·论 著·

miR-6775-3p在乳腺癌细胞中的表达及其对乳腺癌细胞生物学行为的影响

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［摘要］ **背景与目的：**微小RNA（microRNA，miRNA）与肿瘤的发生、发展过程密切相关。探讨miR-6775-3p在乳腺癌细胞系中的表达及其对乳腺癌细胞生物学行为的影响。**方法：**通过实时荧光定量聚合酶链反应（real-time fluorescence quantitative polymerase chain reaction，RTFQ-PCR）检测4种乳腺癌细胞系MDA-MB-231、MDA-MB-453、MDA-MB-468和BT-549中miR-6775-3p的表达水平，选取miR-6775-3p表达水平最低的乳腺癌细胞系过表达miR-6775-3p后，采用细胞计数试剂盒（cell counting kit-8，CCK-8）法检测细胞的增殖情况，同时采用transwell迁移和侵袭实验分别检测细胞迁移和侵袭能力的变化。通过RTFQ-PCR和蛋白［质］印迹法（Western blot）检测miR-6775-3p过表达的乳腺癌细胞系中细胞周期蛋白依赖性蛋白激酶4（cyclin-dependent protein kinase 4，CDK4）和CDK6，以及侵袭转移标志物基质金属蛋白酶（matrix metalloproteinase，MMP）17和MMP24 mRNA以及蛋白的表达变化。**结果：**RTFQ-PCR结果显示，乳腺癌细胞系MDA-MB-453中miR-6775-3p的表达最低，在MDA-MB-453细胞中转染miR-6775-3p mimics后，miR-6775-3p的表达水平明显升高（P<0.001）。CCK-8实验结果显示，MDA-MB-453细胞过表达miR-6775-3p后，细胞的增殖能力明显降低（P<0.01）。Transwell迁移和侵袭实验结果显示，MDA-MB-453细胞过表达miR-6775-3p后，细胞的迁移（P<0.001）和侵袭能力（P<0.01）明显降低。RTFQ-PCR和Western blot实验结果显示，CDK4、CDK6及MMP17、MMP24的mRNA表达和蛋白水平均显著降低（P<0.01）。**结论：**miR-6775-3p可能抑制乳腺癌细胞的增殖、迁移和侵袭能力。

［关键词］ miR-6775-3p；乳腺癌；增殖；迁移；侵袭
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Expression of miR-6775-3p in breast cancer cells and its effect on biological behavior of breast cancer cells

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［Abstract］ **Background and purpose:** MicroRNA (miRNA) is closely related to the occurrence and development of tumors. This study aimed to investigate the expression of miR-6775-3p in breast cancer cells and its effect on the biological characteristics of breast cancer cells. **Methods:** Real-time fluorescence quantitative polymerase chain reaction (RTFQ-PCR) was used to detect the expression level of miR-6775-3p in four breast cancer cell lines MDA-MB-231, MDA-MB-453, MDA-MB-468 and BT-549. After miR-6775-3p was over-expressed in breast cancer cells with the lowest expression level of miR-6775-3p, the proliferation of

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cells was detected by cell counting kit-8 (CCK-8) assay, and the migration and invasion ability of cells were detected by transwell migration and invasion assay. To further explore the molecular mechanism of miR-6775-3p in breast cancer, the expressions of cyclin-dependent protein kinase 4 (CDK4), CDK6 and invasion and metastasis markers matrix metalloproteinase 17 (MMP17) and MMP24 in miR-6775-3p overexpressing breast cancer cells were detected by RTFQ-PCR and Western blot. **Results:** RTFQ-PCR results showed that the expression of miR-6775-3p was the lowest in breast cancer cell line MDA-MB-453, and the expression level of miR-6775-3p was significantly increased after transfection of miR-6775-3p mimics in MDA-MB-453 cells ($P<0.001$). CCK-8 assay results showed that the cell proliferation activity was significantly decreased after miR-6775-3p was overexpressed in MDA-MB-453 cells ($P<0.01$). Transwell migration and invasion assay results showed that the cell migration ($P<0.001$) and invasion abilities ($P<0.01$) were significantly decreased after miR-6775-3p was over-expressed in MDA-MB-453 cells. RTFQ-PCR and Western blot results showed that the expressions of cyclin-dependent protein kinases CDK4 and CDK6, as well as the mRNA and protein levels of cell invasion and metastasis markers MMP17 and MMP24 were significantly decreased ($P<0.01$). **Conclusion:** miR-6775-3p may inhibit the proliferation, migration and invasion of breast cancer cells.

