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·基础研究·

miR-640调控Wnt信号通路对腰椎间盘突出患者髓核细胞衰老及凋亡的影响

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[摘 要] **目的** 探讨miR-640调控Wnt信号通路对腰椎间盘突出患者髓核细胞衰老及凋亡的影响及其机制。**方法** 选取我院32例腰椎间盘突出患者的腰椎间盘组织作为观察组,另选取32例外伤患者未病变的腰椎间盘组织作为对照组,qPCR检测2组miR-640表达水平。分离培养人髓核细胞,培养后将原代培养髓核细胞分为Control组、NC组和miR-640组,NC组和miR-640组分别转染NC质粒和miR-640质粒,通过SA-β-gal实验检测各组细胞衰老水平,流式细胞仪检测各组细胞凋亡水平,免疫荧光检测β-连环蛋白(β-catenin)的表达水平和定位,双荧光素酶报告基因实验检测miR-640和β-catenin的靶向调控关系。**结果** 与对照组比较,观察组腰椎间盘组织miR-640表达水平显著升高($P<0.01$)。与Control组比较,miR-640组细胞衰老水平增加,细胞凋亡率增加,差异均具有统计学意义($P<0.01$),NC组与Control组上述指标比较差异无统计学意义($P>0.05$)。免疫荧光证实miR-640组细胞β-catenin的表达降低,β-catenin入核受到抑制,差异具有统计学意义($P<0.01$)。双荧光素酶报告基因实验证实miR-640和β-catenin具有靶向关系,差异具有统计学意义($P<0.01$)。**结论** 腰椎间盘突出患者中miR-640表达水平升高,miR-640可以促进髓核细胞衰老和凋亡,其机制可能与Wnt信号通路的调控有关。

[关键词] miR-640;人髓核细胞;腰椎间盘突出;衰老;凋亡;Wnt信号通路
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Effects of miR-640 regulating Wnt signaling pathway on senile and apoptosis of nucleus pulposus cells in patients with lumbar disc herniation

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Abstract: Objective To investigate the effects and mechanisms of miR-640 regulating Wnt signaling pathway on senile and apoptosis of nucleus pulposus cells in patients with lumbar disc herniation. **Methods** The lumbar disc tissues of 32 patients with lumbar disc herniation in our hospital were selected as the observation group, and the lumbar disc tissues of 32 patients with trauma without lesions were selected as the control group. The expression level of miR-640 of the two groups was detected by qPCR. The human nucleus pulposus cells were isolated and cultured, and the primary cultured nucleus pulposus cells were divided into the Control group, the NC group and the miR-640 group. The NC group and the miR-640 group were transfected with NC plasmid and miR-640 plasmid, respectively. SA-β-Gal experiment was used to detect the cell senile level in each group, flow cytometry was used to detect the apoptosis level of cells in each group,

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immunofluorescence was used to detect the expression level and localization of β-catenin. Dual luciferase reporter gene experiment was used to detect the targeted regulation relationship between miR-640 and β-catenin. **Results** Compared with the control group, the expression level of miR-640 of the lumbar disc tissue in the observation group increased significantly ($P<0.01$). Compared with the Control group, the cell senile level and the cell apoptosis rate of the miR-640 group increased, and the differences were statistically significant ($P<0.01$). There was no statisticallysignificant difference in the above indicators between the NC group and the Control group ($P>0.05$). Immunofluorescence confirmed that the expression of β-catenin decreased and the nuclear entry of β-catenin was inhibited in the miR-640 group, and the differences were statistically significant ($P<0.01$). Dual luciferase reporter gene experiment confirmed that miR-640 and β-catenin had a targeted relationship, and the difference was statistically significant ($P<0.01$). **Conclusion** The expression level of miR-640 in patients with lumbar disc herniation is increased. miR-640 can promote the senile and apoptosis of nucleus pulposus cells, and its mechanism may be related to the regulation of Wnt signaling pathway.

