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稳定干扰NOR1基因对HeLa细胞的影响

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[摘要] 目的：研究稳定干扰NOR1 基因对宫颈癌HeLa细胞系的影响。**方法：**采用pSUPER.neo+GFP载体构建靶向NOR1基因的shRNA干扰载体pSUPER-shNOR1-1，pSUPER-shNOR1-2以及无关序列对照载体pSUPER-scramble，通过脂质体转染HeLa细胞，经G418筛选获得稳定干扰细胞系。采用RT-PCR 和Western 印迹检测NOR1 mRNA和蛋白表达水平。采用MTT法测定细胞生长曲线。采用H_2O_2处理HeLa细胞，采用Hoechst 33258染色和TUNEL法测定细胞凋亡。Western印迹检测干扰NOR1对HeLa细胞凋亡相关分子Bcl-2，caspase和聚ADP核糖聚合酶(poly ADP-ribose polymerase，PARP)表达的影响。**结果：**稳定感染的shRNA干扰载体pSUPER-shNOR1-1和pSUPER-shNOR1-2抑制了HeLa细胞内源性NOR1的基因表达，成功构建了稳定干扰NOR1基因的HeLa细胞系。MTT生长曲线测定表明：与pSUPER-scramble质粒转染细胞相比，稳定转染pSUPER-shNOR1-1和pSUPER-shNOR1-2质粒促进了HeLa细胞的活力和增殖，并抑制了H_2O_2诱导的HeLa细胞凋亡。Western印迹检测发现稳定干扰NOR1抑制了H_2O_2诱导的HeLa细胞caspase 9和PARP的活化，上调了Bcl-2蛋白的表达。**结论：**稳定干扰NOR1 基因促进了HeLa细胞的活力与生长，并抑制了H_2O_2诱导的细胞凋亡，其机制与干扰NOR1表达引起抗凋亡分子Bcl-2表达增加和抑制caspase 9活化有关。

[关键词] NOR1；宫颈癌；RNAi；凋亡

Effect of NOR1 gene knockdown on the biological behavior of HeLa cells

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ABSTRACT

Objective: To explore the effect of the oxidored nitro domain containing protein 1 (NOR1) gene knockdown on the biological behavior of HeLa cells in cervical carcinoma.

Methods: The recombinant plasmids pSUPER-shNOR1-1, pSUPER-shNOR1-2 and pSUPER-scramble, which targeted to NOR1 gene, were constructed by pSUPER.neo+GFP vector, transfected into HeLa cells respectively using Lipofectamine 2000 reagent, and followed by G418 selection. The expression level of NOR1 mRNA and protein were determined by RT-PCR and Western blotting, respectively. Methyl thiazolyl tetrazolium (MTT) assay was performed to determine the growth curve of cell viability. The stable transfectants were treated with H_2O_2 and cell apoptosis was determined by Hoechst 33258 staining and terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay. The expression levels of Bcl-2, cleaved caspase 9 and poly ADP-ribose polymerase (PARP) were measured by Western blot.

Results: NOR1- knockdown HeLa cells were successfully constructed by transfection of pSUPER-shNOR1-1 or pSUPER-shNOR1-2 plasmids into HeLa cells. MTT assay showed that the silence of endogenous NOR1 in HeLa cells could lead to the increase in cell viability and proliferation, and the inhibition of H_2O_2-induced apoptosis compared with the negative control. Western blot showed that the expression level of active caspase 9 and cleaved PARP was inhibited in NOR1-knockdown cells when they were treated with H_2O_2 while the expression level of Bcl-2 protein increased.

Conclusion: Silence of endogenous NOR1 facilitates the cell viability and growth of HeLa cells, and attenuates HeLa cells apoptosis induced by H_2O_2, which might be mediated by up-regulation of Bcl-2 level and down-regulation of the cleaved caspase 9 cascade.

