

论 著

宫颈癌细胞外泌体hsa_circ_0087432对人脐静脉内皮细胞增殖及迁移的影响

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[摘要] 目的 分析环状RNA 0087432(hsa_circ_0087432)在宫颈癌患者血清外泌体中的表达, 探讨其与宫颈癌转移可能存在的关系。方法 收集广州市番禺区中心医院2019年6月—2020年6月诊断为宫颈鳞癌且术前未行放化疗的30例患者的宫颈癌组织及血清作为实验组, 另将30例未患宫颈癌的女性正常宫颈组织及血清作为对照组。采用实时定量聚合酶链反应(qPCR)检测hsa_circ_0087432在宫颈癌患者与健康人宫颈组织和血清外泌体中的表达水平。通过质粒转染Siha细胞以过表达hsa_circ_0087432, 将Siha细胞依不同处理分为3组: control组(未转染质粒), vector组(转染pLC5-ciR)及hsa_circ_0087432组(转染hsa_circ_0087432-pLC5-ciR), 并提取各组外泌体; 采用透射电镜(TEM)、纳米颗粒跟踪分析技术(NTA)及Western blotting检测外泌体表征。将人脐静脉内皮细胞按不同处理分为3组: control-Exs组(Siha细胞来源外泌体)、vector-Exs组(vector组Siha细胞来源外泌体)、hsa_circ_0087432-Exs组(hsa_circ_0087432组Siha细胞来源外泌体), 采用CCK-8试剂盒检测外泌体对人脐静脉内皮细胞增殖的影响, 采用划痕实验及Transwell检测外泌体对人脐静脉内皮细胞迁移的影响。结果 电镜下观察结果显示, Siha细胞的外泌体大部分呈椭圆形或圆形, 粒径分析显示其直径大小分布在30~150 nm; Western blotting检测结果显示, 两组囊泡中的CD9、CD81及CD63表达均呈阳性; qPCR结果显示, 实验组宫颈组织hsa_circ_0087432表达量明显高于对照组(4.03 ± 1.51 vs. 1.00 ± 0.26 , $P < 0.01$), 且血清外泌体中hsa_circ_0087432表达量也明显高于对照组(1.97 ± 0.04 vs. 1.02 ± 0.23 , $P < 0.01$)。CCK-8检测结果显示, hsa_circ_0087432-Exs可明显促进人脐静脉内皮细胞增殖(1.57 ± 0.04 vs. 1.09 ± 0.11 , $P < 0.05$)。划痕实验结果显示, 干预12 h后, circ_0087432-Exs组的迁移面积占比高于control-Exs组($24.66\% \pm 2.92\%$ vs. $15.01\% \pm 3.12\%$, $P < 0.05$), 且24 h后hsa_circ_0087432-Exs组的迁移面积占比明显高于control-Exs组($74.84\% \pm 13.22\%$ vs. $38.70\% \pm 2.12\%$, $P < 0.01$)。Transwell检测结果也显示, 与vector-Exs组及control-Exs组比较, hsa_circ_0087432-Exs组迁移细胞数量明显增多, 差异有统计学意义[分别为(1218.00 ± 103.53)个、(1044.00 ± 103.79)个、(1755.00 ± 97.35)个, $P < 0.05$]。结论 hsa_circ_0087432在宫颈癌患者血清外泌体中呈高表达, 且过表达hsa_circ_0087432的宫颈癌细胞来源的外泌体可促进人脐静脉内皮细胞增殖与迁移。

[关键词] 环状RNA; 外泌体; 宫颈癌; 增殖; 迁移

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Effect of cervical cancer-derived exosomal hsa_circ_0087432 on the proliferation and migration of HUVECs

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[Abstract] **Objective** To analyze the expression of circRNA0087432 (hsa_circ_0087432) in serum exosomes of patients with cervical cancer, and explore its possible relationship with cervical cancer metastasis. **Methods** The cervical cancer tissue and serum of 30 patients, who were diagnosed as cervical squamous cell carcinoma from June 2019 to June 2020 in Guangzhou Panyu District Central Hospital and untreated with radiotherapy and chemotherapy before surgery, were collected as the experimental group. Normal cervical tissue and serum of 30 women without cervical cancer were used as control group. Real-time quantitative polymerase chain reaction (RT-qPCR) was used to detect the expression level of hsa_circ_0087432 in cervical tissues and serum exosomes of

