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·临床研究·

lncRNA HOTAIR通过miR-519d-3p/CCND1分子轴促进乳腺癌SKBR3细胞的恶性生物学行为

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[摘 要] **目的**:探究lncRNA HOTAIR/miR-519d-3p/CCND1分子轴对乳腺癌细胞增殖和转移的影响及其可能机制。**方法**:收集2017年3月至2019年2月南昌市第三医院乳腺外科手术切除的乳腺癌患者的乳腺癌组织及配对癌旁组织各50例,qPCR检测癌及癌旁组织中HOTAIR的表达水平,乳腺正常上皮细胞及乳腺癌细胞系中HOTAIR和miR-519d-3p的表达水平。将乳腺癌SKBR3细胞分为NC组、si-HOTAIR组、miR-519d-3p mimics组,miR-519d-3p mimics+pcHOTAIR组、miR-519d-3p mimics+pcCCND1组和si-HOTAIR+pcCCND1组,CCK-8法检测各组SKBR3细胞增殖能力、Transwell检测细胞侵袭和迁移能力、Western blotting检测SKBR3细胞中E-cadherin、N-cadherin、Vimentin以及CCND1表达水平。双荧光素酶报告基因检测HOTAIR和miR-519d-3p以及miR-519d-3p和CCND1的靶向关系。**结果**:HOTAIR在癌组织以及乳腺癌细胞系中呈高表达,且在SKBR3细胞系中表达最高。敲降HOTAIR可显著抑制SKBR3细胞增殖、侵袭和迁移,并显著增加E-cadherin的表达水平、降低N-cadherin和Vimentin的表达水平(均$P<0.05$)。双荧光素酶报告基因检测显示,HOTAIR靶向下调miR-519d-3p的表达,miR-519d-3p靶向下调CCND1的表达。敲降HOTAIR可增强miR-519d-3p对CCND1的下调作用,抑制SKBR3细胞EMT、增殖、侵袭和迁移能力(均$P<0.05$)。**结论**:敲降HOTAIR可抑制SKBR3细胞增殖和转移,其机制是通过调控miR-519d-3p/CCND1分子轴实现的。

[关键词] 乳腺癌;SKBR3细胞;lncRNA HOTAIR;miR-519d-3p;细胞周期蛋白D1;增殖;迁移;侵袭
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lncRNA HOTAIR promotes malignant biological behaviors of breast cancer SKBR3 cells through miR-519d-3p/CCND1 axis

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[Abstract] Objective: To explore the effect of lncRNA HOTAIR/miR-519d-3p/cyclin D1 (CCND1) axis on the proliferation and metastasis of breast cancer cells and its underlying mechanism. **Methods:** A total of 50 pairs of breast cancer tissues and corresponding para-cancer tissues resected from breast cancer patients in the Department of Breast Surgery, the Third Hospital of Nanchang from March 2017 to February 2019 were collected for this study. The expression level of HOTAIR in breast cancer tissues and paired para-cancer tissues was detected by qPCR, in addition, the expressions of HOTAIR and miR-519d-3p in normal breast epithelial cells and breast cancer cell lines were also detected. Breast cancer SKBR3 cells were divided into NC group (without any treatment), si-HOTAIR group, mir-519d-3p mimics group, miR-519d-3p mimic+pcHOTAIR group, miR-519d-3p mimic+pcCCND1 group, and si-HOTAIR+pcCCND1 group. The proliferation ability of SKBR3 cells was detected by CCK-8. Invasion and migration of SKBR3 cells were detected by Transwell. The expression levels of E-cadherin, N-cadherin, Vimentin and CCND1 in SKBR3 cells were detected by Western blotting. The targeting relationship between HOTAIR and miR-519d-3p, miR-519d-3p and CCND1 was detected by Dual-luciferase reporter gene system. **Results:** HOTAIR was highly expressed in breast cancer tissues and cell lines, with the highest expression in SKBR3 cells. HOTAIR knockdown significantly inhibited the proliferation, invasion and migration of SKBR3 cells, as well as increased the expression level of E-cadherin and decreased the expression levels of N-cadherin and Vimentin. Dual-luciferase reporter gene assay showed that HOTAIR targetedly down-regulated the expression of miR-519d-3p, and miR-519d-3p targetedly down-regulated the expression of CCND1. Further studies showed that knockout of HOTAIR inhibited the EMT, proliferation, invasion and