KEY WORDS NOR1; cervical cancer; RNAi; apoptosis

NOR1(oxidored-nitro domain containing protein 1）是中南大学肿瘤研究所率先克隆得到的抑瘤基因，在鼻咽癌、宫颈癌等肿瘤组织和细胞系中表达下调[1-4]。NOR1基因上游调控区域包含一典型的CpG岛，在鼻咽癌组织以及髓性白血病细胞中因启动子高甲基化修饰而表达沉默[1,5-6]。NOR1基因启动子区域具有热休克因子1(heat shock factor 1，HSF1)和核呼吸因子1(nuclear respiratory factory 1，NRF1)结合位点，其表达受氧化应激调节[1,7]。在鼻咽癌HNE1细胞中稳定转染NOR1基因，具有抑制HNE1细胞生长的作用。稳定表达NOR1可以抑制HNE1细胞基础水平的自噬以及氧化应激诱导的细胞自噬，促进肿瘤细胞在氧化应激状态下自噬向凋亡的转换[7]。然而，抑制肿瘤细胞内源性表达的NOR1基因对肿瘤细胞生物学行为以及氧化应激的反应尚未清楚。本研究构建靶向NOR1基因的shRNA载体，建立稳定干扰NOR1基因表达的HeLa细胞系，通过MTT法测定稳定干扰NOR1对HeLa细胞活力和生长的影响，研究干扰NOR1对HeLa细胞在氧化应激状态下细胞凋亡的影响。

1 材料与方法

1.1 材料

宫颈癌细胞系HeLa细胞购自美国标准生物品收藏中心(American Type Culture Collection，ATCC)。DMEM (Dulbecco's Modified Eagle's Medium)细胞培养基、胎牛血清购自美国Gibco公司。反转录试剂盒购自美国Fermentas公司。脂质体Lipofectamin 2000和TRIzol 购自美国Life Technologies公司。RNA free DNase购自日本TaKaRa公司。荧光显微镜IX51购自日本Olympus公司。CFX96 real-time PCR仪购自美国BioRad公司。PCR引物由北京六合华大基因科技股份有限公司合成。shRNA 载体pSUPER.neo+GFP购自美国Oligoengine公司。siRNA购自美国siGENOME公司。GAPDH抗体、α-tubulin抗体、鼠二抗、兔二抗购自美国Santa Cruz公司，C-caspase 9 和聚ADP核糖聚合酶(poly ADP-ribose polymerase，C-PARP) 抗体购自美国Cell Signaling Technology(CST)公司，NOR1抗体购自美国Sigma公司。Hoechst33258染色液、TUNEL细胞凋亡检测试剂盒购自中国碧云天生物技术研究所。T4 DNA连接酶购自美国Promega公司。

靶向NOR1基因的siRNAs购自美国Dharmacon公司。

1.2 方法

1.2.1 NOR1 shRNA 载体构建

根据NOR1基因mRNA序列，筛选靶向人NOR1基因的shRNA位点，序列分别为5'-GCGGCAGCTGACAGAAATATA-3'，5'-GCCCAGAATGATGCTGCTAGT-3'。shRNA无关序列为5'-CCTAAGGTTAAGTCGCCCTCG-3'。合成shRNA发卡结构，两端分别携带*Bgl* II和*Hin*d III酶切位点。shRNA发卡连入经*Bgl* II和*Hin*d III双酶切回收的线性化pSUPER.neo+GFP载体，转化大肠杆菌JM109，通过氨苄青霉素筛选获得抗性克隆，抽提质粒测序鉴定。测序正确的NOR1干扰载体分别命名为pSUPER-shNOR1-1，pSUPER-shNOR1-2，无关序列对照载体命名为pSUPER-scramble。

1.2.2 细胞培养和转染

HeLa细胞于转染前1 d接种于含10% 胎牛血清的DMEM完全培养液中，37℃，5% CO_2，饱和湿度的培养箱中培养至细胞融合度达90%，采用脂质体Lipofectamine 2000转染干扰 NOR1质粒pSUPER-shNOR1-1，pSUPER-shNOR1-2 及对照载体pSUPER-scramble至HeLa细胞中，经200 μg/mL G418筛选2周后，抗性克隆扩大培养用于鉴定及后续实验。筛选得到的G418抗性细胞分别命名为HeLa-shNOR1-1，HeLa-shNOR1-2和 HeLa-sh-scramble，其中以HeLa-sh-scramble细胞作为后续研究的对照细胞。

HeLa细胞于转染前1 d接种于含10% 胎牛血清的DMEM完全培养液中，37℃，5% CO_2，饱和湿度的培养箱中培养，采用Lipofectamine RNAiMAX瞬时转染干扰 NOR1 siRNA至HeLa细胞中，继续培养24 h，进行后续检测。

