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·基础研究·

lncRNA HOTAIR促椎间盘髓核细胞凋亡的机制研究

艾力夏提·艾热提,马志刚,李洪伟 (新疆维吾尔自治区人民医院骨科中心脊柱一科,新疆 乌鲁木齐 830000)

[摘 要] **目的** 研究长链非编码RNA(lncRNA)HOX转录反义RNA(HOTAIR)对椎间盘髓核细胞凋亡的调控作用。**方法** 培养人椎间盘髓核细胞系(HNPC),并将HNPC细胞随机分为对照组(Ctrl组)、晚期糖基化终末产物(AGE)组(AGE组)、微小RNA(microRNA,miR)-22的拟似物组(mimic组)、mimic的阴性对照组(mimic-NC组)、lncRNA HOTAIR过表达组(HOTAIR组)、过表达质粒的空载体组(Vector组)、HOTAIR联合mimic处理组(HOTAIR+mimic组)、HOTAIR联合核苷酸结合寡聚结构域样受体家族Pyrin域蛋白3(NLRP3)的抑制剂mcc950处理组(HOTAIR+mcc950组)。qRT-PCR法检测miR-22和lncRNA HOTAIR的表达。双荧光素酶报告基因实验检测lncRNA HOTAIR和miR-22以及miR-22和NLRP3的关系。Western blot检测NLRP3炎症小体相关蛋白的表达。流式细胞术和Hoechst 33342染色检测HNPC细胞的凋亡水平。**结果** 与Ctrl组比较,AGE组lncRNA HOTAIR及NLRP3、Caspase1、含半胱天冬氨酸蛋白酶激活和招募结构域结构的凋亡相关斑点样蛋白(ASC)均上调,细胞凋亡增加,miR-22表达下调,差异均有统计学意义($P<0.05$);lncRNA HOTAIR可以和miR-22的碱基序列结合,且miR-22能和NLRP3 3′UTR的碱基序列结合。与Vector组比较,HOTAIR组的lncRNA HOTAIR及NLRP3、Caspase1、ASC蛋白表达上调,细胞凋亡增加,miR-22表达下调,差异均有统计学意义($P<0.05$)。与HOTAIR组比较,HOTAIR+mimic组的miR-22表达上调,NLRP3、Caspase1、ASC蛋白表达均下调,细胞凋亡数减少,差异均有统计学意义($P<0.05$)。与HOTAIR组比较,HOTAIR+mcc950组的lncRNA HOTAIR和miR-22表达差异不显著($P>0.05$),NLRP3、Caspase1、ASC蛋白表达下调,细胞凋亡数减少,差异均有统计学意义($P<0.05$)。**结论** lncRNA HOTAIR通过靶向调控miR-22激活NLRP3炎症小体促进椎间盘髓核细胞凋亡。

[关键词] 长链非编码RNA;HOX转录反义RNA;miRNA-22;核苷酸结合寡聚结构域样受体家族Pyrin域蛋白3;椎间盘髓核细胞;凋亡

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Study on mechanism of lncRNA HOTAIR promoting apoptosis of intervertebral disc nucleus pulposus cells

Ailixiati Aireti, MA Zhi-gang, LI Hong-wei (First Department of Spine, Orthopedic Center, People's Hospital of Xinjiang Uygur Autonomous Region, Urumqi Xinjiang 830000, China)

