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MicroRNA-215-5p Inhibits the Proliferation and Migration of Wilm's Tumor Cells by Targeting CRK

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Abstract

Objective: Wilm's tumor is a common renal malignancy in childhood with unsatisfactory prognosis. microRNA-215-5p (miR-215-5p) has been reported as a tumor-suppressive miRNA in different types of human cancers, but rarely in the Wilm's tumor. In light of this, we tried to investigate the regulatory role and underlying mechanism of miR-215-5p in the Wilm's tumor. **Methods:** After sample collection and cell culture, the expression of miR-215-5p and CT10 Regulator of Kinase (CRK) was detected. Then rhabdoid tumor cell lines (formerly classified as Wilms' tumor cell lines), G401 and WT-CLS1 cells were transfected with pcDNA3.1, pcDNA3.1-CRK, sh-NC, sh-CRK, agomir NC, miR-215-5p agomir, antagomir NC or miR-215-5p antagomir to explore the function of miR-215-5p and CRK in the Wilm's tumor cell proliferation and migration. Moreover, the relationship between miR-215-5p and CRK was analyzed by dual luciferase reporter gene assay. **Results:** Lowly-expressed miR-215-5p and highly-expressed CRK were observed in the Wilm's tumor tissues and cells. Transfection of pcDNA3.1-CRK or miR-215-5p antagomir could promote G401 and WT-CLS1 cell proliferation and enhance migration ability, while transfection of sh-CRK or miR-215-5p agomir led to opposite results. Additionally, miR-215-5p may bind to CRK. Moreover, transfection of pcDNA3.1-CRK in G401 and WT-CLS1 cells could partially reverse the inhibitory effect of miR-215-5p agomir on the proliferation and migration of Wilm's tumor cells. **Conclusion:** Our study highlighted that miR-215-5p could suppress the proliferation and migration of Wilm's tumor cells by regulating the expression of CRK, providing new ideas for molecular targeted therapy for Wilm's tumor.

Keywords

Wilm's tumor, microRNA-215-5p, CRK, proliferation, migration

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Introduction

Wilm's tumor, also known as nephroblastoma, is the most prevalent renal malignancy that affects childhood, characterized with complex pathogenetic pathways involving diverse epigenetic changes as well as gene mutations.[1] Although remarkable achievement in overall survival rates as high as 90% for large patient subgroups, the outcomes are still unsatisfactory for certain individuals with bilateral or relapsed diseases.[2] Despite targeted therapy has emerged as an alternative option with clinical benefit, scarce refractory or recurrent cases, rapid progression along with genetic heterogeneity in this malignancy all make it a big challenge in the early-phase trials of those therapies.[3] The unsatisfactory prognosis and difficulty in clinical trials forces us to broaden our knowledge of

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germline and somatic genetic alterations which underlie the progression of Wilm's tumor to seek more effective strategies and alternative therapies for those patients.[4]

Fortunately, recent researches have addressed the diagnostic/prognostic value of microRNAs (miRNAs) in a series of pediatric central nervous system tumors including medulloblastoma, pilocytic astrocytoma, glioblastoma, and etc. The etiology of Wilm's tumors can be partially attributable to miRNA mutations; to date, how these mutations give rise to tumorigenesis is not fully understood.[5,6] miRNAs can translationally down-regulate or target the expression of mRNA.[7] miR-215-5p has been reported as a tumor-suppressive miRNA in several types of human cancers *via* targeting different oncogenes. For instance, miR-215-5p targets epiregulin and HOXB9 to restrain proliferation, clonogenicity, and migrating potency of colorectal cancer cells.[8] In multiple myeloma, miR-215-5p arguments cell apoptosis through repression of RUNX1.[9] More recently, Gao *et al* have demonstrated that miR-215-5p can retard aggressive progression of breast cancer by down-regulating Sox9.[10] It has been reported that miR-215-5p expression is obviously reduced in Wilm's tumor,[11] but the mechanism remains unclear. Importantly, a binding relationship between and miR-215-5p and CT10 Regulator of Kinase (CRK) has been obtained in our study from 4 databases including Targetscan, Starbase, MiRanda and DIANA-microT. CRK and CRK-like, 2 cellular counterparts of viral oncogene v-Crk, are critically implicated in the tumor-forming process of tumor cells.[12] A recent study has identified CRK as an oncogene in Wilm's tumor by enhancing cell invasion, migration and inducing mesenchymal-to-epithelial transition.[13] In light of those findings, we speculated that miR-215-5p might target CRK to affect the biological behaviors of cancer cells in Wilm's tumor.

Materials and Methods

Ethical Statements

The study was approved by the Ethics Committee of The First Hospital of Changsha (committee approval number: LC2020016), and written informed consent was obtained from all subjects.

Clinical Sample Collection

Cancer tissues (primary tumor) and non-tumor kidney tissues adjacent to cancer (adjacent tissues) were surgically removed from 13 patients with Wilm's tumor from March 2014 to November 2018 in our hospital. The non-tumor kidney tissue adjacent to cancer were clinically judged initially by the clinician during surgical resection and then histopathologically confirmed by the pathologist. The isolated tissue specimens were sub-packaged into sterile cryopreservation tubes, quickly placed in liquid nitrogen, and then stored in a low temperature refrigerator at $-80^{\circ}C$ for later use. Subsequently, the expression patterns of miR-215-5p and CRK were detected by reverse transcription polymerase chain reaction (RT-PCR).

Cell Culture

Human normal embryonic kidney cells CC-HEK-1, rhabdoid tumor cell lines (formerly classified as Wilms' tumor cell lines) G401 and WT-CLS1, purchased from the Shanghai Cell Bank of the Chinese Academy of Sciences were cultured in minimum Eagle's medium (Thermo Fisher Scientific, Wilmington, DE, USA) containing 10% Fetal Bovine Serum (FBS) (Thermo Fisher Scientific, Wilmington, DE, USA), 100 IU/mL penicillin and 100 μg/ml streptomycin. All the cells were placed in a humidified culture at $37^{\circ}C$ with 5% CO_2, and the cell growth status was observed under a microscope.

