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microRNA-214 Prevents Traits of Cutaneous Squamous Cell Carcinoma via VEGFA and Bcl-2

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Abstract

Background: Dysregulation of microRNA-214 (miR-214) has been indicated in different tumors. The function of miR-214 in cutaneous squamous cell carcinoma (CSCC) is yet to be deciphered. The current study aimed to investigate the specific mechanism underpinning CSCC development with the involvement of miR-214 and its putative targets. **Methods:** Microarray analysis of CSCC and adjacent tissues was carried out to filter the most significant downregulated miRNA. Survival analysis of patients was subsequently implemented, followed by miRNA expression determination in CSCC cells. Gain-of-function assays were performed to evaluate its function on cellular level. The targets of the determined miRNA were predicted and their expression in CSCC and adjacent tissues was evaluated. The targeting relationship was analyzed by dual-luciferase assays. Finally, rescue experiments were conducted. **Results:** miR-214 was reduced in CSCC tissues and cells, and the survival of patients harboring overexpression of miR-214 was higher. miR-214 restoration increased CSCC cell apoptosis, while decreased proliferative, invasive and migratory activities. miR-214 interacted with vascular endothelial growth factor A (VEGFA) and B-cell CLL/lymphoma 2 (Bcl-2). VEGFA and Bcl-2, overexpressed in CSCC tissues and cells, were negatively correlated with miR-214. Moreover, VEGFA and Bcl-2 overexpression reversed the anti-tumor phenotypes of miR-214 on CSCC cells. miR-214 disrupted the Wnt/β-catenin pathway through VEGFA and Bcl-2 in the CSCC cells. **Conclusion:** Our data demonstrates that miR-214 exerts a suppressing role in CSCC. The discovery of novel targets such as miR-214 and VEGFA/Bcl-2 may facilitate the development of therapeutic options.

Keywords

microRNA-214, Cutaneous squamous cell carcinoma, VEGFA, Bcl-2, The Wnt/β-catenin pathway

Abbreviations

ANOVA, analysis of variance; Bcl-2, B-cell CLL/lymphoma 2; CCK-8, cell counting kit-8; CSCC, cutaneous squamous cell carcinoma; DMEM, Dulbecco's modified Eagle's medium; EdU, 5-Ethynyl-2'-deoxyuridine; FBS, fetal bovine serum; FITC, fluorescein isothiocyanate; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; miR-214, microRNA-214; OD, optical density; OE, overexpression; PI, propidium iodide; RT-qPCR, reverse transcription-quantitative PCR; SD, standard deviation; SDS, sodium dodecyl sulfate; VEGFA, vascular endothelial growth factor A

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Introduction

Cutaneous squamous cell carcinoma (CSCC) represents one of the most seen cancers in white populations, and sufferers often develop multiple primary malignancies over time, which further increases the disease burden.[1] CSCC typically illustrates as a range of progressively advanced cancers, from precursor actinic keratosis, squamous cell carcinoma in situ, to invasive CSCC and finally metastatic SCC.[2] Exposure to ultraviolet radiation and sunlight feeds into the greatest risk factor, and incidences of regional and distant metastases as well as disease-specific death caused by CSCC have been reported as 5%, 1%, and 1%, respectively.[3] Therefore, innovative approaches applied for molecular modification pattern detection might result in the development of improved diagnosis and novel therapeutics for CSCC.

microRNAs (miRNAs) are small noncoding RNAs which function by sequence-specific binding to the 3'untranslated region of its target mRNAs, thus degrading or translationally inhibiting the mRNAs.[4] The biogenesis and function of miRNA has been suggested to be related to the mechanisms of different clinical diseases, which could modulate every aspect of cellular activity, including proliferation, apoptosis and tumorigenesis.[5] Moreover, the dysregulation of miRNAs is well-known to be participated in the progression of CSCC.[6] Among them, human miR-214, located in the chromosomal region 1q24.3, exerts essential roles in modulating tumor proliferation, angiogenesis, invasiveness, metastasis as well as resistance to chemotherapy.[7] miR-214 was observed to be deregulated in several other cancers in addition to skin cancers.[8] For instance, miR-214 repressed the growth of renal cell carcinoma cell *in vitro* and *in vivo* through targeting LIVIN.[9] Moreover, miR-214-3p was downregulated in hepatocellular carcinoma tissues versus normal liver tissues, and poor miR-214-3p expression was linked to shorter overall survival rate and recurrence-free survival of hepatocellular carcinoma patients.[10] Nevertheless, no researches concentrated on the relevance of miR-214 in CSCC. miR-214 was observed to be remarkably downregulated in CSCC in the current study by microarray analysis, and to target vascular endothelial growth factor A (VEGFA) and B-cell leukemia 2 (Bcl-2). Invasive CSCC expresses enhanced levels of VEGFA, predominantly in the leading front of the cancer site.[11] Besides, Bcl-2 overexpression in human melanoma cells promoted tumor progression-related properties, including tumor growth and angiogenesis.[12] Intriguingly, miR-214 inhibited cell proliferation, migration, and epithelial-mesenchymal transition of colon cancer via the blockage of the Wnt signaling by targeting BCL9L.[13] This report highlighted the association between miR-214 and VEGFA/Bcl-2 in regulating cell viability, migration, invasion and apoptosis of CSCC cells, explaining the explicit molecular mechanism.

Table 1. Clinicopathological Features of Patients With CSCC.

Clinicopathological features	n = 22
Age (year)	
<50	8
≥50	14
Gender	
Male	12
Female	10
Tumor size (cm)	
<4	9
≥4	13
TNM staging	
III + IV	6
I + II	16

Note: CSCC, cutaneous squamous cell carcinoma; TNM, Tumor, Node, Metastases.

Materials and Methods

Tissue Specimens

Twenty-two CSCC patients were selected from Affiliated Hospital of Beihua University between February 2013 to September 2015. The chemotherapy- and radiotherapy-treated patients were excluded. Their CSCC tissues along with the corresponding adjacent tissues (at least 5 cm away from the tumor lesion) were collected. After collection, the samples were frozen in liquid nitrogen and stored in -80°C for subsequent use. A summary of the demographic and baseline characteristics of patients is in Table 1.

