·论 著·

miR-625-5p通过靶向调控PRKACA促进肺腺癌细胞的增殖和侵袭

胡雅琼[1]，白 俊[1]，陈 琳[1]，陈新璐[1]，张丽萍[1]，周丹丹[1]，王 玉[2]，尹崇高[3]，李洪利[4]，刘雨清[1]

1. 潍坊医学院病理学教研室，山东 潍坊 261053；
2. 潍坊医学院生物科学与技术学院，山东 潍坊 261053；
3. 潍坊医学院护理学院外科护理学教研室，山东 潍坊 261053；
4. 潍坊医学院医学研究实验中心，山东 潍坊 261053

［**摘要**］ **背景与目的：**肺腺癌是非小细胞肺癌的一种亚型，虽在诊断和治疗方面已经取得了很大进展，但晚期肺腺癌临床预后和总生存仍较差。近年来多项研究表明，miRNA在多种癌症中发挥作用，并在细胞增殖、转移、炎症等生物学过程中发挥重要作用。探究miR-625-5p对肺腺癌细胞增殖和侵袭能力的影响及分子机制，旨在为后续肺腺癌的诊断和治疗提供新的思路。**方法：**利用GEO数据库查找肺腺癌组织中差异表达的miRNA。采用实时荧光定量聚合酶链反应（real-time fluorescence quantitative polymerase chain reaction，RTFQ-PCR）检测miR-625-5p在各肺腺癌细胞系中的表达情况，采用EdU细胞增殖实验和transwell侵袭实验研究miR-625-5p对肺腺癌细胞增殖和侵袭能力的影响；通过生物信息学预测miR-625-5p靶向结合的关键基因；采用蛋白质印迹法（Western blot）检测对蛋白激酶cAMP激活催化亚基α（protein kinase cAMP-activated catalytic subunit alpha，PRKACA）在肺腺癌细胞中的表达情况进行验证，采用双荧光素酶报告基因实验和Western blot分析miR-625-5p与PRKACA之间的靶向关系。Western blot检测共转染敲低PRKACA质粒和敲低miR-625-5p质粒后各组细胞PRKACA的表达情况。采用EdU细胞增殖实验和transwell侵袭实验检测共转染以后各组肺腺癌细胞增殖和侵袭能力的改变。**结果：**miR-625-5p在肺腺癌组织（P<0.000 1）和各组肺腺癌细胞（P<0.000 1）中表达上调。EdU细胞增殖实验和transwell侵袭实验结果显示，miR-625-5p能够促进肺腺癌细胞的增殖（P=0.002 3）和侵袭（P=0.000 3）能力。双荧光素酶报告基因实验结果显示，miR-625-5p能与PRKACA靶向结合（P=0.000 8）。在肺腺癌细胞中，miR-625-5p与PRKACA的表达呈负相关（P<0.000 1）。miR-625-5p下调能够逆转敲低PRKACA对A549细胞增殖（P=0.011 9）和侵袭（P=0.001 5）能力的促进作用。**结论：**miR-625-5p在肺腺癌组织和细胞中表达上调，并通过负向调控PRKACA促进肺腺癌细胞增殖和侵袭。

［**关键词**］ miR-625-5p；蛋白激酶cAMP激活催化亚基α；增殖；侵袭；肺腺癌
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miR-625-5p promotes proliferation and invasion of lung adenocarcinoma by targeting PRKACA

HU Yaqiong[1], BAI Jun[1], CHEN Lin[1], CHEN Xinlu[1], ZHANG Liping[1], ZHOU Dandan[1], WANG Yu[2], YIN Chonggao[3], LI Hongli[4], LIU Yuqing[1] (1. Department of Pathology, Weifang Medical University, Weifang 261053, Shandong Province, China; 2. College of Biological Science and Technology, Weifang Medical University, Weifang 261053, Shandong Province, China; 3. Department of Surgical Nursing, College of Nursing, Weifang 261053, Shandong Province, China; 4. Medical Research Center, Weifang Medical University, Weifang 261053, Shandong Province, China)
Correspondence to: LIU Yuqing E-mail: 893326684@qq.com

［**Abstract**］ **Background and purpose:** Lung adenocarcinoma is a subtype of non-small cell lung cancer. Although much progress has been made in the diagnosis and treatment of lung adenocarcinoma, the clinical prognosis and overall survival of advanced lung