Cell Transfection

The G401 and WT-CLS1 cells in the logarithmic phase were transfected with 2 μg pcDNA3.1 (empty vector for overexpression vector), pcDNA3.1-CRK (overexpressed CRK), sh-negative control (NC) (NC for short hairpin RNA), sh-CRK (short hairpin RNA targeting CRK), 100 nM agomir NC (NC for miR-215-5p agonist), miR-215-5p agomir (miR-215-5p agonist), antagomir NC (NC for miR-215-5p antagonist), or miR-215-5p antagomir (miR-215-5p antagonist) (RiboBio Co., Ltd, Guangzhou, China) according to the instructions of lipfectamine 2000 kit (Thermo Fisher Scientific, MA, USA).The transfected cells were cultured in serum-free Dulbecco's modified eagle medium (DMEM) in a 5% CO_2, $37^{\circ}C$ incubator.

3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) Assay

Rhabdoid tumor cell G401 and WT-CLS1 cells were counted and 100 μL of the cell suspension (10^4-10^5 cells) was placed in a 96-well plate and incubated in a 5% CO_2, $37^{\circ}C$ incubator. Three duplicated wells were set. After cell culture for respectively 24 h, 48 h, 72 h and 96 h, 20 μL of MTT solution (5 mg/mL, Sigma, USA) was added into each well for incubation in a 5% CO_2, $37^{\circ}C$ incubator for 4 hours. After the removal of the culture solution, dimethyl sulfoxide (DMSO) was added into each well at a dose of 150 μL for 10 minutes to promote crystal dissolution. The optical density value (OD_{540} value) of each well at a wavelength of 540 nm was measured on the enzyme-linked immunoassay instrument. Then, the MTT curve was constructed with the absorbance value as the ordinate and the time as the abscissa. The OD value was measured repeatedly for 3 times and the average value was taken.

Colony Formation Assay

The monolayer cultured cells in the logarithmic phase were digested with 0.25% trypsin, triturated into single cells, and suspended in the culture medium containing 10% FBS for later use. The cell suspension of G401 and WT-CLS1 cells was respectively inoculated into a dish containing 10 ml of $37^{\circ}C$ pre-warmed culture medium (50, 100, or 200 cells per dish) for incubation at an environment of $37^{\circ}C$ 5% and saturated

Table 1. Primer Sequences.

Name of primer	Sequences (5'-3')
CRK-F	CTATTCGGAGTCTGGAGGGG
CRK-R	GAGGCATGCATGAAAGCTCCA
miR-215-5p-F	UAUGGCUUUUUAUUCCUAUGUGA
miR-215-5p-R	CAGTGCGTGTCGTGGAGT
GAPDH-F	ACCACAGTCCATGCCATCAC
GAPDH-R	TCCACCACCCTGTTGCTGTA
U6-F	TCGCTTCGGCAGCACATATAC
U6-R	GCGTGTCATCCTTGCGCAG

humidity for 2-3 weeks. When there were visible clones in the petri dish, the culture was terminated with the supernatant discarded. After that, the cells were washed carefully with phosphate buffered saline (PBS) twice, fixed with 5 ml of acetic acid/methanol (1:3) for 15 minutes, stained with proper amount of Giemsa dye for 10-30 minutes, washed with running water, and air-dried. The plate was turned upside down and superimposed a transparent film with a grid. The clones were observed with the naked eye, or the number of clones with more than 10 cells was counted on a microscope (low power) followed by calculation of the clone formation rate. Cell experiments were repeated for 3 times.

Scratch Test

The cell scratch test was performed as described previously.[14] Briefly, cells (4×10^5) were placed on a six-well plate, 3 straight lines were drawn in the plate with a 100 μL pipette tip when cell confluence reached 90%. Then, the floating cells were washed with PBS and cultured with the serum-free culture medium. The blank channels between cells were observed under a low-power phase contrast microscope (Olympus MK, Tokyo, Japan). After incubation for 24 hours, the gap changes in the blank channels were further observed. The increased area was calculated by ImageJ, and 3 replicate experiments were set for each type of cell.

RT-PCR

The treated G401 and WT-CLS1 cells were dissolved in 1 ml Trizol (Thermo Fisher Scientific, MA, USA), and RNA was extracted according to Trizol instructions. After quantification, RNA was reversely transcribed into cDNA. Based on the fluorescence quantitative PCR kit instructions (Takara, Dalian, China), the PCR reaction system was configured, and the ABI7500 quantitative PCR instrument (Applied Biosystems, USA) was applied to perform real-time quantitative PCR experiments. The reaction conditions consisted of predenaturation at 95°C for 10 minutes, and a total of 40 cycles of denaturation at 95°C for 10 seconds, annealing at 60°C for 20 seconds, and extension at 72°C for 34 seconds. All RT-PCR reaction primers, synthesized by Genewit Biotechnology Co., Ltd, are shown in Table 1. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and U6 were used as the internal references. The $2^{-\Delta\Delta Ct}$ was used for data analysis. Cell experiments were repeated for 3 times.

Western Blot Analysis

After 48 hours, the treated G401 and WT-CLS1 cells were washed by pre-cooled PBS for 3 times, lysed and centrifuged at 12000 rpm at 4°C for 10 minutes. After that, the supernatant was centrifuged in a 0.5 mL centrifuge tube and stored at −20°C. Next, protein quantification was performed through bicinchoninic acid (BCA) kit (Wanlei Bio, Shenyang, China). Then, the protein was added with 6×SDS loading buffer at 100°C for protein denaturation, separated by SDS electrophoresis, and transferred into membranes for 1.5 hours. After being blocked with the 5% skimmed milk powder configured with Tris-buffered saline containing 0.05% Tween-20 (TBST) for 1 hour, the membrane was incubated with primary antibody of CRK (ab45136, 1:1000) or β-actin (ab4970 s, 1:1000) overnight at 4°C. All the primary antibodies were purchased from Abcam (Cambridge, UK). The membrane was re-probed with goat anti-rabbit IgG or goat anti-mouse IgG at room temperature (1:5000, Beijing ComWin Biosciences Co., Ltd., China) for 2 hours. After TBST washing, electrochemiluminescent substrate A and B (Thermo Scientific Pierce Company, USA) were mixed in a ratio of 1:1 and then dropped on the membrane, followed by color development and exposure. Using β-actin as an internal reference, ImageJ was used to calculate the gray value of each band, and 3 replicate experiments were set for each cell line.