Microarray Platforms

A GeneChip miRNA 3.0 Array (Thermo Fisher Scientific Inc., Waltham, MA, USA) was applied to analyze the differentially expressed miRNAs between CSCC and adjacent tissues collected from Affiliated Hospital of Beihua University. Tissue samples from 6 patients were selected for analysis, including 3 males and 3 females. RNA of the patient tissues was extracted and added to the GeneChip™ miRNA 4.0 Array Plate (Thermo Fisher), and the miRNA was labeled using a FlashTag Biotin HSR RNA Labeling Kit (Thermo Fisher) and hybridized with the array. miRNA expression data was obtained from GeneChip Scanner 3000 7G (Thermo Fisher) at 570 wavelengths. The data were loaded onto Partek Genomic Suite 6.6 software (Partek Incorporated, St. Louis, MI, USA) for statistical filtering, and miRNA arrays were standardized using RMA algorithms. The significant criteria for significantly downregulated miRNAs in CSCC tissues were $|\text{Log}_2\text{Foldchange}| > 1.5$ and p values < 0.05.

Reverse Transcription-Quantitative PCR (RT-qPCR)

After isolation from CSCC cells or clinical tissues with Trizol reagent (Beijing Solabio Life Sciences Co., Ltd., Beijing, China) as per the manufacturer's instructions, the RNA was quantified by Nanodrop2000 (Thermo Fisher). The fragment

Table 2. Primer Sequences for Reverse Transcription-Quantitative PCR.

Targets	Sequence (5'-3')
miR-214	Forward: 5'-TGGCAACAACCGATGAACTGGA-3'
	Reverse: 5'- CCCGAAGTAGAGGCGGAAAG-3'
VEGFA	Forward: 5'-ATGTACCATAGCTACGCTGTCC-3'
	Reverse: 5'- ATAGAGTGGGTGGGACACATAG-3'
Bcl-2	Forward: 5'-GGTGGGGTCATGTGTGTGG-3'
	Reverse: 5'-GGTGGGGTCATGTGTGTGG-3'
U6	Forward: 5'-TCGCTTCGGCAGCACATATAC-3'
	Reverse: 5'-TATGGAACGCTTCACGAATTTG-3'
GAPDH	Forward: 5'-GTCAACGGATTTGGTCGTATTG-3'
	Reverse: 5'- CATGGGTGGAATCATATTGGAA-3'

Note: miR-214, microRNA-214; VEGFA, vascular endothelial growth factor A; Bcl-2, B-cell CLL/lymphoma 2; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

size of the RNA was examined by gel electrophoresis, and quality control (QC) was conducted using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA), where the minimum RNA integrity number value was 7.5, above which the RNA was considered to have passed the QC test. The reverse transcription of mRNA and qPCR were carried out using MMLV First-Strand Kits (Invitrogen Inc., Carlsbad, CA, USA), Oligo (dT) 20 primer (Shanghai Sangon Biological Engineering Technology & Services Co., Ltd., Shanghai, China) and TaqMan Universal Master Mix II (Ambion, Austin, TX, USA). Stratagene Mx3005P real-time fluorescence quantitative PCR instrument (Agilent Technologies) was applied for qPCR reaction. U6 served as the internal reference for miR-214, while glyceraldehyde-3-phosphate dehydrogenase (GAPDH) for VEGFA and Bcl-2 by $2^{-\Delta\Delta Ct}$ method. Primers are exhibited in Table 2.

Cell Culture and Transfection

Human immortal keratinocyte line HaCaT and CSCC cells, including A431, SCC13, HSC-5, SCL-I cells as well as 293T cells were from American Type Culture Collection (Manassas, VA, USA) and grown in Dulbecco's modified Eagle's medium (DMEM; Thermo Fisher) supplemented with 10% fetal bovine serum (FBS, Gibco, Carlsbad, CA, USA) and 1% penicillin/streptomycin (Sigma-Aldrich Chemical Company, St Louis, MO, USA). The cells were kept in a culture environment with 5% CO_2 at 37°C. miR-214 mimic, VEGFA overexpression (OE) and Bcl-2 OE were synthesized by GenePharma Co., Ltd. (Shanghai, China). All fragments were loaded into pCMV-MC plasmids (Biovector Science Lab, Beijing, China) for the following transfection procedure which was carried out using a Cell Line Nucleofector Kit V (Amaxa Biosystems, Koln, Germany). The transfection efficacy was assessed by RT-qPCR.

Cell Proliferation Assays

Cell proliferation was detected by cell counting kit-8 (CCK-8) method. The cells were seeded at 1×10^4 cells/well on a 96-well microporous plate and incubated at 37°C with 5% CO_2 for 0, 24, 48, 72 or 96 h. The medium was renewed with 100 μL growth medium and 10 μL CCK-8 solution (MedChemExpress, Monmouth Junction, NJ, USA) for another 3-h incubation at 37°C. The optical density (OD) value at 450 nm was measured by a spectrometer reader (SpectraMax M2, Molecular Devices, San Jose, CA, USA).

The effect of miR-214, VEGFA or Bcl-2 overexpression on cell proliferation was also determined by Click-iT Plus 5-Ethynyl-2'-deoxyuridine (EdU) Alexa Fluor 1594 Imaging kits (Thermo Fisher). Transfected A431 and SCC13 cells were immobilized with 50 μL cold 4% formaldehyde for 30 min at room temperature. 4',6-diamidino-2-phenylindole (1:2000) was used to stain the nucleus at room temperature for 30 min, and the signals were observed using Olympus FLUOVIEW FV1000 confocal laser-scanning microscope (×100 magnification, Olympus Corporation, Tokyo, Japan).

Cell Apoptosis Assay

The cells were detached with 0.25% trypsin and centrifuged at 111.8 ×g at 4°C for 5 min. Afterwards, the cells were resuspended in 100 μL 1× binding buffer (MultiScience (LIANKE) Biotech, Co., Ltd., Hangzhou, Zhejiang, China). The apoptosis rate was then assessed using a BD Accuri C6Plus flow cytometry (BD Biosciences, San Jose, CA, USA) equipped with a CellQuest software (version 6.1, BD Biosciences) after adding the staining solution in the dark for 15 min at room temperature as per the instructions of Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) apoptosis detection kits (Bestbio, Shanghai, China).

Western Blot

Protease inhibitor cocktail (Roche Applied Science, Mannheim, Germany) containing 50 mM Tris-HCl, 150 mM NaCl, 1% NP-40 and 0.1% sodium dodecyl sulfate (SDS) was added to the radioimmunoprecipitation assay buffer (pH = 8.0, MedChemExpress). The protein was extracted by the addition of mixed solution to the cells. The bicinchoninic acid protein quantification kit (Sigma-Aldrich Chemical Company) was applied for protein quantification. Proteins were then subjected to 10% SDS-polyacrylamide gel electrophoresis and transferred to a 0.45 μm polyvinylidene fluoride membrane (Millipore Corporation, Billerica, MA, USA). The membrane was sealed for 60 min with 5% bovine serum albumin and probed overnight with primary antibodies to Cyclin D1 (sc-8396, Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), β-catenin (ab22656, Abcam, Cambridge, UK) and β-actin (sc-81178, Santa Cruz Biotechnology) at 4°C. The secondary goat anti-mouse antibody (ab205719, Abcam) was used for a 1-h incubation at 25°C. The immune response was detected using Super Signal West Femto Maximum Sensitivity Substrate Kit (Thermo Fisher) and C-DiGit Blot Scanner (Gene Company, HK, China) was used for image acquisition.