Keywords: miR-640; human nucleus pulposus cells; lumbar disc herniation; senile; apoptosis; Wnt signaling pathway

腰椎间盘突出症是较为常见的疾病之一,其主要诱因是腰椎间盘的髓核、纤维环及软骨病变。髓核为椎间盘结构的重要组成部分,位于两软骨板与纤维环之间,不同程度的退行性改变后,如在外力因素的作用下,椎间盘的纤维环破裂,髓核组织从破裂之处突出或脱出于后方或椎管内,会导致相邻脊神经根遭受刺激或压迫,产生腰部疼痛[1-3]。腰椎间盘突出的进展伴随着髓核细胞的衰老[4],研究表明,衰老相关p16蛋白可以通过氧化应激反应和髓核细胞周期调控参与椎间盘退变过程[5];也有研究表明核心蛋白聚糖通过mTOR途径诱导髓核细胞衰老和凋亡[6],但是髓核细胞的衰老和凋亡是否受到非编码RNA,尤其是microRNA的调控,目前尚不清楚。microRNA在细胞生长发育、增殖、衰老、凋亡的过程中发挥重要作用[7]。研究表明,miR-640可通过调节神经元pentraxin-2(NPTX2)、沉默环状RNA circ_0054537抑制肾细胞癌的恶性进展[8]。也有研究表明,miR-640通过Wnt7b/β-连环蛋白(β-catenin)信号通路在乳腺癌中发挥潜在抑制作用[9-10]。Wnt/β-catenin信号通路是保护软骨细胞的重要通路,但miR-640是否能通过靶向Wnt/β-catenin信号通路对髓核细胞的衰老和凋亡发挥作用目前尚不清楚。因此,本研究拟通过组织学明确miR-640在腰椎间盘突出患者中的表达情况,并通过细胞学实验探究转染miR-640对髓核细胞衰老、凋亡、β-catenin表达和细胞定位的影响,为miR-640在髓核细胞中发挥的作用及相关机制的阐明奠定基础。

1 材料与方法

1.1 材料

选取2020年1月至2022年1月我院归档资料完整的32例腰椎间盘突出患者的腰椎间盘组织作为观察组,另选取32例外伤患者未病变的腰椎间盘组织作为对照组,患者平均年龄(50.23±6.74)岁。所有患者均知情本研究并签署知情同意书,本研究通过华北医疗健康集团峰峰总医院医学伦理委员会审核(KYLL20190118)。

1.2 主要器材及试剂

4%多聚甲醛购自碧云天公司,SA-β-gal购自Elabscience公司,Annexin V-FITC Apoptosis Staining/Detection Kit(ab14085)购自美国abcam公司,Lipofectamine™3000 Reagent转染试剂购自美国Thermo Fisher Scientific公司,miR-640质粒及NC质粒由上海吉玛基因有限公司合成,双荧光素酶报告基因检测试剂盒(ab228530)购自美国abcam公司,细胞培养皿等实验耗材购自美国BD公司,倒置显微镜购自德国蔡司公司,XSP-BM-13C型荧光显微镜购自化科有限公司;流式细胞仪FACS Calibur购自美国BD公司。

1.3 qPCR检测腰椎间盘组织mRNA表达。

qPCR检测2组髓核组织的miR-640表达水平。将组织研磨后,无酶条件下加入1 mL TRIzol提取总RNA,反转录合成cDNA后,进行PCR扩增,miR-640引物序列:F, GACAGCGACCCTGT; R, CTTCATGGCG CAAT。U6: F, CAGTTAGCATGTAAGCCCT; R, TAG CTCTTTCGTTTTCA,95 ℃预变性10 min,95 ℃变性30 s,60 ℃退火30 s,72 ℃延伸60 s,共38个循环。每个样品重复检测3次,利用$2^{-\triangle\triangle CT}$相对定量法计算各个指标的相对表达量,采用内参U6对其进行标准化处理。

1.4 细胞培养及质粒转染方法

去除髓核组织周围少许肌肉、骨性结构、纤维环及终板软骨组织等。分离培养人髓核细胞(培养密度:1×10^6个细胞/培养皿);每隔1 d更换培养液,并于第2~3天传代,传代比例为1∶4。将细胞随机分为Control组、NC组和miR-640组,按照Lipofectamine™3000说明书对NC组和miR-640组细胞分别转染NC质粒和miR-640质粒,8 h后更换完全培养基;对照组不做处理。

