蒿本内酯通过lncRNA NKILA对干扰素α诱导的人脑血管外膜成纤维细胞增殖及凋亡的影响

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摘要 该文旨在探讨蒿本内酯对干扰素α(IFN-α)诱导的人脑血管外膜成纤维细胞(HBVAF)增殖及凋亡的影响及其可能作用机制。HBVAF细胞经IFN-α诱导后建立细胞损伤模型, 将不同剂量的蒿本内酯作用于IFN-α诱导的HBVAF细胞后, 采用MTT法、平板克隆形成实验、流式细胞术分别检测细胞增殖、克隆形成及凋亡情况, qRT-PCR法检测lncRNA NKILA的表达量; 为探究蒿本内酯与lncRNA NKILA对IFN-α诱导的HBVAF细胞增殖及凋亡的影响, 将pcDNA、pcDNA-lncRNA NKILA分别转染至HBVAF细胞后加入IFN-α处理24 h, si-NC、si-lncRNA NKILA分别转染至HBVAF细胞后加入蒿本内酯与IFN-α共处理24 h, Western blot测定凋亡相关蛋白表达量。结果显示, 蒿本内酯处理后细胞存活率和lncRNA NKILA的表达量升高(P<0.05), 细胞克隆形成数增多(P<0.05), 细胞凋亡率和Cleaved-caspase3、Cleaved-caspase9蛋白水平降低(P<0.05), 且呈剂量依赖性; 转染pcDNA-lncRNA NKILA后, 细胞存活率升高(P<0.05), 细胞克隆形成数增多(P<0.05), 细胞凋亡率和Cleaved-caspase3、Cleaved-caspase9蛋白水平降低(P<0.05); 转染si-lncRNA NKILA可减弱蒿本内酯对IFN-α诱导的HBVAF细胞增殖、克隆形成及凋亡的作用。总之, 蒿本内酯可通过上调lncRNA NKILA表达而促进IFN-α诱导的脑血管外膜成纤维细胞增殖、克隆形成及抑制细胞凋亡。

关键词 人脑血管外膜成纤维细胞; 干扰素α; 蒿本内酯; lncRNA NKILA; 细胞增殖; 凋亡

The Effect of Ligustilide on the Proliferation and Apoptosis of Human Cerebral Adventitia Fibroblasts Induced by Interferon-α Through lncRNA NKILA

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Abstract The aim of this study is to investigate the effect of ligustilide on the proliferation and apoptosis of HBVAF (human brain vascular adventitia fibroblast) induced by IFN-α (interferon-α) and its possible mechanism. HBVAF cells were induced by IFN-α to establish a cell injury model. The effect of different doses of ligustilide on the proliferation, colony formation and apoptosis of HBVAF cells induced by IFN-α were detected by MTT, plate clone formation assays and flow cytometry, respectively. qRT-PCR was used to detect the expression of lncRNA NKILA. In order to explore the effects of ligustilide and lncRNA NKILA on the proliferation and apoptosis of HBVAF cells induced by IFN-α, HBVAF cells were transfected with pcDNA and pcDNA-lncRNA NKILA, and then treated with IFN-α for 24 h.

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HBVAF cells were transfected with si-NC and si-lncRNA NKILA, and then co-treated with ligustilide and IFN-α for 24 h. Western blot was used to detect the expression of apoptosis-related proteins. The results showed that after ligustilide treatment, the cell survival rate and the expression of lncRNA NKILA were increased (P<0.05); the number of colonies formed was increased (P<0.05); and the apoptosis rate and the protein levels of Cleaved-caspase3, Cleaved-caspase9 were decreased (P<0.05). The effect was dose-dependent. After transfection of pcDNA-lncRNA NKILA, the cell survival rate was increased (P<0.05), the number of colonies formed was increased (P<0.05), and the apoptosis rate and the protein levels of Cleaved-caspase3, Cleaved-caspase9 were decreased (P<0.05). Transfection of si-lncRNA NKILA could attenuate the effects of ligustilide on the proliferation, colony formation and apoptosis of HBVAF cells induced by IFN-α. In conclusion, ligustilide could promote the proliferation, colony formation and inhibit the apoptosis of IFN-α-induced cerebral adventitia fibroblasts by up-regulating the expression of lncRNA NKILA.

