocarcinoma; Grade II) prostate cancer (PCa) cells were obtained from Erzurum Technical University and cultivated in RPMI-1640 media (EcoTech Biotech., Turkey) enriched with 10 % FBS (Gibco), 1 % antibiotics (Invitrogen, Thermo Fisher Sci.) under 5 % CO_2 and 37 °C humidified conditions.

CDR1as siRNA and miR-7-5p mimic transfection

CDR1as siRNA (SISEL SIRNA, Thermo Sci.), non-targeting (nt) siRNA control (SISEL SIRNA, Thermo Sci.), miR-7-5p mimic and nt mimic control (Ambion, USA) reagents were purchased commercially. 3×10^5 PCa cells per well were seeded in six-well plates (Nest Biotech., China) and at the 24th hour after the seeding, relevant siRNA/mimic was transfected into the cells at a concentration of 30 pmoL. Transfection was performed in accordance with the manufacturer's protocols using Opti-MEM (Gibco) media and Lipofectamine 3000 reagent (Invitrogen, Thermo Fisher Sci.). Cells were subsequently maintained at 5 % CO_2 and 37 °C humidified conditions for up to 24–72 h and then cells were collected for the functional *in vitro* assays.

RNA extraction, cDNA synthesis, and qRT-PCR for transfection validation

Total RNAs from cells transfected with siRNA or mimic were isolated using TRIzol (Invitrogen). RNA concentrations and purities were measured with a NanoDrop (ND-2000c) (Thermo Fisher Sci.) spectroscopy and kept at −80 °C till used. qRT-PCR was performed using specific primers to determine the transfection efficacy of CDR1as siRNA and miR-7-5p mimic in cell lines. For the siRNA transfection validation and gene expression evaluation, cDNA synthesis was performed using a total of 1,000 ng RNA with the "RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific)" and qRT-PCR was conducted using "5× HOT FIRE qPCR Mix Plus (Solis Bio-Dyne)" based to the supplier's procedure. GAPDH was used as a housekeeping gene for normalization. For miRNA transfection validation and the evaluation of miR-7-5p expression in CDR1as siRNA and nt siRNA control transfected cells, a total of 30 ng of RNA was employed for cDNA synthesis. cDNA synthesis was performed using the "Taqman Reverse Transcriptase Kit (Thermo Fisher)" and "miRNA RT primers (Thermo Fisher)" following to the instructions provided by the manufacturer. qRT-PCR was carried out on the LightCycler480 (Roche) device using the TaqMan Universal Master Mix (Thermo Fisher) kit and TaqMan miRNA probes (Thermo Fisher). RNU43 probe (Thermo Fisher) was used for normalization. Duplicated

experiments were conducted and the $2^{-\Delta\Delta Ct}$ technique was used to detect relative expression.

Cell viability assay

siRNA or mimic transfection effects on the cell viability were determined using "Cell Viability Detecting Kit-8 (CVDK-8) (EcoTech Biotech.)" according to the producer company's instructions. PCa cells were seeded in 96-well plates in triplicate at 3×10^3 cells per well, and transfections were performed 24 h later. The changes in the cell viability were measured and noted at the following 24th, 48th, and 72 nd h. For measurement, in all cell viability experiments, 10 µl dilution of CVDK-8 kit (EcoTech Biotech.) liquid was applied to the wells and the cells were cultivated at 37 °C for 3 h. Cell viability was measured by the "Multiscan FC Elisa Microplate Reader (Thermo Sci.)" spectrophotometer, which can measure absorbance at 450 nm.

Scratch-wound assay

The scratch assay was carried out to assess the influence of raising the miR-7-5p level and suppressing the CDR1as level on cellular migration. In six-well plates, PCa cells were planted at a density of 3×10^5 cells per well. After the cells reached 80 % confluency, transfection was performed according to the producer's directions using Opti-MEM medium (Gibco) and Lipofectamine 3000 reagent (Invitrogen, Thermo Fisher Sci.). A 200 µL pipette tip is applied to the 100 % confluent cells (approximately 24 h later) to create a uniform scratch. 1 × PBS (phosphate-buffered saline) (Thermo Fisher) was used to eliminate cell residues by washing. The migration potential of the cells (the size of the wound's healing) was evaluated at 100× magnification with an inverted microscope after 48 h, and cell images were captured.