1.5 SA-β-gal实验检测细胞衰老情况

将处于对数生长期的髓核细胞进行胰酶消化后按照每孔2×10^5个细胞的密度接种于6孔板中,每组3个复孔。加入1 mL β-gal染色固定液,室温固定15 min,PBS洗涤3次,每次3 min,按照试剂盒说明书配置染色工作液6 mL,每孔加入1 mL染色工作液,用保鲜膜封住培养板,37 ℃培养过夜,于200倍显微镜下观察。

1.6 流式细胞仪检测细胞凋亡水平

将各组髓核细胞用不含EDTA的胰酶消化后,以1 000 r/min于4 ℃离心5 min,离心半径35 cm,收集细胞;用预冷的PBS洗涤细胞2次,以1 000 r/min于4 ℃离心5 min,离心半径15 cm;收集细胞,加入100 μL 1×Binding Buffer重悬细胞。严格按照试剂盒说明书加入5 μL Annexin V-FITC和10 μL PI染液,轻轻混匀,避光、室温反应10~15 min;加入400 μL 1×Binding Buffer,混匀后置于冰上;在1 h内用流式细胞仪检测。

1.7 免疫荧光检测β-catenin的表达水平及定位

选择处于对数生长期且状态良好的细胞作为实验对象,于小皿中进行培养,待细胞贴壁后,去除旧培养基,PBS清洗2次,室温下甲醇固定15 min,弃去甲醇,每孔加入1% BSA 500 μL,室温下封闭2 h,封闭时在摇床上以50 r/min的速度摇晃。配制一抗,每孔200 μL,4 ℃孵育过夜(13~16 h)。PBST溶液清洗3次,每次10 min,摇床90~100 r/min。配制二抗,按照1∶1 000的比例稀释Alexa488-conjugated,每孔200 μL,室温孵育40~70 min,PBST溶液清洗3次,每次10 min,摇床90~100 r/min。配制DAPI,按照1∶1 000的比例稀释Alexa488-conjugated,每孔200 μL,室温孵育1~5 min,PBST溶液清洗3次,每次10 min,摇床90~100 r/min。封片,荧光显微镜下观察。

1.8 双荧光素酶报告基因实验检测miR-640和β-catenin的靶向关系

构建合成含有β-catenin野生型(WT)和β-catenin突变型(MUT)的荧光素酶报告载体,分别将β-catenin野生型(WT)、β-catenin突变型(MUT)和miR-640共转染至髓核细胞中,用双荧光素酶报告基因测定试剂盒检测荧光素酶活性。

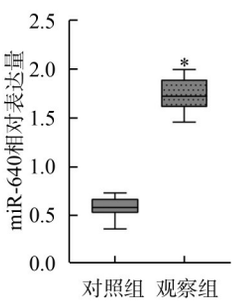
1.9 统计学处理

采用SPSS 25.0统计软件处理数据,Graphpad v9.2软件进行绘图。计量资料以均数±标准差($\bar{x}\pm s$)表示,2组间符合正态分布且满足方差齐性的数据采用双尾独立样本*t-test*,细胞活性与时间的统计采用重复测量分析,$P<0.05$为差异具有统计学意义。

2 结果

2.1 miR-640在腰椎间盘组织的表达水平

采用qPCR检测腰椎间盘组织miR-640的表达水平,结果显示,与对照组比较,观察组患者腰椎间盘组织中miR-640表达水平升高,差异具有统计学意义($P<0.01$),见图1。