Dual Luciferase Reporter Gene Assay

The binding sites between miR-215-5p and CRK were obtained from online prediction software TargetScan and Starbase. Based on the prediction results, the wild type (WT) CRK sequence containing the miR-215-5p binding sites and mutant (MT) sequence containing mutated binding sites were designed respectively which were then cloned and inserted into PGL3-Basic vector (Promega, Madison, USA), named MT-CRK and WT-CRK, respectively. After that, MT-CRK or WT-CRK and miR-215-5p agomir or miR-215-5p antagomir were co-transfected into HEK-293 T cells in 70% cell density. After 48 hours of transfection, the Dual luciferase reporter gene kit (Promega, Madison, USA) was used to detect the luciferase activity. Three duplicated experiments were set.

Statistical Analysis

Data statistical analysis was performed using SPSS 18.0 (IBM Corp., Armonk, NY, USA) and GraphPad Prism 6.0 (GraphPad Software Inc.). The measurement data are displayed in the form of mean ± standard deviation. The comparison between the 2 groups was analyzed by T test, and the comparison between multiple groups was by one-way analysis of variance. The correlation between miR-215-5p and CRK was analyzed using

Pearson correlation coefficient. The significance level of the difference between results was accepted to be 0.05.

Results

Decreased miR-215-5p in Wilm's Tumor Tissues and Cells

To study the functional significance of miR-215-5p in Wilm's tumor, 13 pairs of Wilm's tumor primary tumor tissues and adjacent tissues were collected. The clinical data of children with Wilm's tumor are shown in Table 2. Our results from PCR showed that miR-215-5p was expressed in both Wilm's tumor tissues and adjacent tissues, but the expression of miR-215-5p in Wilm's tumor tissues was remarkably lower than that in adjacent tissues (Figure 1A, $P < 0.01$). Additionally, compared with human normal embryonic kidney cells CC-HEK-1, the expression of miR-215-5p in Wilm's tumor cell lines G401 and WT-CLS1 was significantly reduced (Figure 1B, $P < 0.01$). Then miR-215-5p agomir/antagomir or its corresponding NC was transfected into G401 and WT-CLS1 cells. PCR results depicted that compared with the agomir NC group, the expression of miR-215-5p in the ago-miR-215-5p group was significantly increased. Compared with the antagomir NC group, the miR-215-5p expression in the antago-miR-215-5p group was significantly reduced (Figure 1C and D, $P < 0.01$), indicating that miR-215-5p agomir and miR-215-5p antagomir had good transfection efficiency in Wilm's tumor cells which were suitable for the subsequent experiments.

Table 2. Clinical Characteristics of All the Wilm's Tumor Patients.

Clinical characteristics	Cases		
Gender	male (n = 8)	female (n = 5)	
Age	0-3 years (n = 3)	4-6 years (n = 6)	7-9 years (n = 4)
Location	left kidney (n = 8)	right kidney (n = 5)	
Tumor grade	I-II (n = 9)	III-IV (n = 4)	