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migration of SKBR3 cells through enhancing the inhibitory effect of miR-519d-3p on CCND1 expression (all P<0.05). **Conclusion:** HOTAIR knockdown inhibits proliferation and metastasis of SKBR3 cells by regulating the axis of miR-519d-3p/CCND1.

[Key words] breast cancer; SKBR3 cell; lncRNA HOTAIR; miR-519d-3p; cyclin D1 (CCND1); proliferation; migration; invasion

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乳腺癌是世界范围内女性最常见的恶性肿瘤之一，全球每年有约45万人死于乳腺癌，占女性癌症相关死亡的13.7%，对女性健康造成很大威胁[1-2]。研究[3-5]发现，长链非编码RNA（lncRNA）的异常表达在多种人类癌症中发挥重要作用，其表达失调与乳腺癌的发生发展有关[6]。HOTAIR是一种lncRNA，参与乳腺癌[7]、结肠癌[8]、前列腺癌[9]等癌症的发展。miR-519d-3p是HOTAIR的候选靶基因，有研究[10-11]表明，miR-519d-3p在一些癌细胞及组织中表达异常，可调控肿瘤细胞的生物学行为。细胞周期蛋白D1（cyclin D1，CCND1）为miR-519d-3p的候选靶基因，其在乳腺癌[13]、胶质瘤[14]等癌症细胞中异常高表达，而CCND1过表达或CCND1下游通路异常激活可直接促进肿瘤生长[12]。本文旨在研究lncRNA HOTAIR通过对miR-519d-3p/CCND1分子轴的调控影响乳腺癌细胞增殖和转移及其可能的机制。

1 资料与方法

1.1 临床样本收集

收集2017年3月至2019年2月南昌市第三医院乳腺外科手术切除的乳腺癌组织及配对癌旁组织（距离癌灶边缘5 cm）各50例，患者年龄在31~78岁之间，28例肿瘤直径≤2 cm，其余均>2 cm；根据TNM分期，I期15例、II期26例、III期9例；有淋巴结转移的31例，无淋巴结转移的19例。标本在液氮中存储。纳入标准：既往没有使用抗癌药物或放疗的治疗史；经病理确诊为乳腺癌。排除标准：曾接受抗肿瘤治疗；合并其他恶性肿瘤。研究方案经医院伦理委员会审查并批准，所有患者签署知情同意书。

1.2 细胞株与主要试剂

人正常乳腺上皮细胞MCF10A（货号：CL-0525）和乳腺癌细胞株MCF-7（货号：CL-0149）、ZR-75-30（货号：CL-0248）、SKBR3（货号：CL-0211）均购自武汉普诺赛生命科技有限公司。DMEM培养基、胎牛血清（FBS）、青霉素-链霉素预混液（P/S）购自Thermo Fisher Scientific公司，TRIzol试剂盒购自武汉默沙克生物科技有限公司，Primescript RT试剂盒购自北京智杰远科技有限公司，CCK-8试剂盒购自GLPBIO公司，Lipofectamine 2000转染试剂盒购自上海吉玛制药技术有限公司，Transwell小室购自北京明阳科华生物科技有限公司，细胞总蛋白提取试剂盒和Western blotting（WB）所用一抗（E-cadherin、N-cadherin、Vimentin、CCND1的单抗，均1∶2 000稀释）和二抗（1∶5 000稀释）均购自上海邦奕生物科技有限公司。si-HOTAIR、miR-519d-3p mimics、pcHOTAIR、pcCCND1均由上海吉玛基因公司设计并合成。