1.2.3 RNA 抽提和 RT-RCR

将稳定及瞬时干扰NOR1基因的HeLa细胞接种于含10% 胎牛血清的DMEM完全培养液中，37℃，5% CO_2，饱和湿度的培养箱中培养，用TRIzol 法提取细胞总RNA，DNase消化痕量gDNA。采用美国Fermentas公司反转录试剂盒反转录为cDNA，总反应体积20 μL，反应条件为37℃，60 min；70℃，10 min灭活反转录酶，–20℃保存或立即进行PCR扩增。荧光定量RT-PCR以GAPDH为内参，扩增NOR1、Bax和Bcl基因3' 端mRNA，NOR1引物序列：正链5'-TCAAGGGATTCATCCGAGAC-3'，反链5'-CTGGCCAAGAAATTCAGCTC-3'；Bax 引物序列：正链5'-TTTTGCTTCAGGGTTTCATC-3'，反链5'-GACACTCGCTCAGCTTCTTG-3'；Bcl2 引物序列：正链5'-TTAGAATTCATGGACGGGTCCG-3', 反链5'-AATCTCGAGTCAGCCCATCTTCT-3'；GAPDH引物：正链5'-AACGGATTTGGTCGTATTGG-3'，反链5'-TTGATTTTGGAGGGATCTCG-3'。反应条件设定为95℃ 5 min，95℃ 30 s，60℃ 30 s，40个循环。

1.2.4 Western 印迹

采用DMEM完全培养液正常培养或加入H_2O_2的DMEM完全培养液处理稳定干扰NOR1基因的细胞，细胞裂解液裂解细胞，收集细胞总蛋白，十二烷基硫酸钠聚丙烯酰胺凝胶电泳(sodium dodecyl sulfate-polyacrylamide gel electrophoresis，SDS-PAGE)，通过Western印迹检测细胞中NOR1，c-caspase9 和c-PARP的蛋白质水平，以GAPDH和α-tubulin为内参。

1.2.5 MTT 方法绘制细胞生存曲线

将稳定干扰NOR1基因的HeLa细胞及对照细胞以每孔1 000个细胞接种到96孔板培养，分别取培养1~6 d的细胞，每孔加入5 mg/mL的MTT (methyl thiazolyl tetrazolium) 20 μL，培养 4 h后加150 μL 二甲基亚砜融解结晶，酶标仪检测各孔490 nm 波长处光吸收值，以时间(d)为横坐标、吸光值为纵坐标绘制细胞生长曲线。

1.2.6 凋亡检测

稳定干扰NOR1基因的HeLa细胞接种于含10% 胎牛血清的DMEM完全培养液中，37℃，5% CO_2，饱和湿度的培养箱中培养，培养液中加入500 μmol/L的H_2O_2，以等体积PBS为对照，继续培养24 h，4%多聚甲醛固定细胞，PBS洗细胞，加入适量的Hoechst33258染色液，染色3 min后在荧光显微镜下观察细胞核状态，凋亡细胞的细胞核呈致密浓染，或呈碎块状致密浓染；采用H_2O_2处理稳定干扰NOR1基因的HeLa细胞，12 h后，4%多聚甲醛固定细胞，PBS洗细胞，采用TUNEL试剂盒对凋亡细胞进行染色，使用流式细胞仪测定细胞的荧光强度。

1.3 统计学处理

采用 SPSS 16.0 统计学软件分析，组间比较采用*t*检验或单因素方差分析，数据以均数±标准差($\bar{x}$±s)形式表示，P<0.05为差异有统计学意义。

2 结 果

2.1 NOR1 基因稳定干扰 HeLa 细胞系的建立

Hela细胞中分别稳定转染构建的shRNA载体pSUPER-shNOR1-1，pSUPER-shNOR1-2及shRNA对照质粒 pSUPER-scramble。荧光照片显示：细胞转染率90%以上。RT-PCR检测显示NOR1敲除效率分别为65%和50%，与scramble细胞组比较，差异有统计学意义(P<0.05，P<0.01)。Western印迹结果显示NOR1敲除效率分别为65%和50%(图1)。