Cell Migration and Invasion Assays

The CSCC cells were detached with 0.25% trypsin at 24 h post-transfection and then washed with D-Hanks solution. For invasion assay, the diluted Matrigel (50 mg/L, 1:8, BD Biosciences) was used to submerge the apical chamber of a Transwell culture chamber. Cell suspensions (100 μL) were added to the apical chamber coated with Matrigel, and 500 μL DMEM containing 2.5% FBS was added to the basolateral chamber. In the migration assay, the apical chamber was free of Matrigel, while the basolateral chamber was supplemented with 5% FBS. After 48 h, the chamber was stained using 0.5% crystal violet for 20 min and photographed under a microscope (Zeiss, Oberkochen, Germany).

Analysis of Targeted Genes

StarBase (http://starbase.sysu.EdU.cn/), Targetscan (http://www.targetscan.org/), miRBase (http://www.mirbase.org) and miRWalk (http://mirwalk.umm.uni-heidelberg.de/) were utilized to predict the binding sites between VEGFA and Bcl-2 with miR-214. Wild type (WT) plasmids with predicted miR-214 target binding sequences and mutant (MT) plasmids were synthesized and cloned into psi-CHECK2 vectors (Promega Corporation, Madison, WI, USA). The 293T cells were seeded into 96-well plates at 1×10^4 cells/well. The 293T cells were transfected by Cell Line Nucleofector Kit V (Amaxa) with WT or MT recombinant plasmids in the presence of miR-214 mimic or control after incubation overnight at 37°C. Luciferase activity was measured using a Dual-Luciferase Reporter assay system (Promega) 48 h post-transfection. Correction for differences in transfection efficiency was conducted by normalizing firefly luciferase activity to Renilla luciferase.

Statistical Analysis

Statistical analyses were performed using the SPSS 26.0 software (IBM, Armonk, NY, USA). Measurement data are exhibited as mean ± standard deviation (SD). Differences between 2 groups were compared by unpaired *t* test. Multiple groups were compared by 1-way or 2-way analysis of variance (ANOVA), followed by Tukey's post hoc test. All statistical tests were considered to reflect significant differences if $p < 0.05$.

Results

The Expression of miR-214 in CSCC Is Remarkably Reduced

We first analyzed the clinical data and randomly selected 6 CSCC patients for microarray analysis and plotted a heatmap, in which the colors from blue to white to red represent the gene expression from high to low, respectively. During the analysis, we found 26 upregulated and 32 downregulated miRNAs. miR-214 was the most significant downregulated miRNA in CSCC (Figure 1A). So, we analyzed the expression of miR-214 in cancer and adjacent tissues of all patients, and observed that although there was a slight difference in the miR-214 expression among different patients, the overall expression of miR-214 in CSCC tissues was much lower than that in adjacent tissues. We divided the patients into high miR-214 expression ($n = 10$) and low miR-214 expression groups ($n = 12$) according to the median value (0.11) of the miR-214 expression (Figure 1B). The survival rate of patients with high miR-214 expression was higher than that those with low miR-214 expression (Figure 1C). To test the effects of miR-214, we purchased HaCaT and several CSCC cell lines, involving A431, SCC13, HSC-5 and SCL-I. The expression of miR-214 in each cell line was detected, and the miR-214 expression in the CSCC cells was much lower than that in HaCaT cells, and the down-regulation was most significant in A431 and SCC13 cells (Figure 1D). Therefore, we used these 2 cell lines for following studies (Figure 1E).

miR-214 Inhibits Malignant Aggressiveness in CSCC Cells

We first examined the proliferation ability of cells overexpressing miR-214. Through CCK8 experiments, we found that the increase of miR-214 lowered the OD value of CSCC cells at 450 nm, and the increase rate of OD also slowed down significantly (Figure 2A). Through EdU experiments, we observed that after 24 h of culture, EdU positive cells were decreased significantly (Figure 2B). We then detected the ability of apoptosis. Flow cytometry showed that the number of PI-Annexin V dual positive cells was increased significantly (Figure 2C), which showed that miR-214 reduced the ability of CSCC cells proliferation and enhanced the ability of apoptosis. We further examined cell migratory and invasive abilities. Through Transwell experiments, it was found that after 24 h of culture, the cells that migrated and invaded into the basolateral chamber were significantly reduced (Figure 2D and E). Thus, we believed that miR-214 inhibited CSCC cells activity.

miR-214 Interacts With VEGFA and Bcl-2 in CSCC Cells

To explore the possible targets of miR-214 in CSCC cells, StarBase, miRWalk, miRBase and Targetscan websites were utilized and their prediction results were intersected. A total of 13 genes were screened out (Figure 3A). To screen out genes that play a role in CSCC cells, we examined the difference in the expression of each gene in cancer and adjacent tissues of CSCC patients. It was displayed that the expression of VEGFA and Bcl-2 in CSCC tissues was increased significantly relative to the adjacent tissues (Figure 3B). Moreover, both genes were negatively correlated with miR-214 expression (Figure 3C and D). The target relationships were detected by dual-luciferase report assays, and VEGFA and Bcl-2 both bound to miR-214 (Figure 3E). To detect changes in VEGFA and Bcl-2 expression between A431 and SCC13 cells and HaCaT cells, RT-qPCR was conducted. Significant increases in VEGFA and Bcl-2 expression was observed in CSCC cells (Figure 3F). Patients were divided into high VEGFA/Bcl-2 expression and low VEGFA/Bcl-2 expression groups according to median

