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·基础研究·

# circ_0001821靶向miR-203调控皮肤鳞状细胞癌A431细胞的增殖、凋亡、迁移和侵袭

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[摘 要] **目的:**探讨circ_0001821通过靶向miR-203调控皮肤鳞状细胞癌(cutaneous squamous cell carcinoma,CSCC)A431细胞增殖、凋亡、迁移及侵袭的分子机制。**方法:**选取2018年2月至2019年8月海南医学院第二附属医院收治的39例CSCC患者的癌及配对癌旁组织,以及人CSCC细胞系A431,用qPCR法检测CSCC癌和癌旁组织中circ_0001821的表达水平。利用脂质体转染技术,分别将si-circ_0001821、si-NC、miR-203 mimic、miR-NC、si-circ_0001821与anti-miR-NC、si-circ_0001821与anti-miR-203转染至A431细胞,用qPCR法检测转染细胞中circ_0001821和miR-203的表达水平,MTT法、流式细胞术和Transwell实验分别检测A431细胞的增殖、凋亡、迁移及侵袭能力,WB法检测细胞中增殖、凋亡、迁移及侵袭相关蛋白的表达水平。通过Circinteractome数据库预测circ_0001821与miR-203存在结合位点,双荧光素酶报告基因实验验证circ_0001821与miR-203的靶向关系。**结果:**CSCC组织中circ_0001821的表达水平显著高于癌旁组织($P$<0.01)。转染si-circ_0001821可显著降低细胞中circ_0001821的表达水平($P$<0.01),降低细胞增殖、迁移和侵袭能力(均$P$<0.01),提高细胞凋亡率($P$<0.01)。双荧光素酶报告基因实验证实,circ_0001821可靶向结合miR-203。转染miR-203 mimic可显著降低A431细胞的增殖、迁移和侵袭能力(均$P$<0.01),提高细胞凋亡率($P$<0.01)。共转染si-circ_0001821与anti-miR-203可明显逆转下调circ_0001821表达对A431细胞增殖、迁移、侵袭及凋亡的调控作用。**结论:**circ_0001821通过靶向miR-203调控CSCC细胞A431的增殖、迁移、侵袭能力及细胞凋亡。

[关键词] circ_0001821;miR-203;皮肤鳞状细胞癌;A431细胞;增殖;凋亡;迁移;侵袭
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# circ_0001821 regulates the proliferation, apoptosis, migration and invasion of cutaneous squamous cell carcinoma A431 cells by targeting miR-203

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**[Abstract] Objective:** To explore the mechanism of circ_0001821 regulating the proliferation, apoptosis, migration and invasion of cutaneous squamous cell carcinoma (CSCC) A431 cells by targeting miR-203. **Methods:** The cancer and para-cancerous tissues of 39 patients with CSCC treated in the Second Affiliated Hospital of Hainan Medical University from February 2018 to August 2019, as well as the human CSCC cell line A431, were collected for this study. The qPCR method was used to detect the expression level of circ_0001821 in CSCC tissues and para-cancerous tissues. Using liposome transfection technology, si-circ_0001821, si-NC, miR-203 mimic, miR-NC, si-circ_0001821+anti-miR-NC and si-circ_0001821+anti-miR-203 were transfected into A431 cells, respectively. qPCR was used to detect the expression of circ_0001821 and miR-203 in the transfected cells. MTT method, Flow cytometry and Transwell chamber assay were used to detect the proliferation, apoptosis, migration and invasion of transfected A431 cells. WB was used to detect the expression of proteins related to proliferation, apoptosis, migration and invasion. Circinteractome database was utilized to predict the binding site between circ_0001821 and miR-203, which was further validated by Dual-luciferase reporter gene assay. **Results:** Compared with para-cancerous tissues, the expression level of circ_0001821 in CSCC tissues was significantly increased ($P$<0.01). Transfection of si-circ_0001821 could significantly reduce the expression of circ_0001821 ($P$<0.01), increase the apoptosis rate ($P$<0.01), and reduce the proliferation, migration and invasion abilities of A431 cells (all $P$<0.01). Dual-luciferase report gene assay confirmed that circ_0001821 could targetedly bind with miR-203. Transfection of miR-203 mimics could significantly reduce the proliferation, migration and invasion abilities but increase the apoptosis rate of A431 cells (all $P$<0.01). Co-transfection of si-circ_0001821 and anti-miR-203 could significantly reverse the effects of si-circ_0001821 transfection on the proliferation, migration,