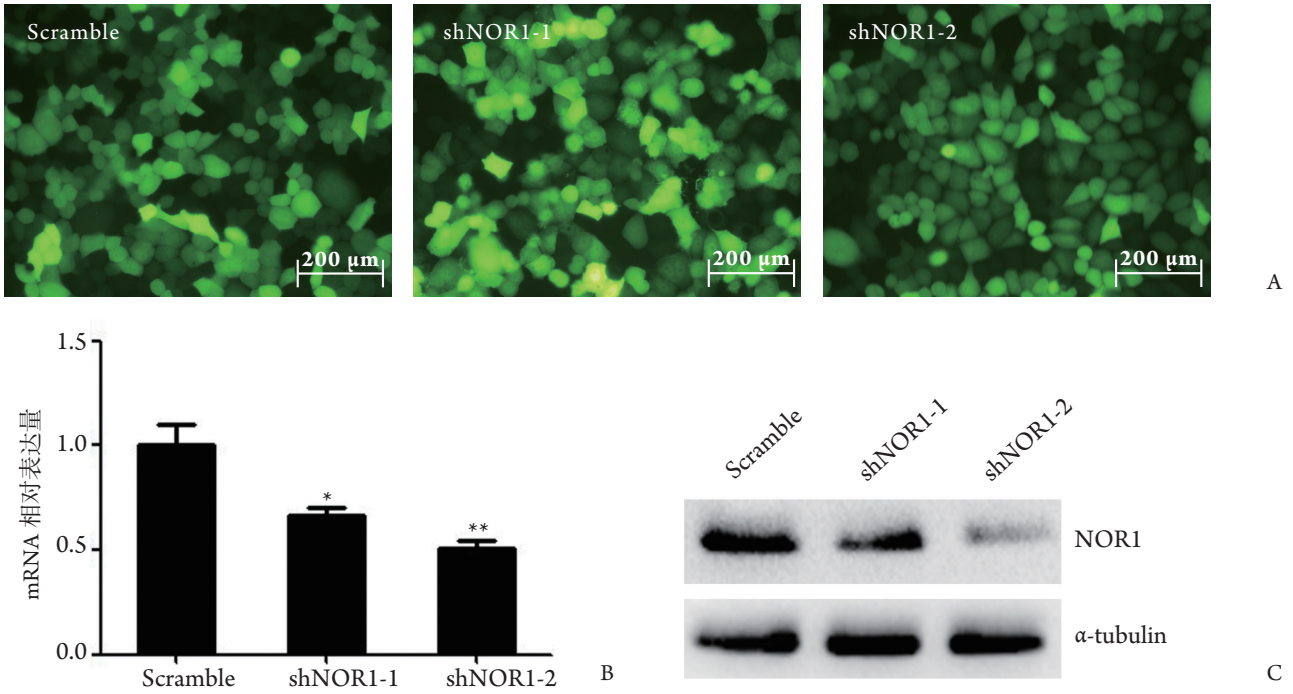

图1 NOR1稳定干扰HeLa细胞系的建立

Figure 1 Establishment of NOR1 stable knockdowned HeLa cells

A: Fluorescent micrographs show the transfection efficiacy of HeLa cells transfected with scrambled or NOR1 shRNA plasmids. Scale bars: 200 μm. B: Expression levels of NOR1 mRNA in HeLa cells transfected with shRNA vectors by quantative RT-PCR. *P<0.05, **P<0.01 vs the pSUPER-scramble cells. C: Expression levels of NOR1 protein in HeLa cells transfected with shRNA vectors by Western blot.

2.2 干扰 NOR1 基因促进 HeLa 细胞生长增殖

检测了NOR1对细胞生长能力的影响。经MTT生长曲线实验检测，以MTT相对吸光值反映细胞活力和相对生长速度，结果发现稳定干扰NOR1基因的HeLa-shNOR1-1和HeLa-shNOR1-2细胞活力较HeLa-sh-scramble细胞增强(图2)。