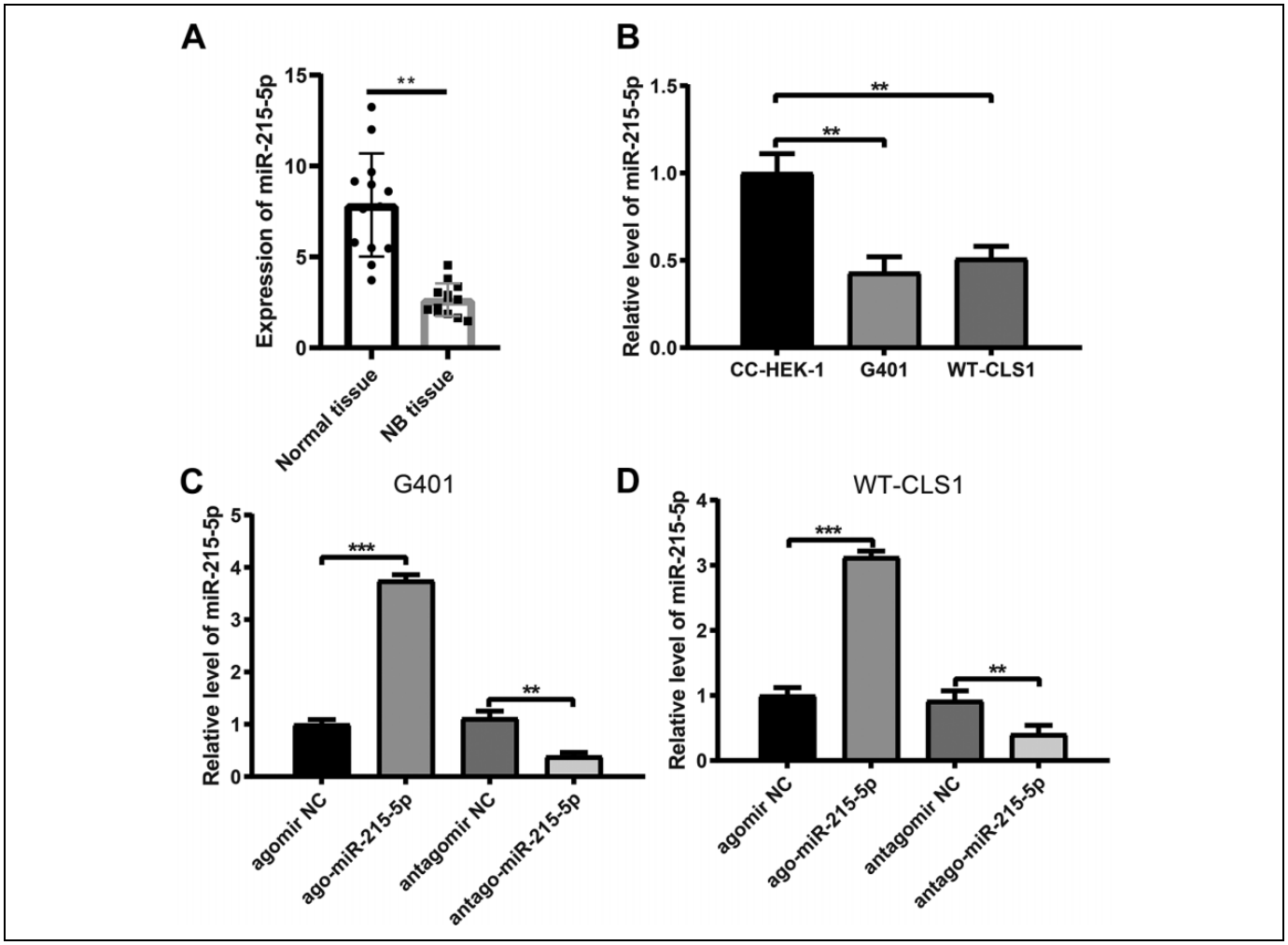

Figure 1. Lowly expressed miR-215-5p in Wilm's tumor tissues and cells. (A) PCR was performed to detect miR-215-5p expression in both Wilm's tumor tissues and adjacent tissues; (B) PCR was performed to detect miR-215-5p expression inhuman normal embryonic kidney cells CC-HEK-1 and rhabdoid tumor cell lines G401 and WT-CLS1 (formerly classified as Wilms' tumor cell lines); (C) PCR was used to detect miR-215-5p expression inG401 cells transfected with miR-215-5p agomir or miR-215-5p antagomir; (D) PCR was carried out to detect miR-215-5p expression inWT-CLS1 cells transfected with miR-215-5p agomir or miR-215-5p antagomir; $^{**}P < 0.01$, $^{***}P < 0.001$.

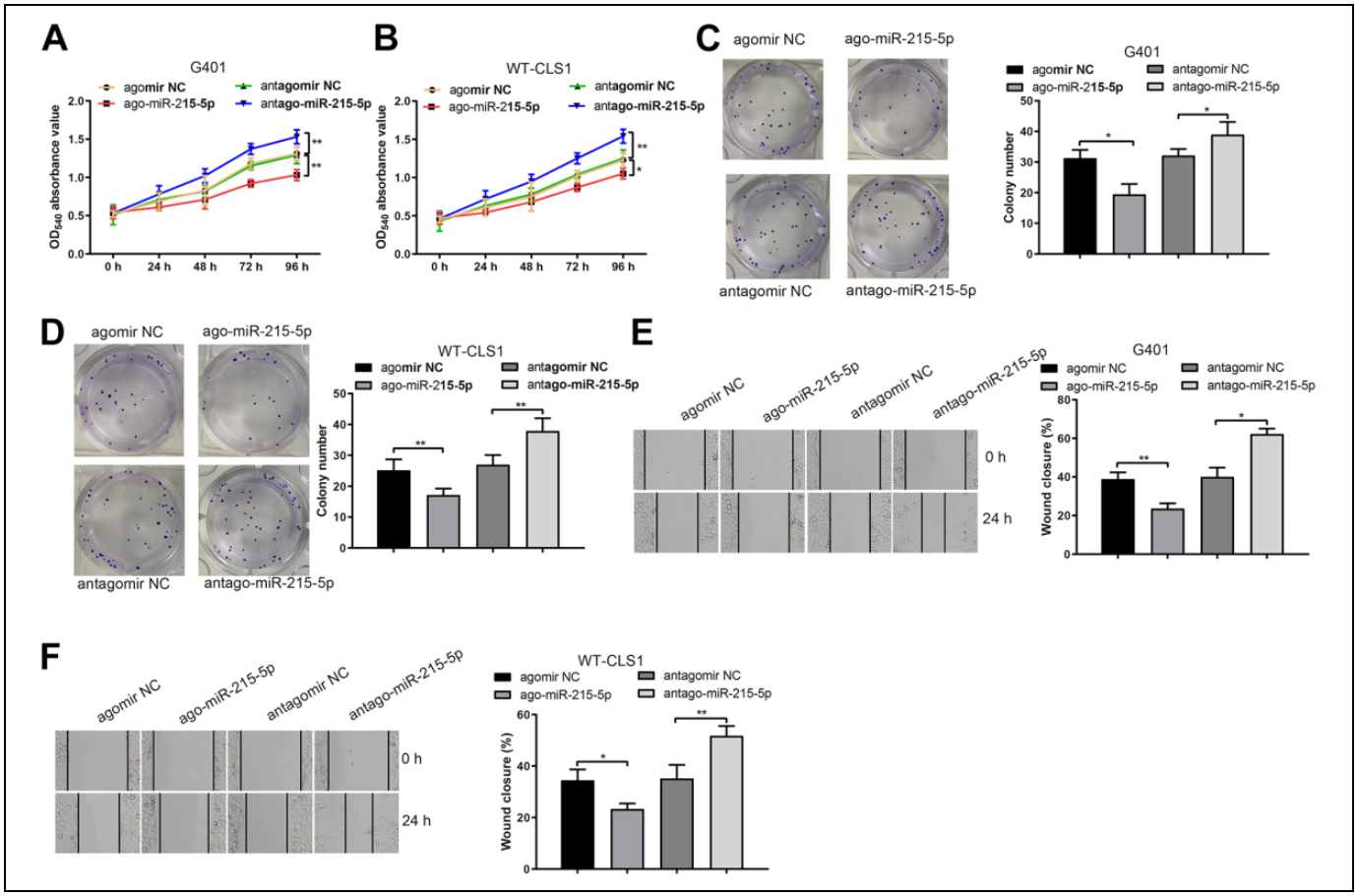

Figure 2. Down-regulation of miR-215-5p enhanced Wilm's tumor cell proliferation and migration. Rhabdoid tumor cell lines G401 and WT-CLS1 (formerly classified as Wilms' tumor cell lines) were transfected with miR-215-5p agomir, miR-215-5p antagomir or corresponding controls. A-B, MTT assay was used to detect the proliferation of G401 (A) and WT-CLS1 (B) cells. C-D, Colony formation assay was performed to detect the colony formation abilities of G401 (C) and WT-CLS1 (D) cells. E-F, Scratch test was applied to test the migration ability of G401 (E) and WT-CLS1 (F) cells; $^{**}P < 0.05$, $^{***}P < 0.01$.

miR-215-5p Suppressed the Proliferation and Migration Abilities of Wilm's Tumor Cells

To explore the role of miR-215-5p in Wilm's tumor cells, miR-215-5p was overexpressed or inhibited in G401 and WT-CLS1 cells. The results of MTT and colony formation assays demonstrated that compared with the agomir NC group, the proliferation and colony formation abilities of cells in the ago-miR-215-5p group were significantly reduced. Compared with the antagomir NC group, the cell proliferation and colony formation abilities in the antago-miR-215-5p group were significantly increased (Figure 2A-D, $P < 0.01$). Additionally, Scratch test revealed that compared with the agomir NC group, the cell migration ability of the ago-miR-215-5p group was significantly reduced. Compared with the antagomir NC group, the cell migration ability of the antago-miR-215-5p group was significantly increased (Figure 2E and F, $P < 0.01$). The above-indicated findings elaborated that miR-215-5p inhibited the proliferative and migratory capabilities of Wilm's tumor cells G401 and WT-CLS1.