Keywords human cerebral adventitia fibroblasts; INF-α; ligustilide; lncRNA NKILA; cell proliferation; apoptosis

人脑血管外膜成纤维细胞属于细胞外膜的重要组成成分, 细胞增殖与凋亡失衡可促进新生内膜形成或病理性血管重构从而导致动脉粥样硬化等血管增生性疾病的发生[1-2]。干扰素α(interferon-α, IFN-α)可通过调控多种基因表达而参与血管增生性疾病发生[3-4]。蒿本内酯是我国中药当归、川芎的主要活性成分, 可以改善血液循环并发挥免疫调节等多重作用。蒿本内酯可以通过抗神经细胞凋亡途径减轻脑损伤[5], 但蒿本内酯对IFN-α诱导的脑血管外膜成纤维细胞损伤的影响尚未可知。长链非编码RNA(lncRNA)可参与多种疾病发生及发展过程, 并发挥重要调控作用。研究表明lncRNA NKILA在创伤性脑损伤中表达下调, 上调其表达可减轻神经元损伤[6]。但lncRNA NKILA在血管增生性疾病进展中的变化机制尚有争议。因此, 本研究通过IFN-α诱导构建人脑血管外膜成纤维细胞(human brain vascular adventitia fibroblast, HBVAF)损伤模型, 探究蒿本内酯对细胞增殖、凋亡进程的影响是否与lncRNA NKILA相关。

1 材料与方法

1.1 材料与试剂

人脑血管外膜成纤维细胞(HBVAF)由上海泽叶生物科技有限公司提供; IFN-α由北京三元基因药业股份有限公司提供; 蒿本内酯(纯度≥98%)由宝鸡市翊瑞生物科技有限公司提供; DMEM培养基/胎牛血清/胰蛋白酶购自美国Gibco公司; Lipofectamine™ 3000 Transfection Reagent转染试剂购自美国Invitrogen公司; Trizol试剂购自美国Thermo Fisher公司; 反转录与荧光定量PCR试剂盒购自北京天根生化科技有限公司; pcDNA/pcDNA-lncRNA NKILA/si-NC/si-lncRNA NKILA由上海吉玛制药技术有限公司提供; 兔抗人Cleaved-caspase3/Cleaved-caspase9抗体购自武汉艾美捷科技有限公司; 内参*GAPDH*抗体/HRP标记的山羊抗兔IgG二抗购自美国Santa Cruz公司; MTT试剂/细胞凋亡检测试剂盒购自北京索莱宝科技有限公司。

1.2 方法

1.2.1 实验处理及分组 HBVAF细胞接种于6孔板(1×10^{5}个/孔)中, 于含有IFN-α的培养基中培养24 h, 浓度为30 μmol/L[7], 记为IFN-α组。同时将正常培养的HBVAF细胞记为con组。细胞于含有不同浓度(5 μmol/L、10 μmol/L、15 μmol/L)蒿本内酯[8]与2 000 U/mL IFN-α的培养基中培养24 h, 分别记为IFN-α+蒿本内酯L组、IFN-α+蒿本内酯M组、IFN-α+蒿本内酯H组。采用脂质体转染法将pcDNA、pcDNA-lncRNA NKILA分别转染至HBVAF细胞, 转染成功后于含有IFN-α(2 000 U/mL)的培养基中培养24 h, 分别记为IFN-α+pcDNA组、IFN-α+pcDNA-lncRNA NKILA组。采用脂质体转染法将si-NC、si-lncRNA NKILA分别转染至HBVAF细胞, 转染成功后于含有蒿本内酯(15 μmol/L)与IFN-α(2 000 U/mL)的培养基中培养24 h, 分别记为IFN-α+蒿本内酯+si-NC组、IFN-α+蒿本内酯+si-lncRNA NKILA组。

1.2.2 MTT检测细胞增殖 HBVAF细胞接种于96孔板中, 每孔300个细胞, 每孔加入20 μL MTT溶液, 继续培养4 h后加入150 μL DMSO, 采用酶标仪测定

波长在490 nm处的吸光度值, 计算细胞增殖抑制率。HBVAF细胞于含有蒿本内酯(0.625、1.25、2.5、5、10、15、30 μmol/L)的培养基中培养24 h, 取正常培养的HBVAF细胞与经蒿本内酯和IFN-α(2 000 U/mL)处理后的细胞, 采用MTT法检测细胞存活率。