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invasion and apoptosis of A431 cells. **Conclusion:** circ_0001821 regulates the proliferation, migration, invasion and apoptosis of CSCC A431 cells by targeting miR-203.

**[Key words]** circ_0001821; miR-203; cutaneous squamous cell carcinoma (CSCC); A431 cell; proliferation; apoptosis; migration; invasion

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皮肤鳞状细胞癌（cutaneous squamous cell carcinoma，CSCC）是常见的一种恶性肿瘤，目前其发病机制尚未阐明，因此亟待研究CSCC的病理机制、寻找新的分子标志物和治疗策略。研究[1-4]显示，环状RNA（circular RNA，circRNA）在肿瘤中异常表达并可作为肿瘤诊断和预后评估的生物标志物，以及肿瘤生物治疗的潜在靶点；circRNA在CSCC中表达上调或下调，并可能充当微小RNA（microRNA, miRNA）的海绵分子，从而调控细胞的增殖、凋亡、迁移及侵袭。circ_0001821又称circPVT1，其在CSCC中表达上调，并可发挥癌基因作用[5]。靶基因预测显示，miRNA-203（miR-203）与circ_0001821存在结合位点，miR-203在非小细胞肺癌中表达下调，其可通过下调RGS17的表达从而抑制癌细胞的增殖、迁移及侵袭[6]。但circ_0001821是否通过靶向miR-203参与CSCC的发生和发展过程尚未可知。鉴于此，本研究主要探讨circ_0001821通过靶向miR-203调控CSCC细胞的增殖、凋亡、迁移及侵袭的作用及其分子机制，旨在为CSCC的靶向治疗提供新的靶标。

## 1　材料与方法

### 1.1　组织标本、细胞系及主要试剂

选取2018年2月至2019年8月海南医学院第二附属医院收治的39例CSCC患者的癌和配对癌旁组织标本，其中男20例、女19例，年龄50~70岁，平均年龄（58.35±9.68）岁。所有患者均经病理学诊断为CSCC，术前均告知并签署知情同意书，切取后的组织标本置于−80 ℃超低温冰箱内保存。

人CSCC细胞系A431购自美国ATCC细胞库。DMEM培养基、胎牛血清购自上海玉博生物科技有限公司，TRIzol试剂、cDNA合成与qPCR试剂购自日本TaKaRa公司，Lipofectamine 2000转染试剂、MTT试剂、细胞凋亡检测试剂盒和基质胶（matrigel）购自北京索莱宝科技有限公司，si-circ_0001821、si-NC、miR-203 mimic、miR-NC、anti-miR-203、anti-miR-NC购自上海吉玛制药技术有限公司，pcDNA-circ_0001821、pcDNA购自广州辉骏生物科技股份有限公司，Transwell小室购自南京迅贝生物科技有限公司，兔抗人cyclin D1、MMP-2、MMP-9、Bcl-2、p21和Bax单克隆抗体购自美国Santa Cruz公司，辣根过氧化物酶（horseradish peroxidase，HRP）标记的山羊抗兔IgG二抗购自美国Abcam公司。

### 1.2　细胞培养、转染及分组

CSCC细胞系A431在培养箱内常规培养，待细胞汇合度达到70%时进行转染，按照Lipofectamine 2000转染试剂说明书分别将si-circ_0001821、si-NC、miR-203 mimic、miR-NC、si-circ_0001821与anti-miR-NC、si-circ_0001821与anti-miR-203转染至A431细胞中，分别记作si-circ_0001821组、si-NC组、miR-203组、miR-NC组、si-circ_0001821+anti-miR-NC组、si-circ_0001821+anti-miR-203组。