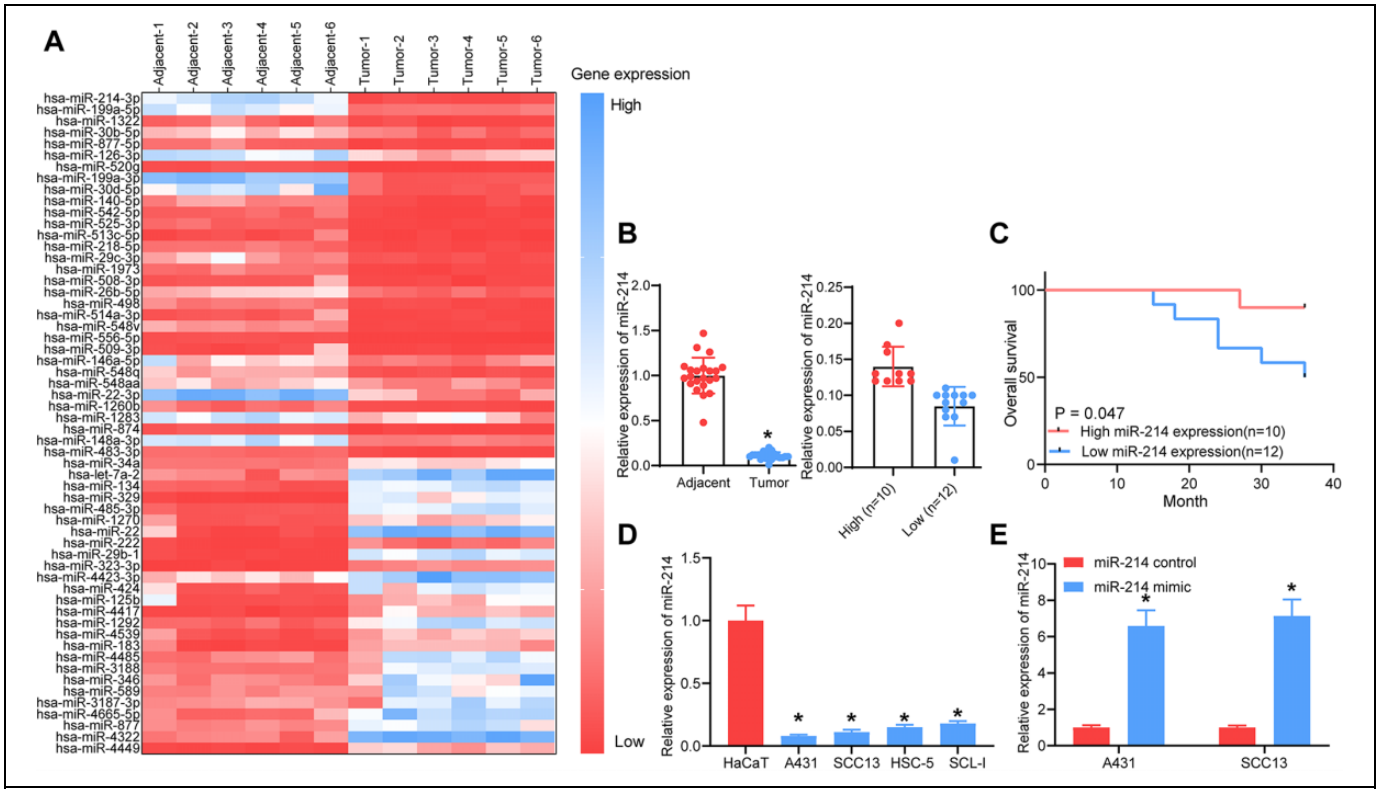

Figure 1. miR-214 is screened out to be significantly reduced in CSCC cells. A, detection of differentially expressed miRNAs by microarray analysis; B, RT-qPCR detection of miR-214 expression in cancer and adjacent tissues (*$p < 0.05$ according to the 2-way ANOVA); C, survival analysis of CSCC patients with different miR-214 expression; D, RT-qPCR detection of miR-214 expression in cancer and HaCaT cells (*$p < 0.05$ according to the 1-way ANOVA); E, RT-qPCR detection of miR-214 expression in CSCC cells overexpressing miR-214 (*$p < 0.05$ according to the 2-way ANOVA).

values of their expression in CSCC patients (VEGFA = 3.65, Bcl-2 = 4.16). The survival of patients with low VEGFA/Bcl2 expression was higher (Figure 3G and H), which made us wonder whether miR-214 functions in CSCC cells by targeting these 2 genes. Consequently, we detected changes in VEGFA and Bcl-2 expression in cells overexpressing miR-214 and found that miR-214 reduced VEGFA and Bcl-2 expression (Figure 3I). Therefore, we believed that in CSCC cells, miR-214 exerts its tumor suppressor role by binding to VEGFA and Bcl-2.

VEGFA or Bcl-2 Reveres the Inhibition of miR-214 in CSCC Cells

To determine whether both VEGFA and Bcl-2 are miR-214 targeting genes in CSCC cells, we simultaneously upregulated miR-214 and VEGFA/Bcl-2. Upon confirmation of successful co-transfection (Figure 4A), we first observed, through CCK-8 assays, that the OD value of cells harboring enhanced miR-214 and VEGFA/Bcl-2 was increased significantly relative to cells overexpressing miR-214 alone (Figure 4B), while the number of EdU positive cells was also elevated (Figure 4C). Similarly, by flow cytometry, we found that the number of cell apoptosis decreased after transfection of VEGFA/Bcl-2 and miR-214 overexpression (Figure 4D). The migration and invasion abilities of cells were subsequently assessed, the cells in the basolateral chamber were photographed and counted, and the number of migratory and invasive cells was enhanced (Figure 4E and F). These series of experiments identified the targeting relationships between miR-214 and VEGFA and Bcl-2, and also identified the effect of VEGFA and Bcl-2 on miR-214 roles during the events of cell viability, apoptosis, migration and invasion.

Wnt/β-Catenin Pathway Is Involved in miR-214-Mediated CSCC Activities

We detected aberrant changes in β-catenin and Cyclin D1 protein expression in miR-214 overexpressed CSCC cells, which made us wonder whether the Wnt/β-catenin pathway influences the process. As a result, we examined the β-catenin and Cyclin D1 protein expression in miR-214-overexpressed cells and found that the pathway activity was decreased following miR-214 overexpression. Meanwhile, miR-214 mimic + VEGFA/Bcl-2 OE significantly increased the pathway activity in CSCC cells compared with over-expression miR-214 mimic alone, which also provides forceful evidence for miR-214 targeting VEGFA and Bcl-2 in CSCC cells (Figure 5).