［**Key words**］ miR-6775-3p; Breast cancer; Proliferation; Migration; Invasion

乳腺癌是全球最常见的女性恶性肿瘤[1]，随着近年来分子生物学技术的不断发展，越来越多的分子靶点被发现与乳腺癌的发生、发展密切相关，其中非编码RNA在乳腺癌研究领域也占据至关重要的地位。

miRNA是一类长度为21～23个核苷酸的内源性非编码小RNA，它主要通过靶向mRNA的3'非翻译区（3'untranslated region，3'UTR）从而抑制靶基因的表达[2-3]。有研究报道，miR-6775-3p可通过靶向MAGE-A家族抑制食管鳞状细胞癌的进展[4]。本研究旨在探讨miR-6775-3p对乳腺癌细胞增殖、迁移和侵袭能力的影响。

1 材料和方法

1.1 细胞系及主要试剂

人乳腺癌细胞系MDA-MB-231、MDA-MB-453、MDA-MB-468和BT-549购自武汉普诺赛生命科技有限公司，RPMI-1640培养基、胎牛血清、胰蛋白酶均购自美国Gibco公司，青链霉素双抗混合液购自上海翊圣生物科技有限公司，TRIzol Reagent购自美国Invitrogen公司，实时荧光定量聚合酶链反应（real-time fluorescent quantitative polymerase chain reaction，RTFQ-PCR）Mix购自美国Promega公司，Hiperfect 转染试剂购自德国QIAGEN公司，transwell小室购自美国Corning公司，Matrigel基质胶购自美国BD公司，兔抗人β-actin、CDK4、CDK6、基质金属蛋白酶（matrix metalloproteinase，MMP）17和MMP24多克隆抗体购自美国Abcam公司，山羊抗兔二抗购自美国Proteintech生物技术有限公司。

1.2 RTFQ-PCR法检测miR-6775-3p在乳腺癌细胞系中的表达

采用RTFQ-PCR法检测4种乳腺癌细胞系MDA-MB-231、MDA-MB-453、MDA-MB-468和BT-549中miR-6775-3p的表达水平。细胞培养至呈对数生长期，采用TRIzol Reagent提取细胞的总RNA。按照Promega反转录和扩增试剂盒说明书冰上制备cDNA模板后进行扩增反应，条件为95 ℃ 5 min；95 ℃ 15 s，58 ℃ 30 s，72 ℃ 30 s，共40个循环。miR-6775-3p和内参基因*U6*的特异性反转录和扩增引物购自广州锐博生物技术有限公司。本实验采用$2^{-\triangle\triangle CT}$法计算miR-6775-3p的平均相对表达量。

1.3 细胞miR-6775-3p mimics转染

对照组（normal control，NC）和miR-6775-3p mimics质粒购自上海吉玛生物技术有限公司。人乳腺癌细胞系MDA-MB-453培养于含10%胎牛血清、1%青链霉素双抗混合液的RPMI-1640培养基中，放置于37 ℃、CO_2体积分数为5%的培养箱中温育。待细胞长至对数生长期时，按1×10^6个/孔的密度种植于6孔板中。培养24 h后，按照Hiperfect Reagent推荐的转染方法，于每孔中分别加入10 μL miR-6775-3p mimics或对照组NC质粒和50 μL无牛清的空RPMI-1640培养基。另将4 μL Hiperfect Reagent加至50 μL无牛清的空

RPMI-1640培养基中，分别混匀静置5 min。再将质粒与转染试剂混合，室温静置20 min后加至每孔使终体积达到2 mL。转染6~8 h后，更换新鲜的完全培养基，继续温育24~48 h。