1.2.3 平板克隆形成实验 收集HBVAF细胞并按每孔500个细胞接种于6孔板中, 接种完毕后置于37 °C培育箱继续培育至出现肉眼可见的细胞克隆团时弃培养基, 采用500 μL甲醇于37 °C固定2 h, 加入400 μL浓度为1%的结晶紫进行染色, 观察细胞克隆形成数。

1.2.4 流式细胞术检测细胞凋亡率 培养48 h后, 收集HBVAF细胞, PBS冲洗后加入500 μL结合缓冲液重悬细胞。根据Annexin V-FITC/PI凋亡检测试剂盒操作说明, 先后加入10 μL的Annexin V-FITC和5 μL的PI, 进行避光染色, 15 min后采用流式仪分析细胞凋亡率。

1.2.5 qRT-PCR检测lncRNA NKILA的表达水平 取HBVAF细胞并向其中加入1 mL Trizol试剂提取各组细胞总RNA, 再用紫外分光光度计进行测量。

建立反转录体系, 采用20 μL的RNase-Free ddH$_2$O作为补足体系。将cDNA作为模板用于后续的qRT-PCR扩增操作, 在95 °C温度下完成预变性、变性反应, 时间分别设定为2 min、30 s, 60 °C温度下进行退火处理, 30 s后在72 °C温度下延伸30 s, 共40次循环。扩增完成后再运用PCR仪检测lncRNA NKILA相对表达量, 以*GAPDH*为内参, 采用$2^{-\Delta\Delta Ct}$法计算。lncRNA NKILA正向引物5′-CTG TCG GGG ACT GGT GTA TT-3′, 反向引物5′-AAT ACA CCA GTC CCC GAC AG-3′; *GAPDH*正向引物5′-GGA GCG AGA TCC CTC CAA AAT-3′, 反向引物5′-GGC TGT TGT CAT ACT TCT CAT GG-3′。

1.2.6 Western blot检测Cleaved-caspase3、Cleaved-caspase9蛋白表达水平 用RIPA裂解液预处理HBVAF细胞以提取细胞总蛋白, 采用BCA法对蛋白浓度定量。随后, 采用SDS-PAGE分离目的蛋白, 并将蛋白转移至PVDF膜。PVDF膜在37 °C下, 经5%的脱脂牛奶封闭1 h后, 加入一抗(1:1 000) 4 °C孵育过夜, 再与二抗(1:3 000)常温孵育2 h。Quantity One软件分析蛋白条带灰度值。

1.3 统计学分析

获取的研究数据均录入SPSS 21.0软件进行统计分析, 计量资料以均值±标准差($\bar{x}\pm s$)形式表示, 两组间比较采用独立样本t检验, 多组间比较采用单因素方差分析, P<0.05为差异具有统计学意义。

2 结果

2.1 蒿本内酯促进干扰素α诱导的HBVAF存活

随着蒿本内酯给药剂量的增加, IFN-α组细胞存活率逐渐升高, 而con组细胞存活率无明显变化(图1)。

2.2 蒿本内酯促进干扰素α诱导的HBVAF增殖, 抑制HBVAF凋亡

如图2所示, 与con组比较, IFN-α组细胞存活率降低(P<0.05), 细胞凋亡率和Cleaved-caspase3、Cleaved-caspase9蛋白水平升高(P<0.05), 细胞克隆形成数减少(P<0.05); 与IFN-α组比较, IFN-α+蒿本内酯L组、IFN-α+蒿本内酯M组、IFN-α+蒿本内酯H组细胞存活率升高(P<0.05), 细胞凋亡率和Cleaved-caspase3、Cleaved-caspase9蛋白水平降低(P<0.05), 细胞克隆形成数增多(P<0.05), 且呈剂量依赖性(图2)。