Abstract: Objective To study the regulatory effect of long non-coding RNA (lncRNA) HOX transcript antisense intergenic RNA (HOTAIR) on the apoptosis of nucleus pulposus cells of intervertebral disc. **Methods** Human intervertebral disc nucleus pulposus cell line (HNPC) was cultured and randomly divided into control (Ctrl) group, advanced glycation end products (AGE) group (AGE group), microRNA(miR)-22 mimic group(mimic group), mimic negative control group(mimic-NC group), lncRNA HOTAIR overexpression group (HOTAIR group), empty vector of overexpression plasmid group (Vector group), HOTAIR combined with mimic treatment group(HOTAIR+mimic group), and HOTAIR combined with the inhibitor of nucleotide-binding oligomerization domain-like receptor family pyrin domain-containing protein 3 (NLRP3)mcc950 group (HOTAIR+mcc950 group). The expression of miR-22 and lncRNA HOTAIR was detected by qRT-PCR. The relationship between lncRNA HOTAIR and miR-22, as well as miR-22 and NLRP3, was detected by dual luciferase reporter gene assay. The expression of NLRP3 inflammasome-related proteins was detected by Western blot. And the apoptosis of HNPC cells was detected by flow cytometry and Hoechst 33342 staining. **Results** Compared with the Ctrl group, the expression of lncRNA HOTAIR, NLRP3, Caspase1, and apoptosis-associated speck-like protein containing a caspase activation and recruitment domain (ASC) protein was up-regulated, cell apoptosis was increased, and the expression of miR-22 was down-regulated in AGE group, the differences were significant ($P<0.05$). In addition, lncRNA HOTAIR could bind to the base sequence of miR-22, and miR-22 could bind to the base sequence of NLRP3 3′UTR. Compared with the Vector group, the expression of lncRNA HOTAIR, NLRP3, Caspase1, and ASC protein was up-regulated, cell apoptosis was increased, and the expression of miR-22 was down-regulated in the HOTAIR group, the differences were significant ($P<0.05$). Compared with the HOTAIR group, the expression of miR-22 was up-regulated, and the protien expression of NLRP3, Caspase1, and ASC was down-regulated, and cell apoptosis was decreased in the HOTAIR+mimic group, the differences were significant ($P<0.05$). Compared

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[通信作者] 李洪伟,E-mail: dr_lihongwei@163.com

with the HOTAIR group, there was no significant difference in the expression of lncRNA HOTAIR or miR-22 in the HOTAIR+mcc950 group (P>0.05), and the protein expression of NLRP3, Caspase1, and ASC was down-regulated, and cell apoptosis was decreased, the differences were significant (P<0.05). **Conclusion** lncRNA HOTAIR activates NLRP3 inflammasome and promotes the apoptosis of intervertebral disc nucleus pulposus cells by targeting miR-22.

Keywords: long non-coding RNA; HOX transcript antisense intergenic RNA; miR-22; nucleotide-binding oligomerization domain-like receptor family pyrin domain-containing protein 3; intervertebral disc nucleus pulposus cell; apoptosis

椎间盘退变(intervertebral disc degeneration,IDD)是下腰痛最常见的原因之一,表现为细胞外基质的退化、细胞凋亡、炎症反应增加[1]。当前IDD的治疗主要为缓解疼痛和控制症状,较少干扰其病理生理过程,虽然已有一些缓解轻度和中度IDD的临床策略,但仍需进一步探讨[2-3]。因此,深入研究IDD的发病机制对研发新型治疗方法有帮助。长链非编码RNA(long non-coding RNA,lncRNA)是一类长度超过200个核苷酸,且无编码蛋白功能的RNA。越来越多的证据表明,lncRNA在人类退行性髓核组织中存在差异表达,并可能在调节IDD的进展中发挥关键作用[4-5]。有研究发现,核苷酸结合寡聚结构域样受体家族Pyrin域蛋白3(nucleotide-binding oligomerization domain-like receptor family pyrin domain-containing protein 3,NLRP3)在IDD中表达明显增高,且与椎间盘退变程度呈正相关,而且IDD的髓核细胞中NLRP3炎症小体被激活[6-8]。另外,已有研究表明,lncRNA HOX转录反义RNA(HOX transcript antisense intergenic RNA,HOTAIR)与NLRP3炎症小体的激活有关[9]。lncRNA HOTAIR可通过内源竞争性RNA(competing endogenous RNA,ceRNA)竞争机制来控制微小RNA(microRNA,miR)-22的表达和功能[10-11],而且lncRNA HOTAIR还可以调控IDD的进展[12]。然而,lncRNA HOTAIR在IDD中对髓核细胞的炎症小体和细胞凋亡的具体作用尚不清楚。故本研究探讨了lncRNA HOTAIR对椎间盘髓核细胞中炎症小体激活的调节作用和机制。

1 材料与方法

1.1 人椎间盘髓核细胞系培养

人椎间盘髓核细胞系(human intervertebral disc nucleus pulposus cell line,HNPC)购自武汉普诺赛生物科技有限公司。HNPC细胞在含有10%胎牛血清(美国GIBCO公司)、100 mg/mL链霉素和100 U/mL青霉素的杜氏改良培养基中培养,并置于37 ℃的湿润培养箱[95%(V/V)空气和5%(V/V) CO_2]中培养,培养基每隔2 d更换1次。细胞每隔3 d传代1次,将传代2次后的对数生长期细胞用于后续实验。

