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Over-expression of mir-124 inhibits MMP-9 expression and decreases invasion of renal cell carcinoma cells

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Abstract. – OBJECTIVE: JAK-STAT3 signaling pathway is related to tumor invasion and metastasis that can regulate matrix metalloproteninase-9 (MMP-9) expression. MicroRNA-124 (MiR-124) was found downregulated in renal cell carcinoma. Bioinformatics analysis revealed the complementary binding site between miR-124 and 3'-UTR of signal transducers and activators of transcription 3 (STAT3). This study investigated the role of miR-124 in regulating Janus kinase (JAK)-STAT3 activity, MMP-9 expression, and renal cell carcinoma invasion.

MATERIALS AND METHODS: Lucifer say was used to test the targeting regul fect of miR-124 on STAT3. MiR-124, STAT3, s- phorylated STAT3 (p-STAT3), and MMP- pressions were compared in HK-2, 769-P, OS-RC-2 cells. Transwell assay was applied evaluate cell invasion. OS-RC-2 ere divid ed into control, miR-NC, miR STAT3 inhibition, and miR-124 mim Statti ups.

RESULTS: MiR-124 tar d inhib STAT3 expression. OS-RC-2 c hibite gest invasive ability, f low P and 2 cells. STAT3, p-ST and expressions were highest in OS 2 cells, f by 769-P and HK-2 cells. M 4 demonstra oppo- site expression -124 mimic a or Stat- tic treatment uat invasion through reducing STAT3, p-STAT3, MP-9 levels.

CONCL ONS: MiR-124 regulation was associa with renal cancer OS-RC-2 invasion e iceme Over-expression of miR-124 atte ed OS 2 cell invasion by down-regulat AT3 MMP-9

Key Wo
-124, M , Renal cell carcinoma, In-

Introduction

enal cell carcinoma (RCC) is a common type o gnancy that is the major type of primary t r in kidney[1]. Its morbidity accounts for the secon y system on after bladder cancer[2]. ause satisfactory effect of radiotherapy and chem py, surgical resection is t treatment f for RCC, in spite o igh postoperative recurrence rate at 20-6[3]. In additi RCC is featured as rapid gress, invasi and metastasis, resulting in therapeutic ect and prognosis. Janus kinase (JAK)-sig transducer and activator of trans AT) signaling pathway widely exists in various tissues and cells. It participates in the regulation of cell survival, apoptosis[4], migration and invasion[6]. Signal transducers and activators of transcription 3 (STAT3), the most important one in STAT transcription factor family, is thought to be a proto-oncogene[7]. Matrix metalloproteninases-9 (MMP-9) is a member of MMP family with largest molecular weight. It over-expression plays a critical role in promoting tumorigenesis, progress, and metastasis[8,9]. Several studies reported that Janus kinase (JAK)-STAT3 signaling pathway plays an important role in regulating MMP-9, and mediating multiple tumor invasion and metastasis, such as nasopharyngeal carcinoma[10] and ovary cancer[11]. However, its role in RCC invasion and metastasis has not been clarified. MiRNA is a type of endogenous single-stranded non-coding RNA at the length of 22-25 nt. It plays a degrading or inhibiting role on mRNA by binding with the 3'-UTR. Abnormal microRNA (miRNA) expression and function is closely related to the pathogenesis of RCC[12,13]. It was revealed that miR-124 significantly declined in RCC tissue, suggesting its potential suppression role in RCC[14,15]. Bioinformatics analysis revealed the complementary binding site between miR-124 and 3'-UTR of STAT3. This study investigated the role of miR-124 in regulating JAK-STAT3 activity, MMP-9 expression, and renal cell carcinoma invasion.