1.3 细胞培养及转染

将正常乳腺上皮细胞（MCF10A）和其他常见乳腺癌细胞系（MCF-7、ZR-75-30、SKBR3）接种于DMEM培养基（含10% FBS+1% P/S），置于37 ℃、5% CO_2培养箱中培养。取处于对数生长期的SKBR3细胞，用胰蛋白酶消化后，将细胞以1×10^{5}个/ml的密度接种于6孔板，按照Lipofectamine 2000转染试剂说明书将si-HOTAIR、miR-519d-3p mimics、HOTAIR+miR-519d-3p mimics、miR-519d-3p mimics+CCND1、si-HOTAIR+CCND1转染于SKBR3细胞中，于37 ℃、5% CO_2培养箱中培养培养48 h待用。

1.4 qPCR检测乳腺癌组织或细胞中HOTAIR、miR-519d-3p和CCND1的表达

采用TRIzol一步法分别提取待测组织和细胞中总RNA，用Primescript RT试剂逆转录成互补脱氧核糖核酸（cDNA），取2 μl逆转录产物为模板行qPCR，预变性95 ℃、5 min，94 ℃变性30 s、60 ℃退火30 s、72 ℃延伸1 min，循环50次后进行终延伸完成PCR，采用$2^{-\Delta\Delta Ct}$法计算目的基因（HOTAIR、miR-519d-3p和CCND1）表达量。以U6和GAPDH为内参照，引物序列见表1，实验重复3次。

表1 qPCR引物序列

Tab.1 Primer sequences of qPCR

Gene	Sequence
U6	F: 5'-TGCGGGTGCTCGCTTCGGCAGC-3'
	R: 5'-CCAGTGCAGGGTCCGAGGT-3'
GAPDH	F: 5'-CGCTCTCTGCTCCTCCTGTTC-3'
	R: 5'-ATCCGTTGACTCCGACCTTCAC-3'
HOTAIR	F: 5'-GGGTGGCTCACTCTTCTGGC-3'
	R: 5'-TGGCCTTGCCCGGGCTTGTC-3'
miR-519d-3p	F: 5'-TGCGGCAAAGTGCCTCCCTTTAG-3'
	R: 5'-CCAGTGCAGGGTCCGAGGT-3'
CCND1	F: 5'-ACCTGAGGAGCCCCAACAA-3'
	R: 5'-TCTGCTCCTGGCAGGCC-3'

1.5 CCK-8法检测lncRNA HOTAIR/miR-519d-3p/CCND1轴对SKBR3细胞增殖的影响

收集各转染组处于对数生长期的待测细胞，以

未经转染的细胞为对照，用胰蛋白酶消化后以1×10^{5}个/ml的密度接种至96孔板，置于37 ℃、5% CO_2培养箱中培养24 h。分别在培养0、1、2、3、4 d时向每孔加入10 μl CCK-8溶液，相同条件下继续培养2 h，用酶标仪测定在450 nm处的光密度（*D*）值，以*D*值代表细胞增殖水平。

1.6　Transwell实验检测lncRNA HOTAIR/miR-519d-3p/CCND1轴对SKBR3细胞侵袭和迁移能力的影响

将Matrigel胶置于4 ℃冰箱融化，用无血清DMEM培养基以1∶8的比例稀释，铺于Transwell上室，37 ℃放置4 h使Matrigel聚合成凝胶，用于细胞侵袭实验。迁移实验不用预铺Matrigel胶。将密度为2×10^{5}个/ml的细胞悬液100 μl分别接种于铺有基质胶和未铺基质胶的Transwell上室，加100 μl胎牛血清培养基，下室加入500 μl含10%胎牛血清的DMEM培养液。将细胞置于37℃、5% CO_2培养箱中培养24 h后取出，PBS漂洗3遍，用棉签去除上表面细胞，5%戊二醛固定30 min，用0.1%结晶紫染色30 min。最后用40倍倒置显微镜观察拍照和计数。