1.2 细胞的分组与处理

用晚期糖基化终末产物(advanced glycation end products,AGE)诱导HNPC细胞退变。首先将HNPC细胞分为AGE(200 μg/mL,上海远慕生物有限公司)处理组(AGE组)和对照组(与AGE等体积的生理盐水处理细胞,Ctrl组)。其次,将HNPC细胞分为miR-22的拟似物(20 μg/mL,北京生工生物技术公司)组(mimic组)、mimic的阴性对照(20 μg/mL,北京生工生物技术公司)组(mimic-NC组)、lncRNA HOTAIR过表达质粒(10 μg/mL,上海吉玛公司)组(HOTAIR组)、过表达质粒的空载体(10 μg/mL,上海吉玛公司)组(Vector组)、10 μg/mL HOTAIR联合20 μg/mL mimic处理组(HOTAIR+mimic组)、10 μg/mL HOTAIR联合50 μg/mL NLRP3抑制剂mcc950处理组(HOTAIR+mcc950组)。各组细胞均处理24 h用于后续实验。

1.3 流式细胞术检测HNPC细胞的凋亡率

用Annexin V-FITC凋亡试剂盒(美国Sigma公司)评估AGE组、Ctrl组、HOTAIR组以及Vector组细胞凋亡率。各组HNPC细胞在结合缓冲液中洗涤2次后,以500 g离心5 min。然后置于100 μL Annexin V-FITC和10 μL PI的1×结合缓冲液中避光培养。采用流式细胞仪检测细胞凋亡率。

1.4 Western blot检测炎症小体蛋白标志物

检测AGE组、Ctrl组、HOTAIR组、Vector组、HOTAIR+mimic组、HOTAIR+mcc950组中炎症小体相关蛋白NLRP3、Caspase1、含半胱天冬酶激活和募集结构域的凋亡相关斑点样蛋白(apoptosis-associated speck-like protein containing a caspase activation and recruitment domain,ASC)的表达。用RIPA裂解缓冲液裂解细胞提取总蛋白。用二喹啉甲酸试剂盒测定蛋白浓度。此外,使用12% SDS-PAGE凝胶分离蛋白,并将其转移到聚偏二氟乙烯膜上。将膜用5%的脱脂牛奶封闭2 h,然后在4 ℃下与一抗(均购于美国Abcam公司)孵育10 h。免疫复合物用辣根过氧化物酶偶联的抗兔IgG抗体(1∶5 000)孵育。使用Western-Light化学发光检测系统捕获蛋白信号表达。所有实验均重复3次。抗体为NLRP3(1∶500)、Caspase1(1∶1 000)、

ASC(1∶600)。

1.5 qRT-PCR法检测lncRNA HOTAIR和miR-22的表达

根据试剂盒说明书,使用TRIzol试剂(美国Invitrogen公司)和RNeasy mini kit(美国Qiagen公司)从各组HNPC细胞中提取总RNA或者总miRNA。通过分光光度法测定总RNA或者总miRNA的浓度和纯度,并使用PrimeScript RT逆转录酶试剂盒(日本Takara公司)按照说明书合成互补DNA。使用SYBR Premix Ex Taq试剂盒(日本Takara公司)和StepOnePlus实时PCR系统(美国Applied Biosystems)进行qRT-PCR分析。lncRNA HOTAIR表达以GAPDH为内参,miR-22表达以U6为内参,用$2^{-\Delta\Delta Ct}$法测定基因的相对表达量。目的基因引物由上海生工生物技术有限公司设计。引物序列:lncRNA HOTAIR正向5′-CCTTATAAGCTCATCGGAGCA-3′,反向5′-CATTTCTGGGTGGTTCCTTT-3′;miR-22正向5′-GGTTAAGCTGCCAGTTGAA-3′,反向5′-CCAGTGCGTGTCGTGGAGT-3′;GAPDH正向5′-CTCACTGCTACATCCAGTACAC-3′,反向5′-CCTGCCTACCATGTTTACCG-3′;U6正向5′-CTCGCTTCGGCAGCACA-3′,反向5′-AACGCTTCACGAATTTGCGT-3′。