### 1.3　qPCR法检测CSCC组织和A431细胞中circ_0001821和miR-203的表达水平

采用TRIzol法提取CSCC组织和癌旁组织、各转染组A431细胞中的总RNA，用cDNA合成试剂盒将总RNA逆转录合成cDNA，置于−20 ℃冰箱内保存。qPCR反应体系按照试剂盒说明书配置。qPCR反应条件：95 ℃预变性2 min，95 ℃变性15 s、60 ℃退火30 s、72 ℃延伸30 s，共进行40个循环。circ_0001821引物序列上游为CCCCGACTCTTCCTGGTGAA，下游为CAGGCACAGCCATCTTGAGG；GAPDH引物序列：上游为AACGGATTTGGTCGTATTG，下游为GGAAGATGGTGATGGGATT；miR-203引物序列：上游为GATCGATCACCAGGATTTG，下游为GTATCCAGTGCGAATACCTC；U6引物序列：上游为CTCGCTTCGGCAGCACA，下游为AACGCTTCACGAATTTGCGT。用$2^{-\triangle\triangle Ct}$法计算基因的相对表达量。

### 1.4　MTT法检测A431细胞的增殖能力

取各组A431细胞接种于96孔板（$5\times10^3$个/孔），培养24 h后加入MTT试剂（20 μl/孔），继续培养4 h后弃上清，加入DMSO（150 μl/孔），应用酶标仪检测各孔波长在490 nm处的光密度（*D*）值，以*D*值代表细胞的增殖能力。

### 1.5　流式细胞术检测A431细胞的凋亡率

取各组A431细胞加入0.25%胰蛋白酶消化，1 000×*g*离心6 min，弃上清，预冷PBS洗涤，加入500 μl结合缓冲液，加入5 μl Annexin Ⅴ-FITC与5 μl PI，振荡摇晃10 min，应用FACSCalibur流式细胞仪检测各组细胞的凋亡率。

### 1.6　Transwell实验检测A431细胞的迁移与侵袭能力

细胞侵袭实验前需使用培养基稀释matrigel后加入小室的上室（20 μl/孔）并晾干，置于培养箱5 h后取出，上室分别加入各组A431细胞悬浮液（$3\times10^4$个/ml，200 μl/孔），下室加入含有10%胎牛血清的培养液

（600 μl/孔），继续培养24 h后使用PBS洗涤，4%多聚甲醛溶液固定10 min后再用PBS洗涤，吸干上室固定液，用0.1%结晶紫染液染色10 min，用棉签轻轻擦拭未侵袭的细胞，光学显微镜下观察侵袭细胞数。细胞迁移实验：无需预铺matrigel，在Transwell上室直接加入各组A431细胞悬浮液（密度为3×10⁴个/ml，200 μl/孔），后续实验步骤同侵袭实验。

### 1.7 双荧光素酶报告基因实验验证circ_0001821与miR-203的靶向关系

通过Circinteractome数据库预测显示，circ_0001821与miR-203之间存在结合位点。因此，分别构建野生型载体WT-circ_0001821与突变型载体MUT-circ_0001821，分别将miR-NC、miR-203 mimic与WT-circ_0001821、MUT-circ_0001821共转染至A431细胞，培养24 h后，用双荧光素酶报告基因实验检测转染细胞的相对荧光素酶活性。

### 1.8 WB法检测A431细胞中增殖、凋亡、迁移与侵袭相关蛋白的表达

用RIPA裂解液提取各组A431细胞总蛋白，用BCA试剂盒提测定蛋白含量。取40 μg蛋白样品，进行SDS-PAGE分离，转膜后用5%脱脂奶粉封闭2 h，分别加入均以1∶1 000稀释的cyclin D1、MMP-2、MMP-9、Bcl-2、p21和Bax一抗，4 ℃孵育过夜。清洗后，加入HRP标记的山羊抗兔IgG二抗（1∶2 000），4 ℃下孵育1 h，滴加化学发光液进行发光，显影后，应用ImageJ软件分析各蛋白条带的灰度值。