1.4　采用细胞计数试剂盒（cell counting kit-8，CCK-8）检测乳腺癌细胞的增殖能力

采用CCK-8（美国MCE公司）检测乳腺癌细胞系MDA-MB-453的增殖能力。人乳腺癌细胞系MDA-MB-453培养于含10%胎牛血清、1%青链霉素双抗混合液的RPMI-1640培养基中，放置于37 ℃、CO_2体积分数为5%的培养箱中温育。待细胞长至对数生长期时，按照1×10^3个/孔的密度种植于96孔板中，每孔加入100 μL细胞悬液。分别于第0、1、2、3、4、5 d时在每孔轻轻加入10 μL CCK-8试剂，避免产生气泡，振荡器摇匀5 min后置于37 ℃、CO^2体积分数为5%的细胞培养箱中温育1~2 h。采用Magellan for F50酶标系统测量细胞的450 nm吸光度（*D*）值。

1.5　Transwell小室迁移和侵袭实验检测乳腺癌细胞的迁移和侵袭能力

采用transwell小室法检测乳腺癌细胞系MDA-MB-453的迁移和侵袭能力。将低温融化的Matrigel基质胶与无血清RPMI-1640培养液按照1：7稀释，震荡混匀后于24孔板中加入20 μL，之后放在37 ℃、CO_2体积分数为5%的细胞培养箱中过夜凝固。选取对数生长期的乳腺癌细胞系MDA-MB-453重悬于200 μL无血清的空RPMI-1640培养基中，按照2×10^5个/孔的密度种植于transwell小室上，而24孔板的每孔中加入800 μL带牛清的RPMI-1640完全培养基。注意24孔板中加Matrigel基质胶时检测细胞的侵袭能力，不加Matrigel基质胶时则检测细胞的迁移能力。细胞培养箱中温育48 h后，用4%多聚甲醛固定20 min，然后结晶紫染色10 min，在普通光学显微镜下观察计数穿膜的细胞数目并拍照。

1.6　采用蛋白［质］印迹法（Western blot）检测蛋白质的表达水平

转染48 h后的对照组和实验组细胞用PBS缓冲液清洗2~3次，细胞刮刀收集至1.5 mL离心管中，8 000 r/min离心5 min。弃去上清液，在细胞沉淀中加入RIPA裂解液和适量蛋白酶抑制剂PMSF（1：100），吹打混匀后冰上裂解1~2 h。12 000 r/mim，4 ℃离心30 min后吸取上清液。加入适量蛋白变性缓冲液后行10% SDS-PAGE，然后经低温电转移至PVDF膜，5%脱脂奶粉室温摇床封闭60 min。用TBST洗膜3次后加入β-actin、CDK4、CDK6、MMP17和MMP24一抗（1：1 000），4 ℃摇床温育过夜。次日，用TBST洗膜3次，加入二抗37 ℃温育1 h，TBST洗3次后滴加ECL发光液进行显色，采用Image J软件进行蛋白条带灰度分析。

1.7　统计学处理

采用SPSS 22.0对实验结果进行统计分析。计量资料的数据用$\bar{x} \pm s$表示，组间的两两比较采用Student *t*检验。$P<0.05$为差异有统计学意义。

2　结　　果

2.1　miR-6775-3p在乳腺癌细胞系中的表达情况

本实验选取4种人乳腺癌细胞系MDA-MB-231、MDA-MB-453、MDA-MB-468和BT-549，采用RTFQ-PCR法检测这4种细胞系中miR-6775-3p的表达水平。结果显示，乳腺癌细胞系MDA-MB-231、MDA-MB-453、MDA-MB-468和BT-549中miR-6775-3p的相对表达量分别为（1.02 ± 0.04）、（0.80 ± 0.04）、（0.65 ± 0.07）和（0.43 ± 0.04）（图1）。其中，MDA-MB-453细胞中miR-6775-3p的表达水平最低，故接下来选用MDA-MB-453细胞进行miR-6775-3p的过表达实验。