1.6 双荧光素酶报告实验

通过双荧光素酶报告实验检测lncRNA HOTAIR与miR-22以及miR-22与NLRP3的3′-UTR的结合作用。通过PCR扩增HOTAIR中包含预测miR-22结合位点的cDNA片段。然后将结合位点的假定序列克隆到pGL3双荧光素酶miRNA-基因表达载体(美国Promega公司)中,构建报告载体pGL3-HOTAIR-wild-type(WT-lncRNA HOTAIR)。另外,使HOTAIR基因中miR-22的结合位点发生突变,并命名为pGL3-HOTAIR-mut(MUT-lncRNA HOTAIR)。随后,根据说明书,使用Lipofectamine 2000将1 μg WT-lncRNA HOTAIR或1 μg MUT-lncRNA HOTAIR分别与mimic转染的HNPC细胞(5×10⁴个/孔)共转染(mimic组),或使用Lipofectamine 2000将1 μg WT-lncRNA HOTAIR或1 μg MUT-lncRNA HOTAIR分别与mimic-NC转染的HNPC细胞(5×10⁴个/孔)共转染(mimic-NC组)。双荧光素酶报告系统(美国Promega公司)检测荧光素酶活性。

另外,与上述方案一致,构建报告载体pGL3-NLRP3-3′UTR-wild-type (WT-NLRP3-3′UTR)。使NLRP3-3′UTR中miR-22的结合位点发生突变,并命名为pGL3-NLRP3-3′UTR-mut(MUT-NLRP3-3′UTR)。随后,根据说明书,使用Lipofectamine 2000将1 μg WT-NLRP3-3′UTR或1 μg MUT-NLRP3-3′UTR分别与mimic转染的HNPC细胞(5×10⁴个/孔)共转染(mimic组),或使用Lipofectamine 2000将质粒1 μg WT-NLRP3-3′UTR或1 μg MUT-NLRP3-3′UTR分别与mimic-NC转染的HNPC细胞(5×10⁴个/孔)共转染(mimic-NC组)。双荧光素酶报告系统(美国Promega公司)检测荧光素酶活性。

1.7 Hoechst 33342染色检测HNPC细胞的凋亡率

将Vector组、HOTAIR组、Ctrl组、HOTAIR+mimic组、HOTAIR+mcc950组HNPC细胞过夜培养。用PBS轻轻冲洗细胞,同时加入0.5 mL Hoechst 33342(美国Thermo Fisher公司)和PI(美国Invitrogen公司)工作液。将细胞置于室温培养箱中培养15 min。PBS冲洗后,用荧光显微镜观察分析,统计图中HNPC细胞的凋亡数。

1.8 统计学分析

使用GraphPad PRISM 5.01进行数据录入和统计学分析,正态分布的数据以均数±标准差($\bar{x}\pm s$)表示。2组间比较采用*Student t*检验,多组间比较采用单因素方差分析(*ANOVA*),进一步两两比较采用*LSD-t*检验。检验水准为α=0.05,P<0.05为差异有统计学意义。