### 1.9 统计学处理

qPCR、MTT、流式细胞术、Transwell和WB等实验均重复3次。采用SPSS21.0统计学软件对实验数据进行统计学分析。呈正态分布的计量数据以$\bar{x}\pm s$表示，两组间比较采用独立样本$t$检验，多组间比较采用单因素方差分析，以$P<0.05$或$P<0.01$表示差异具有统计学意义。

## 2 结 果

### 2.1 circ_0001821在CSCC组织中高表达

qPCR法检测结果显示，CSCC组织中circ_0001821的表达水平显著高于癌旁组织（5.96±0.02 *vs* 1.00±0.03，$P<0.01$）。

### 2.2 转染si-circ_0001821可抑制A431细胞的增殖能力

转染si-circ_0001821后，qPCR、MTT和WB法（图1）检测结果显示，与si-NC组比较，si-circ_0001821组A431细胞中si-circ_0001821表达水平显著降低（0.47±0.04 *vs* 1.02±0.02，$P<0.01$）、细胞增殖能力显著降低（0.49±0.03 *vs* 0.85±0.03，$P<0.01$），细胞中cyclin D1表达水平显著下调（0.36±0.03 *vs* 0.78±0.02，$P<0.01$）、p21蛋白表达水平显著上调（0.83±0.0 *vs* 0.42±0.02，$P<0.01$）。结果表明，转染si-circ_0001821后，CSCC A431细胞的增殖能力显著降低。

### 2.3 抑制circ_0001821表达可降低A431细胞的迁移、侵袭能力并促进其凋亡

转染si-circ_0001821后，Transwell、流式细胞术和WB法检测结果（图2，表1）显示，与si-NC组比较，si-circ_0001821组A431细胞的迁移和侵袭能力均显著降低、细胞凋亡率显著升高（均$P<0.01$），细胞中MMP-2、MMP-9和Bcl-2蛋白的表达水平均显著下调（均$P<0.01$）、Bax蛋白的表达水平显著上调（$P<0.01$）。结果表明，抑制si-circ_0001821表达可显著降低A431细胞的迁移、侵袭能力并促进细胞凋亡。

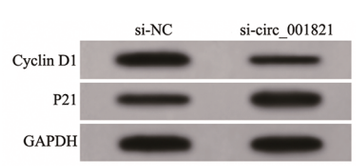
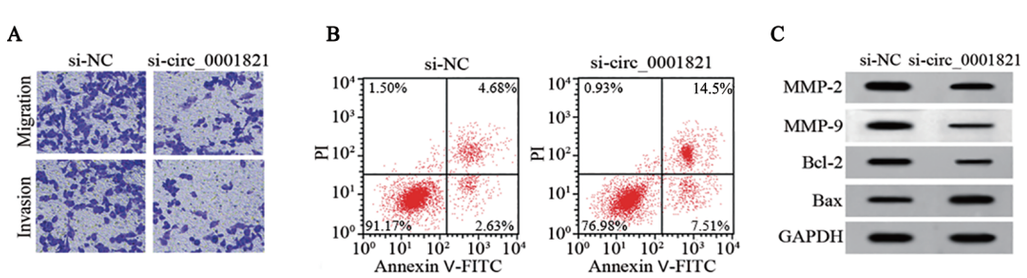



**图1 抑制circ_0001821表达对A431细胞中cyclin D1和p21蛋白表达的影响**

**Fig.1 Effect of circ_0001821 inhibition on the expression of cyclin D1 and p21 protein in A431 cells**




A: Transwell assay was used to detect the migration and invasion of A431 cells (×200); B: Apoptosis level of A431 cells was detected by Flow cytometry; C: The expression of migration, invasion and apoptosis related proteins was detected by WB method