Caspase-3 activity assay

2×10^5 PCa cells were planted in six-well plates to evaluate the effect of the siRNA or mimic transfection on apoptosis. CDR1as siRNA, nt siRNA control, mimic of the miR-7-5p, and nt mimic (as a control) were transmitted via Lipofectamine-3000 reagent according to the supplier's procedure, and cells were collected 24 h after transfection. Caspase-3 activity was measured with the Human Caspase 3 Instant ELISA Kit (Invitrogen) and the manufacturer's protocol was applied. The Caspase-3 level measurement was carried out at a 450 nm absorbance utilizing the microplate reader.

In silico analysis to identify potential targets of miR-7-5p

Potential targets of miR-7-5p were defined using the miRNet tool [17]. In the miRNet tool, the "human, miRbase, miRTarbase, and Tarbase" choices were selected to search. Since miR-7-5p is a tumor-suppressing miRNA, its potential targets are expected to be oncogenic properties. Therefore, genes with oncogenic properties found to be elevated in prostate cancer were chosen from the potential target genes in the miRNet tool. After thoroughly reviewing the literature, these selected genes were included in the *in vitro* study. Protein-protein interaction (PPI) was examined via the online tool "STRING" [18]. The miRNet tool was used to investigate the potential interaction with transcription factors.

Expression analysis of the possible targets of miR-7-5p

Using a total of 1 µg RNA isolated from the cells, cDNA synthesis was performed with the "RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific")" and qRT-PCR was performed using "5× HOT FIRE qPCR Mix Plus (Solis Bio-Dyne)" according to the manufacturer's protocol. The *GAPDH* gene was utilized for normalization. Duplicate experiments were undertaken, and quantitation analysis was performed as relative via the $2^{-\Delta\Delta Ct}$ method.

Western blot analysis

Total protein lysates were generated by harvesting in cultivated cells using RIPA Lysis Buffer (EcoTech Biotech.) containing 1 % Phenylmethylsulfonyl fluoride (PMSF) and 1X protease inhibiting mix (Thermo Sci.). Protein concentrations were measured using ClearBand Bradford Reagent (EcoTech Biotech.). Proteins were then mixed with 10X Laemmli Buffer (EcoTech Biotech.) and prepared at 100 °C for 5 min. Equal quantities of protein samples were separated by molecular weight in "sodium dodecyl sulfate-polyacrylamide gel" and transferred to "ClearBand Nitrocellulose Membranes (EcoTech Biotech.)". Then, membrane was coated with 5 % milk powder prepared with 1 × PBST buffer (EcoTech Biotech.) for 1 h and incubated with anti-β-actin (1:500, Santa Cruz Biotech), anti-*IGF1R* (1:200 Thermo

Sci.) and anti-EIF4E (1:200 Thermo Sci.) antibodies overnight at 4 °C. β-Actin was used as an internal control. PBST solution was used three times for 10 min to wash antibodies that were not tightly bound to the membrane or not bound at all. Following washing, the membranes were treated with secondary antibodies (anti-rabbit) linked with horse-radish peroxidase (dilution with PBST 1:2000, Santa Cruz Biotech.) for 1 h at room temperature. After secondary antibody therapies, the membranes were rinsed again. Signals of immuno-reactive bands were detected by "ClearBand Western Blotting Substrate (EcoTech Biotech.)" according to the producer's protocol. Bands were observed using the "ChemiDoc-MP (BioRad) image system".

3′UTR cloning for luciferase assay

IGF1R 3′UTR sequence including the estimated miR-7-5p binding sequence was amplified using forward and reverse primers with NheI and XhoI restriction sites incorporated at the 5′ and 3′ ends, respectively. The PCR product was cloned into the "LightSwitch 3′ UTR-Reporter GoClone Collection (SwitchGear Genomics)" plasmid using NheI and XhoI restriction digestion. Correct colonies carrying the "*IGF1R* 3′ UTR sequence (LightSwitch Reporter) were chosen by colony PCR, and plasmids were isolated using the EcoSpin Plasmid Extraction Kit according to the producer's protocol. The plasmids were then analyzed to ensure that they did not include mutated sequences. Primer sequences used for cloning are given in Supplementary File, Table S1.