2 结果

2.1 髓核细胞中lncRNA HOTAIR、miR-22、NLRP3的表达

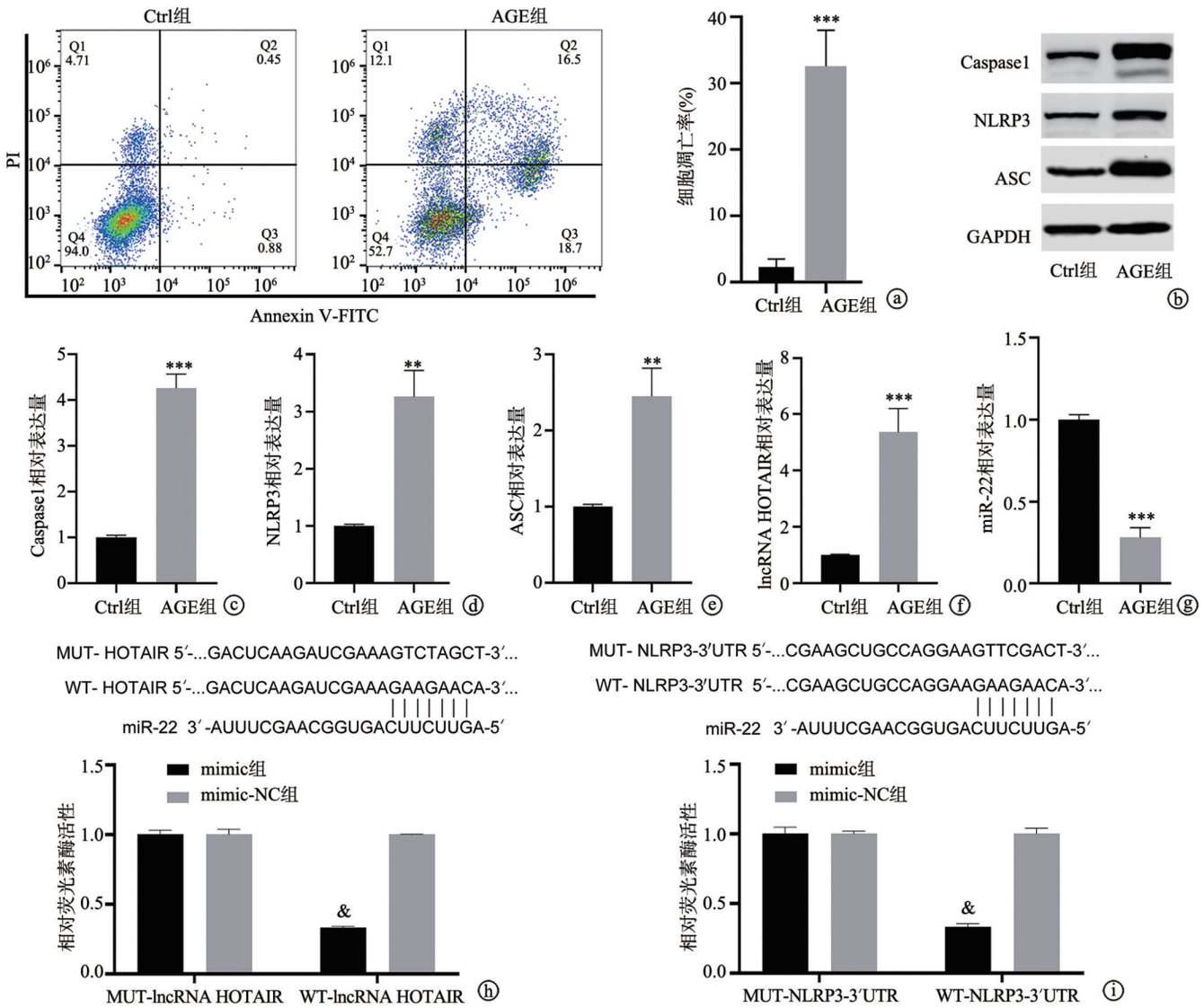
使用200 μg/mL的AGE诱导髓核细胞退变。与Ctrl组比较,AGE组细胞凋亡率明显升高(P<0.05),而且AGE组NLRP3、ASC、Caspase1的表达均上调(P<0.05)。与Ctrl组比较,AGE组髓核细胞中lncRNA HOTAIR的表达上调(P<0.05),而miR-22下调(P<0.05)。双荧光素酶报告实验检测发现,lncRNA HOTAIR可直接结合miR-22,且miR-22可与NLRP3的3′UTR结合,见图1。

2.2 过表达lncRNA HOTAIR促进炎症小体的激活和细胞凋亡

与Vector组比较,HOTAIR组的lncRNA HOTAIR表达显著增加(P<0.05)。与Vector组比较,HOTAIR组NLRP3、ASC、Caspase1蛋白表达均上调(P<0.05),且髓核细胞的凋亡率显著升高(P<0.05),miR-22表达显著降低(P<0.05),见图2。

2.3 lncRNA HOTAIR通过抑制miR-22激活NLRP3促进的细胞凋亡

与Ctrl组比较,HOTAIR组细胞凋亡数明显增加



a:流式细胞术检测AGE诱导下髓核细胞凋亡水平;b~e:Western blot检测AGE诱导下髓核细胞NLRP3炎症小体标志物的表达;f~g:qRT-PCR法检测AGE诱导下lncRNA HOTAIR和miR-22的表达;h:双荧光素酶基因报告实验检测lncRNA HOTAIR和miR-22之间的调控关系;i:双荧光素酶基因报告实验检测NLRP3和miR-22之间的调控关系 **:与Ctrl组比较,$P<0.01$;***:与Ctrl组比较,$P<0.001$;&:与mimic-NC组比较,$P<0.01$