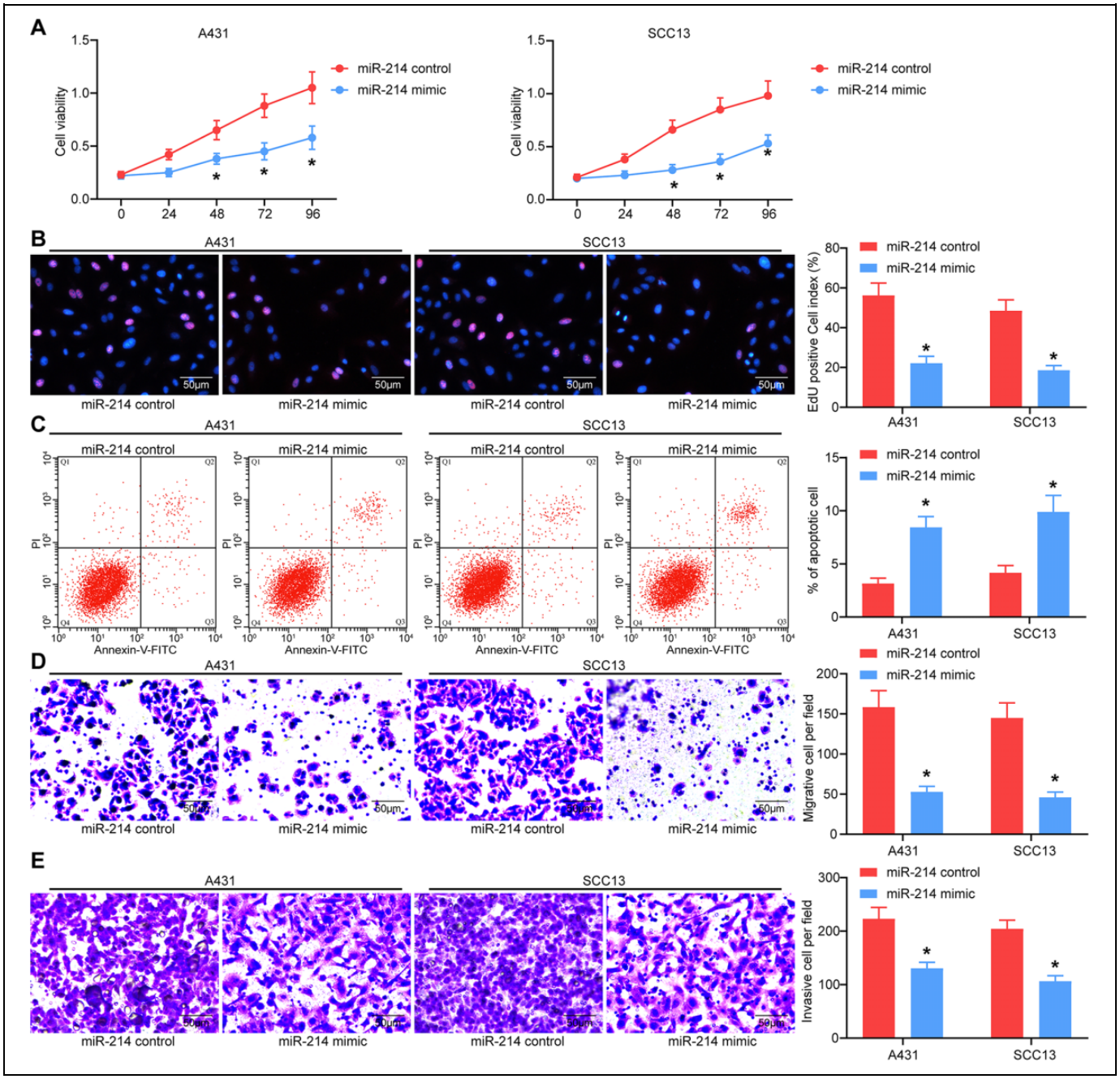

Figure 2. miR-214 overexpression retards the growth, migration and invasion capacities of CSCC cells. CSCC cells were transfected with miR-214 mimic or control. A, CSCC cell viability evaluated by CCK-8 assay (*$p < 0.05$ according to the 2-way ANOVA); B, EdU detection of CSCC cell proliferative activity (*$p < 0.05$ according to the 2-way ANOVA); C, changes of apoptosis ability of CSCC cells detected by flow cytometry (*$p < 0.05$ according to the 2-way ANOVA); D, CSCC cell migration evaluated by Transwell assay (*$p < 0.05$ according to the 2-way ANOVA); E, CSCC cell invasion evaluated by Transwell assay (*$p < 0.05$ according to the 2-way ANOVA).

Discussion

miRNAs have been increasingly recognized as important players in the molecular pathogenesis of CSCC.[14] In the current study, miR-214 expression was reduced in A431 and SCC13 cell lines, while the restoration of miR-214 suppressed the proliferation, migration and invasion, yet enhanced the apoptosis of A431 and SCC13 cells. Furthermore, we also discovered that VEGFA and Bcl-2 were putative targets of miR-214, which could be diminished by the upregulation of miR-214 and therefore disrupted the Wnt/β-catenin pathway in A431 and SCC13 cells. Altogether, our studies imply that miR-214 targeted the VEGFA/Bcl-2-Wnt/β-catenin axis to compromise cell proliferation, migration and invasion capacities in CSCC.

Our findings demonstrated that miR-214 was decreased in CSCC tissues relative to the matched adjacent tissues. In addition, a positive correlation was witnessed between the miR-214