CRK Was the Potential Target for miR-215-5p

To further explore the mechanism of miR-215-5p, the 4 most widely used biological online prediction databases Targetscan (http://www.targetscan.org/), Starbase (http://starbase.sysu.edu.cn/), MiRanda (http://microma.sanger.ac.uk/), and DIANA-microT (http://diana.cslab.ece.ntua.gr/) were applied for predicting the target of miR-215-5p. In the Venn diagram, we found 24 genes could interact with miR-215-5p (Figure 3A). Additionally, the mutual binding site of miR-215-5p and CRK was observed in Figure 3B. In order to verify the interaction between miR-215-5p and CRK, the expression of CRK in Wilm's tumor cell lines G401 and WT-CLS1 was firstly detected, and the result found that compared with human normal embryonic kidney cells CC-HEK-1, the expression of CRK in G401 and WT-CLS1 was significantly increased (Figure 3C and D, $P < 0.01$). Consistently, higher expression level of CRK was also observed in Wilm's tumor primary tumor tissues when compared with that in adjacent tissues (Figure 3E, $P < 0.01$). Correlation analysis also demonstrated a negative relation between miR-215-5p expression and CRK expression (Figure

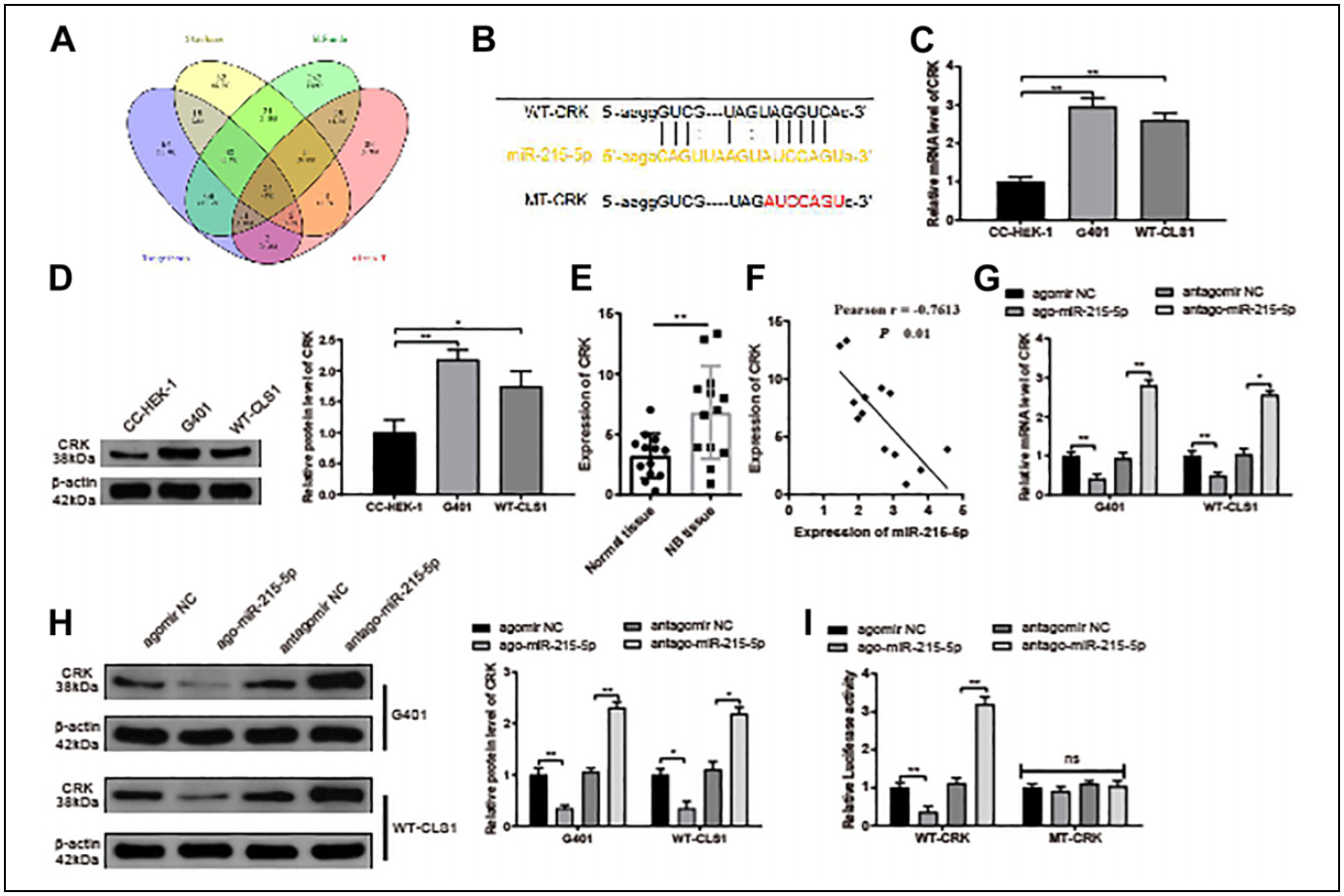

Figure 3. CRK was the potential target of miR-215-5p. (A) Venn analysis was used for analyzing the prediction results of 4 online softwares Targetscan, Starbase, Miranda and DIANA-microT; (B) targeting relationship between miR-215-5p and CRK was predicted by online prediction software; (C) PCR was performed to detect CRK expression inhuman normal embryonic kidney cells CC-HEK-1 and Wilm's tumor cell lines G401 and WT-CLS1; (D) Western blot analysis was performed to detect CRK expression in human normal embryonic kidney cells CC-HEK-1 and rhabdoid tumor cell lines G401 and WT-CLS1 (formerly classified as Wilms' tumor cell lines); (E) PCR was performed to detect CRK expression in both Wilm's tumor tissues and adjacent tissues; (F) the correlation between miR-215-5p and CRK was analyzed by Pearson; (G) PCR was performed to detect CRK expression in G401 and WT-CLS1; (H) Western blot analysis was performed to detect CR expression in Wilm's tumor cell lines G401 and WT-CLS1; (I) luciferase reporter gene assay was applied for verifying the targeting relationship between miR-215-5p and CRK; $^{**}P < 0.05$, $^{***}P < 0.01$, ns: no significant.