图1 lncRNA HOTAIR、miR-22、NLRP3炎症小体在AGE诱导的髓核细胞退变中的表达

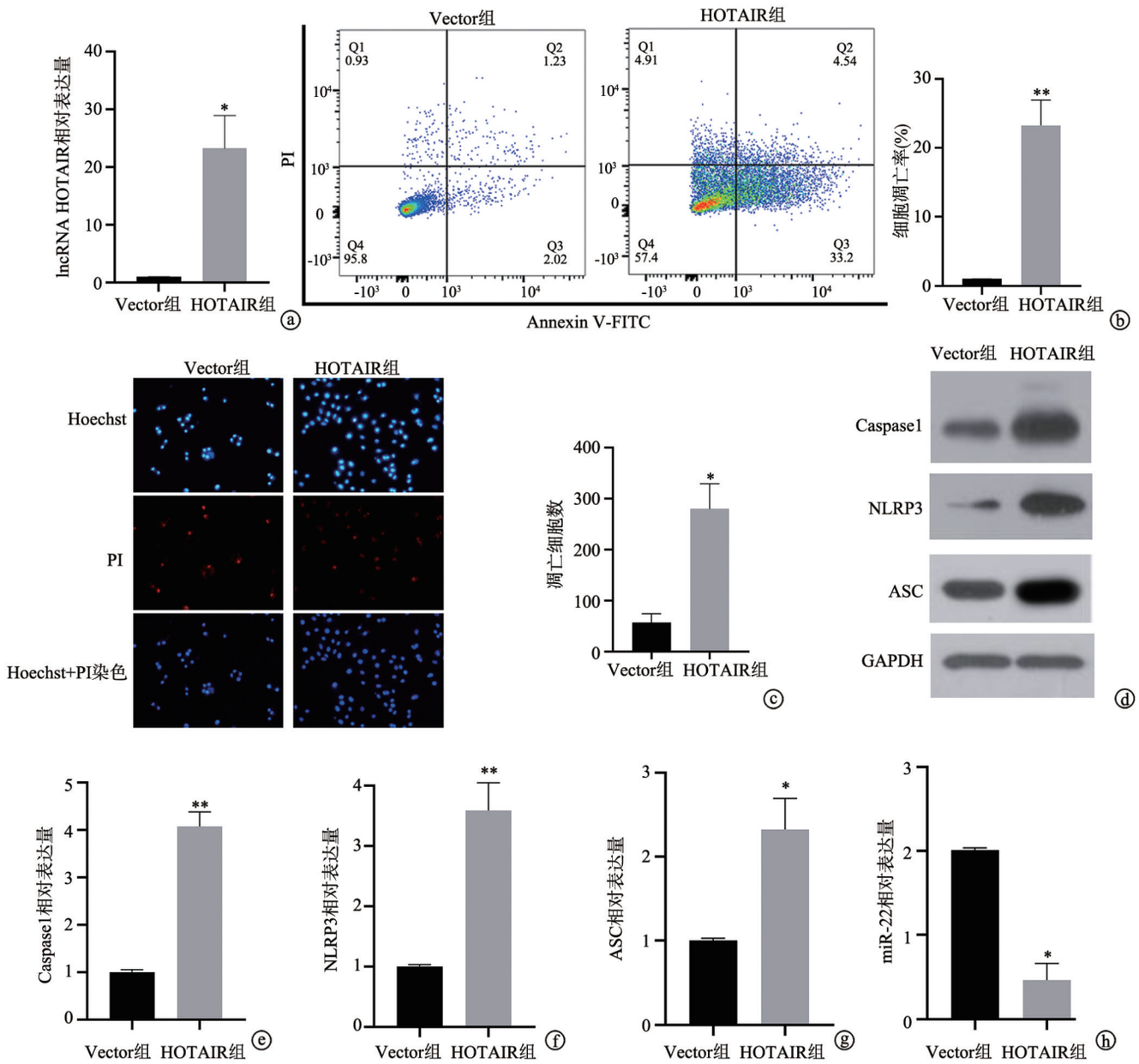
($P<0.05$);与HOTAIR组比较,HOTAIR+mimic组及HOTAIR+mcc950组细胞凋亡数均显著减少($P<0.05$),见图3。

2.4 lncRNA HOTAIR通过抑制miR-22激活NLRP3介导的炎症小体

与Ctrl组比较,HOTAIR组lncRNA HOTAIR以及NLRP3、ASC、Caspase1蛋白表达均上调($P<0.05$),而miR-22的表达显著下调($P<0.05$)。与HOTAIR组比较,HOTAIR+mimic组lncRNA HOTAIR的表达无显著变化($P>0.05$),miR-22的表达显著上调($P<0.05$),而NLRP3、ASC、Caspase1蛋白表达均显著下调($P<0.05$)。与HOTAIR组比较,HOTAIR+mcc950组lncRNA HOTAIR和miR-22表达均无显著变化($P>0.05$),而NLRP3、ASC、Caspase1蛋白表达均显著下调($P<0.05$),见图4。