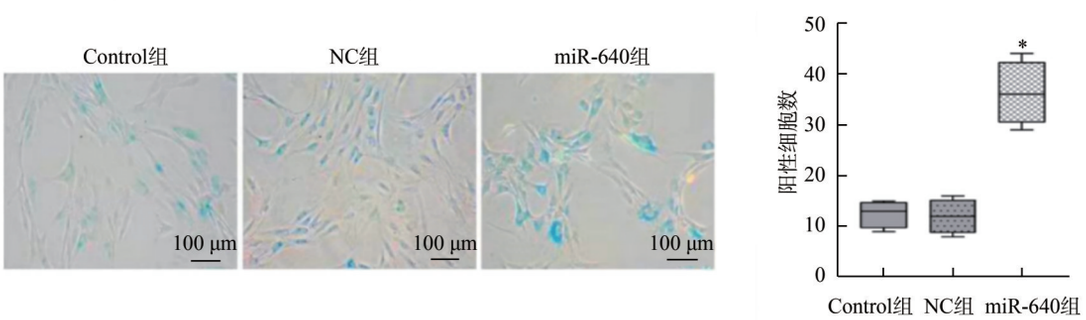


*:与对照组比较,$P<0.01$

图1 miR-640在腰椎间盘组织的表达水平

2.2 miR-640对人髓核细胞衰老的影响

通过SA-β-gal确定miR-640对人髓核细胞衰老的影响,结果显示,miR-640组细胞衰老水平高于Control组,差异具有统计学意义($P<0.01$),而NC组与Control组细胞衰老水平比较差异无统计学意义($P>0.05$),见图2。

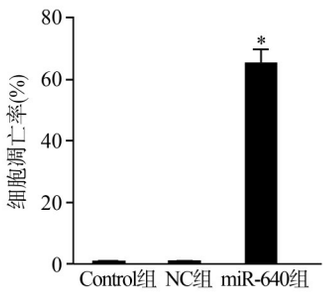


*:与Control组比较,$P<0.01$

图2 miR-640对人髓核细胞衰老的影响

2.3 miR-640对人髓核细胞凋亡的影响

通过流式细胞分析miR-640对人髓核细胞凋亡的影响，结果显示，与Control组比较，miR-640组细胞凋亡率增加，差异具有统计学意义（$P<0.01$），而NC组与Control组上述指标比较差异无统计学意义（$P>0.05$），见图3。



*：与Control组比较，$P<0.01$

图3 miR-640对人髓核细胞凋亡的影响

2.4 miR-640对人髓核细胞β-catenin蛋白的影响

免疫荧光结果显示，与Control组比较，miR-640组细胞β-catenin的表达降低，β-catenin入核受到抑制，差异具有统计学意义（$P<0.01$），而NC组与Control组比较差异无统计学意义（$P>0.05$），见图4。

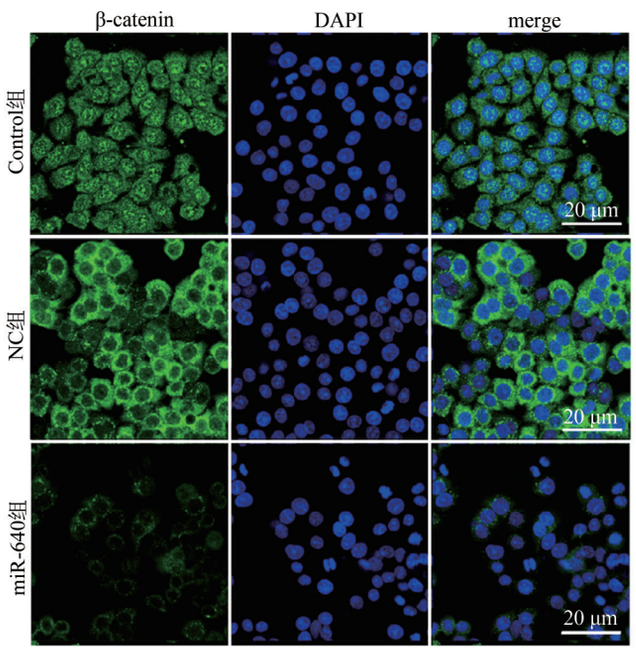
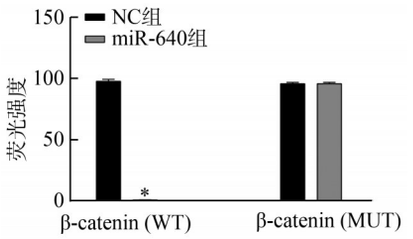


图4 miR-640对人髓核细胞β-catenin蛋白的影响

2.5 miR-640与β-catenin的靶向关系

通过双荧光素酶报告基因实验检测miR-640与β-catenin的靶向关系，结果显示，β-catenin是miR-640的靶基因，差异具有统计学意义（$P<0.01$），见图5。