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patients with cervical cancer and healthy people; Siha cells were transfected with plasmid to overexpress hsa_circ_0087432. According to different treatments, Siha cells were divided into three groups: control group (untransfected plasmid), vector group (transfected with pLC5-ciR) and hsa_circ_0087432 group (transfected with hsa_circ_0087432-pLC5-ciR). The exosomes were collected and extracted, and the exosome were characterized by transmission electron microscope (TEM), nanoparticle tracking analysis (NTA) and Western blotting; Endothelial cells were divided into three groups according to different treatments: control-Exs group (extracted exosomes from control group), vector-Exs group (extracted exosomes from vector group) and hsa_circ_0087432-Exs group (extracted exosomes from hsa_circ_0087432 group). CCK-8 kit was used to detect the effect of exosomes on proliferation of endothelial cells. The effect of exosomes on the migration of endothelial cells were detected by scratch test and Transwell. **Results** The results of TEM showed that most of the exosomes of Siha cells were elliptical or round. The particle size analysis showed that their diameters ranged of 30-150 nm. Western blotting showed that the expressions of CD9, CD81 and CD63 in the two groups of vesicles were positive. The qPCR results showed that the expression level of hsa_circ_0087432 in cervical tissue was significantly higher in experimental group than that in control group (4.03 ± 1.51 vs. 1.00 ± 0.26 , $P < 0.01$), and the expression level of hsa_circ_0087432 in serum exosomes was also obviously higher in experimental group than that in control group (1.97 ± 0.04 vs. 1.02 ± 0.23 , $P < 0.01$); CCK-8 results showed that, compared with the control-Exs group, the proliferation of human umbilical vein endothelial cells was significantly promoted in hsa_circ_0087432-Exs group (1.57 ± 0.04 vs. 1.09 ± 0.11 , $P < 0.05$). Scratch experiments showed that, 12 hours after treatment, the healing degree was better in hsa_circ_0087432-Exs group than that in control-Exs group ($24.66\% \pm 2.92\%$ vs. $15.01\% \pm 3.12\%$, $P < 0.05$); and 24 hours after scratching, the healing degree was even better in hsa_circ_0087432-Exs group than that in control-Exs group ($74.84\% \pm 13.22\%$ vs. $38.70\% \pm 2.12\%$, $P < 0.01$). Transwell also showed that the counted number of endothelial cells increased obviously in hsa_circ_0087432-Exs group than that in vector-Exs group and control-Exs group (1755.00 ± 97.35 vs. 1218.00 ± 103.53 vs. 1044.00 ± 103.79) with significant difference ($P < 0.05$). **Conclusion** The hsa_circRNA 0087432 is highly expressed in serum exosomes of patients with cervical cancer, and the exosomes derived from cervical cancer cells which over-expressing the hsa_circ_0087432 can promote the proliferation and migration of human umbilical vein endothelial cells.

[Key words] circRNA; exosome; cervical cancer; proliferation; migration

宫颈癌是最常见的恶性肿瘤之一，发病率在女性生殖系统恶性肿瘤中居第二位，严重危害女性健康^[1]。环状RNA(circular RNA, circRNA)是一类广泛存在于哺乳动物细胞中的内源性RNA分子，转录后具有调控基因表达的作用^[2-4]。circRNA在人体细胞中广泛稳定表达^[5]，且在疾病发展过程中呈特异性表达，因此可成为肿瘤早期诊断与预后评估的标志物^[6]。外泌体(exosome, EXs)是细胞内起源的纳米级细胞外脂质双层囊泡，其内携带有多种活性物质，如circRNA、miRNA、mRNA及特异性的蛋白质，具有广泛的生物学活性^[7-8]。此外，大量研究表明，外泌体作为信号分子载体，可参与细胞间信息传递，并与肿瘤的转移密切相关^[9-10]。有研究表明，外泌体中含有比分泌细胞更多的circRNA，且这一分泌可被miRNA调控^[11-12]。因此，外泌体中circRNA的多样性和特异性使其能够用于肿瘤的辅助诊断，也可用于监控肿瘤的进展，近年来备受关注。本研究探讨了hsa_circ_0087432在宫颈鳞状细胞癌发生发展中的作用，提出hsa_circ_0087432可能是通过癌细胞分泌的外泌体促进宫颈鳞癌侵袭与转移的假设，以此来探讨外泌体中的circRNA对宫颈鳞癌诊疗的意义。