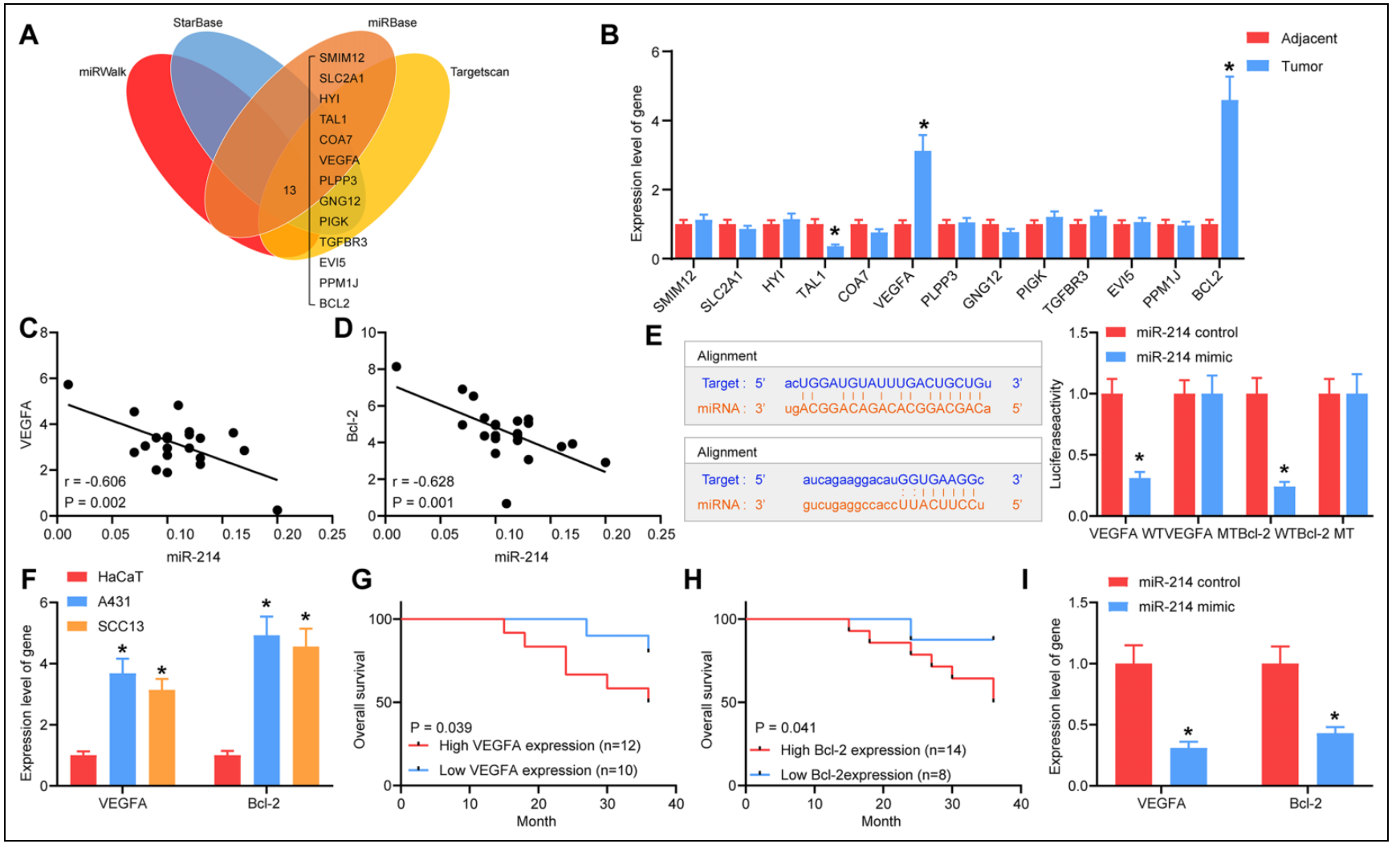

Figure 3. miR-214 directly targets VEGFA and Bcl-2. A, genes targeted by miR-214 screened out by StarBase, miRWalk, miRBase and Targetscan websites; B, RT-qPCR detection of expression of each gene in cancer tissues and adjacent tissues (*$p < 0.05$ according to the 2-way ANOVA); C, correlation analysis of miR-214 and VEGFA; D, correlation analysis of miR-214 and Bcl-2; E, targeting relationship between miR-214 and VEGFA/Bcl-2 (*$p < 0.05$ according to the 2-way ANOVA); F, RT-qPCR detection of VEGFA and Bcl-2 expression in CSCC cells and normal cells (*$p < 0.05$ according to the 2-way ANOVA); G, survival analysis of CSCC patients with different VEGFA expression; H, survival analysis of CSCC patients with different Bcl-2 expression; I, RT-qPCR detection of expression of VEGFA and Bcl-2 after overexpression of miR-214 (*$p < 0.05$ according to the 2-way ANOVA).