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通信作者：刘雨清 E-mail：893326684@qq.com

adenocarcinoma are still poor. In recent years, a number of studies have shown that miRNA can play a role in a variety of cancers, and play an important role in cell proliferation, metastasis, inflammation and other biological processes. This study aimed to explore the effect of miR-625-5p on the proliferation and invasion ability of lung adenocarcinoma cells and its molecular mechanism, so as to provide a new idea for the diagnosis and treatment of lung cancer. **Methods:** GEO database was used to search for differentially expressed miRNA in lung adenocarcinoma. Real-time fluorescence quantitative polymerase chain reaction (RTFQ-PCR) was used to detect the expression of miR-625-5p in various lung adenocarcinoma cell lines. The effects of miR-625-5p on the proliferation and invasion of lung adenocarcinoma cells were investigated by EdU cell proliferation assay and transwell invasion assay. Key genes of miR-625-5p targeted binding were predicted by bioinformatics. Western blot experiment validated the expression of protein kinase cAMP-activated catalytic subunit alpha (PRKACA) in lung adenocarcinoma cells. The targeted relationship between miR-625-5p and PRKACA was analyzed by double luciferase assay and Western blot. Western blot assay was used to detect the expression of PRKACA after co-transfection of si-PRKACA and si-miR-625-5p in each group. The effect of miR-625-5p on the proliferation and invasion ability of lung adenocarcinoma cells by targeting PRKACA was observed by EdU cell proliferation assay and transwell invasion assay. **Results:** The expression of miR-625-5p was up-regulated in lung adenocarcinoma tissues (P<0.000 1) and cells (P<0.000 1). The results of EdU cell proliferation assay and transwell invasion assay showed that miR-625-5p promoted the proliferation (P=0.002 3) and invasion (P=0.000 3) of lung adenocarcinoma cells. Double luciferase assay showed that miR-625-5p could target and bind to PRKACA (P=0.000 8). In lung adenocarcinoma cells, miR-625-5p was negatively correlated with PRKACA expression (P<0.000 1). Down-regulation of miR-625-5p reversed the promotion of the proliferation (P=0.011 9) and invasion (P=0.001 5) ability of A549 cells by knockout of *PRKACA*. **Conclusion:** MiR-625-5p is up-regulated in lung adenocarcinoma and promotes proliferation and invasion of lung adenocarcinoma tissues and cells by negatively regulating PRKACA.

［**Key words**］ miR-625-5p; Protein kinase cAMP-activated catalytic subunit alpha; Proliferation; Invasion; Lung adenocarcinoma

在过去的几十年里，肺癌已经成为全球常见的癌症之一[1]。而非小细胞肺癌发生率约占肺癌的85%，其中肺腺癌是非小细胞肺癌最常见的亚型[2]。癌症防治的进展使得近年来肺癌的死亡率大幅下降，但肺腺癌仍然是影响癌症死亡率的重要因素[3-4]。因此，研究探讨肺腺癌增殖和侵袭的分子机制，寻找靶向治疗肺腺癌的指标，对肺腺癌的治疗具有重要意义。

miRNA是一种约22 nt的非编码RNA。在癌症中，基因组的不稳定性、表观遗传修饰和转录因子的改变影响miRNA，从而在影响细胞发育、分化、增殖、生存和死亡的许多基因中起调控作用[5-6]。miRNA的失调与多种人类癌症有关，作为典型的癌基因或抑癌基因发挥作用，在癌症的发生、发展中起着重要作用[7]。前期相关研究[7-10]证明，miR-625-5p可以影响多种类型的癌症细胞的增殖和侵袭。但是，miR-625-5p在肺腺癌发展进程中产生的作用尚不清楚。

*PRKACA*基因位于人类第19号染色体p13.1，长约26 000个核苷酸，是环磷酸腺苷依赖性蛋白激酶A催化亚基的编码基因[11]。蛋白激酶A在细胞信号转导中第二信使cAMP的关键效应中起到核心作用，是为了应对由多种激素和神经递质引起的G蛋白偶联受体激活而产生的[12]。已有研究[13-14]证明，PRKACA在多种癌症的发生、发展中起到重要作用，但是PRKACA与miR-625-5p之间的关系及其影响肺腺癌发生、发展的分子机制仍不清楚，需要我们进行深入的研究与探索。