1.7　双荧光素酶报告基因检测HOTAIR与miR-519d-3p、miR-519d-3p以及CCND1之间的靶向关系

使用StarBase预测HOTAIR和miR-519d-3p以及miR-519d-3p和CCND1的结合位点，扩增HOTAIR和CCND1基因片段，插入荧光素酶报告基因PmirGLO中，构建HOTAIR和CCND1野生型质粒（PmirGLO-HOTAIR-Wt、PmirGLO-CCND1-Wt），利用基因突变技术构建HOTAIR和CCND1突变型质粒（PmirGLO-HOTAIR-Mut、PmirGLO-CCND1-Mut），将miR-519d-3p mimics和HOTAIR或CCND1的突变型或野生型质粒转染至SKBR3细胞中，置于37 ℃、5% CO_2培养箱中培养48 h，用双荧光素酶报告基因试剂盒检测各组荧光素酶活性。

1.8　WB检测lncRNA HOTAIR/miR-519d-3p/CCND1轴对SKBR3细胞中CCND、E-cadherin、N-cadherin和Vimentin表达水平的影响

提取各组细胞总蛋白，BCA法测定蛋白浓度。每组取50 μg进行SDS-PAGE分离蛋白条带，用电转移法将目的蛋白转移至PVDF膜上，5%脱脂奶粉封闭1 h，加入一抗，4 ℃孵育过夜；次日，TBST洗涤3次，加入HRP标记的二抗，室温孵育2 h；TBST洗涤3次后加入ECL化学发光液进行显影，然后在凝胶成像仪上观察拍照。用Image J灰度分析软件分析灰度值，计算目的蛋白相对表达量。

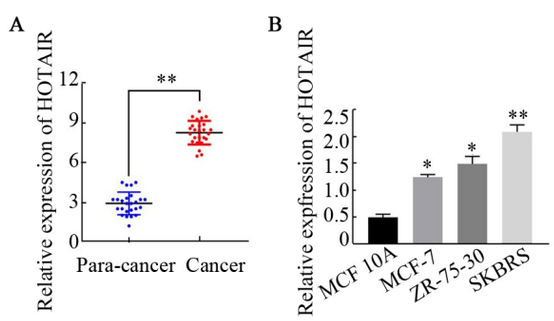
1.9　统计学处理

使用SPASS 20进行实验数据分析，采用GraphPad 7.0软件进行相关图片绘制。所有实验独立重复3次，呈正态分布的计量数据采用$\bar{x}\pm s$表示，两组间比较采用*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　HOTAIR在乳腺癌组织及细胞中呈高表达

采用qPCR分析50例乳腺癌组织及配对癌旁组织中HOTAIR的表达，结果（图1A）显示，HOTAIR在乳腺癌组织中表达显著高于配对的癌旁组织（*P*<0.01）。qPCR检测人正常乳腺上皮细胞MCF10A和其他常见乳腺癌细胞系（MCF-7、ZR-75-30、SKBR3）中HOTAIR的表达水平，结果（图1B）显示，HOTAIR在乳腺癌细胞中表达显著高于乳腺上皮细胞（*P*<0.05或*P*<0.01），且在SKBR3细胞系中表达最高，故选用SKBR3细胞进行后续实验。