Luciferase reporter assay

The Luciferase reporter test was utilized to validate the direct binding of miR-7-5p to the putative 3′ UTR binding site of *IGF1R*. Briefly, 1.5×10^5 HEK-293 cells were placed into each well of the six-well plate and incubated for 24 h prior to transfection. Cells were subsequently transfected with the "LightSwitch *IGF1R* 3′ UTR Reporter (SwitchGear Genomics) with the *IGF1R* 3′UTR sequence containing the predicted miR-7-5p binding site. Then, cells were transfected with either 100 pmol miR-7-5p mimic or non-targeting mimic control. All transfection experiments were carried out using Lipofectamine-3000 reagent under the instructions given by the manufacturer.

Statistical analysis

The data were provided as the mean minus the standard deviation from at least 2 to 3 separate assessments. The Student's *t*-test was used to establish statistical significance, and a p-value of less than 0.05 was considered significant. Utilizing GraphPad Prism (version 10.2), figures were created. Relative quantification analysis was performed using the $2^{-\Delta\Delta Ct}$ method. Public databases such as miRNet, miRTarbase, and TarBase were used in the study.

Results

According to the CircInteractome tool, miR-7-5p, which has the most binding sites on CDR1as, was chosen as the most suitable candidate for *in vitro* research (Supplementary File, Figure S1). The miRNet tool includes numerous putative miR-7-5p target genes, such as *IGF1R, EIF4E, SKP2, KLF4, SP1, OGT*, and *BCL2* (Figure 1A). According to the literature, these seven genes act as oncogenes in numerous malignancies, including prostate cancer. Furthermore, they are involved in some other malignancies by being targeted by miR-7-5p. We hypothesized that these possible miR-7-5p targets would be significant in prostate cancer, so we included them in the *in vitro* investigation. The STRING tool analysis revealed that the interaction between these seven genes was higher than expected (Figure 1B). This can be considered an important finding as it indicates that genes can function together. The enrichment analysis determined that *IGF1R*, among these seven genes, may be associated with many transcription factors (Figure 1C). It was established that transfection occurred significantly in the cells transfected by CDR1as siRNA and miR-7-5p mimic (Figure 2A and B, respectively). Increasing the expression level of miR-7-5p significantly reduced the viability of PCa cells transfected by siRNA and mimic ($p<0.05$) (Figure 3). The reduction of cell migration in PCa cells by elevation of the miR-7-5p level was identified. The scratch wound area showed less healing in groups transfected with CDR1as siRNA and miR-7-5p mimic in comparison to the control groups (Figure 4). It was determined that transfection of the CDR1as siRNA and the miR-7-5p mimic increased cell apoptosis by significantly inducing Caspase 3 levels in PCa cells ($p<0.05$) (Figure 5). Supplementary File Table S2 lists all primer sequences of potential miR-7-5p target genes, including OGT, SKP2, EIF4E, IGF1R, BCL2, KLF4, and SP1 [19–26].

The expressions of the target genes *OGT*, *EIF4E*, and *IGF1R* were found to be substantially reduced in both CDR1as siRNA transfected or miR-7-5p mimic transfected PCa cells (Figure 6). The expressions of *EIF4E* and *IGF1R* genes, which were found to be significantly suppressed at the mRNA level in cells transfected by miR-7-5p or si-CDR1as comparison to the control group, were examined at the protein level. Western blotting demonstrated a substantial reduction in