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Materials and Methods

Main Reagents and Materials

Normal human proximal convoluted tubule epithelial cell HK-2, low invasive RCC cell 769-P, and high invasive RCC cell OS-RC-2 were bought from Mengwei Biotechnology Ltd, Co. (Shanghai, China). Keratinocyte Serum Free Medium and 1640 medium were purchased from Gibco BRL. Co. Ltd. (Grand Island, NY, USA). Fetal bovine serum (FBS), penicillin, and streptomycin were purchased from Lonza Inc. (Allendale, NJ, USA). RNA extraction kit Rneasy MiNi Kit and Effectene Transfection Reagent were obtained from Qiagen (Hilden, Germany). PrimeScript™ RT reagent Kit and SYBR Green were purchased from TaKaRa (Dalian, China). miR-NC, miR-124 mimic, and miR-124 inhibitor were bought from Ribobio (Tokyo, Japan). Mouse anti human STAT3 and p-STAT3 primary antibodies were purchased from Abcam Biothechnology (Cambridge, MA, USA). Rabbit anti human MMP-9 primary and horseradish peroxidase (HRP) labeled secondary antibodies were obtained from Cell Signaling Tech[illegible] Inc. (Beverly, MA, USA). si-NC and si[illegible] were purchased from Santa Cruz Biothec[illegible]gy (Santa Cruz, CA, USA). pGRE-luc luci[illegible] reporter gene plasmid was got from BioVec[illegible] Science Lab Inc. (Beijing, Chi[illegible]). Lucifera[illegible] activity detection kit was pur[illegible]m Beyotime Biotech. (Shanghai, C[illegible]). ST[illegible] inhibitor Stattic was bought f[illegible] Selleck[illegible]micals Inc. (Houston, TX, USA[illegible]rigel[illegible] from BD Bioscience[illegible] (Sa[illegible]CA, USA). Transwell chamber[illegible]s got f[illegible]orning Costar (Corning, CA[illegible]SA).

Cell Culture

HK-2 cells were main[illegible]d in Keratinocyte Serum F[illegible] medium, while [illegible] and OS-RC-2 cells w[illegible] cultured in 1640 me[illegible]um containing 10% [illegible] and [illegible] penicillin-streptomycin. The cel[illegible]e pa[illegible]ed at 1:[illegible]. The cells in logarithmic p[illegible] used f[illegible]periments. This study [illegible] appr[illegible]y the [illegible]ical Committee of Heze [illegible]ipal H[illegible]andong, China).

Du[illegible]uciferase Reporter Gene Assay

[illegible]ducts containing the full length [illegible]TAT3 gene 3'-UTR or mutant segment were [illegible]le digested and connected to pGRE-luc. [illegible] was transformed to DH5α competent cells[illegible]d sequenced to select the plasmid with correct sequence, namely pGRE-STAT3-wt and pGRE-STAT3-mut, respectively. Th[illegible] STAT3-wt (or pGRE-STAT3-mut) [illegible]co-tra[illegible] fected to HEK293T cells using [illegible]tene Transfection Reagent together with r[illegible]4 mimic (or miR-NC). The luciferase activit[illegible] detected after cultured for 48 h.

Cell Grouping

OS-RC-2 cells we[illegible]vided [illegible] control, miR-NC, miR-124 mimic, [illegible]T3 [illegible]bition, [illegible]miR-124 mimic + St[illegible] gr[illegible]e cells[illegible]e used for the followi[illegible]xperime[illegible]r 4[illegible].

Quantit[illegible] Time-PCR (RT-PCR)

Total[illegible]A wa[illegible]cted using Rneasy MiNi Kit and reverse trai[illegible]d to complementary DN[illegible]A) using PR[illegible]cript™ RT reagent K[illegible]. The reverse transcription condition was [illegible]C for 15 min[illegible]d 98°C for 5 min. The PCR [illegible]ction was c[illegible]osed of 95°C pre-denaturation for 5 min[illegible]owed by 40 cycles of 95°C d[illegible]uration f[illegible]s, 60°C annealing for 30 s, and [illegible]tion for 30 s. Real-time PCR was performed on CFX96 Touch™ to test the [illegible]lative expression. The primers used were [illegible]. miR-124P_F: 5'-CGGTAAGGCACG-[illegible]GCA-3', miR-124P_R: 5'-AGTGCGAACTGT-GGCGAT-3'; U6P_F: 5'-ATTGGAACGATACA-GAGAAGATT-3', U6P_R: 5'-GGAACGCTTCAC-GAATTTG-3'; STAT3P_F: 5'-ATCACGCCTTC-TACAGACTGC-3', STAT3P_R: 5'-CATCCTG-GAGATTCTCTACCACT-3'; MMP-9P_F: 5'-TG-TACCGCTATGGTTACACTCG-3', MMP-9P_R: 5'-GGCAGGGACAGTTGCTTCT-3'; β-actinP_F: 5'-GAACCCTAAGGCCAAC-3', β-actinP_R: 5'-TGTCACGCACGATTTCC-3'.