1 材料与方法

1.1 实验标本及材料 收集广州市番禺区中心医

院2019年6月—2020年6月诊断为宫颈鳞癌且术前未行放化疗的30例患者的宫颈鳞癌组织及血清作为实验组，30例未患宫颈癌的女性正常宫颈组织及血清作为对照组。本研究经过番禺区中心医院伦理委员会批准，所有患者均签署知情同意书。Siha细胞(人宫颈鳞癌细胞)、人脐静脉内皮细胞购自武汉普诺赛(Procell)生命科技有限公司。胎牛血清、0.25% EDTA胰蛋白酶溶液、青链霉素、DMEM培养基、PBS均购自美国Gibco公司；MEM培养基购自美国Hyclone公司；Lipofectamine 3000试剂、Western blotting相关试剂及BCA蛋白定量试剂盒、荧光定量PCR仪(ABI 7500)购自美国ThermoFisher公司；CD9、CD63和CD81抗体购自美国Abcam公司；实时定量聚合酶链反应(qPCR)试剂盒购自广州瑞真生物技术有限公司；4%多聚甲醛购自武汉赛维尔生物技术有限公司；外泌体RNA提取试剂盒购自北京全式金生物技术有限公司；细胞外泌体提取试剂购自广州市锐博生物技术有限公司；PKH-26染料购自美国Sigma公司；hsa_circ_0087432过表达质粒载体hsa_circ_0087432-pLC5-ciR和其空载体pLC5-ciR购自广州吉赛生物科技股份有限公司；GAPDH、hsa_circ_0087432引物由上海生工生物工程公司合成。

1.2 方法

1.2.1 细胞培养及质粒转染 人脐静脉内皮细胞用

含10%胎牛血清、1%青链霉素双抗的DMEM高糖完全培养基培养, 宫颈癌细胞株(Siha)用含10%胎牛血清、1%青链霉素双抗的MEM完全培养基培养, 置入37℃、5% CO₂培养箱中, 每2 d换液1次, 待细胞密度达90%时进行传代培养或质粒转染。Siha细胞按不同处理分为3组: control组(未转染质粒), vector组(转染pLC5-ciR)和hsa_circ_0087432组(转染hsa_circ_0087432-pLC5-ciR), 转染流程按照说明书进行操作。转染24~48 h, 荧光显微镜观察转染效率, 拍照。qPCR验证转染后hsa_circ_0087432的表达。

1.2.2 外泌体分离、鉴定及内皮细胞对其摄取的检测 细胞转染成功后, 弃去培养基, PBS洗3次, 更换为10%无外泌体血清的完全培养基, 24 h后收集细胞上清, 以备后续提取外泌体。参照外泌体提取试剂说明书提取外泌体, 适量PBS重悬外泌体, 取10 μl采用BCA蛋白定量试剂盒测定蛋白含量, 其余置入-80℃冰箱保存。采用透射电子显微镜鉴定外泌体形态, 纳米颗粒跟踪分析(nanoparticle tracking analysis, NTA)技术测定外泌体粒径范围。Western blotting检测外泌体生物标志物CD9、CD63及CD81的表达情况。提取外泌体总蛋白, 以每孔20 μg上样电泳, 完成后将蛋白转移至0.45 μm PVDF膜上, 封闭后加入一抗, 置于4℃冰箱孵育过夜。第2天孵育二抗1 h, TBST洗膜3次后, 加显影液曝光蛋白条带, 统计分析灰度值。

参照PKH26染料说明书按比例标记外泌体, 提纯再次重悬后, 加入培养基中与内皮细胞避光共培养24 h, PBS洗3次; 4%多聚甲醛溶液固定后, PBS洗3次; DAPI染色5~10 min, PBS洗3次; 抗荧光淬灭封片剂封片后, 荧光显微镜下拍照。

1.2.3 血清外泌体RNA的提取 血清按收集的来源不同分为宫颈癌组与对照组。提取方法参照血清外泌体RNA提取试剂盒说明书, 完成后用反转录试剂盒将RNA反转录为cDNA, 最后采用SYBR®Green法进行实时定量PCR检测。