本研究旨在探索miR-625-5p对肺腺癌细胞增殖和侵袭能力的影响及分子机制，为后续肺腺癌的诊断和治疗提供新的思路。

1 材料和方法

1.1 主要试剂和仪器

人正常肺上皮细胞BEAS-2B，肺腺癌细胞系A549、H1299购自美国典型培养物保藏中心（American Type Culture Collection，ATCC），RPMI-1640培养基、DMEM培养基、胎牛血清、新生牛血清购自美国HyClone公司，F12-K培养基购自美国Sigma公司。β-actin（ab8226）、PRKACA（ab32376）抗体购自英国Abcam公司，Lipofectamine™2000购自美国Invitrogen公司，miR-625-5p敲低及对照质粒、蛋白激酶

cAMP激活催化亚基α（protein kinase cAMP-activated catalytic subunit alpha，PRKACA）基因敲低及对照质粒、荧光素酶报告基因载体均购自上海吉凯基因医学科技股份有限公司。

1.2 网上在线数据库

采用GEO数据库（https://www.ncbi.nlm.nih.gov/）查找肺腺癌组织与正常肺组织差异表达的miRNA，GSE74190数据集是包含36例肺腺癌组织和44例相邻正常组织的miRNA表达谱，运用GEO2R进行分析，以$\log_2$FC>1、P<0.05为筛选阈值，选出差异表达的上调miRNA。采用Starbase数据库（http://starbase.sysu.edu.cn/index.php）分析miR-625-5p在肺腺癌组织中的表达情况，查找与miR-625-5p相关的靶蛋白并分析*hub*基因在肺腺癌中的表达情况。

1.3 细胞培养及转染

所有细胞均按照ATCC提供的培养条件进行培养。细胞培养24 h后进行转染，饥饿细胞30 min，将对照质粒NC及敲低miR-625-5p质粒用Lipofectamine^{TM}2000转染细胞。将细胞分组：① si-NC/A549组，转染敲低miR-625-5p质粒的对照质粒的A549细胞；② si-miR-625-5p/A549组，转染敲低miR-625-5p质粒的A549细胞；③ si-control/A549组，转染敲低PRKACA质粒的对照质粒的A549细胞；④ si-PRKACA/A549组，转染敲低PRKACA质粒的A549细胞；⑤ si-PRKACA+si-NC/A549组，共转染敲低PRKACA质粒和敲低miR-625-5p质粒的对照质粒的A549细胞；⑥ si-PRKACA+si-miR-625-5p/A549组，共转染敲低PRKACA质粒和敲低miR-625-5p质粒的A549细胞。相关质粒由上海吉凯基因医学科技股份有限公司构建获得，miR-625-5p敲低质粒序列si-miR-625-5p：5’-GGACTATAGAACTTTCCCCCT-3’；miR-625-5p敲低对照质粒序列si-NC：5’-AGGUCTAAGUUCUAUGCACC-3’；PRKACA敲低质粒序列si-PRKACA：5’-GATAATCAGAGGGACAGAAAC-3’；PRKACA敲低对照质粒序列si-control：5’-TTCTCCGAACGTGTCACGT-3’。

1.4 实时荧光定量聚合酶链反应（real-time fluorescence quantitative polymerase chain reaction，RTFQ-PCR）检测

将各组生长良好的细胞培育24 h，TRIzol提取细胞总RNA，并合成cDNA，以cDNA为模板，采用*U6*作为内参，检测细胞中miR-625-5p表达量。RTFQ-PCR反应条件为95 ℃ 30 s，95 ℃ 5 s，65 ℃ 30 s，72 ℃ 30 s，循环35次。miR-625-5p上游引物为5’-CGCGAGGGGGAAAGTTCTA-3’，下游引物为5’-AGTGCAGGGTCCGAGGTATT-3’，茎环结构为5’-GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACGGACTA-3’。运用$2^{-\triangle\triangle Ct}$计算结果。

1.5 EdU细胞增殖实验

整个实验按照BeyoClick^{TM}EdU-594细胞增殖检测试剂盒说明书进行操作，于显微镜下随机选取5个视野拍照计数，计算结果。

1.6 Transwell侵袭实验

取转染后的细胞悬液200 μL，4×10^4个细胞，分别添加到铺有Matrigel基质胶的小室上室中，下室加入含有10%胎牛血清的培养液500 μL，培养24 h。24 h后用甲醇固定20 min，PBS清洗3次，Giemsa染液染色35 min，PBS清洗3次，吸弃PBS晾干后于显微镜下随机选取5个视野拍照计数，取平均值为最终结果。