Western Blot

Total protein was extracted by radioimmunoprecipitation assay (RIPA) and centrifuged at 10000 r/min for 15 min. A total of 50 μg protein were separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to membrane. Next, the membrane was blocked and incubated in primary antibody at 4°C overnight (STAT3, p-STAT3, MMP-9, and β-actin at 1:400, 1:200, 1:300, and 1:800, respectively). Next, the membrane was incubated in secondary antibody (1:10000) for 60 min after washed by PBS Tween-20 (PBST) for three times. At last, the protein expression was detected by enhanced chemiluminescence (ECL).

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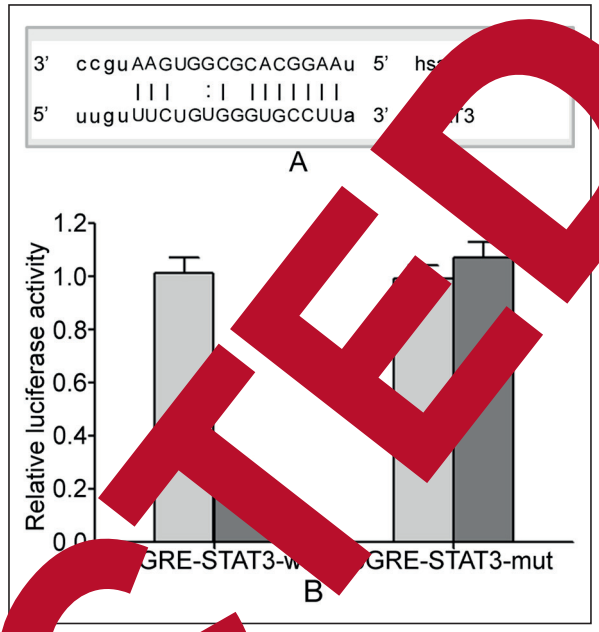
Transwell Assay

The Matrigel was unfrozen at 4°C and diluted by serum-free 1640 medium. It was paved on the upper chamber of transwell and put into the incubator. OS-RC-2 cells were re-suspended in serum-free medium and seed on the upper chamber, while 600 µl complete 1640 medium containing 10% FBS was put into the lower chamber. After cultured for 48 h, the Matrigel and unpenetrated cells were removed. At last, the membrane was fixed by 4% paraformaldehyde and stained by 0.1% crystal violet. The penetrated cell number was counted under the microscope within 5 random views.

Statistical Analysis

All data analyses were performed on SPSS 18.0 software (SPSS, Inc., Chicago, IL, USA). The measurement data were depicted as mean ± standard deviation (SD). The Student's *t*-test was used to compare the differences between two groups. The Tukey's post-hoc test was used to validate the ANOVA for comparing measurement data between groups. $p < 0.05$ was considered statistically significant.



ure 1. MiR-124 eted regulated STAT3 expression. he binding site een miR-124 the 3'-UTR of STAT3 *(B)* Dual l ase assay. *$p < 0.05$, compared with mi

Results

The Targeted Regulatory Relationship Between miR-124 and ST

microRNA.org online pr ion ved the targeted binding site bet miR- and 3'-UTR of STAT3 mRNA ure l ciferase assay reveale that mimic significantly declined ive luc activity in HEK293 cells (F 1B), indica e regulatory relations en miR-124 STAT3 mRNA.

MiR-124 wnregulation STAT3 Overe ession Were Asso iated With High vasive s of OS-RC-2 Cells

vell y dem trated that OS-RC-2 cells he str st invasive ability, followed by and -2 cells (Figure 2A, B). CR si STAT3 and MMP-9 mRNA ls were ificantly higher, while miR-124 ression was significantly lower in 769-P ce with HK-2 cells (Figure 2C). T3 and MMP-9 mRNA levels were markedly er, while miR-124 expression was apparently l OS-RC-2 cells compared with 769-P cells igure 2C). Western blot revealed that d MMP-9 protein expressions in 769-P ns e significantly higher than that in HK-2 cells but lower than in OS-RC-2 cells (Figure 2D).