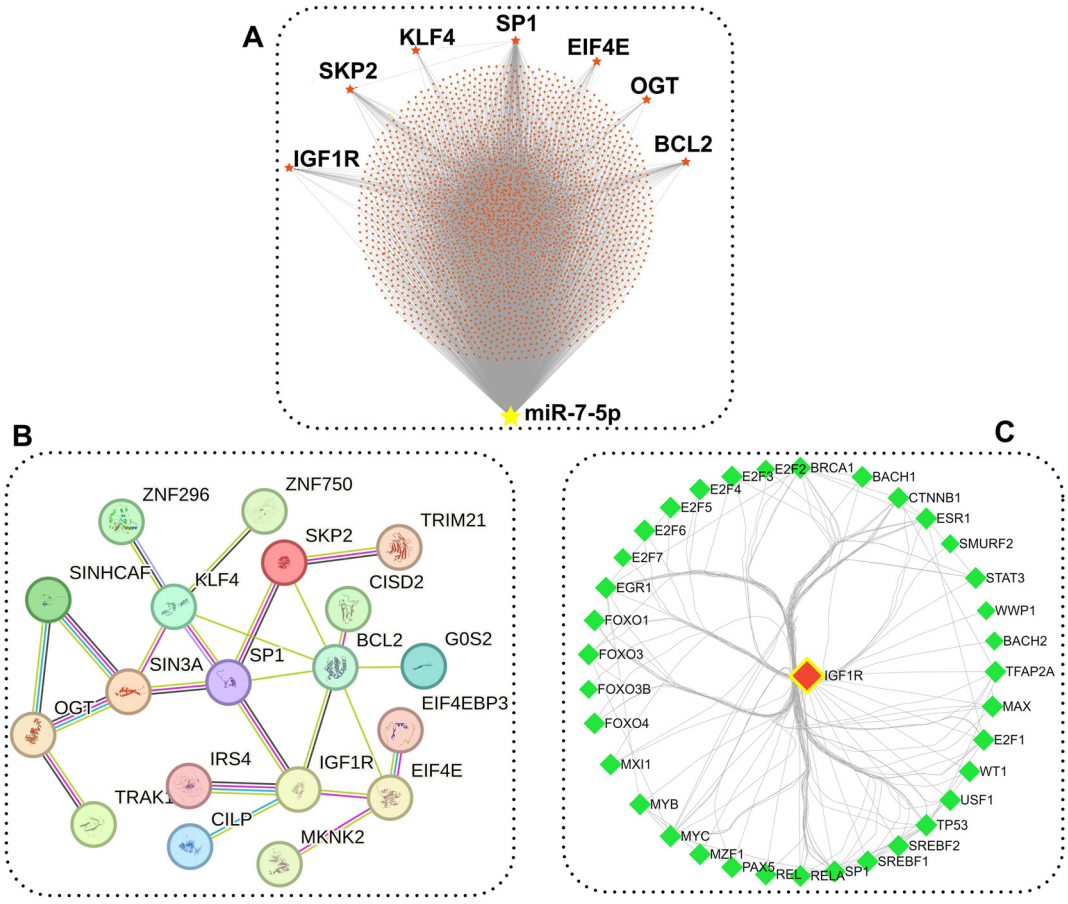

Figure 1: *In silico* associations of possible miR-7-5p target genes. (A) 3,069 genes, including seven genes (*IGF1R*, *EIF4E*, *SKP2*, *KLF4*, *SP1*, *OGT*, and *BCL2*) identified as possible targets of miR-7-5p using the miRNet tool. The number of interactions between these genes is (edge) 12237. (B) Interactions of seven genes selected according to the STRING database (number of nodes: 22, number of edges: 24, average node degree: 2.18, average local clustering coefficient: 0.598) expected number of edges: 5 (PPI enrichment p-value = 2.68e-10). (C) 36 transcription factors may be associated with the *IGF1R* gene according to miRNet (RegNetwork, human, gene names were chosen in miRNet tool for transcription factor association analysis).