*：与NC组比较，$P<0.01$

图5 双荧光素酶报告实验荧光素酶活性测定结果

3 讨论

随着社会的发展，腰椎间盘突出症患者不断增多。腰椎间盘突出症是在退行性变基础上积累伤所致，积累伤又会加重椎间盘的退变，影响患者的正常生活，因此预防的重点在于减少积累伤[11-12]。髓核是位于软骨板和纤维环中间，由纵横交错的纤维网状结构即软骨细胞和蛋白多糖黏液样基质构成的弹性胶冻物质。发育成熟的髓核是软骨样细胞，分散在细胞间质内，占椎间盘横断面积的50%～60%；由于其含水量很高，并与软骨终板紧密接触，是渗透交换营养的主要部分，髓核与包裹其上下面的软骨终板及周围的纤维环共同构成对抗重力和张力的闭合缓冲系统，避免外力作用时腰椎损伤[13-14]。腰椎间盘突出患者通常伴随髓核损伤，髓核的生物学功能受到microRNA的调控，细胞学实验研究表明，自噬激活的髓核细胞通过靶向MMP-13传递microRNA以防止细胞外基质降解[15]，microRNA过表达通过靶向Toll样受体4抑制脂多糖诱导的髓核细胞凋亡和细胞外基质降解[16]。由此可见microRNA具有重要的临床意义，但目前对于腰椎间盘突出患者髓核组织中miR-640的表达尚不清楚。本研究通过组织学实验证实腰椎间盘突出患者髓核组织中miR-640表达水平显著高于正常组织，提示miR-640可能在腰椎间盘突出中具有调控作用。

细胞衰老和凋亡是细胞在执行生命活动过程中，随着时间的推移，细胞增殖与分化能力和生理功能逐渐发生衰退的过程，涉及一系列基因的激活、表达以及调控等作用，髓核细胞的功能异常伴随着细胞衰老和凋亡过程。研究表明，FAM134B介导的内质网吞噬上调减轻AGEs诱导的人髓核细胞衰老与凋亡[17]，巨噬细胞移动抑制因子（macrophage migration inhibitory factor，MIF）通过抑制有丝分裂吞噬作用加重超负荷压迫诱导的髓核细胞氧化损伤，进而干预髓核细胞的衰老与凋亡[18]，但是髓核细胞的衰老与凋亡是否受到microRNA的调控目前尚不清楚。因此，本研究结合临床组织学miR-640高表达的结果，通过细胞学实验证

实过表达miR-640会显著促进髓核细胞的衰老,抑制细胞存活,促进细胞凋亡,提示miR-640可能参与了细胞衰老与凋亡动态平衡的过程,从而对腰椎间盘组织,尤其是髓核细胞的生存产生调控作用,因此,miR-640在腰椎间盘突出及退行性病变中高表达,从而促进了髓核细胞的凋亡和组织损伤。β-catenin是Wnt信号通路的重要信号分子,发挥转录因子的作用,启动促细胞分裂过程。研究表明,Wnt/β-catenin途径促进人髓核细胞凋亡,抑制蛋白多糖合成和细胞外基质水平[19],人骨髓间充质干细胞衍生的细胞外小泡通过SOX4/Wnt/β-catenin轴促进髓核细胞增殖和细胞外基质合成[20]。由此可见Wnt信号通路在髓核细胞发展中发挥重要调控作用。本研究通过免疫荧光和双荧光素酶报告基因实验证实了miR-640过表达可以显著抑制β-catenin表达,并且干预β-catenin的入核,抑制细胞增殖信号转导过程,且miR-640对β-catenin的调控具有靶向作用。由此,本研究推测miR-640是通过抑制β-catenin表达和入核定位,发挥促进髓核细胞衰老和凋亡的作用。

综上,miR-640具有抑制人髓核细胞存活,促进人髓核细胞凋亡和衰老的作用,其机制与抑制Wnt/β-catenin信号通路活性相关。未来可进一步通过临床样本进行验证,探究Wnt/β-catenin蛋白在髓核细胞组织中的表达,从原位上证实miR-640对髓核细胞的调控作用,为临床诊断提供理论基础。

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