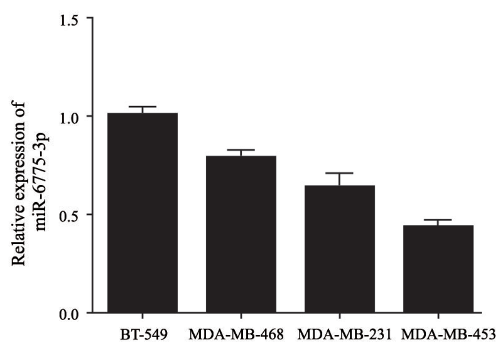

图1　miR-6775-3p在乳腺癌细胞系MDA-MB-231、MDA-MB-453、MDA-MB-468和BT-549中的表达

Fig. 1　miR-6775-3p expression levels in four human breast cancer cell lines MDA-MB-231, MDA-MB-453, MDA-MB-468 and BT-549

2.2 转染miR-6775-3p mimics对乳腺癌细胞MDA-MB-453中miR-6775-3p表达的影响

本实验利用miR-6775-3p mimics模拟物转染乳腺癌细胞MDA-MB-453后，通过RTFQ-PCR法检测细胞中miR-6775-3p的转染效率。结果显示，miR-6775-3p mimics转染组的MDA-MB-453细胞中miR-6775-3p表达水平比NC对照组明显升高，差异有统计学意义（1 198.00±27.47 *vs* 1.00±0.05，P<0.001，图2）。以上数据表明，MDA-MB-453细胞已经成功实现了miR-6775-3p的过表达。

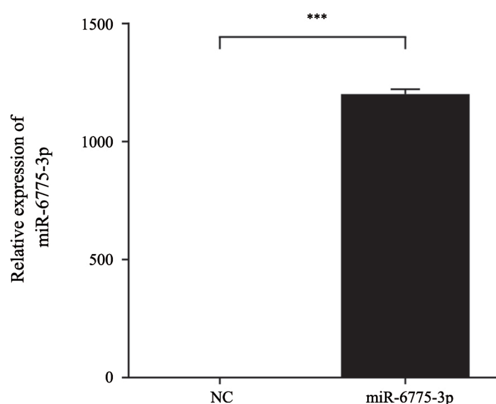

图 2 RTFQ-PCR法检测miR-6775-3p mimics转染后MDA-MB-453细胞中miR-6775-3p 的表达变化

Fig. 2 The expression of miR-6775-3p in MDA-MB-453 cells transfected with miR-6775-3p mimics or normal control (NC) group was detected by RTFQ-PCR

The results showed that miR-6775-3p was highly expressed in MDA-MB-453 cells after miR-6775-3p mimics transfection. ***: P<0.001, compared with NC group

2.3 miR-6775-3p可能抑制乳腺癌细胞的增殖能力

本实验将miR-6775-3p mimics转染至乳腺癌细胞MDA-MB-453后，采用CCK-8法检测miR-6775-3p对乳腺癌细胞增殖能力的影响。本研究采用第1、2、3、4天的$D_{450\ nm}$与第0天的$D_{450\ nm}$的比值为纵坐标绘制增殖曲线，结果见图3，在转染第2、3、4天后，miR-6775-3p过表达组中乳腺癌细胞MDA-MB-453的增殖能力与NC对照组相比明显降低（第2天/第0天：3.34±0.74 *vs* 2.15±0.25，P<0.05；第3天/第0天：5.45±0.97 *vs* 3.41±0.56，P<0.01；第4天/第0天：6.78±1.21 *vs* 3.99±0.81，P<0.01），差异均有统计学意义。

2.4 miR-6775-3p可能抑制乳腺癌细胞的迁移和侵袭能力

本实验进一步采用transwell迁移和侵袭实验检测miR-6775-3p对乳腺癌细胞MDA-MB-453迁移和侵袭能力的影响。结果显示，转染miR-6775-3p mimics组中迁移和侵袭的细胞数目均比NC对照组显著减少（迁移：105.60±8.69 *vs* 223.40±13.41，P<0.001；侵袭：22.20±3.31 *vs* 54.80±5.60，P<0.01），差异有统计学意义（图4）。以上数据表明miR-6775-3p可能抑制乳腺癌细胞的迁移和侵袭能力。