MiR-124 Overexpression Attenuated OS-RC-2 Cell Invasion By Reducing STAT3 and MMP-9 Expressions

MiR-124 mimic transfection significantly downregulated STAT3, p-STAT3, and MMP-9 expressions in OS-RC-2 cells (Figure 3A, B), and attenuated cell invasion (Figure 2C), indicating that downregulated miR-124 promoted OS-RC-2 cell invasion by elevating STAT3 and MMP-9. STAT3 inhibitor Stattic intervention showed no statistical impact on STAT3 protein expression, but markedly restrained STAT3 phosphorylation, declined MMP-9 expression (Figure 3A, B), and weakened OS-RC-2 cell invasion (Figure 3C).

Discussion

JAK-STAT signaling pathway is found to play an important role in cell proliferation, differentiation, apoptosis, migration, invasion, and immunoregulation[16]. JAK and STAT are the major components of JAK-STAT signaling pathway[17]. Extracellular signal molecules bind with cell

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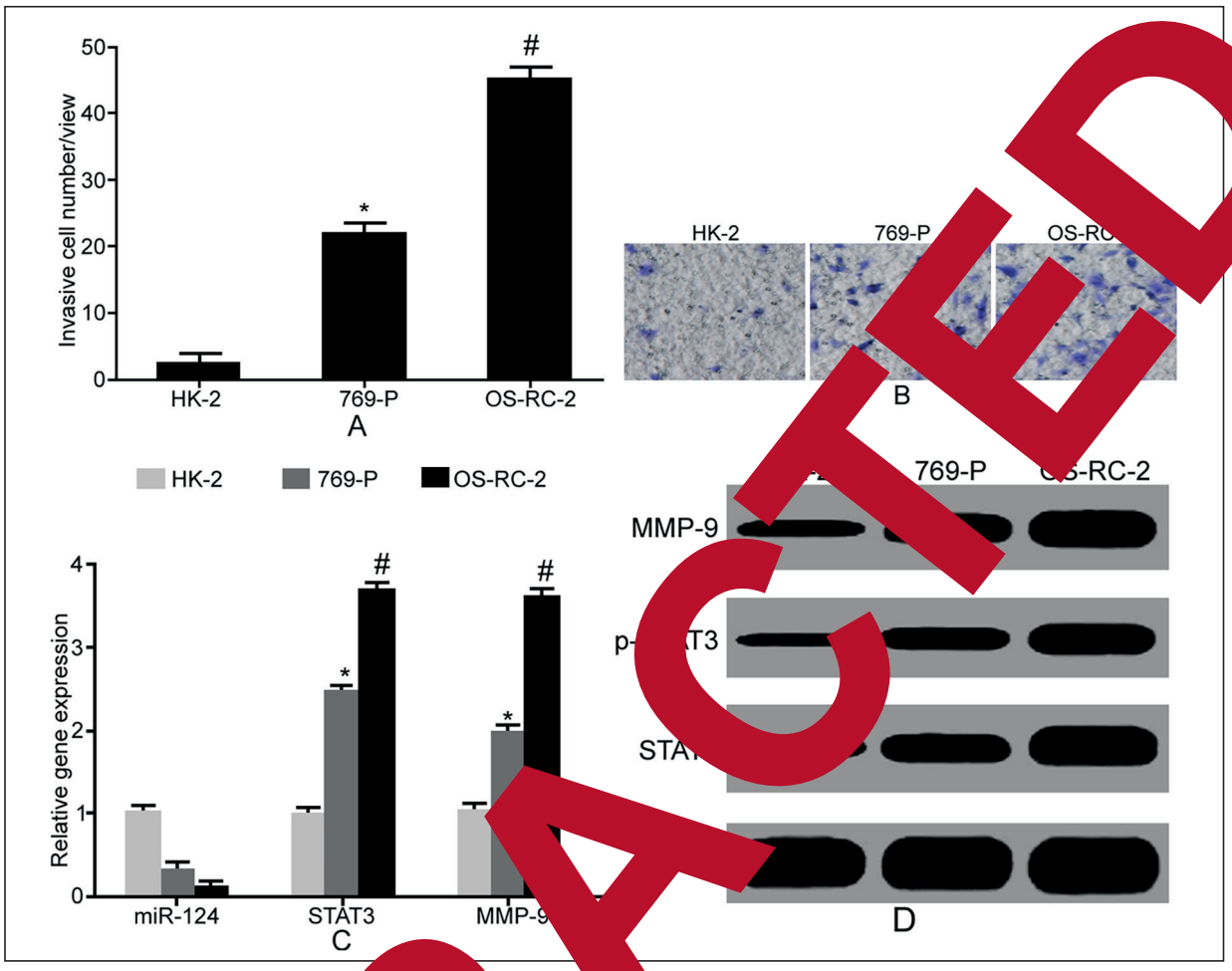