1.2.4 宫颈组织RNA的提取 宫颈组织按收集的来源不同分为宫颈癌组与对照组。采用Trizol法提取组织中的总RNA, 称取组织, 每10 mg组织加入1 ml Trizol试剂, 加入研磨钢珠后放在研磨机内研磨, 全程冰上操作。然后参照细胞总RNA提取操作步骤, 得到总RNA, 测定浓度后, 根据试剂盒说明将RNA反转录为cDNA, 最后采用SYBR®Green法进行实时定量PCR。

1.2.5 qPCR检测 采用SYBR®Green法, 操作步骤按照试剂盒说明, 配制总反应体积为20 μl的反应体系, 以GAPDH为内参。引物序列(5'-3')如下: GAPDH

正义AGAAGGCTGGGGCTCATTTG, 反义GCAGGAGGCATTGCTGATGAT; hsa_circ_0087432正义CGCATTATGCCTGATTCCAA, 反义CTGCTGGACTAGGGTGA AAA; hsa_circ_0081672正义TTTACCAACGCCATCACCGA, 反义CCCAGTGTGTGGTCTTCTTGT; hsa_circ_0075430正义CAGCACACGGCGGAAGAAA, 反义CCCCTGATTGTGACCTTCGT。qPCR结果采用2^{-ΔΔCt}方法进行计算。

1.2.6 CCK-8法检测细胞增殖情况 人脐静脉内皮细胞提前1 d接种于96孔板上, 按不同处理分为3组: control-Exs组(siha细胞外泌体)、hsa_circ_0087432-Exs组(hsa_circ_0087432组siha细胞外泌体)、vector-Exs组(vector组siha细胞外泌体), 每组6个复孔, 细胞数3 × 10³个/孔。细胞贴壁后, 按照分组加入外泌体干预48 h, 更换含有CCK-8工作液的培养基(CCK-8工作液与培养基配制比例为1:10), 每孔100 μl, 37℃避光孵育1.5~2.0 h, 用酶标仪检测450 nm处吸光度值。

1.2.7 划痕实验检测细胞迁移情况 分组同1.2.6。提前1 d接种人脐静脉内皮细胞于6孔板上, 每孔保持等量的细胞悬液, 待细胞生长至对数期, 融合至90%时, 取200 μl无菌枪头, 在6孔板上划线, PBS洗去细胞碎片, 更换含1%血清的完全培养基, 同时加入外泌体, 置于37℃、5% CO₂的培养箱中培养。划痕后0、12、24 h后分别对划痕处进行拍照。实验重复3次。

1.2.8 Transwell小室检测细胞迁移能力 分组同1.2.6。细胞同步化12 h后, 胰酶消化, 计数细胞, 小室内加入200 μl细胞悬液及外泌体, 培养液为含0.5%血清的完全培养基, 下室加入400 μl含20%血清的完全培养基。常规培养24~48 h, 用棉签擦去内室的细胞, PBS清洗后用4%多聚甲醛固定30 min, 0.1%结晶紫染色, 显微镜下计数小室底部滤膜下层穿过的细胞数, 每组计数5个视野。实验重复3次。

1.3 统计学处理 采用SPSS 21.0软件进行统计分析。计量资料以 $\bar{x} \pm s$ 表示, hsa_circ_0087432在宫颈癌和正常组织及血清外泌体中的表达差异采用两独立样本t检验进行分析; 符合方差齐性, 多组间比较采用单因素方差分析, 进一步两两比较采用LSD-t检验。P<0.05为差异有统计学意义。

2 结果

2.1 两组宫颈组织和血清外泌体中hsa_circ_0087432的表达水平比较 qPCR结果显示, 实验组患者宫颈组织中hsa_circ_0087432表达量(4.03 ± 1.51)明显高于对照组(1.00 ± 0.26, P<0.01); 两组hsa_circ_0081672及hsa_circ_0075430的表达量差异无

统计学意义($P>0.05$, 图1A)。此外, 实验组血清外泌体中hsa_circ_0087432的表达量(1.97 ± 0.04)明显高于对照组(1.02 ± 0.23), 差异有统计学意义($P<0.01$, 图1B)。

2.2 外泌体形态、特征蛋白鉴定及细胞摄取 分别收集control组、vector组及hsa_circ_0087432组Siha细胞的上清提取外泌体。电镜下观察发现Siha细胞的胞外囊泡大部分呈椭圆形或圆形(图2A), 粒径分析显示上清中提取的囊泡颗粒直径大小分布在30~150 nm(图2B), Western blotting结果显示control组及hsa_circ_0087432组囊泡中的CD9、CD81及