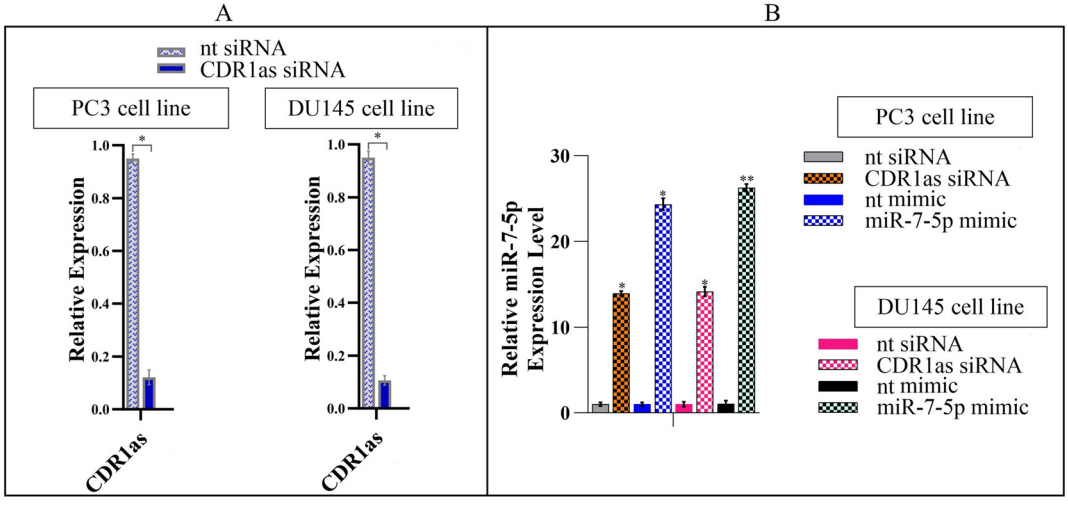

Figure 2: Transfection validation. (A) Decrease in CDR1as expression was confirmed in CDR1as siRNA transfected cells compared to the control group. (B) Increased miR-7-5p expression was confirmed in CDR1as siRNA and miR-7-5p mimic transfected cells compared to the control group (nt: non-targeting; *p<0.05; **p<0.01).

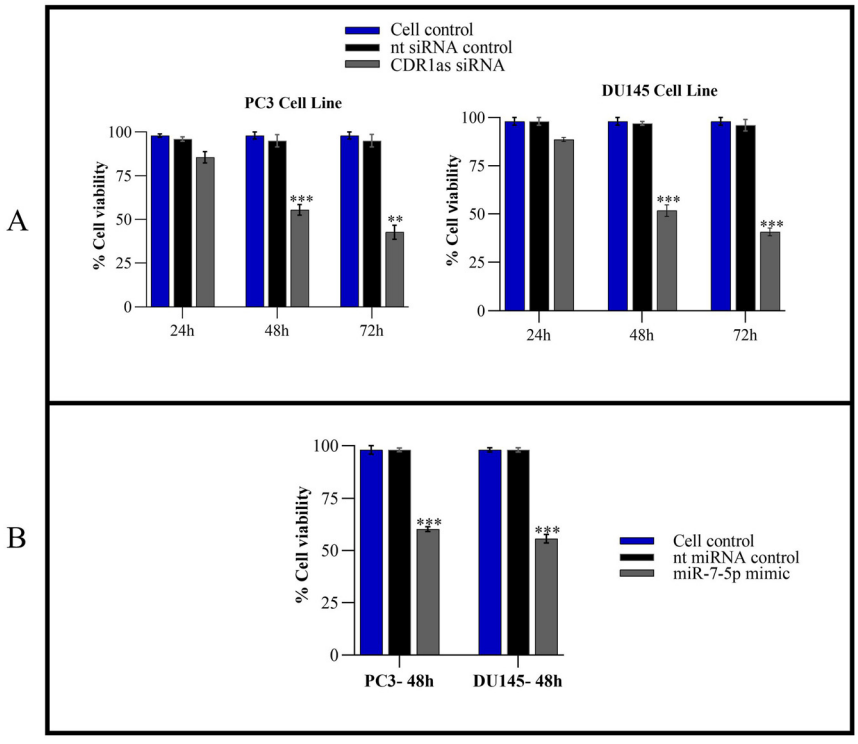

Figure 3: Effects of CDR1as siRNA and miR-7-5p mimic transfection on cell viability in PCa cells (A) CDR1as siRNA transfection reduced cell viability at 48 and 72 h, and (B) miR-7-5p mimic transfection reduced cell viability at 48 h (**p<0.01;***p<0.001). PCa, prostate cancer.