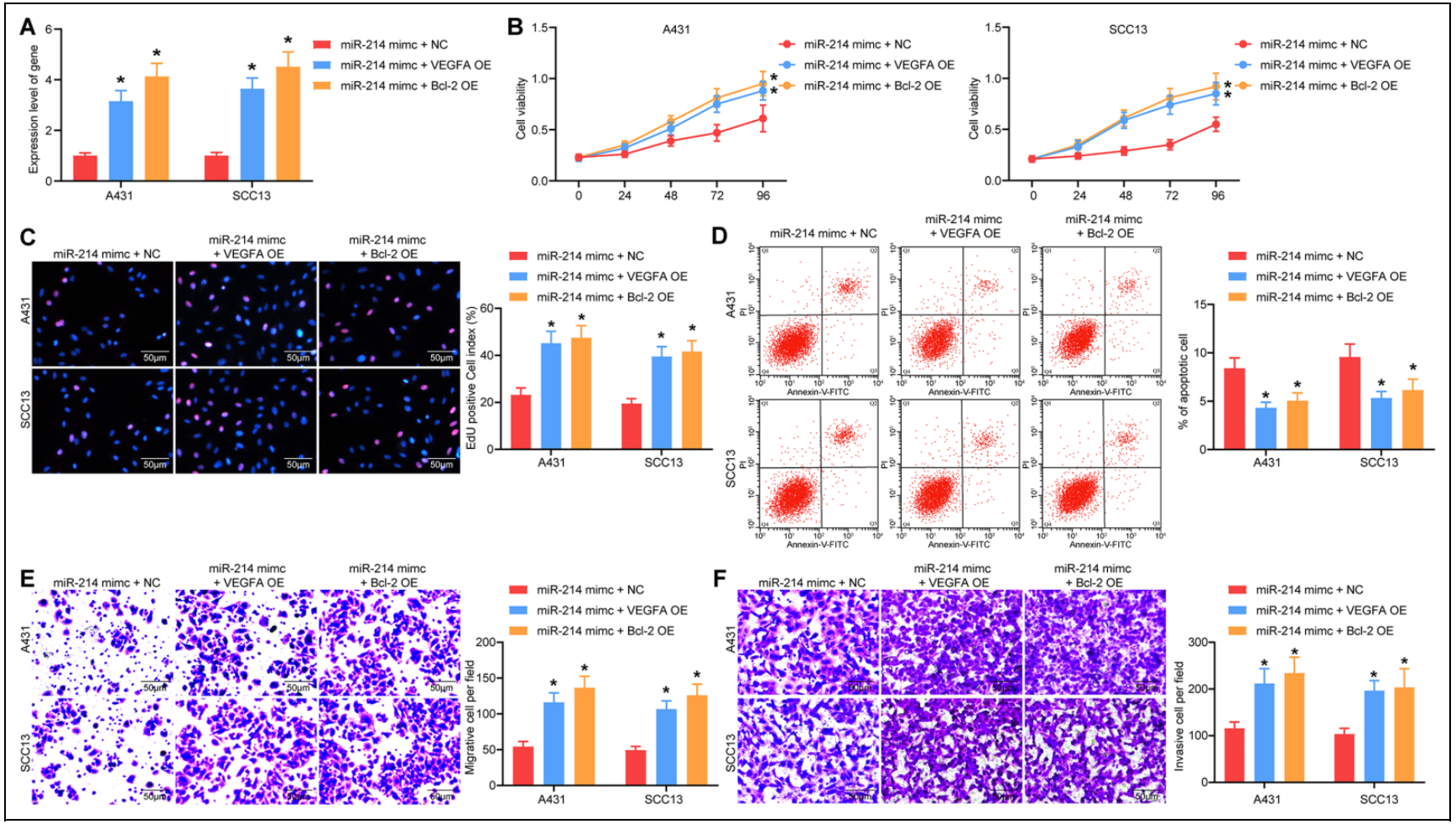

Figure 4. Overexpression of VEGFA or Bcl-2 restores viability of CSCC cells. CSCC cells were transfected with VEGFA OE, Bcl-2 OE or NC in the presence of miR-214 mimic. A, RT-qPCR detection of expression of VEGFA and Bcl-2 after co-transfection (*$p < 0.05$ according to the 2-way ANOVA); B, CSCC cell viability evaluated by CCK-8 assay (*$p < 0.05$ according to the 2-way ANOVA); C, EdU detection of CSCC cell proliferative activity (*$p < 0.05$ according to the 2-way ANOVA); D, changes of apoptosis ability of CSCC cells detected by flow cytometry (*$p < 0.05$ according to the 2-way ANOVA); E, CSCC cell migration evaluated by Transwell assay (*$p < 0.05$ according to the 2-way ANOVA); F, CSCC cell invasion evaluated by Transwell assay (*$p < 0.05$ according to the 2-way ANOVA).

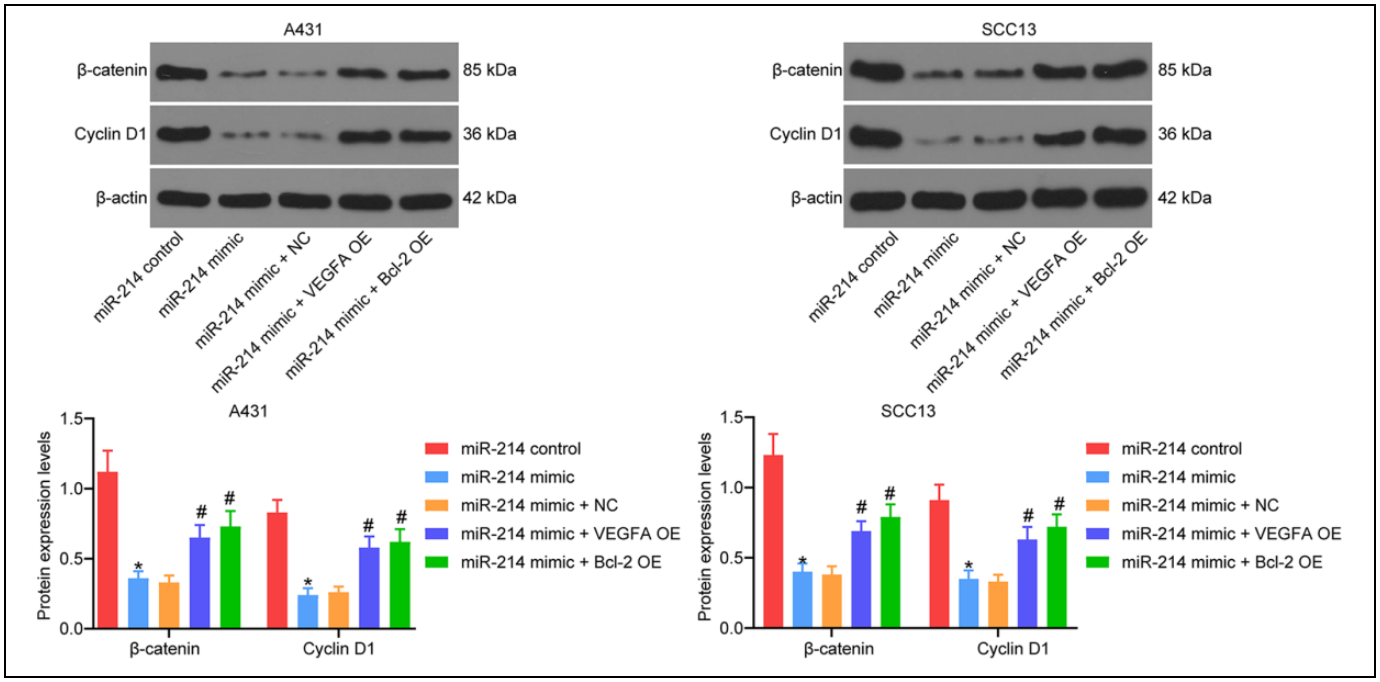

Figure 5. miR-214 impairs the Wnt/βcatenin pathway by interacting with VEGFA/Bcl-2. The protein expression of βcatenin and Cyclin D1 in CSCCs transfected with miR-214 alone or with VEGFA OE/Bcl-2 OE assessed by western blot (*$p < 0.05$ *vs.* miR-214 control treatment, #$p < 0.05$ *vs.* miR-214 mimic + NC treatment according to the 2-way ANOVA).

expression pattern and the overall survival of CSCC patients. In line with our data, the overall survival of patients with multiple myeloma harboring high expression of miR-214 was significantly improved.[15] Yamane et al established that expression of miR-214 was remarkably reduced in CSCC *in vitro* and *in vivo*.[16] For the identification of the functional relevance of miR-214 in the mediation of the properties of CSCC cells, we elevated the miR-214 expression in A431 and SCC13 cells and observed that the proliferation, invasion and migration rates were depressed, while the apoptosis was expedited. Consistently, miR-214-5p enhancement resulted in declines in the osteosarcoma cell proliferation, migration and invasion, and miR-214-5p inhibition contributed to the opposite trends.[17] Meanwhile, miR-214-3p was notably decreased in colon cancer, and upregulation of miR-214-3p repressed the proliferation and migration of colon cancer cells *in vivo* and *in vitro*.[18] Considering the aforementioned findings, we speculated that the downregulated miR-214 might be a cause encouraging the development of CSCC.