1.7 蛋白质印迹法（Western blot）检测

将转染后的各组细胞裂解，收集悬液，提取总蛋白质，检测蛋白质浓度并进行SDS-PAGE、转膜、封闭、一抗4℃过夜、1×TBST洗膜、二抗常温温育1 h、显影、曝光，分析灰度值，计算结果。抗体配制如下：PRKACA抗体（1∶500），采用β-actin（1∶1 000）作为内参。

1.8 双荧光素酶报告基因实验

将A549细胞接种于24孔板中培养24 h，饥饿30 min后，将miR-625-5p过表达质粒及对照质粒和PRKACA的野生型质粒PRKACA-3’-UTR-Wt、突变型质粒PRKACA-3’-UTR-Mut共转染入A549细胞，培养48 h后，PLB裂解液裂解15 min提取悬液，测定荧光素酶活性，分析相对荧光强度，

计算结果。

1.9 统计学处理

每个实验重复3次，用SPSS 17.0软件对数据进行统计学分析，计量结果使用$\bar{x} \pm s$表示，两组间均数比较采用独立样本t检验，P<0.05为差异有统计学意义。

2 结 果

2.1 miR-625-5p在肺腺癌组织及细胞中表达上调

通过分析GSE74190数据集，筛选其中符合$\log_2$FC>1、P<0.05条件的miRNA，发现miR-625-5p的$\log_2$FC=1.93（P<0.000 1），在肺腺癌组织与正常肺组织中的表达差异明显（图1A），因此我们选择miR-625-5p为研究对象。运用Starbase数据库分析miR-625-5p的表达情况，发现在20例正常肺组织和512例肺腺癌组织中，miR-625-5p在肺腺癌组织中表达明显上调（图1B）。RTFQ-PCR显示，miR-625-5p在肺腺癌细胞A549、H1299中的表达（3.20 ± 0.12、2.18 ± 0.01）相比在BEAS-2B细胞中显著升高（图1C，P<0.000 1），结果表明，miR-625-5p在肺腺癌的发展中可能起着癌基因的作用。由于在A549细胞的表达差异更为明显，故采用A549细胞进行后续研究。