2.5 miR-6775-3p抑制乳腺癌细胞中CDK4、CDK6、MMP17和MMP24的表达

为探究miR-6775-3p抑制乳腺癌细胞增殖、迁移和侵袭能力的分子机制，本研究采用TargetScan在线数据库预测得出，miR-6775-3p与细胞周期蛋白依赖性蛋白激酶CDK4和CDK6，以及细胞侵袭转移标志物MMP17和MMP24的3'UTR存在结合位点（表1）。为验证上述结合位点的活性，我们接下来采用RTFQ-PCR法检测MDA-MB-453细胞转染miR-6775-3p mimics后CDK4、CDK6、MMP17和MMP24的mRNA表达变化，结果如图5A所示，转染miR-6775-3p mimics的MDA-MB-453细胞中CDK4、CDK6、MMP17和MMP24 mRNA的表达水平均比对照

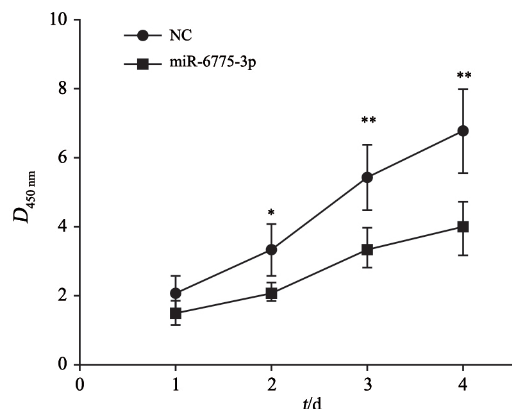

图 3 miR-6775-3p对乳腺癌细胞MDA-MB-453增殖能力的影响

Fig. 3 The effect of miR-6775-3p on the proliferative capacity of breast cancer cell MDA-MB-453 was measured by CCK-8 assay

*: P<0.05, compared with NC group; **: P<0.01, compared with NC group

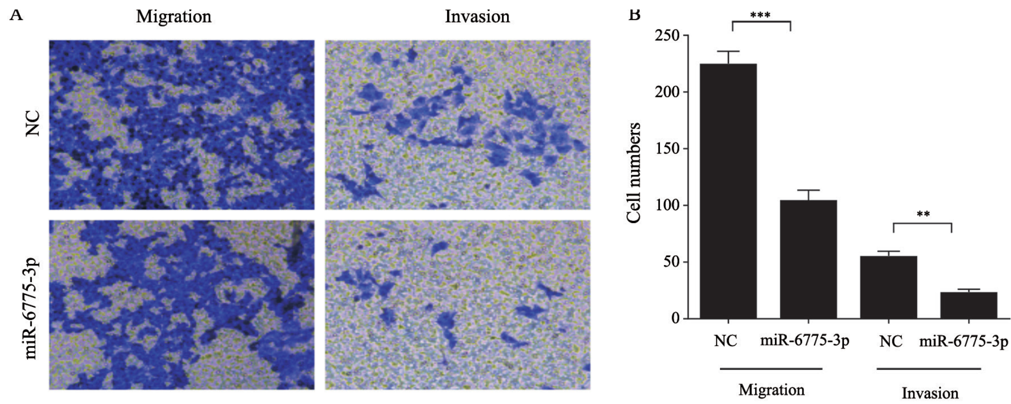

图 4 Transwell实验检测转染miR-6775-3p mimics后乳腺癌细胞MDA-MB-453的迁移和侵袭能力

Fig. 4 The migration and invasion capacity of MDA-MB-453 cells after transfection with miR-6775-3p mimics was detected by transwell migration and invasion assay

A: Transwell assay, crystal violet staining (×200); B: The results showed that the migration and invasion capacity of MDA-MB-453 cells was significantly decreased after miR-6775-3p mimics transfection. ***: P<0.001, compared with NC group; **: P<0.01, compared with NC group