Figure 2. MiR-124 down-regulat[ed] and S[TAT3] over-expre[ssion] were associated with high invasiveness of OS-RC-2 cells. (A) Comparison of invasive cell [number]. ***(B)*** T[ranswell assay] d[etection] of cell invasive ability. ***(C)*** qRT-PCR detection of gene expression. ***(D)*** Western blot d[etection] of pr[oteins]. $^{*}p < 0.05$, compared with HK-2 cells. $^{\#}p < 0.05$, compared with 769-P cells.

membrane recept[ors] may cause recep[tor] dimerization, leading to [JAK] binding site combines with JH6 and [JH7] re[gion] binding domain on JAK and activates JAK [kinase]. Activated JAK phosphor[ylates] the tyrosine [residue] on receptor to form docking site with surrounding amino acid [sequence], recruiting STAT protein with SH2 dom[ain] to JAK, phosphorylates STAT with specific SH2 [domain] through its SH1 domain. Activated STAT pr[otein] enters the nucleus to bind with [DNA] in form, so as to regulate gene transcription and expression[16,18]. STAT3 is one of the most important members of STAT family, considered as a proto-oncogene[19,20]. STAT3 expression and activity is extremely low under physiological condition, while its abnormal [activation] and sustained activation may cause dysregulation of JAK-STAT3 signaling pathway, thus participating in multiple tumors occurrence, progress, migration, and invasion, such as intestinal cancer[21], lung cancer[22], gastric cancer[23], and breast cancer[24]. Tumor cells must penetrate basement membrane and extracellular matrix (ECM) to complete invasion and metastasis. ECM degradation is the initial factor and primary step in tumor cell invasion and metastasis. MMPs are a kind of protease family widely exists in connective tissue that can degrade various components in ECM, such as collagen, fibronectin, and gelatin. MMP-9 is a member of MMPs with the largest molecule weight. It mainly degrades collagen IV, which widely exists in numerous cells ECM. MMP-9 activation plays a crucial role in tumor occurrence, progress, and metastasis[8,9]. Multiple studies reported that JAK-STAT3 signaling pathway is involved in mediating nasopharyn-