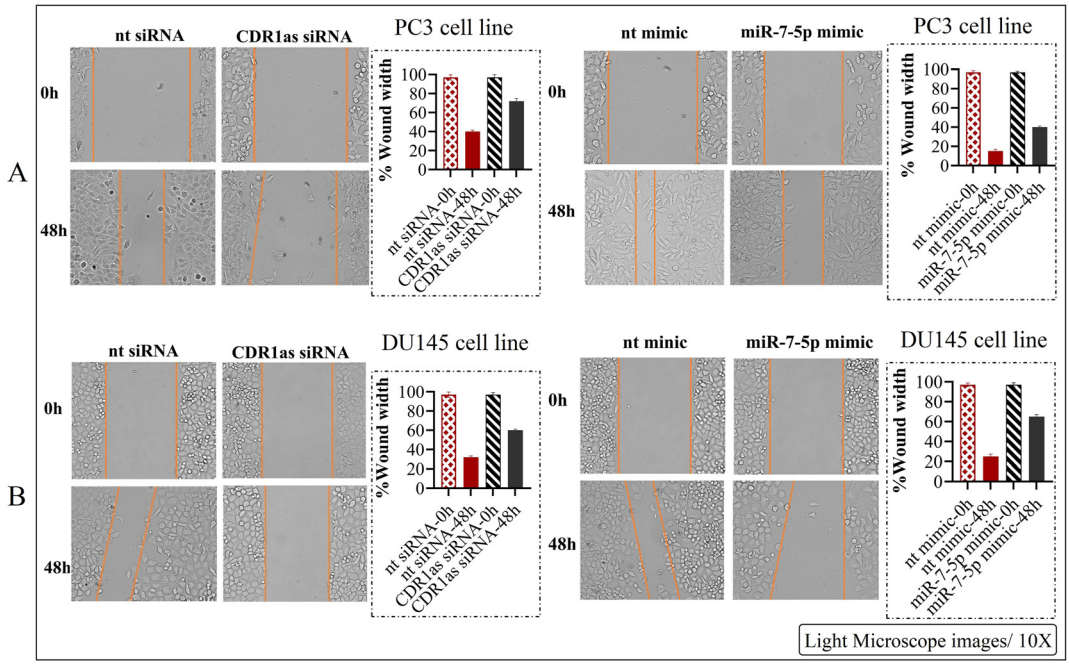

Figure 4: CDR1as siRNA and miR-7-5p mimic transfection inhibit cell migration in (A) PC3 and (B) DU145 cells (light microscope images 10×).

the *IGF1R* protein level in mimic and siRNA-transfected cells comparison to controls. Nevertheless, the level of *EIF4E* protein in the experimental groups did not discernibly alter (Figure 7A). *IGF1R* 3′UTR region contains miR-7-5p binding site (Figure 7B). The miRNA-target verification was performed using the Luciferase reporter test for the *IGF1R* gene, which was revealed to be more reduced in miR-7-5p elevated cells at both the RNA and protein levels. Decreased luciferase

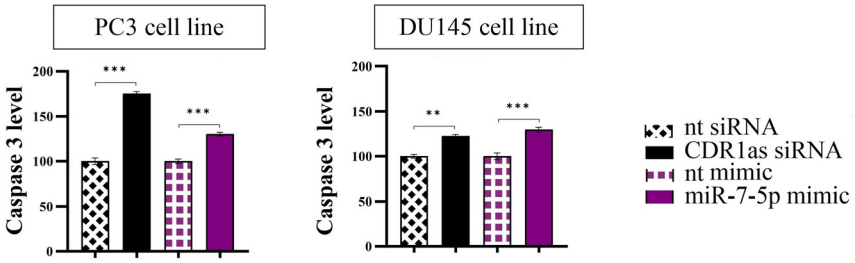

Figure 5: Evaluation of the influence of CDR1as siRNA and miR-7-5p mimic transfection on cell death by Caspase-3 level (**p<0.01;***p<0.001).