*P<0.05, **P<0.01 *vs* Para-cancer tissues group or MCF10A cells group

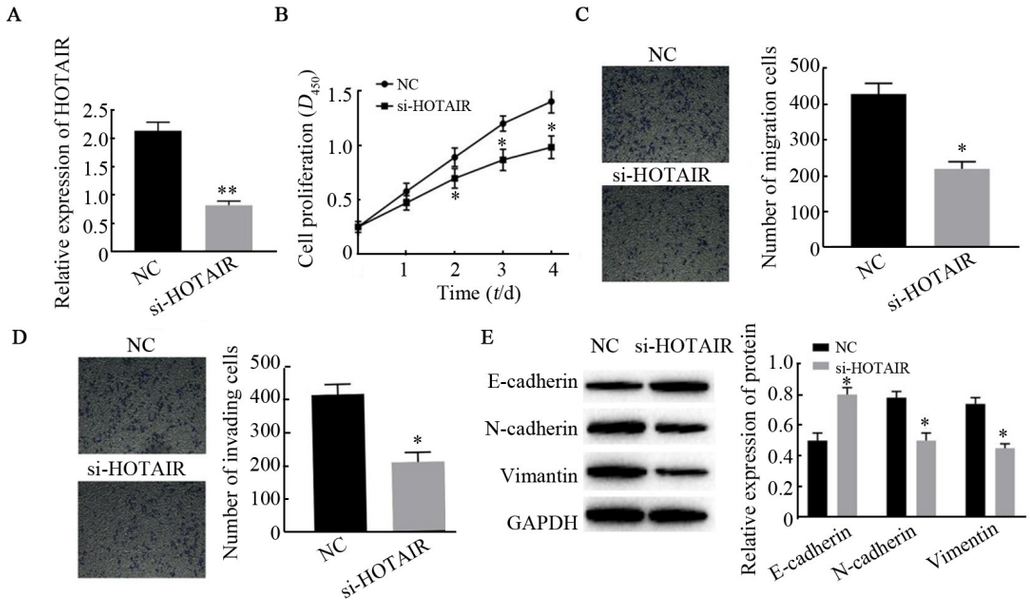
A: qPCR detected the expression of HOTAIR in breast cancer tissues and paired para-cancer tissues; B: qPCR detected the expression of HOTAIR in mammary epithelial cells breast cancer cell lines

图1　HOTAIR在乳腺癌组织和细胞中高表达

Fig.1　High expression of HOTAIR in breast cancer tissues and cell lines

2.2　敲降HOTAIR抑制SKBR3细胞的增殖、侵袭和迁移

qPCR检测结果（图2A）显示，与NC组相比，si-HOTAIR组SKBR3细胞中HOTAIR的表达显著降低（*P*<0.01）。CCK-8检测结果（图2B）显示，相比于NC组，si-HOTAIR组SKBR3细胞增殖能力显著降低（*P*<0.05）。Transwell实验检测结果（图2C、D）显示，si-HOTAIR组SKBR3细胞侵袭和迁移的穿膜细胞数均显著低于NC组（均*P*<0.05）。WB检测结果（图2E）显示，si-HOTAIR组SKBR3细胞中E-cadherin的表达水平显著增加（*P*<0.05），N-cadherin和Vimentin的表达水平显著降低（均*P*<0.05）。



*P<0.05, **P<0.01 *vs* NC group

A: qPCR was used to detect the transfection efficiency of si-HOTAIR; B: The proliferation activity of SKBR3 cells was detected by CCK-8; C and D: Number of migration and invasion SKBR3 cells were detected by Transwell assay (×40); E: WB detected the expression levels of E-cadherin, N-cadherin and Vimentin

图2 敲降HOTAIR抑制SKBR3细胞增殖、迁移和侵袭

Fig.2 HOTAIR knockout inhibited proliferation, migration and invasion of SKBR3 cells

2.3 HOTAIR调控miR-519d-3p/CCND1分子轴

通过生物学信息库StarBase 2.0预测，结果（图3A）显示miR-519d-3p可能是HOTAIR的靶基因。双荧光素酶报告基因检测miR-519d-3p和HOTAIR的靶向关系，结果（图3B）显示，转染miR-519d-3p mimics显著降低HOTAIR野生型载体的的荧光素酶活性（P<0.05），而对HOTAIR突变型载体荧光素酶活性无明显影响。qPCR检测HOTAIR对miR-519d-3p的调控结果（图3C）显示，敲降HOTAIR可显著增加miR-519d-3p的表达水平（P<0.05）。由此可知，HOTAIR靶向负调控miR-519d-3p。