**图2 抑制circ_0001821表达对A431细胞迁移、侵袭和凋亡的影响**

**Fig.2 Effect of circ_0001821 inhibition on migration, nvasion and apoptosis of A431 cells**

表1 抑制circ_0001821表达对A431细胞迁移、侵袭和凋亡的影响（*n*=9）

Tab.1 Effect of circ_0001821 inhibition on migration, invasion and apoptosis of A431 cells (*n*=9)

| Group | Migration cells | Invasion cells | Apoptosis (%) | MMP-2 | MMP-9 | Bcl-2 | Bax |
|---|---|---|---|---|---|---|---|
| si-NC | 101.90±2.04 | 86.84±2.97 | 7.31±0.01 | 0.84±0.03 | 0.67±0.03 | 0.59±0.03 | 0.26±0.03 |
| si-circ_0001821 | 52.19±2.97 | 39.58±2.13 | 22.10±0.04 | 0.38±0.03 | 0.28±0.03 | 0.22±0.01 | 0.69±0.03 |
| *t* | 13.820 | 12.920 | 355.000 | 9.426 | 10.060 | 10.320 | 10.600 |
| *P* | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 |

2.4　circ_0001821靶向调控miR-203的表达

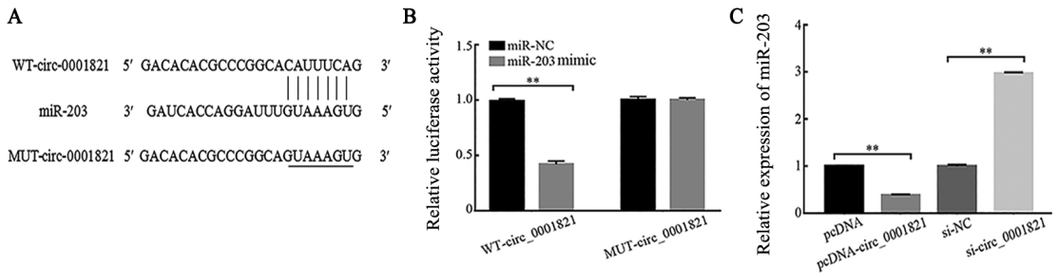
Circinteractome数据库预测结果显示，circ_0001821与miR-203存在结合位点（图3A）。双荧光素报告基因实验结果（图3B）显示，miR-203过表达可显著降低WT-circ_0001821组细胞的荧光素酶活性（*P*<0.01），而对MUT-circ_0001821组细胞的荧光素酶活性无明显影响（*P*>0.05）。qPCR法检测结果（图3C）显示，与pcDNA组比较，pcDNA-circ_0001821组A431细胞中miR-203的表达水平显著降低（*P*<0.01）；与si-NC组比较，si-circ_0001821组细胞中miR-203的表达水平显著升高（*P*<0.01）。实验结果表明，circ_0001821可靶向调控miR-203的表达。

2.5　miR-203过表达可显著降低A431细胞增殖、迁移、侵袭能力并促进细胞凋亡

转染miR-203 mimic后，qPCR、MTT、Transwell和WB法等检测结果（图4，表2）显示，与miR-NC组比较，miR-203 mimic组A431细胞中miR-203表达水显著升高（*P*<0.01），细胞增殖活力、迁移及侵袭能力均显著降低（均*P*<0.01），细胞凋亡率显著升高（*P*<0.01）；细胞中cyclin D1、MMP-2、MMP-9、Bcl-2蛋白表达水平均显著降低（均*P*<0.01），而p21和Bax蛋白表达水平显著升高（均*P*<0.01）。

2.6　下调miR-203表达可逆转抑制circ_0001821表达对A431细胞增殖、迁移、侵袭和凋亡的作用

共转染si-circ_0001821和anti-miR-203后，MTT、Transwell和WB法等检测结果（图5，表3）显示，与si-circ_0001821+anti-miR-NC组比较，si-circ_0001821+anti-miR-203组A431细胞中miR-203表达水平显著降低（*P*<0.01），细胞增殖活力、迁移及侵袭能力均显著升高（均*P*<0.01），细胞凋亡率显著降低（*P*<0.01），细胞中cyclin D1、MMP-2、MMP-9、Bcl-2蛋白表达水平均显著上调（均*P*<0.01），而p21和Bax蛋白表达水平显著下调（均*P*<0.01）。结果表明，下调miR-203表达可逆转抑制circ_0001821表达对A431细胞增殖、迁移、侵袭和凋亡的作用。