CD63表达均呈阳性(图2C), 表明提取的细胞外囊泡为外泌体。PKH-26标记的外泌体与人脐静脉内皮细胞共培养, 免疫荧光染色后发现, 外泌体进入细胞内部, 在细胞核周围分布, 说明外泌体可以很好地被细胞摄取(图2D)。

2.3 过表达hsa_circ_0087432的外泌体对人脐静脉内皮细胞增殖的影响 qPCR结果显示, 与vector-Exs组(2.29 ± 0.21)相比, hsa_circ_0087432-Exs组hsa_circ_0087432表达量(11.39 ± 1.71)明显升高($P<0.001$), 提示成功转染。CCK-8检测结果显示, hsa_circ_0087432-Exs组细胞活力(1.57 ± 0.04)

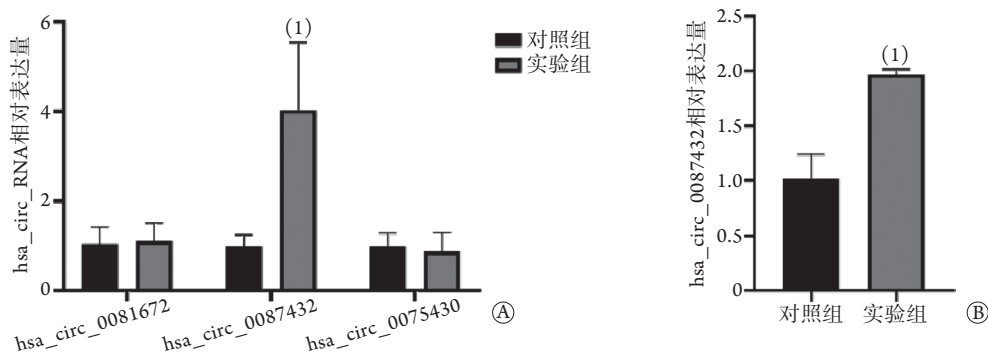


图1 实验组与对照组宫颈组织(A)及血清外泌体(B)中hsa_circ_0087432的表达水平

Fig.1 The expression level of hsa_circ_0087432 in cervical tissue (A) and serum exosomes (B) in experimental group and control group

与对照组比较, (1) $P<0.01$ 。

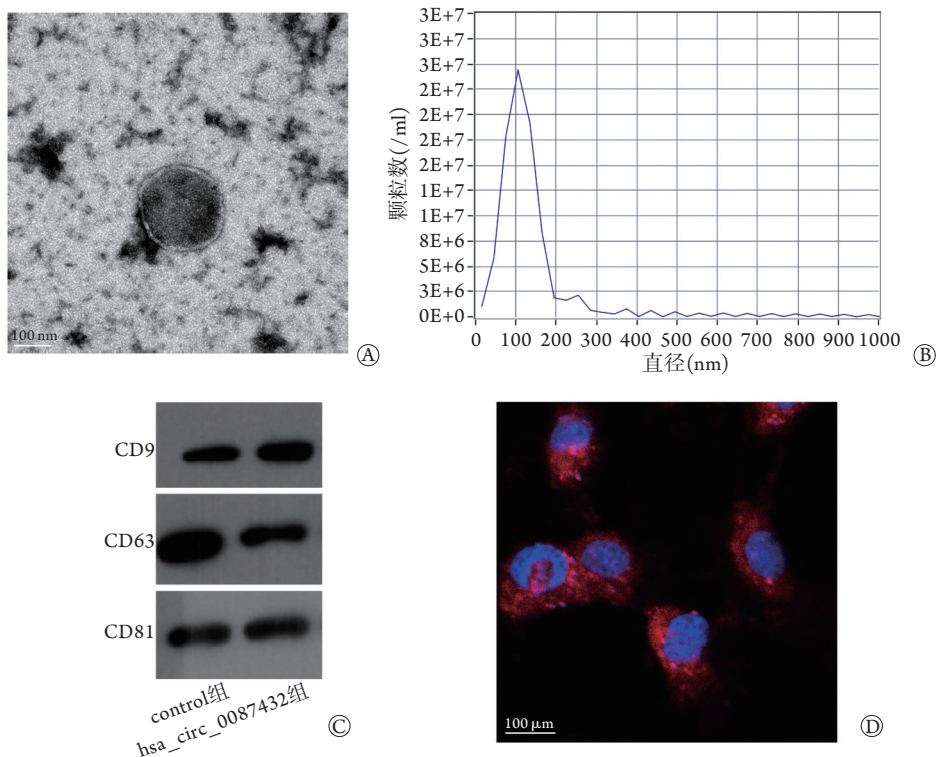


图2 外泌体电镜图、粒径分析、特征蛋白的鉴定及细胞内摄取

Fig.2 Exosomes, particle size, identification of characteristic proteins and intracellular uptake