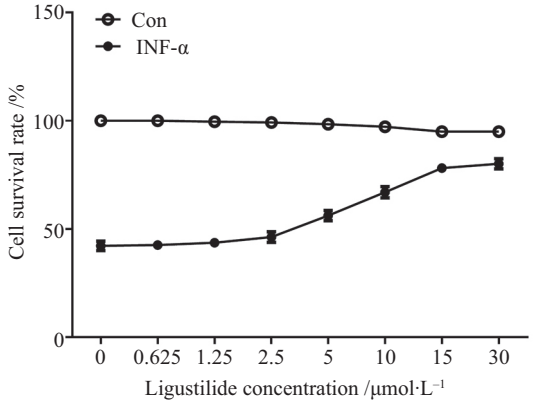
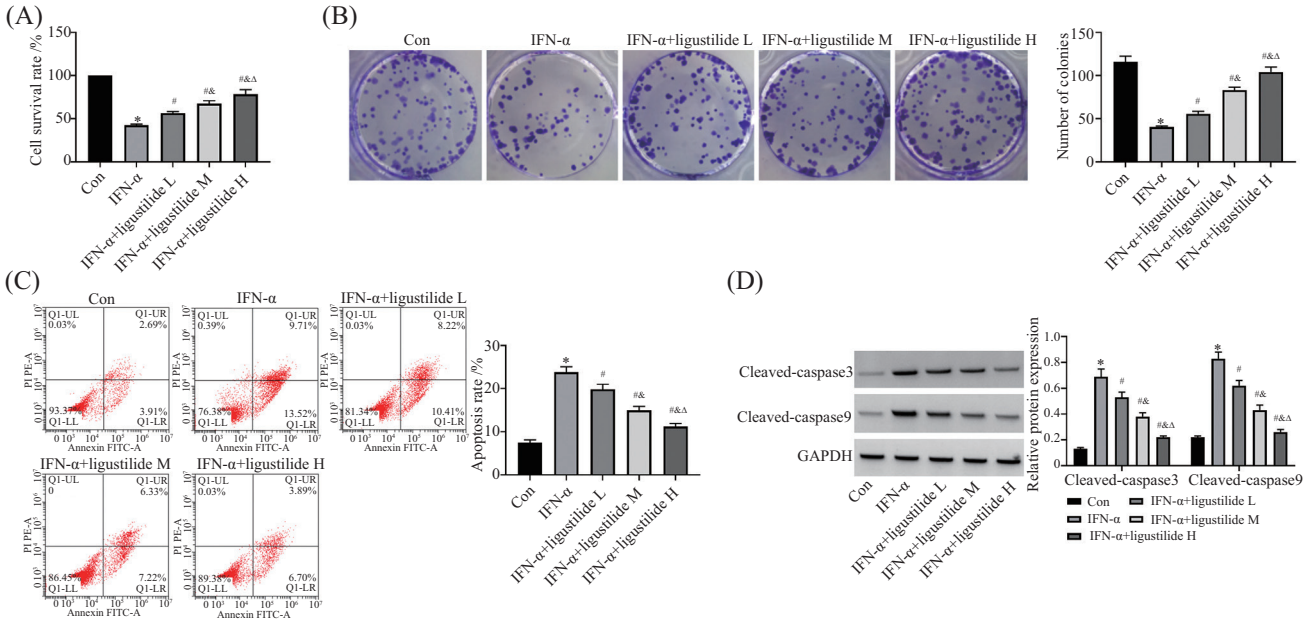


图1 蒿本内酯对HBVAF存活率的影响

Fig.1 Effects of ligustilide on survival rate of HBVAF

2.3 蒿本内酯上调lncRNA NKILA在干扰素α诱导的HBVAF中的表达

与con组比较，IFN-α组lncRNA NKILA的表达量降低(P<0.05, 图3)；与IFN-α组比较，IFN-α+蒿本内酯L组、IFN-α+蒿本内酯M组、IFN-α+蒿本内酯H组中lncRNA NKILA的表达量升高(P<0.05, 图3)，且呈剂量依赖性(图3)。