**P<0.01

A: Circinteractome database was used to predict the binding site between circ_0001821 and miR-203;

B: Dual-luciferase reporter gene assay; C: The expression of miR-203 was detected by qPCR

图3 circ_0001821的序列中含有与miR-203互补的核苷酸序列

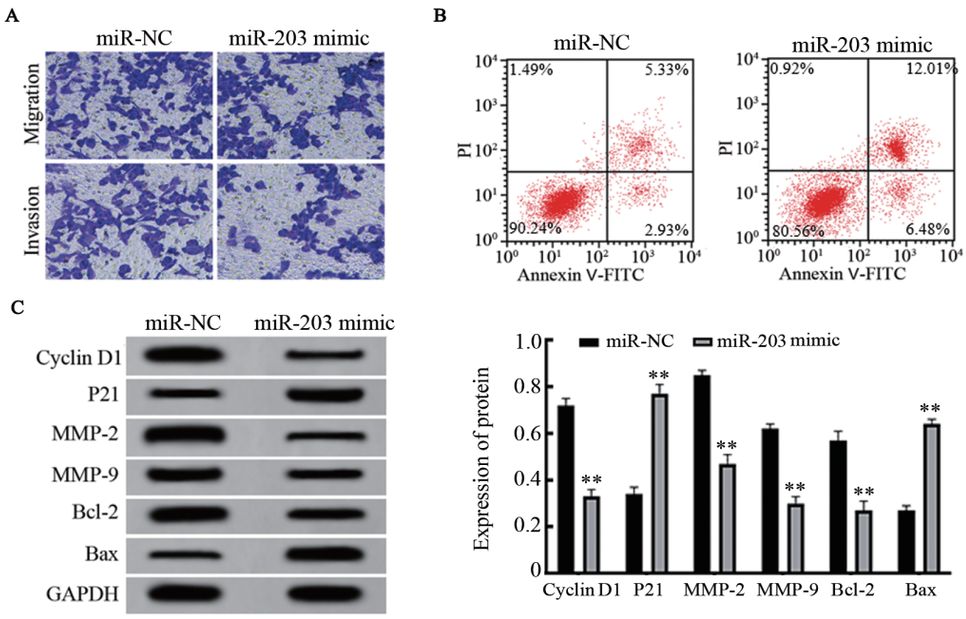
Fig.3 The sequence of circ_0001821 contains nucleotide sequences complementary to miR-203

## 3　讨　论

CSCC是一种常见的皮肤恶性肿瘤。既往研究显示，miRNA在CSCC中可发挥癌基因或抑癌基因作用，miR-221通过靶向PTEN促进CSCC的进展[7]；miR-497通过FAM114A2促进CSCC的进展[8]。LINC00963充当miR-1193的竞争性内源RNA并通过靶向SOX4抑制CSCC的进展[9]。但miRNA上游基因（circRNA或lncRNA）与CSCC关系的研究相对较少，因而本研究探寻新型circRNA分子及其在CSCC发生及发展过程中的作用机制。

circ_0001821（circPVT1）在胃癌中呈高表达，并可能作为胃癌诊断及判断患者预后的生物学标志物[10]。circ_0001821通过充当miR-125b的海绵分子