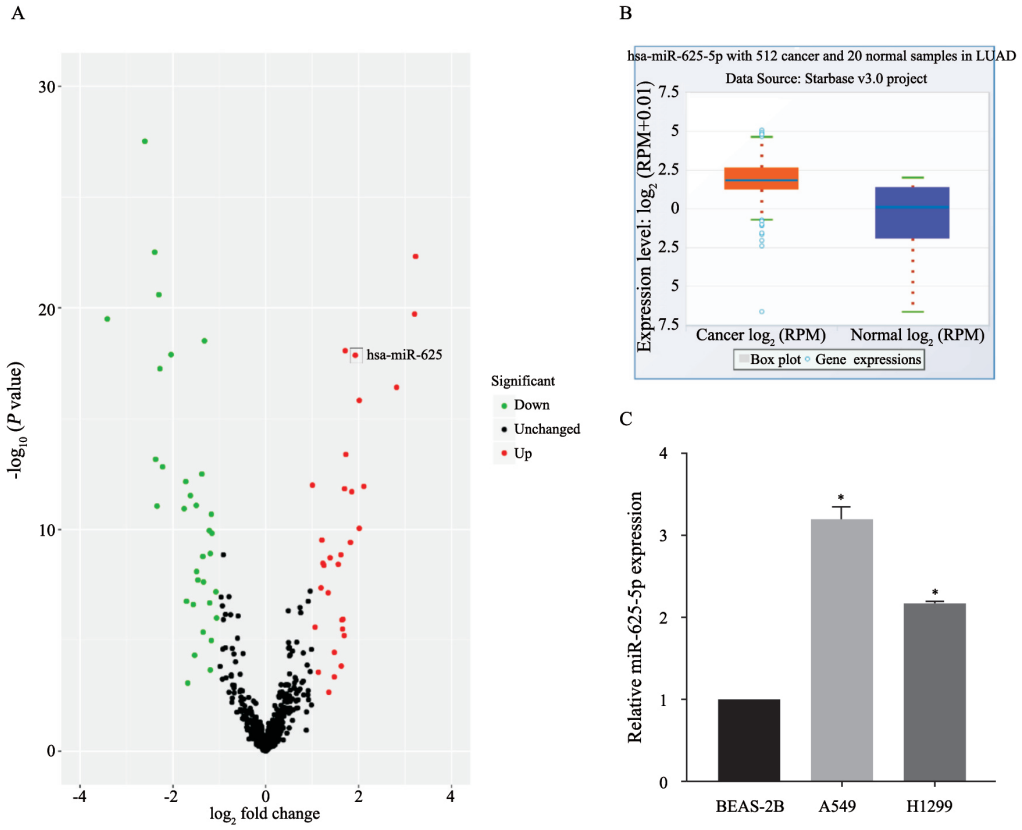

图 1 miR-625-5p在肺腺癌组织及细胞中的表达情况

Fig. 1 The expression of miR-625-5p in lung adenocarcinoma tissues and cells

A: The volcano map of GSE74190; B: The expression of miR-625-5p in lung adenocarcinoma tissues was analyzed by the Starbase database; C: The expression of miR-625-5p in various lung adenocarcinoma cells and BEAS-2B cells; *: P<0.05, compared with BEAS-2B cells

2.2 miR-625-5p促进肺腺癌细胞的增殖和侵袭

RTFQ-PCR检测结果显示，转染干扰质粒后的A549细胞中miR-625-5p表达（0.30 ± 0.05）明显下调（图2A，P<0.000 1），提示转染成功。EdU细胞增殖实验结果显示，si-miR-625-5p/A549组的EdU细胞增殖比为0.30 ± 0.03，与si-NC/A549组（0.60 ± 0.05）相比显著降低，证明

下调miR-625-5p对A549细胞具有增殖抑制作用（图2B，P=0.002 3）。Transwell侵袭实验结果显示，与si-NC/A549组的细胞数（348.00 ± 7.26）相比，si-miR-625-5p/A549组穿膜的细胞数（237.67 ± 10.62）明显减少（图2C，P=0.000 3），证明敲低miR-625-5p后，A549细胞侵袭能力降低。以上结果表明，miR-625-5p能够促进A549细胞的增殖和侵袭能力。

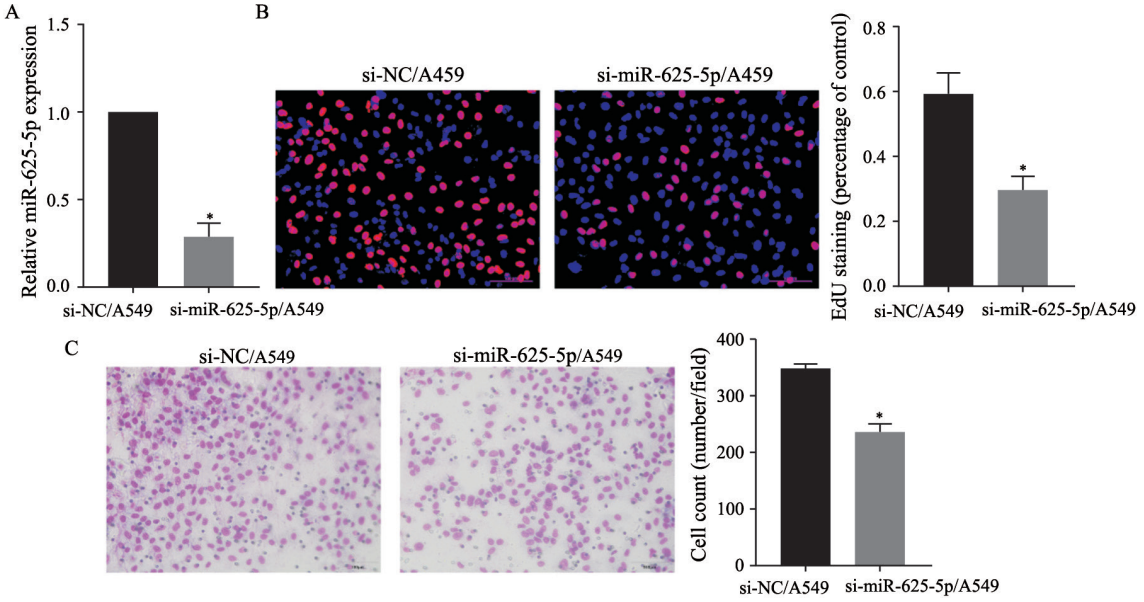

图 2　miR–625–5p促进肺腺癌细胞增殖和侵袭

Fig. 2　miR-625-5p promoted the proliferation and invasion of lung adenocarcinoma cells