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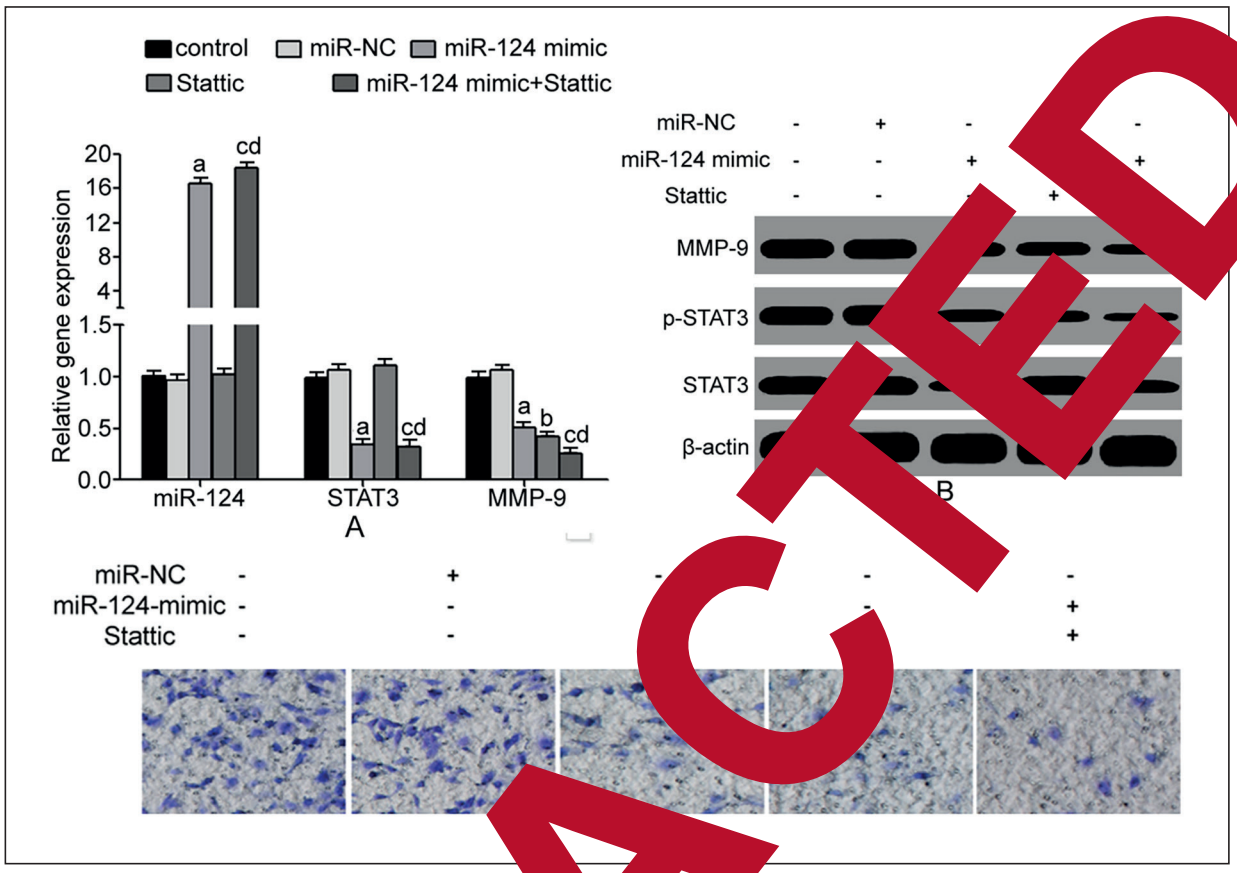

Figure 3. MiR-124 over-expression attenuated OS-[illegible] invasion by reducing STAT3 and MMP-9 expressions. ***(A)*** qRT-PCR detection of gene expression. (B) Western blot[illegible]n of protein expression. ***(C)*** Transwell assay detection of cell invasion. [a]$p < 0.05$, compared with mi[illegible] < 0.05, co[illegible]d with control. [c]$p < 0.05$, compared with miR-NC. [d]$p < 0.05$, compared with Stattic group.

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geal carcinoma[10] and [illegible]ary [illegible]vasion and metastasis. Howeve[illegible] regula[illegible]ole in RCC invasion and me[illegible]sis is still u[illegible] It was found that mi[illegible]s significant[illegible]educed in RCC tiss[illegible]ndic[illegible] its potential tumor suppressor [illegible]unction in [illegible][15]. Bioinformatics analysis [illegible]led the comple[illegible]y binding site betwee[illegible]R-124 and 3'-UTR [illegible]f STAT3. This study [illegible]estigat[illegible]e role of miR-124 in regulating STA[illegible]ctivity, MMP-9 expression, and renal [illegible]noma [illegible]sion. Dual luciferase [illegible]porter [illegible]assay [illegible]monstrate that miR-124 [illegible]trans[illegible]nificantly declined relative luci[illegible]e activ[illegible]n HEK293 cells, confirming [illegible]targeted regulatory relationship between mi[illegible]e 3'-UTR of STAT3 mRNA. [illegible]swell assay revealed that OS-RC-2 cells ex[illegible]ted the strongest invasive ability, followed b[illegible]P and HK-2 cells. It was showed that miR[illegible] expression was markedly lower, whereas STAT3, p-STAT3, and MMP-9 levels were apparently higher in OS-RC-2 and 769-P cells compared with HK-2 cells. It suggested that miR-124 down-regulation may play a role in elevating STAT3 expression, enhancing STAT3 phosphorylation activity, and accelerating RCC occurrence. Butz et al[14] exhibited that miR-124 level was significantly lower in RCC tissue compared with normal control, while its reduction was correlated with worse progression free survival and overall survival. Gebauer et al[15] demonstrated that miR-124 promoter methylation level was significantly stronger in RCC tissue compared with normal kidney, suggesting that miR-124 downregulation may be involved in RCC occurrence. In this study, miR-124 level markedly declined in RCC, which was similar with Butz et al[14] and Gebauer et al[15]. MiR-124 level was apparently lower, while STAT3, p-STAT3, and MMP-9 expressions was significantly higher in OS-RC-2 cells than that in