3F, $P < 0.01$). Subsequently, we tested whether miR-215-5p could regulate the expression of CRK in G401 and WT-CLS1. Compared with the agomir NC group, the expression of CRK in the ago-miR-215-5p group was significantly lower. The expression of CRK in the antago-miR-215-5p group was significantly increased when compared with the antagomir NC group (Figure 3G and H, $P < 0.05$), suggesting that miR-215-5p negatively regulated the expression of CRK. To verify the binding of miR-215-5p to CRK, we constructed a wild-type CRK luciferase plasmid (named WT-CRK) and a mutant CRK luciferase plasmid (named MT-CRK, red base is a mutant sequence, Figure 3B). Following luciferase reporter gene assay, we found that when WT-CRK and ago-miR-215-5p were co-transfected, the luciferase activity was significantly reduced when compared with the corresponding control ($P < 0.01$). When WT-CRK was co-transfected with antago-miR-215-5p, the luciferase activity was significantly higher than that of the corresponding control ($P < 0.01$). When MT-CRK was co-transfected with ago-miR-215-5p or antago-miR-215-5p, there was no significant difference in luciferase activity compared with the corresponding control (Figure 3I), suggesting that there might exist a mutual binding between CRK and miR-215-5p. Above all, miR-215-5p may target CRK.

CRK Promoted the Proliferation and Migration Abilities of Wilm's Tumor Cells

In order to investigate the role of CRK in Wilm's tumor cells, CRK was overexpressed or silenced in Wilm's tumor cells G401 and WT-CLS1. PCR and Western blot analysis confirmed the overexpression efficiency and silencing efficiency of CRK (Figure 4A and B). The results of MTT and colony formation assays displayed that compared with the pcDNA3.1 group, the pcDNA3.1-CRK group showed enhanced cell