表1 miR-6775-3p与细胞周期蛋白依赖性蛋白激酶CDK4和CDK6，以及细胞侵袭转移相关指标MMP17和MMP24的结合位点

Tab.1 The binding sites of miR-6775-3p with the 3'UTR of the cyclin-dependent protein kinases CDK4 and CDK6, as well as the cell invasion and metastasis related markers MMP17 and MMP24

Binding sites	Predicted consequential pairing of target region (top) and miRNA (bottom)
Position 626-632 of CDK4 3' UTR hsa-miR-6775-3p	5' ...AGACUGGUUAAAUUA**CAGGGCC**U... 3' GACCCCGUCUCCU**GUCCCGG**A
Position 1470-1476 of CDK6 3' UTR hsa-miR-6775-3p	5' ...CUUUAGCUAUGUUUU**AGGGCC**AU... 3' GACCCCGUCUCCUG**UCCCGG**A
Position 377-383 of MMP17 3' UTR hsa-miR-6775-3p	5' ...GCUCCCGCCGGCCCA**CAGGGCC**U... 3' GACCCCGUCUCCU**GUCCCGG**A
Position 528-535 of MMP17 3' UTR hsa-miR-6775-3p	5' ...GGCUCCCUGGUGCUC**CAGGGCC**A... 3' GACCCCGUCUCCU**GUCCCGG**A
Position 1141-1147 of MMP17 3' UTR hsa-miR-6775-3p	5' ...GCCGAUGCCACUCUG**CAGGGCC**C... 3' GACCCCGUCUCCU**GUCCCGG**A
Position 1484-1490 of MMP17 3' UTR hsa-miR-6775-3p	5' ...CCAGGAUGAGGCUGC**CAGGGCC**G... 3' GACCCCGUCUCCU**GUCCCGG**A
Position 2030-2036 of MMP17 3' UTR hsa-miR-6775-3p	5' ...UGCUGGGUCCUGGGG**CAGGGCC**U... 3' GACCCCGUCUCCU**GUCCCGG**A
Position 89-96 of MMP24 3' UTR hsa-miR-6775-3p	5' ...CUGGCCAGUGCUCAC**CAGGGCC**A... 3' GACCCCGUCUCCU**GUCCCGG**A
Position 98-104 of MMP24 3' UTR hsa-miR-6775-3p	5' ...GCUCAC**CAG**GGCCAG**CAGGGCC**C... 3' GACCCC**GUC**UCCU--**GUCCCGG**A
Position 1025-1032 of MMP24 3' UTR hsa-miR-6775-3p	5' ...A**GGCAGAG**AUGGCUG**CAGGGCC**A... 3' GA**CCCCGUC**UCCU-----**GUCCCGG**A

组明显下降（$P<0.01$）。采用Western blot实验检测CDK4、CDK6、MMP17和MMP24蛋白表达的变化，结果显示，转染miR-6775-3p mimics的MDA-MB-453细胞中CDK4、CDK6、MMP17和MMP24蛋白的表达水平均比对照组明显下降（$P<0.01$，图5）。