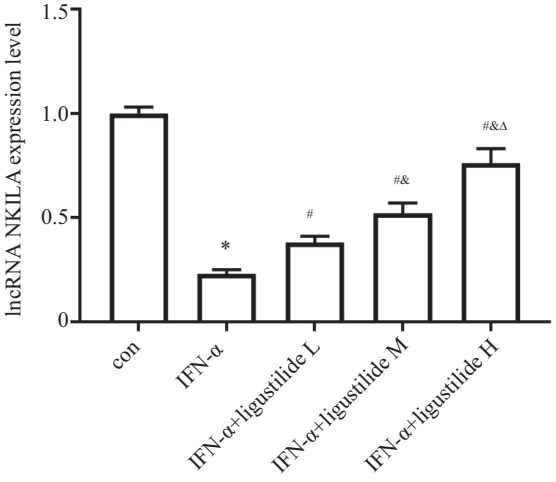


A: 蒿本内酯对干扰素α诱导的HBVAF存活率的影响; B: 蒿本内酯对干扰素α诱导的HBVAF克隆形成能力的影响; C: 蒿本内酯对干扰素α诱导的HBVAF凋亡率的影响; D: 蒿本内酯对干扰素α诱导的HBVAF凋亡相关蛋白表达的影响。*P<0.05, 与con组相比; $^{\#}P$<0.05, 与IFN-α组相比; $^{\&}P$<0.05, 与IFN-α+蒿本内酯L组相比; $^{\triangle}P$<0.05, 与IFN-α+蒿本内酯M相比。

A: the effect of ligustilide on the survival rate of HBVAF induced by INF-α; B: effects of ligustilide on INF-α-induced HBVAF colony-forming ability; C: the effect of ligustilide on the apoptosis rate of HBVAF induced by INF-α; D: effects of ligustilide on the expression of apoptosis-related proteins in HBVAF induced by INF-α; *P<0.05 compared with con group; $^{\#}P$<0.05 compared with IFN-α group; $^{\&}P$<0.05 compared with IFN-α+ligustilide L group; $^{\triangle}P$<0.05 compared with IFN-α+ligustilide M.

图2 蒿本内酯促进干扰素α诱导的HBVAF增殖, 抑制HBVAF凋亡

Fig.2 Ligustilide could promote INF-α-induced HBVAF proliferation and inhibits HBVAF apoptosis



*P<0.05, 与con组相比; $^{\#}P$<0.05, 与IFN-α组相比; $^{\&}P$<0.05, 与IFN-α+蒿本内酯L组相比; $^{\triangle}P$<0.05, 与IFN-α+蒿本内酯M组相比。

*P<0.05 compared with con group; $^{\#}P$<0.05 compared with IFN-α group; $^{\&}P$<0.05 compared with IFN-α+ligustilide L group; $^{\triangle}P$<0.05 compared with IFN-α+ligustilide M group.

图3 qRT-PCR检测lncRNA NKILA的表达

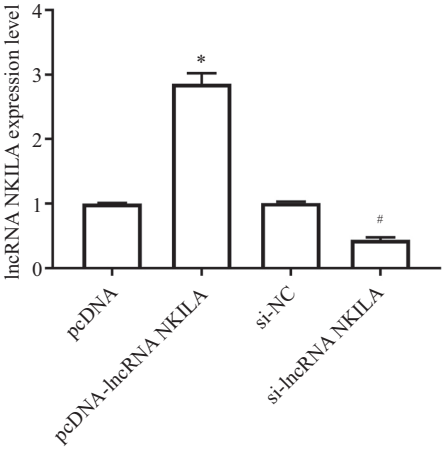
Fig.3 The expression of lncRNA NKILA was detected by qRT-PCR

2.4 lncRNA NKILA处理后转染效率的检测

与pcDNA组比较, pcDNA-lncRNA NKILA组lncRNA NKILA的表达量升高(P<0.05, 图4); 与si-NC组比较, si-lncRNA NKILA组lncRNA NKILA的表达量降低(P<0.05, 图4)。

2.5 过表达lncRNA NKILA逆转了干扰素α诱导的HBVAF损伤

与IFN-α+pcDNA组比较, IFN-α+pcDNA-lncRNA NKILA组细胞存活率升高(P<0.05, 图5), 细胞凋亡率和Cleaved-caspase3、Cleaved-caspase9蛋白水平降低