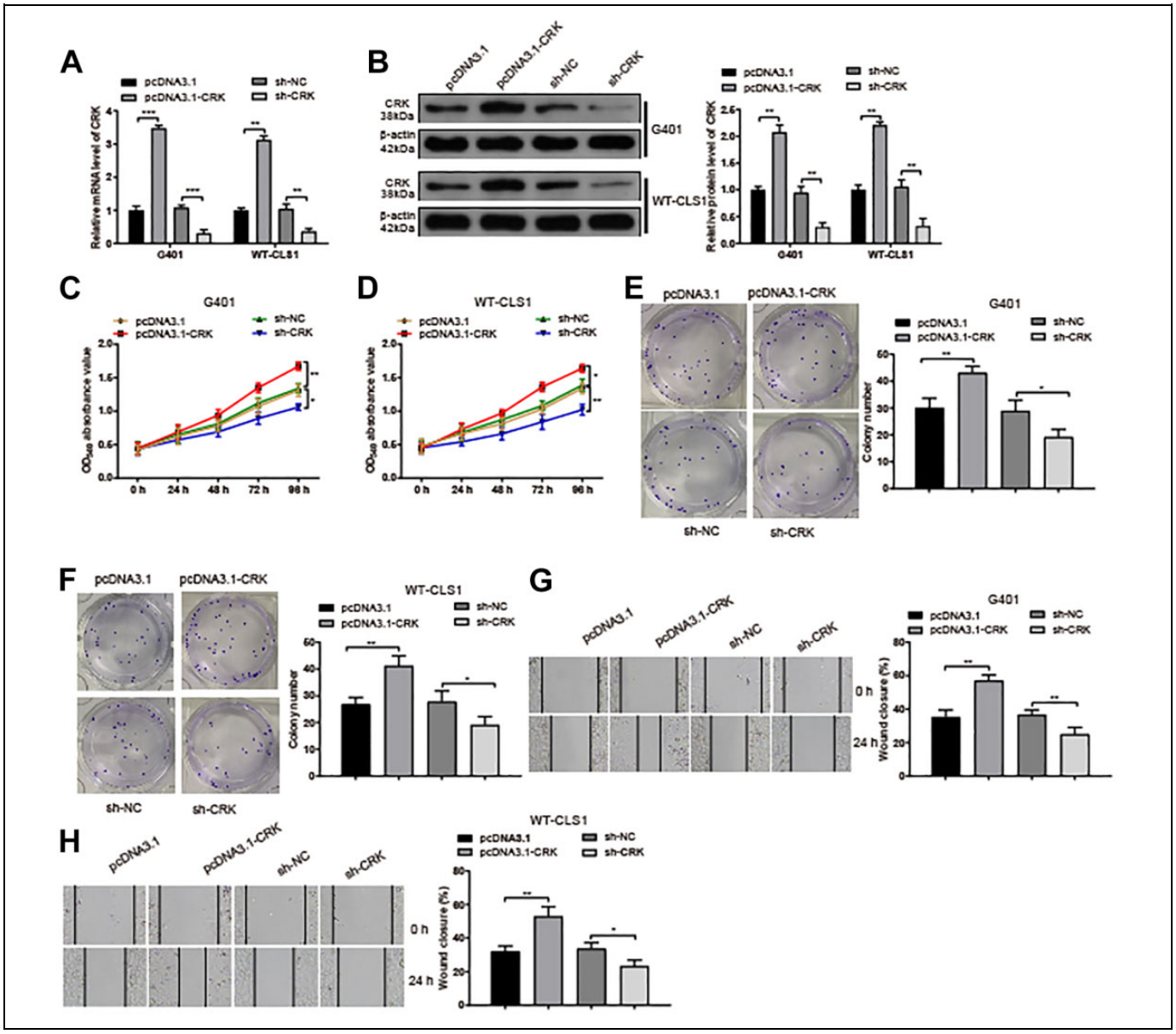

Figure 4. CRK promoted the proliferation and migration of Wilm's tumor cells lines G401 and WT-CLS1. Rhabdoid tumor cell lines G401 and WT-CLS1 (formerly classified as Wilms' tumor cell lines) were transfected with pcDNA3.1-CRK, sh-CRK or corresponding controls. A, PCR was performed to detect CRK expression in Wilm's tumor cell lines G401 and WT-CLS1. B, Western blot analysis was performed to detect CRK expression in Wilm's tumor cell lines G401 and WT-CLS1. C-D, MTT assay was used to detect the proliferation of G401 (C) and WT-CLS1 (D) cells. E-F, Colony formation assay was performed to detect the colony formation abilities of G401 (E) and WT-CLS1 (F) cells. G-H, Scratch test was applied to test the migration ability of G401 (G) and WT-CLS1 (H) cells; $^{**}P < 0.05$, $^{***}P < 0.01$.

proliferation and colony formation abilities. Compared with the sh-NC group, the cell proliferation and colony formation abilities in the sh-CRK group were significantly reduced (Figure 4C-F, $P < 0.01$). Moreover, the results of the scratch test found that compared with the pcDNA3.1 group, the cell migration ability in the pcDNA3.1-CRK group was enhanced, and compared with the sh-NC group, the cell migration ability in the sh-CRK group was weakened (Figure 4G and H, $P < 0.01$). The aforementioned results concluded that CRK promoted the proliferation and migration of Wilm's tumor cells lines G401 and WT-CLS1.

miR-215-5p Resulted in Suppressed Proliferative and Migratory Potentials of Wilm's Tumor Cells by Targeting CRK

To further explore whether miR-215-5p could inhibit the proliferation and migration of Wilm's tumor cells by regulating CRK, we transfected ago-miR-215-5p or pcDNA3.1-CRK or the corresponding negative control in Wilm's tumor cells G401 and WT-CLS1, the results showed that compared with the agomir NC + pcDNA3.1 group, the cell proliferation and colony formation abilities in the ago-miR-215-5p + pcDNA3.1

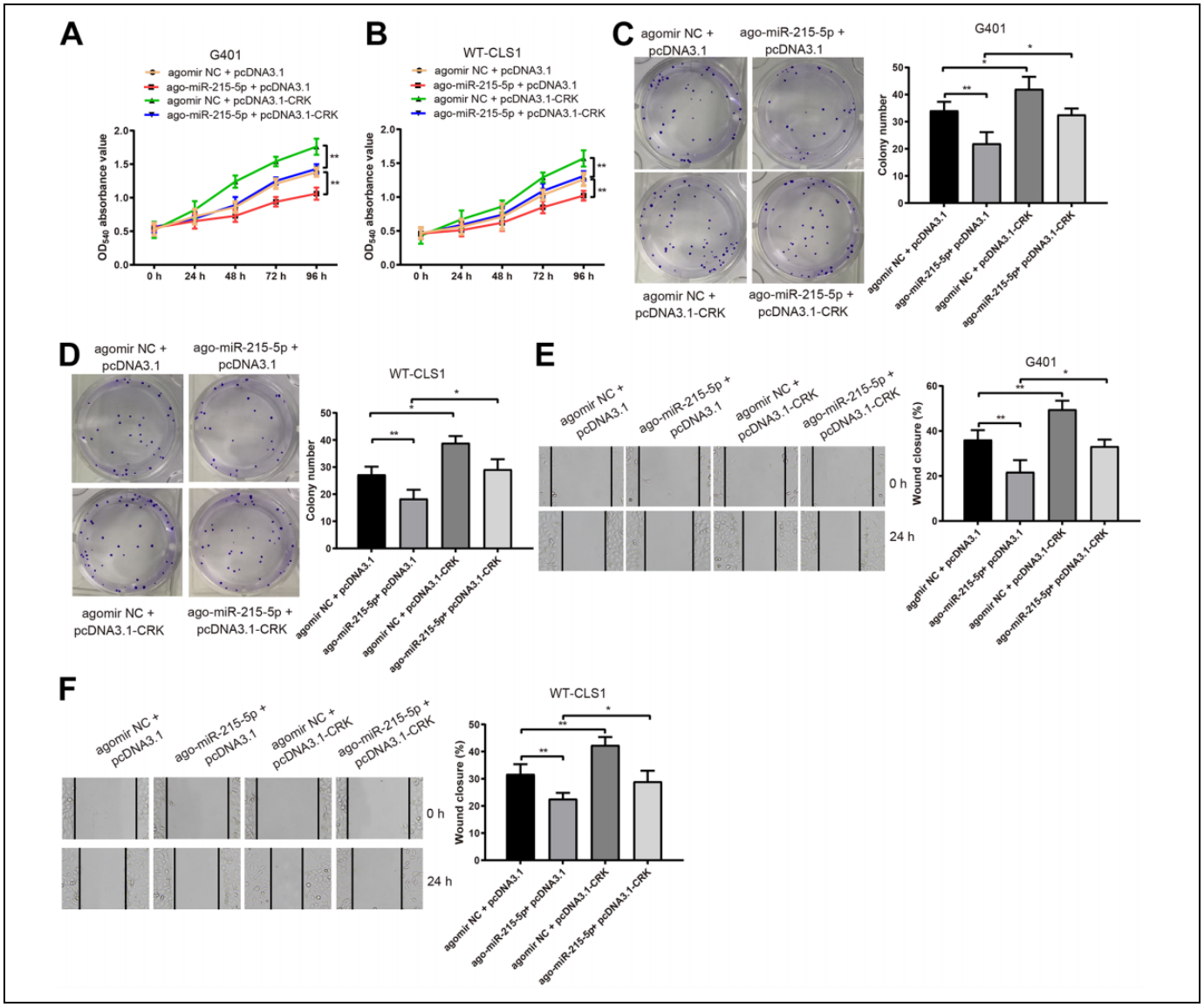

Figure 5. miR-215-5p regulated the proliferation and migration of Wilm's tumor cells through CRK. Rhabdoid tumor cell lines G401 and WT-CLS1 (formerly classified as Wilms' tumor cell lines) were transfected with ago-miR-215-5p, pcDNA3.1-CRK or corresponding controls. A-B, MTT assay was used to detect the proliferation of G401 (A) and WT-CLS1 (B) cells. C-D, Colony formation assay was performed to detect the colony formation abilities of G401 (C) and WT-CLS1 (D) cells. E-F, Scratch test was applied to test the migration ability of G401 (E) and WT-CLS1 (F) cells; $^{**}P < 0.05$, $^{***}P < 0.01$.