A. Siha细胞来源的外泌体电镜图; B. NTA分析Siha细胞的外泌体粒径; C. 外泌体表面蛋白CD9、CD81和CD63的表达; D. 人脐静脉内皮细胞摄取外泌体实验

明显高于control-Exs组(1.09 ± 0.11)及vector-Exs组(1.27 ± 0.02), 差异有统计学意义(P<0.001, P<0.05)。

2.4 过表达hsa_circ_0087432的外泌体对人脐静脉内皮细胞迁移的影响 划痕实验结果显示, 干预12 h后, hsa_circ_0087432-Exs组的迁移面积占比(24.66% ± 2.92%)高于control-Exs组(15.01% ± 3.12%)及vector-Exs组(18.07% ± 4.34%);

干预24 h后, hsa_circ_0087432-Exs组的迁移面积占比(74.84% ± 13.22%)明显高于control-Exs组(38.70% ± 2.12%)及vector-Exs组(35.02% ± 4.43%, P<0.05或P<0.01, 图3A、C)。Transwell检测结果显示, 与vector-Exs组[(1218.00 ± 103.53)个]及control-Exs组[(1044.00 ± 103.79)个]比较, hsa_circ_0087432-Exs组迁移细胞数量[(1755.00 ± 97.35)个]明显增多, 差异有统计学意义(P<0.05, 图3B、D)。

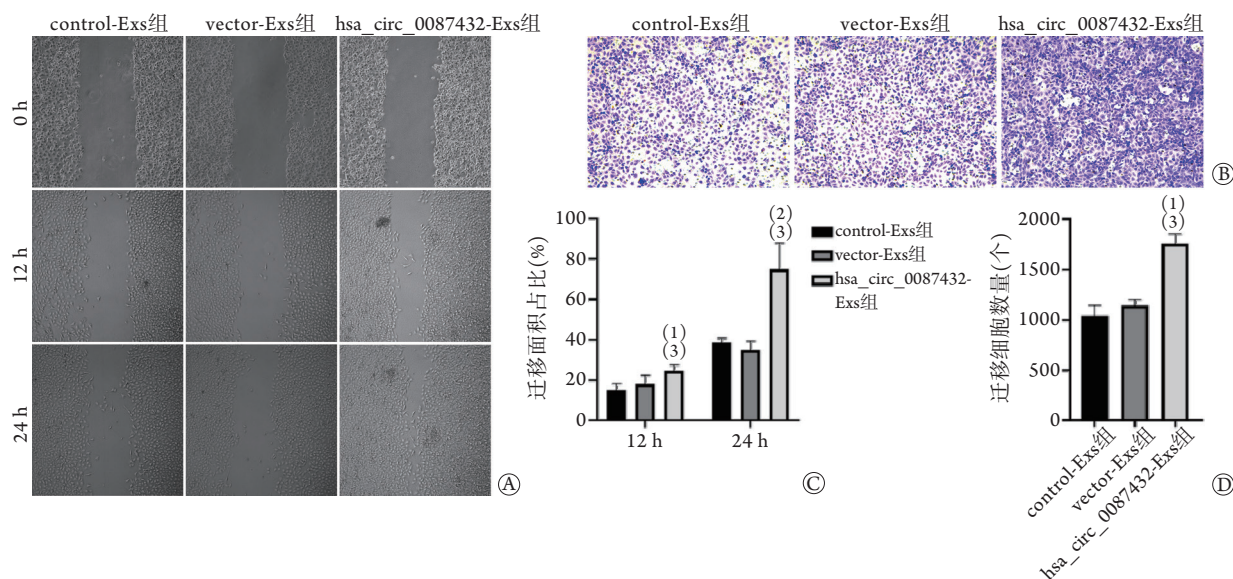


图3 过表达hsa_circ_0087432后细胞分泌的外泌体对人脐静脉内皮细胞迁移的影响

Fig.3 Exocrine derived from the cells which over-expressing the hsa_circ_0087432 can promote the proliferation and migration of human umbilical vein endothelial cells