及激活E2F2信号通路而促进非小细胞肺癌细胞的增殖和侵袭[11]。circ_0001821过表达可促进口腔鳞状细胞癌细胞增殖[12]。circ_0001821通过调节miR-205-5p/c-FLIP轴促进骨肉瘤细胞侵袭和转移[13]。本研究结果显示，CSCC组织中circ_0001821的表达水平高于癌旁组织，提示circ_0001821在CSCC发生过程中可能发挥重要调控作用；抑制circ_0001821表达后可降低细胞增殖活力，以及抑制cyclin D1的表达、促进p21的表达，提示抑制circ_0001821表达可抑制CSCC细胞增殖。Bcl-2/Bax比例失衡可调控细胞凋亡，而MMP-2、MMP-9表达水平升高可促进细胞转移[14-15]。本研究结果发现，抑制circ_0001821表达可明显提高细胞凋亡率及减少迁移、侵袭细胞数，提示抑制circ_0001821表达可促进CSCC细胞凋亡及抑制细胞迁移及侵袭。circ_0001821可充当miR-203的海绵分子，miR-203通过靶向SOCS3调节卵巢癌细胞的增殖和凋亡[16]。miR-203靶向Twist1抑制膀胱癌细胞的增殖[17]。本研究结果提示，抑制miR-203表达可明显逆转抑制circ_0001821表达对CSCC细胞增殖、凋亡、迁移及侵袭的作用。




$^{**}P$<0.01 *vs* miR-NC group

A: Transwell assay was used to detect the migration and invasion of A431 cells (×200); B: Flow cytometry was used to measure the apoptosis rate of A431 cells; C: WB assay was used to detect the expression of proteins related to proliferation, migration, invasion and apoptosis in A431 cells

**图4 miR-203过表达对A431细胞迁移、侵袭和凋亡及其相关蛋白表达的影响**

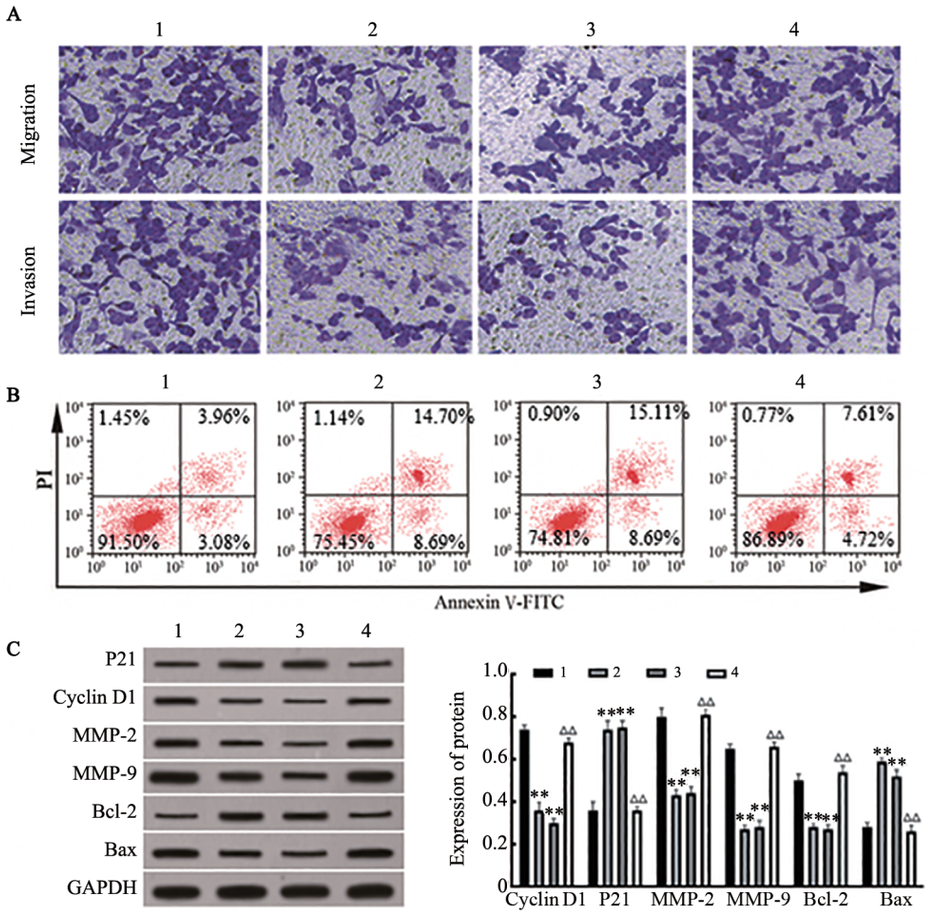
**Fig.4 Effect of miR-203 overexpression on the migration, invasion, apoptosis, and the expression of related proteins in A431 cells**