进一步通过生物学信息库StarBase 2.0预测miR-519d-3p的靶基因，发现CCND1是miR-519d-3p的候选靶基因，预测序列如图3D所示。双荧光素酶报告基因检测miR-519d-3p和CCND1的靶向关系，结果（图3E）显示，转染miR-519d-3p mimics显著降低CCND1野生型载体的荧光素酶活性（P<0.05），而对CCND1突变型载体荧光素酶活性无明显影响。WB检测结果（图3F）显示，转染miR-519d-3p mimics可显著降低CCND1的表达水平（P<0.05）。由此可知，miR-519d-3p靶向负调控CCND1。

2.4 HOTAIR通过调控miR-519d-3p/CCND1分子轴影响SKBR3细胞恶性生物学行为

WB检测结果（图4A）显示，miR-519d-3p mimics组CCND1表达水平较NC组显著降低（P<0.05），而回复实验组（miR-519d-3p mimic+pcHOTAIR共转染组、miR-519d-3p mimic+pcCCND1共转染组、si-HOTAIR+pcCCND1共转染组）中CCND1表达水平较miR-519d-3p mimics组显著升高（P<0.05），且与NC组无显著差异。CCK-8（图4B）和Transwell（图4C、D）检测结果显示，miR-519d-3p mimics组SKBR3细胞增殖（P<0.05）、侵袭（P<0.05）和迁移（P<0.05）较NC组受到显著抑制；而回复实验组中SKBR3细胞增殖（P<0.05）、迁移（P<0.05）和侵袭（P<0.05）能力较miR-519d-3p mimics组显著上调，且与NC组无明显差异。WB检测结果（图4E）显示，与NC组相比，miR-519d-3p mimics组E-cadherin表达水平显著增加（均P<0.05），N-cadherin和Vimentin的表达水平显著降低（均P<0.05）；而回复实验的各组中E-cadherin表达较miR-519d-3p mimics组显著降低（均P<0.05），N-cadherin和Vimentin的表达水平显著增加（均P<0.05），且均与NC组无显著差异。

3 讨 论

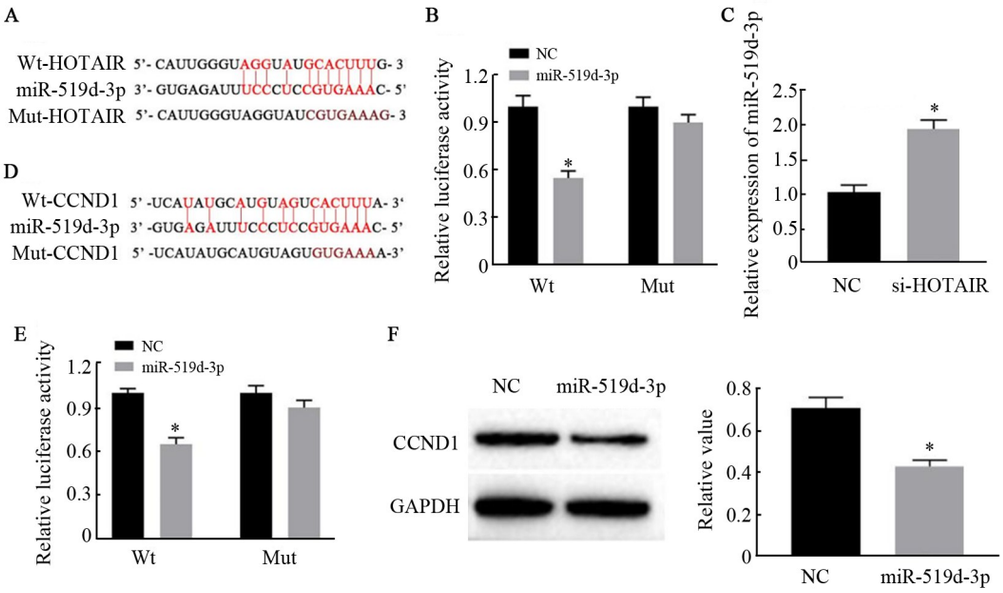
研究[15-16]发现，lncRNA参与并影响多种疾病的发展进程。例如，结直肠癌细胞系中lncRNA FGD5-