A、C. 人脐静脉内皮细胞12、24 h划痕实验; B、D. 人脐静脉内皮细胞迁移实验; 与control-Exs组比较, (1)P<0.05, (2)P<0.01; 与vector-Exs组比较, (3)P<0.01。

3 讨论

circRNA是一种内源性RNA, 可作为miRNA海绵或竞争性内源性RNA参与各种疾病的发生发展^[13]。Zhang等^[14]发现, hsa_circ_0000069可通过海绵作用吸附miR-873-5p, 进而调节靶基因TUSC3的表达, 参与宫颈癌的增殖、侵袭和转移。Ma等^[15]也发现hsa_circ_0005576可与miR-153相互作用, 上调KIF20A的表达, 以此促进宫颈癌的进展。目前, circRNA已被证实在肿瘤组织及细胞中呈异常表达, 极可能参与肿瘤的发生发展^[12]。另外, circRNA还可通过调控Wnt信号通路、上皮-间质转化(epithelialmesenchymal transition, EMT)^[16]等影响肿瘤的发生发展、侵袭转移及耐药性。Wang等^[17]研究发现, circRNA_PVT1可通过靶向miR-1286诱导宫颈癌细胞的EMT, 促进宫颈癌的迁移侵袭。朱小青和罗枫^[18]在GEO数据库中筛选出宫颈鳞状细胞癌组织与正常宫颈组织中119种差异表达的circRNA, 分析发现宫颈鳞状细胞癌的发生、发展

可能与hsa_circ_0031027/hsa-miR-132-3p/KLHL11及hsa_circ_0075341/hsa-miR-4747-5p/LY6G6C两条调控途径有关。Jiao等^[19]发现, 上调E-cadherin可降低hsa_circ_0000745的表达水平, 从而抑制宫颈癌细胞的增殖、迁移和侵袭。

外泌体内含有大量生物活性物质, 如circRNA、miRNA、mRNA及蛋白质等, 可作为细胞间信息交流传递的载体。有学者研究发现, 肿瘤外泌体与肿瘤的侵袭和转移密切相关, 这提示其有望成为临床诊疗的指标^[20-21]。Zhou等^[22]发现, 循环外泌体中的miR-221-3p可下调VASH1的表达, 促进淋巴、血管发生, 与宫颈鳞状细胞癌患者的癌区淋巴管密度及淋巴结转移数目有关。还有学者通过对血浆外泌体miRNA测序及组织PCR进行研究发现, 宫颈癌患者血浆外泌体中let-7d-3p、miR-30d-5p与健康人的表达存在差异, 可作为诊断生物标志物用于宫颈癌及其前体的非侵入性筛查指标^[23]。崔虎军等^[24]发现外泌体miRNA29在宫颈癌转移中有重要作用。

本研究通过qPCR法证实了hsa_circ_0087432在

宫颈癌患者癌组织与正常组织中的表达存在差异,且在患者与健康人血清外泌体中的表达也存在差异,提示hsa_circ_0087432可能是通过癌细胞分泌至外泌体,并参与肿瘤的侵袭转移。根据这一结果,本研究通过质粒转染Siha细胞,过表达了细胞中的hsa_circ_0087432,收集其细胞上清后提取外泌体,并在内皮细胞中进行了验证。CCK-8检测结果证实,来源于过表达hsa_circ_0087432的细胞的外泌体可更明显地提高内皮细胞的增殖能力。划痕实验及Transwell迁移实验也证实,过表达hsa_circ_0087432后提取的外泌体可更好地促进内皮细胞的迁移。以上结果均提示宫颈癌患者外泌体中的hsa_circ_0087432参与了宫颈癌的侵袭及转移。

综上所述,本研究结果显示,hsa_circ_0087432在宫颈癌患者组织和血清中高表达,且有可能通过外泌体的方式促进宫颈癌细胞的转移,为以后的研究提供了新的线索和思路。但以上数据仅表明hsa_circ_0087432参与了宫颈癌的发展进程,并未深入探讨其对宫颈癌侵袭、转移等的具体机制,后续将进一步进行深入研究。

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