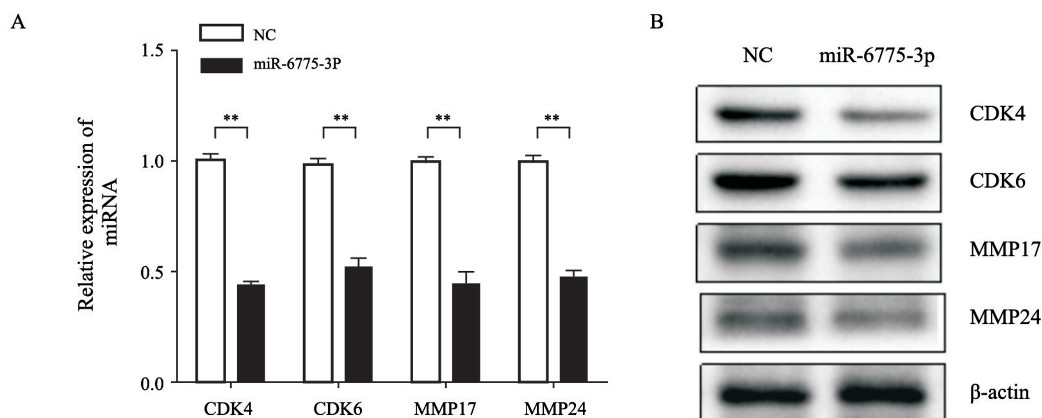

图5 RTFQ-PCR（A）和Western blot（B）检测miR-6775-3p mimics转染MDA-MB-453细胞后CDK4、CDK6、MMP17和MMP24的mRNA和蛋白表达水平

Fig. 5 The expressions of CDK4, CDK6, MMP17 and MMP24 in MDA-MB-453 cells transfected with miR-6775-3p mimic were detected by RTFQ-PCR (A) and Western blot (B)

NC plasmid was transfected into MDA-MB-453 cells as the negative control group. The results showed that the expressions of CDK4, CDK6, MMP17 and MMP24 in MDA-MB-453 cells were down-regulated after miR-6775-3p mimics transfection. **: $P<0.01$, compared with NC group

3 讨　　论

miRNA是近几年研究较为广泛的一类真核生物体内普遍存在的非编码RNA分子，一般在转录后水平上调节其下游靶基因的表达水平[5-7]。乳腺癌是女性常见的一种恶性疾病，目前乳腺癌的治疗以手术为主，根据乳腺癌的不同分期及组织分型联合放疗、化疗、内分泌治疗和生物靶向治疗等综合治疗手段，以达到最佳的治疗效果，提高患者生存率[8-9]。而现在人们对乳腺癌的肿瘤发生与进展转移的分子生物学机制尚不清楚，严重影响乳腺癌患者的诊断及治疗，故寻找更多更有效的分子靶点，是当前解决这一医学难题的重中之重。

本研究结果表明，miR-6775-3p能够抑制乳腺癌细胞的增殖、迁移和侵袭能力。为了进一步探究miR-6775-3p抑制乳腺癌细胞增殖、迁移和侵袭能力的分子机制，我们采用了TargetScan 7.2在线数据库对miR-6775-3p下游的靶基因进行预测，结果发现miR-6775-3p与细胞周期蛋白依赖性蛋白激酶CDK4和CDK6，以及细胞侵袭转移标志物MMP17和MMP24的3’UTR存在结合位点。

细胞周期蛋白依赖性蛋白激酶（cyclin-dependent protein kinase，CDK）属于丝氨酸/苏氨酸蛋白激酶, 分为CDK1~CDK13共13个成员[10]，能够与细胞周期蛋白（cyclin）协同发挥作用，是尤为重要的细胞周期调节因子[11]。有研究表明，CDK4和CDK6可促进细胞由G_1期进入S期，进而促进细胞的有丝分裂过程，在恶性肿瘤的增殖方面起关键作用[12]。MMP家族属于一种锌依赖性蛋白水解酶，能够降解细胞外基质，进而破坏肿瘤细胞浸润要突破的组织屏障，在癌症的侵袭转移方面发挥重要功能[13-14]。MMP17是MMP家族的一个重要成员，能够激活蛋白聚糖酶血小板反应蛋白解整合素金属肽酶4（a disintegrin and metalloproteinase with thrombospondin motifs 4，ADAMTS4），进一步水解蛋白聚糖而导致细胞外基质降解[15]。MMP24属于一种膜型基质金属蛋白酶（membrane-type matrix metalloproteinase，MT-MMP）家族成员，不仅在正常组织中可检测到其表达，在多种类型的肿瘤中也发现其不同程

度的表达[16-17]。

我们的研究发现，miR-6775-3p能通过结合细胞周期蛋白依赖性蛋白激酶CDK4和CDK6，以及细胞侵袭转移标志物MMP17和MMP24的3'UTR，负向调控它们在乳腺癌细胞中mRNA表达和蛋白质水平，从而抑制乳腺癌细胞的增殖、迁移和侵袭能力。

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