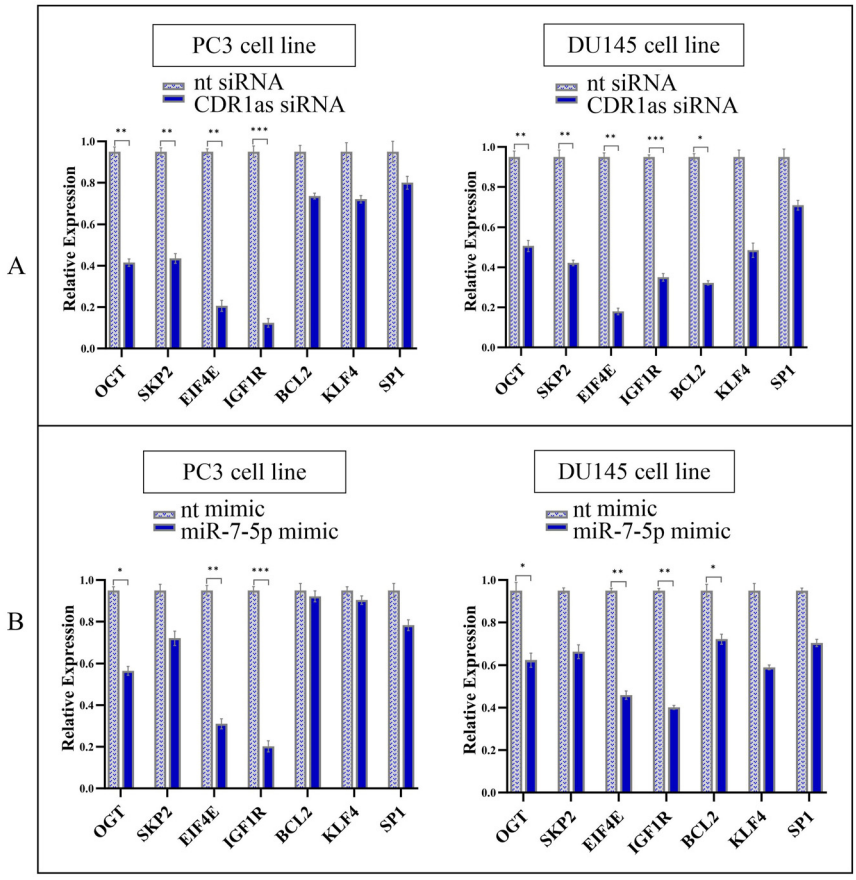

Figure 6: The effect of CDR1as siRNA and miR-7-5p mimic transfection on the expression levels of possible target genes of miR-7-5p in PC3 and DU145 cells (A and B, respectively), (*p<0.05;**p<0.01;***p<0.001).

activity detection in the mimic transfected group showed a significant association between miR-7-5p and 3′ UTR of the *IGF1R* (Figure 7C).

Discussion

Increasing evidence over the last decade has shown that circRNAs may act as regulators of mRNA function through the sponging of miRNAs. Therefore, the "circRNA/miRNA/mRNA" axis has been highlighted as an underlying molecular mechanism of tumor progression in various cancers, which influences tumor pathogenesis at the transcriptional level [27]. circRNAs have been shown to play a critical role in regulating the functions of miRNAs in PCa as well. For example, He et al. demonstrated that CircSCAF8 enhances the growth and metastasis of PCa via the "miR-140-3p/miR-335-*LIF*" axis [28]. Dai et al. reported that CircDHRS3 reduced PCa growth and migration via the "miR-421/MEIS2" pathway [29]. Another study revealed that the "CircUBAP2/miR-1244/*MAP3K2*" axis accelerates the growth of PCa cells [30]. Furthermore, Gao et al. demonstrated that the down-regulation of hsa_circ_0000735 increased the response rate to docetaxel in PCa by targeting miR-7-5p [31].

CDR1as is one of the well-known circRNAs that play an oncogenic role in many cancers. The role of CDR1as in prostate cancer processes is still unclear. In one of the few studies on the prostate cancer/CDR1as relationship, the

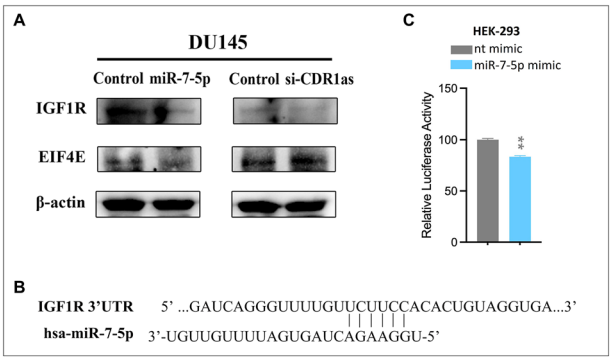

Figure 7: The effect of CDR1as/miR-7-5p axis on *IGF1R* expression. (A) The effect of miR-7-5p restoration on *IGF1R*, *EIF4E*, and β-actin genes' protein expression levels. (B) The predicted target sequence of miR-7-5p on the *IGF1R*. (C) the miR-7-5p mimic transfected cells showed considerably lower luciferase activity than the control group (**p<0.01).