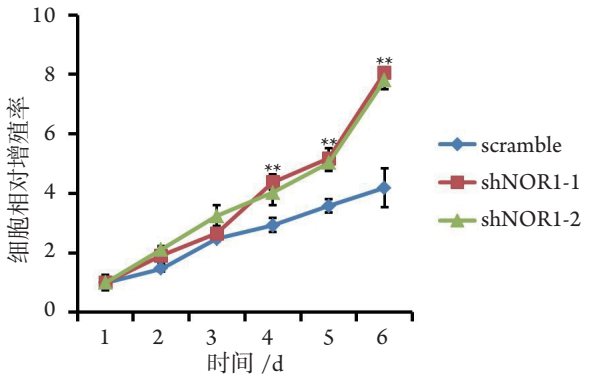

图2 稳定干扰 NOR1 表达提高了细胞活力并促进细胞生长

Figure 2 HeLa cells viability and growth induced by knockdown of NOR1

Viability of HeLa cells stablely transfected with NOR1 shRNA or control vector was determined by MTT assay. Viability of HeLa cell increased and the cells proliferation could be found by the knockdown of NOR1, **P<0.01 vs the scramble sequence transfectants.

2.3 干扰 NOR1 减少细胞对氧化应激的敏感性

采用500 μmol/L的H_2O_2处理稳定干扰NOR1基因表达的HeLa细胞HeLa-shNOR1-1和HeLa-shNOR1-2及对照细胞系HeLa-sh-scramble，处理12 h后，通过Hoechst33258染色、TUNEL等方法检测细胞凋亡。结果显示，光学显微镜下细胞Hoechst 33258染色图片及TUNEL实验证实稳定干扰细胞NOR1后，细胞对H_2O_2的敏感性明显降低(图3)。

2.4 NOR1 对 H_2O_2 处理后 caspase 活化的影响

Western印迹结果表明：H_2O_2处理后稳定干扰NOR1基因的HeLa-shNOR1-1和HeLa-shNOR1-2细胞中caspase 9和 PARP活化水平低于HeLa-sh-scramble细胞，即稳定干扰NOR1后抑制了H_2O_2诱导的caspase 9及PARP活化。