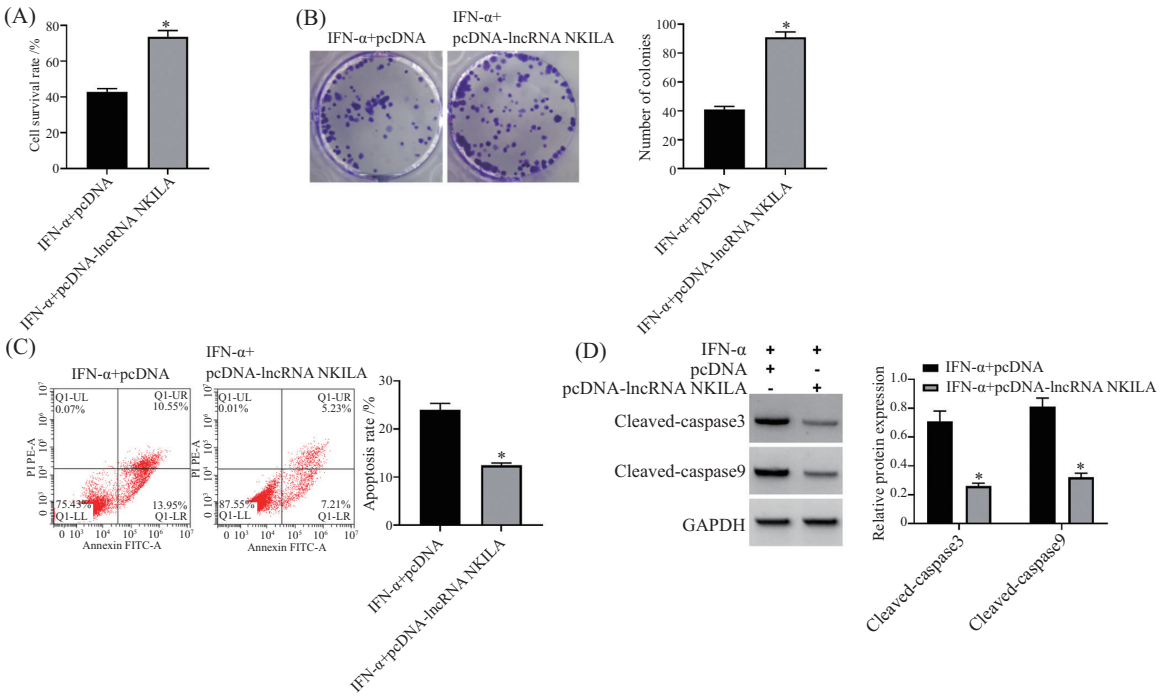


*P<0.05, 与pcDNA组相比; #P<0.05, 与si-NC组相比。

*P<0.05 compared with pcDNA group; #P<0.05 compared with si-NC group.