For exploring the mechanism through which miR-214 affects the properties of CSCC cells, we identified that VEGFA and Bcl-2 are 2 targets of miR-214 and could predict dismal survival of patients with CSCC. The Cancer Genome Atlas database disclosed that upregulation of VEGFA was related with a significant decline in overall survival for patients with glioblastoma.[19] Further, we discovered that the OD values, number of migration and invasion rates were elevated in A431 and SCC13 cells harboring overexpressing miR-214 and VEGFA/Bcl-2 compared to those cells overexpressing miR-214 only. In non-small cell lung cancer, VEGFA silencing reversed the stimulative role of circ0021205 in cell proliferative, migratory, and invasive abilities.[20] Furthermore, VEGFA was identified as a putative target of miR-205 in ovarian cancer and colorectal cancer, whereas VEGFA silencing markedly diminished colorectal cancer cell viability, migration and invasion.[21,22] Most importantly, miR-652-5p targeted VEGFA to suppress esophageal squamous cell carcinoma cell proliferation and metastasis.[23] On the other hand, miR-365 was found to compromised the migration and invasion, whereas enhanced apoptosis in melanoma cell lines, which was at least partially due to downregulation of the oncogene Bcl-2.[24] miR-1290 accelerated asiatic acid-induced apoptosis through decreasing the protein expression of Bcl-2.[25]

This study specified that miR-214 could negatively target VEGFA and Bcl-2 and the subsequent activation of the Wnt/βcatenin pathway in CSCC cells. The significance of the Wnt/β-catenin signaling in CSCC initiation and progression has been underscored,[26] and knockdown of β-catenin led to a marked decline in the colony-forming activity of SCC12 cells.[27] circ_CHFR knockdown was observed to impede the β-catenin expression via promoting miR-214-3p.[28] However, the association between miR-214 and the Wnt/β-catenin pathway in CSCC cells remains an enigma. Our results showed that miR-214 mimic remarkably reduced the protein expression of β-catenin and Cyclin D1, while the addition of VEGFA and Bcl-2 overexpression restored the activity of the Wnt/βcatenin pathway. Mechanistically, a previously report proposed that VEGFA activates VEGFR2 and generates the VEGFA/VEGFR2/VE-cadherin/β-catenin/actin axis, thus involving in the tumor microenvironment.[29]

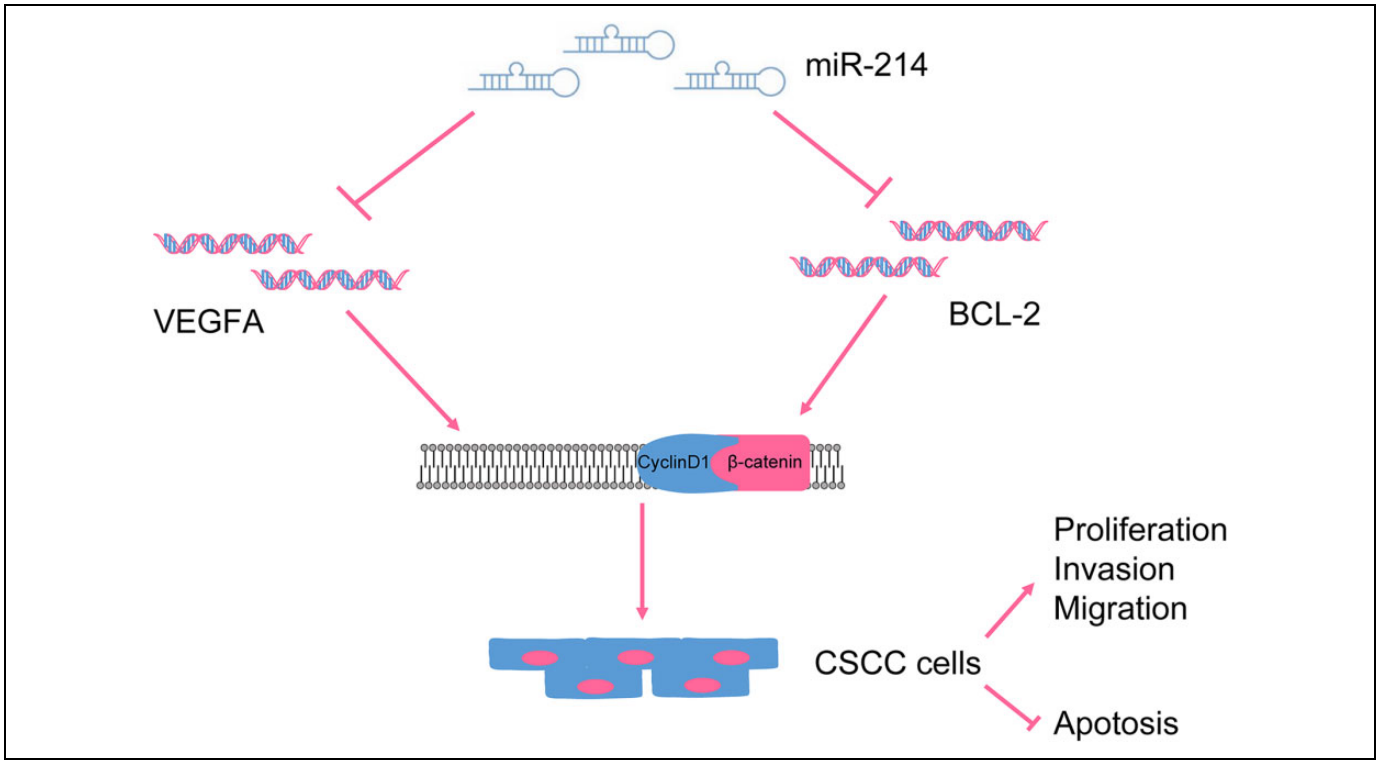

Figure 6. A schematic representation of the miR-214-VEGFA/Bcl-2-Wnt/βcatenin cascade in CSCC cells. miR-214 bound to VEGFA and Bcl-2 in a direct manner and inhibited their transcription, and VEGFA and Bcl-2 affect the Wnt/β-catenin pathway activation, thus influencing proliferation and apoptosis abilities.

Conclusion

In conclusion, our study provided robust evidence that miR-214 blunted the Wnt/β-catenin pathway by inhibiting VEGFA and Bcl-2, contributing to impeded CSCC progression (Figure 6), which offers a potent therapeutic target for CSCC. A potential pitfall of this work may be the limited sample size for the microarray. Due to funding constraints, we were only able to perform microarray analysis on 6 pairs of samples. We will pay more attention to this in subsequent studies and continue to enrich and validate our microarray results. Moreover, additional studies validating the therapeutic potential of this newly identified target *in vivo* will have significant clinical implications.

Authors' Note

Xianpeng Ma and Di Wu contributed equally to this work. The collection of specimens for this study was explicitly approved by the Institutional Review Board of Affiliated Hospital of Beihua University (No. 2013c0110) and in accordance with the *Declaration of Helsinki*. Written informed consent was acquired from each participant.

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Declaration of Conflicting Interests

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