A: RTFQ-PCR assay detected the transfection efficiency of miR-625-5p interfering plasmid; B: EdU assay was used to detect the proliferation ability of different groups of cells (× 20); C: Knockdown of miR-625-5p inhibited the invasion of lung adenocarcinoma cells (× 20); *: P<0.05, compared with each other, scale bar=100 μm

2.3　miR–625–5p靶向结合PRKACA

运用Starbase数据库预测与miR-625-5p结合相关的靶基因，使用Cytoscape 3.6.1软件对相关靶基因进行分析，根据cytoHubba计算各节点degree得分筛选*hub*基因并构建蛋白互作网络图（图3A）。Western blot检测结果显示，肺腺癌细胞A549中PRKACA的表达（0.47 ± 0.04）明显低于正常肺上皮细胞BEAS-2B（1.00 ± 0.05，图3B，P=0.000 4），证明PRKACA在肺腺癌细胞中表达下调。因此，我们考虑*PRKACA*可能为miR-625-5p靶向结合的关键基因。Western blot检测结果显示，si-miR-625-5p/A549组中PRKACA的表达（2.40 ± 0.09）明显高于si-NC/A549组（1.00 ± 0.06，图3C，P<0.000 1），证实miR-625-5p与PRKACA在A549细胞中表达呈负相关。双荧光素酶报告基因实验结果显示，PRKACA-3'-UTR-Wt/miR-625-5p组A549细胞的相对荧光素酶活性（0.48±0.06）明显低于PRKACA-3'-UTR-Wt/miR-NC组（1.00 ± 0.05，图3D，P=0.000 8），而PRKACA-3'-UTR-Mut/miR-625-5p组和PRKACA-3'-UTR-Mut/miR-NC组之间差异无统计学意义（P=0.927 9）。该结果表明，PRKACA-3'-UTR区是miR-625-5p的结合位点。以上结果表明，miR-625-5p通过靶向结合PRKACA负向调控肺腺癌细胞。

2.4　miR–625–5p靶向调控PRKACA促进肺腺癌细胞的增殖

Western blot检测结果显示，与si-control/A549组相比，si-PRKACA/A549组PRKACA的表达明显下调（P=0.005 3），而与si-PRKACA+si-NC/A549组相比，si-PRKACA+si-miR-625-5p/A549组PRKACA的表达显著上调（P=0.001 5），结果证实，敲低miR-625-5p能抑制PRKACA干扰质粒对A549细胞PRKACA表达的抑制作用（图4A）。EdU细胞增殖实验检测发现，si-PRKACA/A549组EdU细胞增殖（0.52 ± 0.04）比si-control/A549组（0.37 ± 0.02）明显增多（P=0.009 7），而

si-PRKACA+si-miR-625-5p/A549组EdU细胞增殖（0.36 ± 0.02）比si-PRKACA+si-NC/A549组（0.46 ± 0.03）明显减少（图4B，P=0.011 9）。结果表明，PRKACA下调能够促进A549细胞增殖，而miR-625-5p下调能够逆转敲低PRKACA对肺腺癌细胞A549增殖能力的促进作用。由此可见，miR-625-5p负向调控PRKACA促进肺腺癌细胞增殖。