图4 qRT-PCR检测lncRNA NKILA转染效率

Fig.4 The transfection efficacy of lncRNA NKILA was detected by qRT-PCR

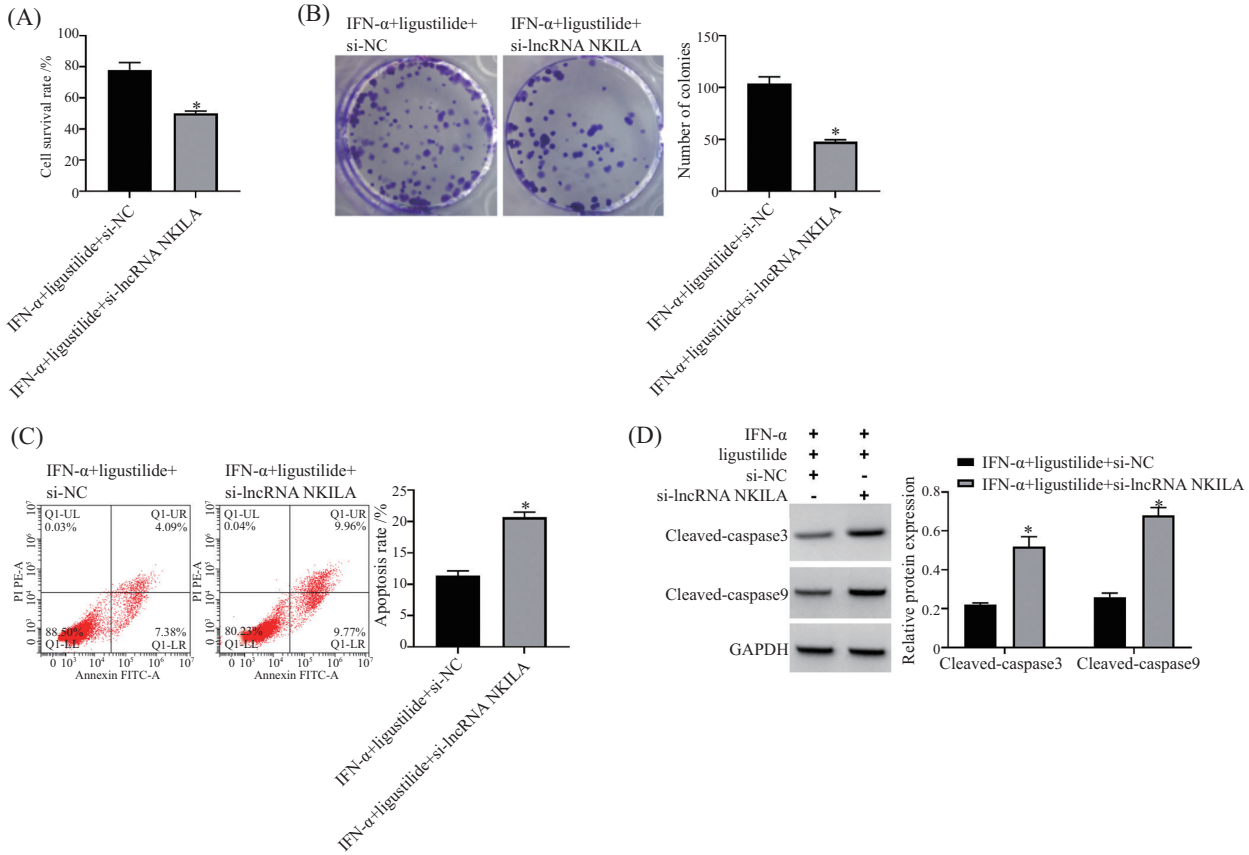


A: lncRNA NKILA对干扰素α诱导的HBVAF存活率的影响; B: lncRNA NKILA对干扰素α诱导的HBVAF克隆形成能力的影响; C: lncRNA NKILA对干扰素α诱导的HBVAF凋亡率的影响; D: lncRNA NKILA对干扰素α诱导的HBVAF凋亡相关蛋白表达的影响。*P<0.05, 与IFN-α+pcDNA组相比。

A: the effect of lncRNA NKILA on the survival rate of HBVAF induced by INF-α; B: the effect of lncRNA NKILA on INF-α-induced HBVAF colony-forming ability; C: the effect of lncRNA NKILA on the apoptosis rate of HBVAF induced by INF-α; D: the effect of lncRNA NKILA on the expression of apoptosis-related proteins in HBVAF induced by INF-α. *P<0.05 compared with IFN-α+pcDNA group.

图5 过表达lncRNA NKILA逆转了干扰素α诱导的HBVAF损伤

Fig.5 Overexpression of lncRNA NKILA could reverse INF-α-induced HBVAF damage



A: 抑制lncRNA NKILA对蒿本内酯处理的干扰素α诱导的HBVAF存活率的影响; B: 抑制lncRNA NKILA对蒿本内酯处理的干扰素α诱导的HBVAF克隆形成能力的影响; C: 抑制lncRNA NKILA对蒿本内酯处理的干扰素α诱导的HBVAF凋亡率的影响; D: 抑制lncRNA NKILA对蒿本内酯处理的干扰素α诱导的HBVAF凋亡相关蛋白表达的影响。*P<0.05, 与IFN-α+蒿本内酯+si-NC组相比。

A: effects of inhibition of lncRNA NKILA on the survival rate of ligustilide-treated HBVAF exposed to INF-α; B: effects of inhibition of lncRNA NKILA on the colony-forming ability of ligustilide-treated HBVAF exposed to INF-α; C: effects of inhibition of lncRNA NKILA on the apoptosis rate of ligustilide-treated HBVAF exposed to INF-α; D: effects of inhibiting lncRNA NKILA on the expression of apoptosis-related proteins in ligustilide-treated HBVAF exposed to INF-α. *P<0.05 compared with IFN-α+ligustilide+si-NC group.