**表2 miR-203过表达对A431细胞增殖、迁移、侵袭和凋亡的影响（*n*=9）**

**Tab.2 Effect of miR-203 overexpression on the proliferation, migration, invasion and apoptosis of A431 cells (*n*=9)**

| Group | miR-203 | Proliferation ($D_{450}$) | Migration cells | Invasion cells | Apoptosis rate (%) |
|---|---|---|---|---|---|
| miR-NC | 1.01±0.02 | 0.89±0.02 | 103.30±1.88 | 89.31±3.26 | 8.27±0.39 |
| miR-203 mimic | 3.63±0.04 | 0.55±0.02 | 63.29±2.77 | 49.41±1.33 | 18.50±0.25 |
| *t* | 59.900 | 11.040 | 11.960 | 11.350 | 22.070 |
| *P* | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 |

综上所述，circ_0001821在CSCC组织中呈高表达，抑制circ_0001821表达可抑制CSCC细胞增殖、迁移、侵袭及促进细胞凋亡，其作用机制与上调miR-203有关，因而circ_0001821可作为CSCC治疗的潜在靶点，但关于circ_0001821如何调控miR-203的靶基因表达及其可能作用机制仍需进一步研究。




$^{**}P$<0.01 *vs* si-NC group; $^{\triangle\triangle}P$<0.01 *vs* si-circ_0001821+anti-miR-NC group or si-circ_0001821 group

1: si-NC group; 2: si-circ_0001821 group: 3: si-circ_0001821+anti-miR-NC group; 4: si-circ_0001821+anti-miR-203

A: Transwell assay was used to detect the migration and invasion ability of cells (×200); B: Flow cytometry was used to measure the apoptosis rate of cells; C: WB assay was used to detect the expression of proteins related to proliferation, migration, invasion, and apoptosis in cells

**图5　下调miR-203表达逆转了敲减circ_0001821表达对A431细胞增殖、迁移、侵袭和凋亡的调控作用**

**Fig.5　Down-regulation of miR-203 expression reversed the regulatory effects of circ_0001821 knockout on proliferation, migration, invasion and apoptosis of A431 cells**

**表3　下调miR-203表达逆转敲减circ_0001821表达对A431细胞增殖、迁移、侵袭和凋亡的调控作用（*n*=9）**

**Tab. 3　Down-regulating miR-203 expression reversed the regulatory effects of circ_0001821 knockout on proliferation, migration, invasion and apoptosis of A431 cells (*n*=9)**

| Group | miR-203 | Prolieration ($D_{450}$) | Migration cells | Invasion cells | Apoptosis rate (%) |
|---|---|---|---|---|---|
| si-NC | 1.02±0.04 | 0.87±0.02 | 96.52±3.61 | 80.77±2.24 | 7.04±0.24 |
| si-circ_0001821 | 2.91±0.04$^{**}$ | 0.52±0.01$^{**}$ | 54.60±0.98$^{**}$ | 40.21±1.51$^{**}$ | 23.41±0.59$^{**}$ |
| si-circ_0001821+anti-miR-NC | 2.96±0.04 | 0.53±0.01 | 53.00±1.21 | 36.90±2.16 | 24.28±0.40 |
| si-circ_0001821+anti-miR-203 | 1.61±0.03$^{\triangle\triangle}$ | 0.81±0.02$^{\triangle\triangle}$ | 83.27±1.32$^{\triangle\triangle}$ | 73.49±1.22$^{\triangle\triangle}$ | 12.33±0.74$^{\triangle\triangle}$ |
| *F* | 590.200 | 135.900 | 108.200 | 151.000 | 257.500 |
| *P* | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 |

$^{**}P$<0.01 *vs* si-NC group; $^{\triangle\triangle}P$<0.01 *vs* si-circ_0001821+anti-miR-NC group or si-circ_0001821 group

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