AS1表达明显高于正常细胞系，敲降FGD5-AS1可抑制结直肠癌细胞增殖、迁移、侵袭，并加速细胞凋亡[17]；lncRNA SPRY4-IT1在胃癌细胞及胃癌患者血清外泌体中表达上调，敲降SPRY4-IT1可通过引起G1停滞而抑制细胞增殖和促进细胞凋亡[18]。同时，HOTAIR已被证实与各种人类恶性肿瘤的细胞凋亡和增殖有关[19]。研究[20]发现，lncRNA HOTAIR在胃癌组织和细胞中表达上调，过表达HOTAIR可促进胃癌细胞增殖。本研究发现，HOTAIR在乳腺癌细胞及组织中表达上调，敲降HOTAIR后可抑制乳腺细胞增殖、侵袭和迁移能力，同时也抑制EMT过程。

研究[21]证明lncRNA具有内源性竞争性RNA（ceRNAs）的功能，并通过与常见的miRNA竞争性结合而与miRNA相互作用，从而调控细胞功能。例如，lncRNA MT1JP可通过靶向调控miRNA-423-3p抑制子宫内膜癌细胞侵袭能力和伤口愈合率[21]；miR-519d-3p作为癌相关靶基因在胃癌、卵巢癌及乳腺癌中表达水平下调，且调节细胞增殖、肿瘤生长和上皮间充质转化[22]。本研究发现HOTAIR与miR-519d-3p竞争性结合而控制miR-519d-3p的表达水平，从而影响乳腺癌细胞的生物学功能。

由于CCND1基因染色体异位导致在多种肿瘤中被扩增或过度表达，而CCND1是细胞周期蛋白家族中的一员，可通过结合和激活CDK4和CDK6激酶的功能转变G1/S细胞周期，进而影响肿瘤细胞生物学功能[23-25]。本研究表明，miR-519d-3p靶向下调CCND1的表达，从而抑制乳腺癌细胞恶性生物学行为。

综上所述，HOTAIR能够通过内源性竞争结合miR-519d-3p，降低miR-519d-3p的表达水平，上调CCND1表达，以此促进乳腺癌细胞增殖和转移。本研究初步探索了HOTAIR影响乳腺癌细胞恶行生物学行为的机制，为乳腺癌的生物治疗找到了潜在的靶点分子。