图6 抑制lncRNA NKILA逆转了蒿本内酯对干扰素α诱导的HBVAF损伤的保护作用

Fig.6 Inhibition of lncRNA NKILA could reverse the protective effect of ligustilide on INF-α-induced HBVAF injury

(P<0.05, 图5), 细胞克隆形成数增多(P<0.05, 图5)。

2.6 抑制lncRNA NKILA逆转了蒿本内酯对干扰素α诱导的HBVAF损伤的保护作用

与IFN-α+蒿本内酯+si-NC组比较, IFN-α+蒿本内酯+si-lncRNA NKILA组细胞存活率降低(P<0.05, 图6), 细胞凋亡率和Cleaved-caspase3、Cleaved-caspase9蛋白水平升高(P<0.05, 图6), 细胞克隆形成数减少(P<0.05, 图6)。

3 讨论

lncRNA可以作为miRNA的竞争性内源RNA从而对靶基因表达及细胞凋亡等生理进程发挥一定的调控作用, 并可参与心肌细胞、神经细胞损伤等过程, 还可能作为细胞损伤的潜在治疗靶点[9-11]。

蒿本内酯可减轻心肌缺血再灌注损伤, 其作用机制可能与抑制PI3K/AKT/mTOR的活化有关[12]。蒿本内酯可通过抑制炎症反应从而减轻人脐静脉内皮细胞损伤[13]。但关于蒿本内酯在IFN-α诱导构建的脑血管外膜成纤维细胞损伤模型中的作用机制却鲜有报道。本次研究发现, IFN-α诱导的脑血管外膜成纤维细胞存活率降低, 细胞克隆形成数减少, 而蒿本内酯处理后细胞存活率升高, 细胞克隆形成数增多, 提示蒿本内酯可促进IFN-α诱导的脑血管外膜成纤维细胞增殖及克隆形成。Caspase9、caspase3被激活后分别形成Cleaved-caspase9、Cleaved-caspase3进而促进细胞凋亡[14]。本研究结果显示, IFN-α诱导的脑血管外膜成纤维细胞凋亡率和Cleaved-caspase3、Cleaved-caspase9蛋白水平

升高, 而随着蒿本内酯浓度的增加, 细胞凋亡率和Cleaved-caspase3、Cleaved-caspase9蛋白水平降低, 提示蒿本内酯可抑制IFN-α诱导的脑血管外膜成纤维细胞凋亡。

lncRNA NKILA过表达可促进骨关节炎软骨细胞增殖并抑制细胞凋亡[15]。lncRNA NKILA过表达可通过抑制NF-κB信号通路而减轻心肌缺血损伤[16]。lncRNA NKILA表达上调可抑制内皮细胞炎症反应[17]。本研究结果显示, IFN-α诱导的脑血管外膜成纤维细胞中lncRNA NKILA的表达量降低, 蒿本内酯呈浓度依赖性上调lncRNA NKILA的表达, 提示蒿本内酯可能通过上调lncRNA NKILA表达而发挥作用。同时本研究结果显示, lncRNA NKILA过表达可增强IFN-α诱导的脑血管外膜成纤维细胞增殖及克隆形成能力, 并可抑制细胞凋亡, 而抑制lncRNA NKILA表达可拮抗蒿本内酯对IFN-α诱导的脑血管外膜成纤维细胞增殖、克隆形成的促进作用及其对细胞凋亡的抑制作用。这提示蒿本内酯可通过促进lncRNA NKILA表达而减轻IFN-α诱导的脑血管外膜成纤维细胞损伤。

综上所述, 蒿本内酯可促进IFN-α诱导的脑血管外膜成纤维细胞增殖及克隆形成, 并抑制细胞凋亡, 其作用机制与促进lncRNA NKILA表达有关, 提示蒿本内酯可作为治疗血管增生性疾病的潜在药物。然而关于其具体作用机制仍需进一步探究。

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