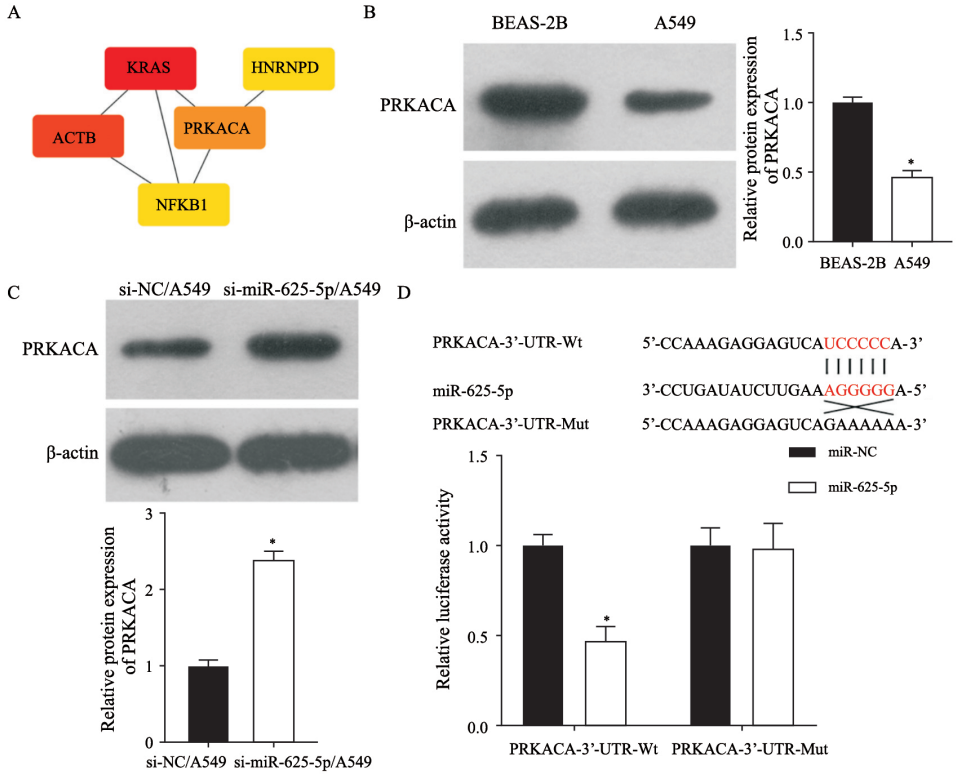

图 3 miR–625–5p靶向结合PRKACA

Fig. 3 miR-625-5p targeted PRKACA

A: The protein interaction network of top 5 target genes associated with miR-625-5p; B: Western blot analysis showed the expression of PRKACA in A549 cells and BEAS-2B cells; C: The expression of PRKACA in different groups of cells; D: Luciferase activity was detected in different groups of cells. *: P<0.05, compared with each other

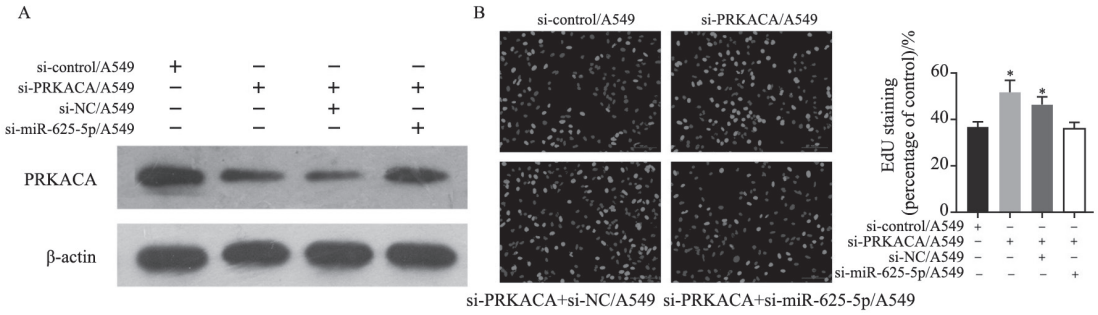

图 4 miR–625–5p靶向调控PRKACA促进肺腺癌细胞的增殖

Fig. 4 miR-625-5p promoted the proliferation of lung adenocarcinoma cells by targeting PRKACA

A: Western blot analysis showed the expression of PRKACA in different groups of cells; B: The effect of miR-625-5p and PRKACA co-transfection on the proliferation ability of lung adenocarcinoma cells (× 20); *: P<0.05, compared with each other, scale bar=100 μm

2.5 miR–625–5p靶向调控PRKACA促进肺腺癌细胞的侵袭

通过transwell侵袭实验检测miR-625-5p通过靶向结合PRKACA对肺腺癌的侵袭能力的影响，结果显示，与si-control/A549组（265.33 ± 16.21）相比，si-PRKACA/A549组穿膜的细胞数（345.00 ± 17.28）明显增多（P=0.004 7），而与si-PRKACA+si-

NC/A549组（339.67±10.78）相比，si-PRKACA+si-miR-625-5p/A549组穿膜的细胞数（233.67±0.04）明显减少（图5，*P*=0.001 5），证实下调PRKACA对A549细胞具有侵袭促进作用，而miR-625-5p下调则可以逆转敲低PRKACA对A549细胞侵袭能力的影响。由此可见，miR-625-5p通过负向调控PRKACA促进肺腺癌细胞侵袭。