3 讨论

IDD是一种常见的退行性疾病,lncRNA在其发病机制中起到重要作用[10-13]。2014年,Wan等[14]首次报道了人类IDD中lncRNA的表达谱,与健康人群相比,IDD人群中lncRNA HOTAIR(NR_003716)的表达差异显著。本研究发现,在AGE诱导髓核细胞退变过程

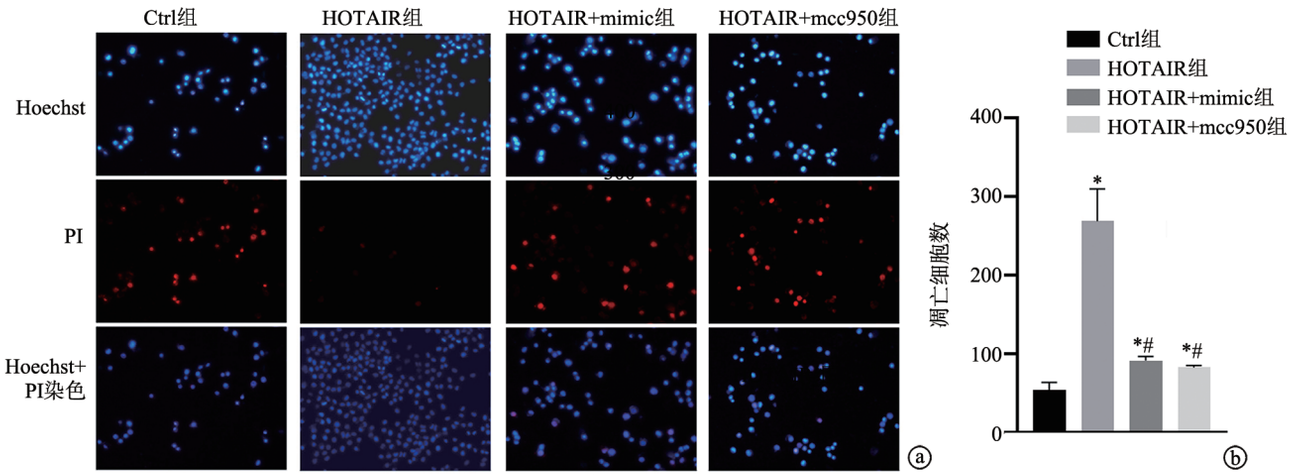


a:qRT-PCR 法检测 lncRNA HOTAIR 的表达;b:流式细胞术检测髓核细胞的凋亡情况;c:Hoechst 33342+PI 染色检测髓核细胞的凋亡情况(×200);d~g:Western blot 检测髓核细胞 NLRP3 炎症小体标志物的表达;h:qRT-PCR 法检测 miR-22 的表达 *:与 Vector 组比较,$P<0.01$;**:与 Vector 组比较,$P<0.001$

图2 lncRNA HOTAIR 过表达对 NLRP3 炎症小体和髓核细胞凋亡的影响

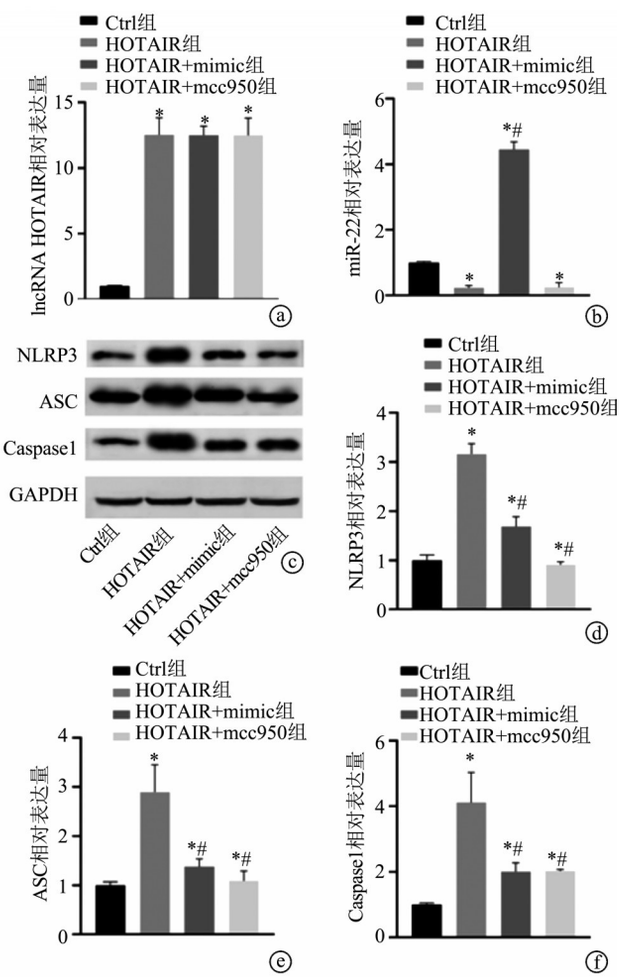
中,lncRNA HOTAIR 表达显著上调,并且进一步证明其具有促进 HNPC 细胞凋亡的能力。此外,本研究发现,lncRNA HOTAIR 通过与 miR-22 和靶基因 NLRP3 的直接靶向结合,调控 NLRP3 炎症小体的激活。这些发现为以 lncRNA HOTAIR/miR-22/NLRP3 轴作为 IDD 的治疗靶点提供了新线索。