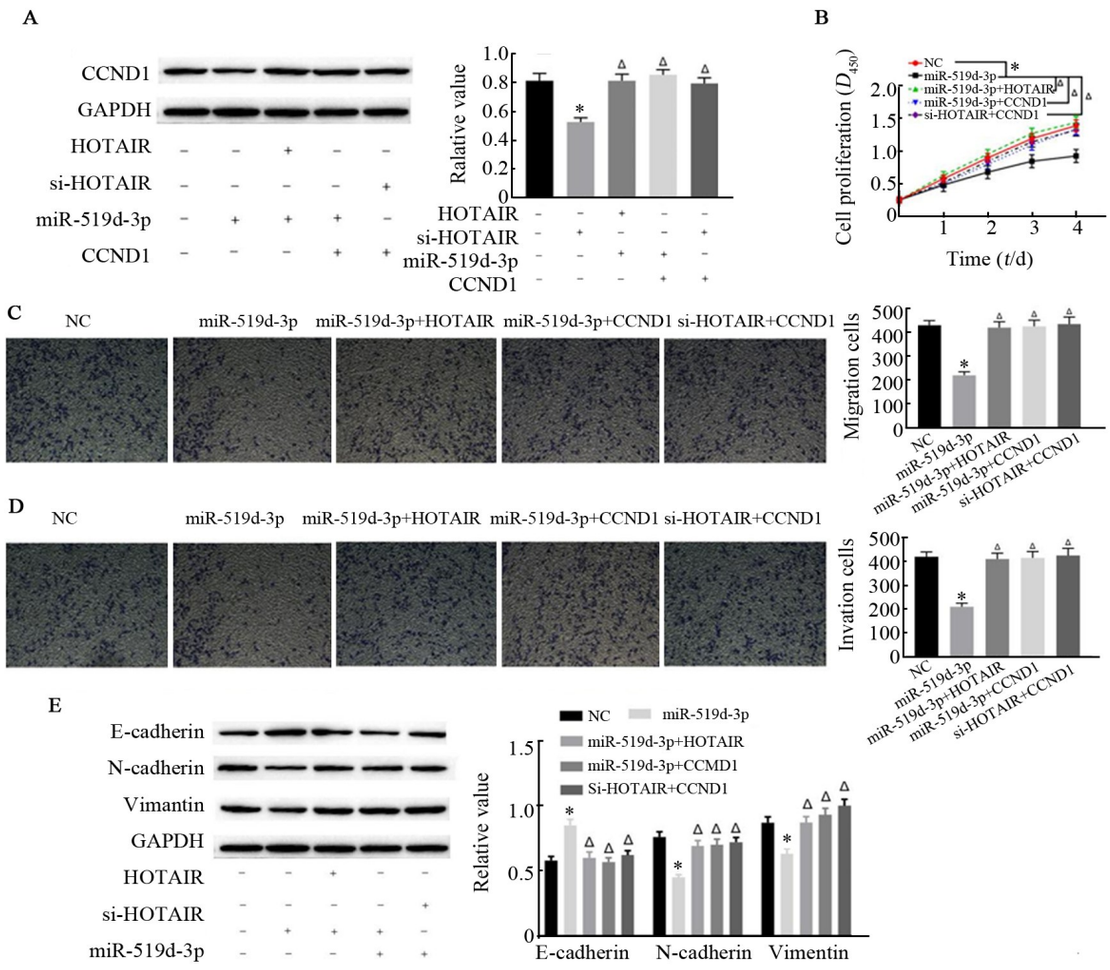


*P<0.05 *vs* NC group

A: The targeting relationship between HOTAIR and miR-519d-3p predicted by StarBase 2.0; B: Dual-luciferase reporter gene verified the relationship between HOTAIR and miR-519d-3p; C: The expression of miR-519d-3p after si-HOTAIR transfection was detected by qPCR; D: StarBase 2.0 predicted the targeting relationship between miR-519d-3p and CCND1; E: The targeting relationship between miR-519d-3p and CCND1 detected by Dual-luciferase reporter gene; F: CCND1 expression was detected by WB after transfection of miR-519d-3p mimics

图3　HOTAIR调控miR-519d-3p/CCND1分子轴

Fig.3　HOTAIR regulated the molecular axis of miR-519d-3p/CCND1



$^{*}P<0.05$ *vs* NC group, $^{\Delta}P<0.05$ *vs* miR-519d-3p group

A: CCND1 expression was detected by WB; B: The proliferation activity of SKBR3 cells was detected by CCK-8; C, D: Invasion and migration of SKBR3 cell were detected by Transwell assay (×40); E: Expression levels of E-cadherin, N-cadherin and Vimentin were detected by WB

图4 HOTAIR可通过调控miR-519d-3p/CCND1分子轴影响SKBR3细胞增殖、侵袭和迁移

Fig.4 HOTAIR affected proliferation, invasion and migration of SKBR3 cells by regulating the miR-519d-3p/CCND1 molecular axis

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