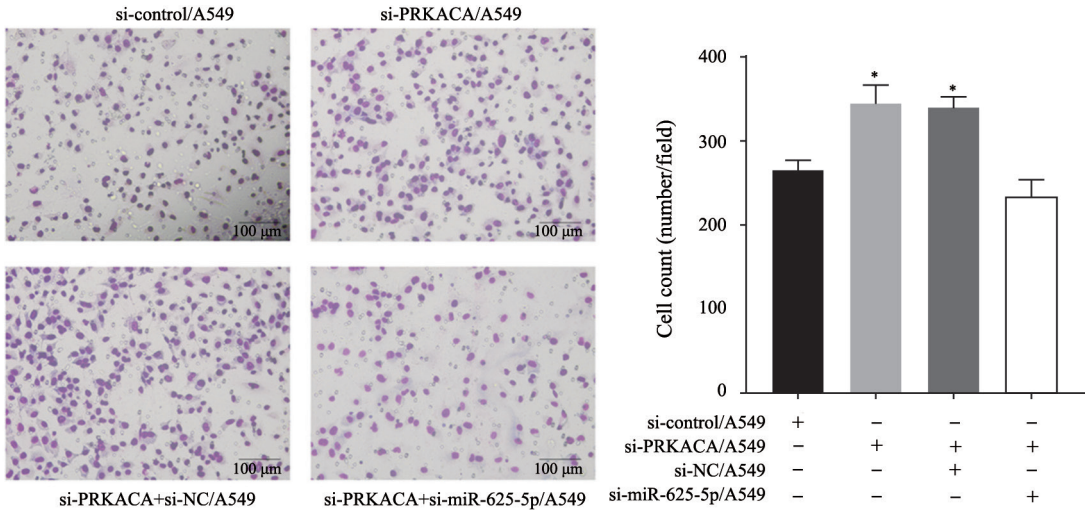

图 5　miR–625–5p靶向调控PRKACA促进肺腺癌细胞侵袭、迁移

Fig. 5　miR-625-5p promoted the invasion and migration of lung adenocarcinoma cells by targeting PRKACA *vs* si-control (×20)

*: *P*<0.05, compared with each other, scale bar=100 μm

3　讨　论

大量研究表明，miRNA控制多种生物学功能，如细胞增殖、分化和凋亡[15-17]。miRNA是可以在外周血中发现和测量的稳定分子，显示出作为检测、分类、预后的生物标志物的潜力[18]。miR-625-5p已被证实与癌症密切相关，能够抑制宫颈癌和胶质瘤细胞增殖、迁移和侵袭[19-20]。本研究通过GEO数据库确定miR-625-5p为研究对象。上述实验表明，miR-625-5p在肺腺癌细胞中表达升高并促进肺腺癌的增殖与侵袭能力，证实miR-625-5p能促进肺腺癌细胞A549增殖和侵袭，是参与肺腺癌发生、发展的关键分子。

PRKACA是蛋白激酶催化亚单位的一种主要的亚型，在大多数组织中表达[11]。许多研究[14, 21-22]表明，PRKACA可在某种程度上影响癌症的发展。由此我们可以推断PRKACA可用作癌症的生物标志物及预后预测工具[23]。近年来已经进行了大量涉及miRNA治疗剂的临床前研究，但到目前为止，只有少数miRNA治疗剂进入了临床开发。开发基于miRNA的治疗药物的最大挑战之一是为每种疾病类型确定最佳的miRNA靶标。本研究通过生物信息学预测与miR-625-5p结合的相关靶基因。双荧光素酶报告基因实验显示，PRKACA与miR-625-5p靶向结合，并通过实验证明miR-625-5p可通过靶向调控PRKACA促进肺腺癌的增殖和侵袭，提示*PRKACA*基因为miR-625-5p靶向结合的关键基因。前期相关研究表明，miR-625可以通过靶向HOXB5激活Wnt/β-catenin途径来抑制非小细胞肺癌的进展[24]。本研究中，我们了解到miR-625-5p影响肺腺癌增殖和侵袭的分子机制，但是miR-625-5p靶向PRKACA究竟通过哪种信号转导通路影响肺腺癌的发生、发展过程尚未可知。为了更加深入地了解miR-625-5p对肺腺癌发展进程的影响，对信号转导通路的研究将成为我们后续研究的重点。

综上所述，miR-625-5p在肺腺癌中表达上调，通过负向调控PRKACA促进肺腺癌的增殖和侵袭。

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