769-P cells. It indicated that miR-124 down-regulation mediated STAT3 and MMP-9 elevation may be related to the invasiveness enhancement of RCC. Butz et al[14] reported that miR-124 level in metastatic lesion was markedly declined compared with primary lesion of RCC. Gebauer et al[15] showed that miR-124 promoter methylation level in RCC tumor tissue with distant metastasis, lymph node metastasis, higher pathological grading, and progressive stage was significantly higher than that in RCC tumor without distant or lymph node metastasis, low pathological grading, and local lesion, suggesting miR-124 down-regulation may be associated with RCC invasion, metastasis, and progression. Zell et al[25] discovered that hypoxic treatment markedly induced epithelial-to-mesenchymal transition (EMT) in renal proximal tubular epithelial cells (RPTEC), accelerated cell migration, and declined miR-124 expression, revealing that miR-124 may be related to migration ability enhancement in RPTEC cells. All of these results indicated that miR-124 downregulation is a facilitator of cell invasion, which was similar with our results. Further investigation showed that miR-124 elevation dec[illegible]d STAT3, p-STAT3, and MMP-9 expressi[illegible] weakened OS-RC-2 cell invasion, indicati[illegible]at STAT3 regulated MMP-9 up-regulation wa[illegible] sociated with high invasiveness of OS-RC-2 c[illegible] though Stattic cannot change STAT3 protein e[illegible] pression, it markedly suppre[illegible]T3 phosphorylation and down-regul[illegible]MM[illegible]expression, thus play an inhibito[illegible]e in O[illegible]C-2 cell invasion. MiR-124 mim[illegible]nsfec[illegible] with Stattic treatmen[illegible]xhi[illegible]ger inhibitory effect on OS-[illegible]L cell i[illegible]a compared with single miR-1[illegible]imic transfe[illegible]r Stattic treatment. Butz[illegible]owed that mi[illegible]4 overexpression m[illegible]dly [illegible]ted RCC 786-O and Caki-2 cell[illegible]igration an[illegible]ion, reduced cell ratio in S[illegible]d G2/M phases,[illegible]veakened cell prolife[illegible]n. Lon[illegible] reported tha[illegible]miR-124 mimic trans[illegible]on sig[illegible]antly promoted Caki-2 doxoru[illegible]esist[illegible]ell apo[illegible]sis, targeted inhibited FZD5[illegible]n, de[illegible]d downstream P-glycoprotein[illegible]ssion[illegible]d enhanced RCC drug [illegible]ity[20]. [illegible]aled the tumor suppressor effe[illegible]f miR-1[illegible]p-regulation in attenuating RCC[illegible]alignancy, which was in accordance with ou[illegible] et al[25] showed that miR-124 re[illegible]ned renal tubular epithelial cell migration by [illegible]eting MMP-2 to suppress EMT. This study [illegible]he impact of miR-124 over-expression in alle[illegible]ng RCC invasion.

Conclusions

We showed that the miR-124 do[illegible]egulat[illegible] was associated with renal cance[illegible]l OS-RC-2[illegible] invasion enhancement. The [illegible]pression of miR-124 attenuated OS-RC-2 ce[illegible]asion by down-regulating STAT3 a[illegible]MMP-9[illegible]

Conflict of Interest

The Authors declare tha[illegible]ave[illegible]onflict o[illegible]ests.

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