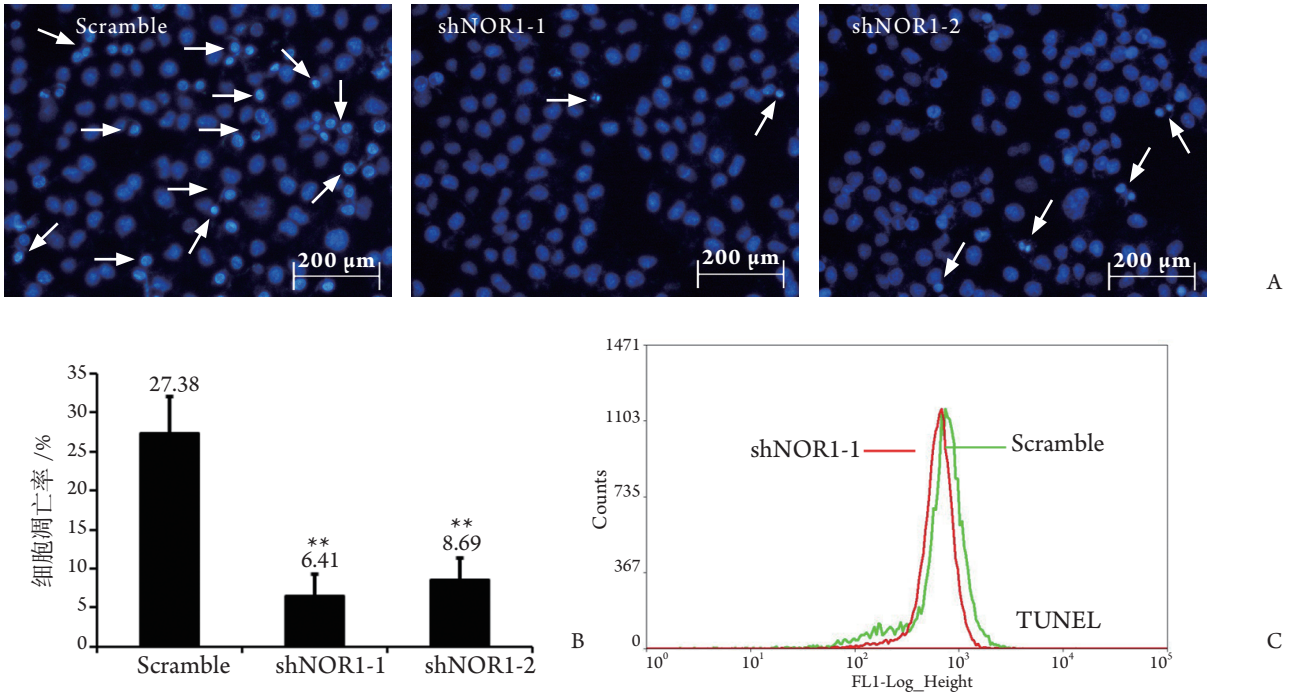

图3 稳定干扰NOR1 抑制H_2O_2诱导的HeLa细胞凋亡

Figure 3 Stable knockdown of NOR1 in HeLa cells inhibits the H_2O_2-induced apoptosis

A: Fluorescent images of HeLa cells transfected with scrambled or NOR1 shRNA vector by Hoechst 33258 staining. Cells were treated with H_2O_2 (500 μmol/L) for 12 h and then subjected to Hoechst 33258 staining. Arrows denote the apoptotic cells defined by nuclear fragmentation. Scale bars: 200 μm. B: Apoptotic rate was determined by comparing the number of apoptotic cells with the total cells from Hoechst 33258 staining. $^{**}P<0.01$ vs the scramble cells. C: Cells were treated with 500 μmol/L H_2O_2 for 24 h and subjected to TUNEL-flow cytometry assay.

2.5 稳定干扰 NOR1 上调 HeLa 细胞 Bcl-2 蛋白表达

siRNA瞬时干扰HeLa细胞中的NOR1基因，定量RT-PCR检测发现Bcl-2 mRNA上调，与scramble细胞相比，差异有统计学意义($P<0.05$)。而Bax mRNA无明显变化，提示干扰NOR1促进Bcl-2表达。进一步通过Western印迹检测发现，HeLa-shNOR1-1，HeLa-shNOR1-2细胞中Bcl-2蛋白水平较HeLa-sh-scramble细胞增加(图4，5)。

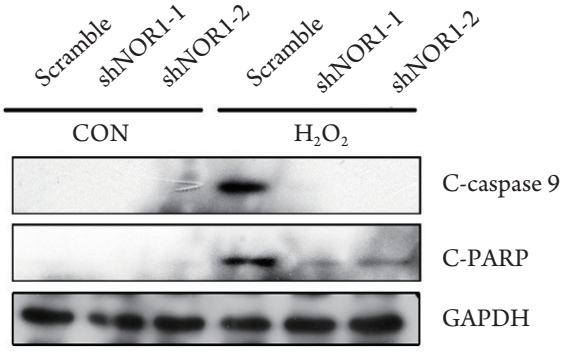

图4 稳定干扰NOR1 表达抑制氧化应激诱导的caspase 9活化

Figure 4 Knockdown of NOR1 inhibits the H_2O_2-induced caspase 9 activation

NOR1 knockdowned HeLa cells and the scamble control cells were treated with 500 μmol/L H_2O_2 for 24 h, the total protein was extracted and subjected to SDS-PAGE, and the expression level of active caspase 9 and PARP were determined by Western blot. CON: Cells were cultured in the complete growth media; H_2O_2: Cells were cultured in the complete growth media combined with 500 μmol/L H_2O_2.