先前的研究表明,ceRNA 调控网络在 IDD 中具有重要作用[15-19]。lncRNA HOTAIR 可通过与 miR-34a、miR-22 等 miRNAs 相互作用来调控髓核细胞凋亡[20-21],且 miR-22 是一种被充分证明受 ceRNA 网络调控并参与介导多种细胞过程的 miRNA[22],其可促进 HNPC 细胞增殖,抑制 HNPC 细胞的退行性改变并减缓细胞衰老[23]。本研究证实了 miR-22 在 AGE 诱导后的 HNPC 细胞中显著降低,而且过表达 lncRNA HOTAIR 显著抑制了 miR-22 的表达,表明 lncRNA HOTAIR 可以负向调控 miR-22。有趣的是,我们进一步研究发现,lncRNA HOTAIR 与 miR-22 直接靶向结合。此外,lncRNA HOTAIR 过表达可以促进 HNPC 细胞凋亡并抑制 miR-22 的表达,过表达 miR-22 后明显逆转了这种促进作用,表明 HNPC 细胞凋亡受 lncRNA HOTAIR/



a:Hoechst 33342+PI 染色检测髓核细胞的凋亡情况(×200);b:细胞凋亡数统计 *:与 Ctrl 组比较,$P<0.05$;#:与 HOTAIR 组比较,$P<0.05$

图3 lncRNA HOTAIR通过抑制miR-22激活NLRP3促进的细胞凋亡



a:qRT-PCR 法检测 lncRNA HOTAIR 的表达;b:qRT-PCR 法检测 miR-22 的表达;c~f:Western blot 检测髓核细胞 NLRP3 炎症小体标志物的表达 *:与 Ctrl 组比较,$P<0.05$;#:与 HOTAIR 组比较,$P<0.05$

图4 髓核细胞中lncRNA HOTAIR、miR-22以及NLRP3炎症小体标志物的表达

miR-22轴的调控。

另外,本研究进一步发现,miR-22与NLRP3直接靶向结合。AGE诱导后NLRP3炎症小体明显被激活,且当lncRNA HOTAIR过表达后可以显著上调NLRP3、ASC、Caspase1蛋白,从而促进NLRP3炎症小体激活。当mcc950抑制NLRP3后,明显逆转了lncRNA HOTAIR对凋亡和炎症小体的促进作用。过表达miR-22后明显逆转了这种促进作用,表明HNPC的细胞凋亡和炎症小体都受lncRNA HOTAIR/miR-22/NLRP3轴的调控。

尽管在体外实验中观察到了lncRNA HOTAIR/miR-22/NLRP3轴在促进HNPC细胞凋亡和炎症小体激活中的重要作用,但仍需要进一步研究来验证这种调控机制在体内是否能逆转IDD的发生和发展。例如,开展动物模型实验和临床研究来评估针对lncRNA HOTAIR/miR-22/NLRP3轴的治疗策略是否能够有效改善IDD患者的病情。

综上所述,lncRNA HOTAIR通过调控miR-22和NLRP3的表达,参与了椎间盘髓核细胞凋亡和炎症小体的激活。本研究表明,以lncRNA HOTAIR/miR-22/NLRP3轴作为IDD的治疗靶点非常有前景,可用于IDD治疗的候选药物的研究中。

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