group were significantly reduced, while the cell proliferation and colony formation abilities in the agomir NC + pcDNA3.1-CRK group were significantly enhanced. Compared with the ago-miR-215-5p + pcDNA3.1 group, the ago-miR-215-5p + pcDNA3.1-CRK group had significantly enhanced cell proliferation and colony formation abilities (Figure 5A-C, $P < 0.01$). The findings uncovered that overexpression of CRK could eliminate the inhibitory effect of ago-miR-215-5p on the proliferation of Wilm's tumor cells. Besides, the cell scratch test showed that compared with the agomir NC + pcDNA3.1 group, the cell migration ability of the ago-miR-215-5p + pcDNA3.1 group was weakened, and the cell migration ability of the agomir NC + pcDNA3.1-CRK group was enhanced. Compared with the ago-miR-215-5p + pcDNA3.1 group, the ago-miR-215-5p + pcDNA3.1-CRK group had enhanced cell migration ability (Figure 5E and F, $P < 0.01$), indicating that overexpressed CRK could partially eliminate the inhibitory effect of ago-miR-215-5p on the migration of Wilm's tumor cells. Based on the above results, overexpression of CRK could eliminate the inhibitory effect of overexpression of miR-215-5p on the proliferation and migration of Wilm's tumor cells, suggesting that miR-215-5p regulated the proliferation and migration of Wilm's tumor cells through CRK.

Discussion

Molecular-targeted therapy attracts great attention of more and more researchers to improve the outcomes for children with tumors.[15] The specificity of any single agent to interfere the target and the existence of multiple genetic abnormalities limited the efficacy of targeted therapy, which forces us to conduct further exploration of the complex pathogenesis of Wilm's tumor. This study mainly suggested the antitumor role of miR-215-5p in Wilm's tumor based on the results of *in vitro* experiments. Furthermore, miR-215-5p may bind to CRK and downregulation of CRK is suggested to be the possible mechanism which underlies the role of miR-215-5p in this malignancy.

The most important finding of our study is that miR-215-5p acts to repress the proliferative and migratory properties of Wilm's tumor cells. A group of miRNAs have recently emerged as promising therapeutic targets for Wilm's tumor by serving as either tumor suppressor or promoter. On the one hand, miR-613 plays an inhibitory role in the growth, proliferation, invasion, and migration processes of Wilm's tumor cells.[16] Additionally, enhancement of miR-539 contributes to preventing the development of Wilm's tumor *in vitro*.[17] On the other hand, miR-190b functions as an onco-miRNA in Wilm's tumor through inhibiting cell apoptosis.[18] Although the role of miR-215-5p in Wilm's tumor is scarcely mentioned, the tumor-suppressive role of miR-215 in other human cancers has been widely reported, such as epithelial ovarian cancer,[19] and non-small cell lung cancer.[20] Similar to our findings, miR-215-5p mimic transfection results in repression of cell proliferation in multiple myeloma.[9] Furthermore, miR-215-5p exerts significant cell killing and pro-apoptotic effects on an aggressive malignancy malignant pleural mesothelioma, demonstrating its therapeutic significance in anticancer treatment.[21] According to our findings, miR-215-5p agomir attenuated the viability, colony-forming and migrating abilities of G401 and WT-CLS1 cells, suggesting the tumor-suppressive function of miR-215-5p *in vitro*, which warrants further investigation in animal models in the future.

Subsequent mechanistic investigation identified that miR-215-5p might bind to CRK and inhibited CRK expression, whereby suppressing cancer progression in Wilm's tumor. Leblanc *et al* illustrated that miR-215-5p could reduce breast cancer cell proliferation and migration.[22] They identified Pax-5 not only as a target gene of miR-215-5p but also an effective modulator of miR-215-5p, which is partially similar to but slightly different with the mechanism defined in this study. Recent studies have reported an interesting role of CRK adaptor proteins (CRKI, CRKII and CRKL) in integrating signals for migrating and invading functions of breast cancer cells.[23,24] Furthermore, CRK proteins are implicated in the aggressiveness of human malignancies, such as lung and gastric adenocarcinomas, sarcomas and gliomas.[25] Also, CRKII-deficient prostate cancer cells exhibit impaired colony formation.[26] Kumar and his team have evidenced that CRK drives tumor growth and metastasis through its Tyr239 phosphorylation.[27] In pancreatic cancer, loss of CRK contributes to suppressed cancer cell growth as well as prolongs survival of mice bearing orthotopic xenograft.[28] In addition to the aforementioned tumors, CRK knockdown can reverse the malignant behaviors of Wilm's tumors enhanced by miR-194-5p inhibitor,[13] which is particularly consistent with our findings. By conducting rescue experiments, our study substantiated that restoration of CRK could rescue the suppressed cell proliferation and migration induced by miR-215-5p overexpression in Wilm's tumor. Hence, it is reasonable to conclude that miR-215-5p delayed the progression of Wilm's tumor by targeting CRK.

Taken together, our data demonstrate that miR-215-5p confers prevention against the progression of Wilm's tumor through repression of CRK. This finding highlights the promise of miR-215-5p and CRK as therapeutic targets for this malignancy. However, there still existed some limitations. Due to the limited clinical sample size, the relatively early time for collection of children's data and incomplete efficacy evaluation standard data for solid tumors, we cannot detect the expression of miR-215-5p between responders and non-responders to treatment, as well as between patients with low-stage and high-stage disease. Additionally, one thing should bear in mind when interpreting the results of our study that the cell lines used in current study are rhabdoid tumor cell lines (WT-CLS1 and G401 cell lines) and are formerly classified as Wilms' tumor cell lines. Besides, the role of other target genes of miR-215-5p in the context of Wilms' tumor needs further evaluation.

Authors' Note

The study was approved by the Ethics Committee of The First Hospital of Changsha (committee approval number: LC2020016), and written informed consent was obtained from all subjects.

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