CDR1as expression was demonstrated significantly higher in PCa compared to BPH. In addition, that study showed that PCa patients over the age of 60 had higher CDR1as expression compared to patients under the age of 60 [15]. Recently, CDR1as has been shown to serve as a strong miR-7-5p sponge, suggesting the new molecular mechanisms regulating miR-7-5p function in many cancers. CDR1as binds many miRNAs, including miR-7-5p, miR-1290, and miR-135a, and participates in the regulation of genes that target these miRNAs [32]. In addition, CDR1as is an RNA molecule recently identified as playing a critical role in cancer progression. When CDR1as binds to miR-7-5p, it prevents the interaction of miR-7-5p with target mRNAs. miR-7-5p has been reported to have tumor suppressor effects in many cancers. It is known to regulate various signaling pathways, particularly the p53 pathway [33]. For instance, CDR1as has an oncogenic function in pancreatic cancer through regulation of the miR-7-5p mediated EGFR/STAT3 signaling pathway [34]. The CDR1as/miR-7-5p/*E2F3* axis has been reported to promote cell growth in nasopharyngeal carcinoma [35]. Another study has shown that; the CDR1as/miR-7-5p/*KLF4* axis is critical in hepatoblastoma pathogenesis by affecting hepatoblastoma stem cells [36]. Li et al. found that silencing CDR1as in breast cancer cells increased cisplatin sensitivity by down-regulating *REGγ* via miR-7 [37]. Furthermore, Li et al. have shown that the CDR1as/miR-7/*REGγ* axis leads to gastric cancer cell death by increasing Diosbulbin-B sensitivity [38].

Despite all these studies, the role of the CDR1as/miR-7-5p axis remains largely unclear in prostate cancer. Here, we aimed to investigate the putative involvement of the "CDR1as/miR-7-5p/*IGF1R*" axis in diagnosing and treating PCa. First, CDR1as siRNA and miR-7-5p mimic were transfected into PCa cells and their effects on cellular processes were investigated. After transfection of CDR1as siRNA and miR-7-5p, we showed that CDR1as expression was reduced and miR-7-5p expression was raised in transfected PCa cells. Consistent with the literature, we demonstrated that transfection of CDR1as siRNA and miR-7-5p dramatically reduced cell viability and migration, and increased apoptosis in PCa cell lines. To reveal the underlying molecular mechanism of miR-7-5p action, we identified the potential target genes of miR-7-5p using miRNet database, after which qRT-PCR validation revealed that *IGF1R*, *EIF4E* and *OGT* expression levels are decreased in the CDR1as siRNA and miR-7-5p mimic transfected groups compared to the corresponding controls. In addition, in the present study, we confirmed *IGF1R* as a direct target of miR-7-5p using a luciferase reporter assay.

IGF1R is a protein that belongs to the receptor tyrosine kinase family and is responsible for transducing signals through the IGF1/*IGF1R* axis, serving as a receptor for IGF1 and IGF2 [39, 40]. Aberrant *IGF1R* signaling has been identified in a variety of human malignancies, including PCa [39, 41]. A recent report demonstrated that circVAPA accelerates small-cell lung cancer progression by regulating the miR-377-3p and "miR-494-3p/IGF1R/AKT" axis [42]. Furthermore, overexpression of circRNA CASC11 increased the aggressiveness of PCa cells by regulating the "miR-145/*IGF1R*" axis via leading to activation of the "PI3K/AKT/mTOR" signaling pathway [43]. Similarly, AFAP1-AS1/miR-15b/*IGF1R* [44] and "MDA-9/Syntenin/*IGF1R/STAT3*" [45] axis have been implicated in the pathogenesis of PCa.

In this study, we demonstrate the role of CDR1as concerning miR-7-5p and its direct target *IGF1R* in the progression of PCa cells. We found that the downregulation of CDR1as and upregulation of miR-7-5p significantly affected cellular phenotypes related to PCa progression through the "CDR1as/miR-7-5p/*IGF1R*" axis. Considering the results of our study, the "CDR1as/miR-7-5p/*IGF1R*" axis has the potential to open new horizons in the diagnosis and treatment of PCa.

This study focused on CDR1as/miR-7-5p/*IGF1R* effects on only prostate cancer cellular level. Our study findings could be confirmed in further studies with tissue level and *in vivo* experiments.

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Research ethics: Not applicable.

Informed consent: Not applicable.

Author contributions: MK and IS contributed to the conception/ design of the present study. IS, MK, AA, and OFK performed the experiments. MK, IS, SO, SP, and KC were responsible for the data analysis. All authors performed data

interpretation. MK, IS, and AA drafted the article. All authors revised the manuscript. All authors have accepted responsibility for the entire content of this manuscript and approved its submission.

Use of Large Language Models, AI and Machine Learning Tools: None declared.

Conflict of interest: The authors state no conflict of interest.

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Data availability: Not applicable.

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