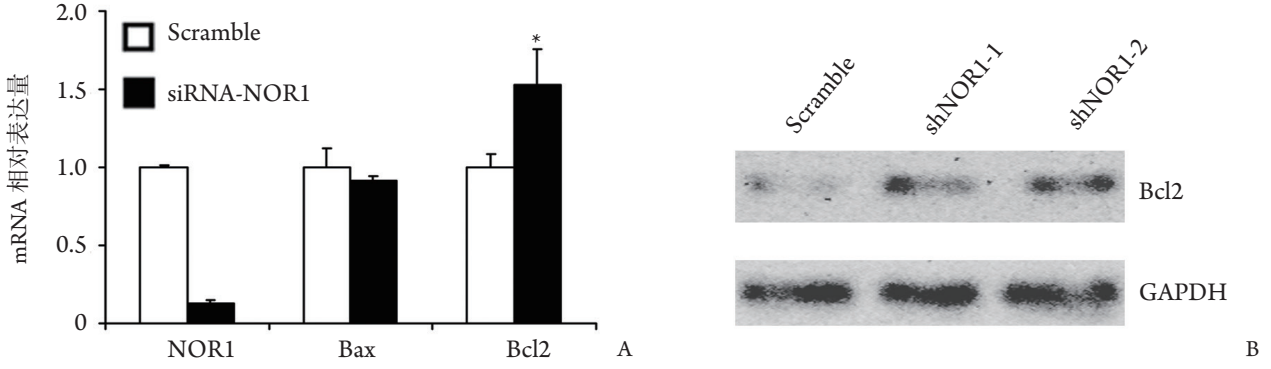

图5 瞬时干扰NOR1 降低 Bax/Bcl-xL比率

Figure 5 Transient knockdown of NOR1 decreases the Bax/Bcl-2 ratio of HeLa cells

A: HeLa cells were transiently trasfected with NOR1 specific siRNAs and the scamble control sequences, the total RNA was extracted and the expression levels of NOR1 mRNA in the transfected HeLa cells were determined by quantative RT-PCR. *P< 0.05 vs the scramble transfected cells. B: Expression levels of Bcl-2 protein in the transfected HeLa cells was determined by Western blot.

3 讨 论

RNA干扰是研究基因功能的有效工具。本研究构建了靶向NOR1基因的shRNA载体，转染HeLa细胞，建立了稳定干扰NOR1基因的细胞系。稳定干扰NOR1基因提高了HeLa细胞的活力、促进其生长，并且抑制了H_2O_2诱导的HeLa细胞凋亡，其分子机制与稳定干扰NOR1上调抗凋亡蛋白Bcl-2蛋白表达和抑制caspase 9活化有关。

NOR1基因是最先从鼻咽癌中克隆得到的抑瘤基因[8]。NOR1基因定位于染色体1p34区域，该区域在鼻咽癌组织、睾丸癌等多种肿瘤中存在杂合性缺失[9-12]。除了在鼻咽癌、肺癌、睾丸癌等肿瘤组织中表达下调[1,3-4,13]，欧阳珏等[2]研究发现NOR1基因在宫颈癌组织中也表达下调。作者研究发现宫颈癌HeLa细胞系中仍可检测到NOR1蛋白表达，因此通过设计shRNA干扰载体，抑制HeLa细胞中NOR1表达。MTT生长曲线测定表明抑制NOR1表达提高了HeLa细胞活力和生长速度，表明NOR1基因表达降低可促进宫颈癌HeLa细胞增殖。

氧化应激NOR1基因编码一个可溶性蛋白，部分定位于线粒体，可能与细胞氧化应激相关[14]。氧化应激是指活性氧生成与清除之间的不平衡状态，导致活性氧(reactive oxygen species，ROS)与活性氮自由基(reactive ntrogen species，RNS)在体内或细胞内蓄积而引起的氧化损伤过程。氧化应激是癌变过程的重要病理生理状态，参与肿瘤的始动与进展[15]。氧化应激可以诱导细胞凋亡，肿瘤组织中虽然存在较高强度的氧化应激，但是却并不引起细胞凋亡[16]，这表明在肿瘤发生过程中与氧化应激相关的凋亡调节因子存在异常。在鼻咽癌细胞中过表达NOR1可以促进H_2O_2引起的细胞凋亡[7]。在本研究中，进一步证实干扰内源性NOR1表达可以抑制H_2O_2引起的HeLa细胞凋亡，表明内源性NOR1蛋白是一个氧化应激相关的调节分子，NOR1表达降低可能是导致HeLa细胞对氧化应激诱导的凋亡耐受增强的原因之一。

过表达NOR1蛋白促进氧化应激引起的细胞凋亡与线粒体凋亡途径有关[2,7]。在本研究中，稳定干扰NOR1引起HeLa细胞线粒体抗凋亡蛋白Bcl-2蛋白表达水平增加。在H_2O_2处理后，caspase 9活化水平和凋亡标志C-PARP表达增加。本研究进一步证明了NOR1可通过影响线粒体凋亡途径分子的表达调节肿瘤细胞凋亡。

本研究首次构建了稳定干扰NOR1表达的HeLa细胞系。稳定干扰NOR1可促进了HeLa细胞的活力和增殖，同时可引起抗凋亡蛋白Bcl-2表达增加，且可抑制H_2O_2诱导的HeLa细胞凋亡。

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