doi: 10.3978/j.issn.2095-6959.2018.12.002
View this article at: http://dx.doi.org/10.3978/j.issn.2095-6959.2018.12.002

ERCC1 siRNA 对鼻咽癌细胞 HNE–1/DDP 顺铂耐药的影响及机制

陆晓明[1]，王丹[1]，万欢[2]

(武汉市第五医院 1. 耳鼻咽喉科；2. 肿瘤放疗科，武汉 430050)

[摘　要]　**目的：**研究切除修复交叉互补基因1(excision repair cross complement 1，ERCC1)siRNA对鼻咽癌细胞HNE-1/顺铂(cisplatin，DDP)耐药的影响及机制。**方法：**以qRT-PCR和Western印迹法测定人鼻咽癌细胞HNE-1/DDP和HNE-1细胞中ERCC1表达水平。人鼻咽癌细胞HNE-1/DDP转染ERCC1 siRNA重组慢病毒载体和阴性对照载体，以qRT-PCR和Western印迹法测定干扰效果。用DDP处理转染ERCC1 siRNA的HNE-1/DDP细胞，MTT检测细胞增殖变化，克隆形成实验检测细胞克隆形成能力，流式细胞术检测细胞凋亡变化，Western印迹法检测细胞中激活型caspase-3(C-caspase-3)、激活型caspase-9(C-caspase-9)蛋白水平，同时用Western印迹法检测细胞质和线粒体中细胞色素C(cytochrome C)蛋白水平。**结果：**HNE-1/DDP细胞中ERCC1表达水平高于HNE-1细胞。ERCC1 siRNA可明显下调HNE-1/DDP细胞中ERCC1的表达和转录。DDP和ERCC1 siRNA可以降低HNE-1/DDP细胞增殖能力和克隆形成能力，提高细胞凋亡率，促进细胞中C-caspase-3，C-caspase-9蛋白表达，提高细胞质中cytochrome C蛋白水平，降低线粒体中cytochrome C蛋白水平。DDP处理转染ERCC1 siRNA的HNE-1/DDP细胞，细胞的增殖和克隆能力下降更多，细胞凋亡率升高更多，细胞中C-caspase-3，C-caspase-9蛋白水平更高，细胞质中cytochrome C蛋白水平也更高，线粒体中cytochrome C蛋白水平下降更多。**结论：**ERCC1 siRNA能够逆转鼻咽癌细胞HNE-1/DDP对DDP耐药，作用机制可能与激活线粒体凋亡途径有关。

[关键词]　鼻咽癌细胞；顺铂耐药；切除修复交叉互补基因1；凋亡

Effect of ERCC1 siRNA on cisplatin resistance in nasopharyngeal carcinoma cell line HNE-1/DDP and its mechanism

LU Xiaoming[1], WANG Dan[1], WAN Huan[2]

(1. Department of Otolaryngology; 2. Department of Tumor Radiotherapy, Fifth Hospital of Wuhan, Wuhan 430050, China)

Abstract　**Objective:** To study the effect and mechanism of excision repair cross complement 1 (ERCC1) siRNA on cisplatin resistance in nasopharyngeal carcinoma cell line HNE-1/DDP. **Methods:** The expression level of ERCC1 in human nasopharyngeal carcinoma cells HNE-1/DDP and HNE-1 cells was measured by qRT-PCR

收稿日期 (Date of reception)：2018–08–06
通信作者 (Corresponding author)：陆晓明，Email: 1442064173@qq.com
基金项目 (Foundation item)：湖北省卫生和计划生育委员会科研项目 (WJ2017F125)。This work has been supported by the Hubei provincial health and Family Planning Commission, China (WJ2017F125).

and Western blot. Human nasopharyngeal carcinoma cell HNE-1/DDP was transfected with ERCC1 siRNA recombinant lentiviral vector and negative control vector. The interference effects were measured by qRT-PCR and Western blot. HNE-1/DDP cells transfected with ERCC1 siRNA were treated with cisplatin (DDP). MTT detection of cell proliferation, colony formation assay was used to detect cell clone formation ability, flow cytometry was used to detect the changes of cell apoptosis, Western blot was used to detect the level of C-caspase-3 and C-caspase-9 protein in the cells, at the same time, and the level of cytochrome C protein in cytoplasm and mitochondria. **Results:** The expression level of ERCC1 in HNE-1/DDP cells was higher than that in HNE-1 cells. ERCC1 siRNA significantly reduced the expression and transcription of ERCC1 in HNE-1/DDP cells. Both DDP and ERCC1 siRNA reduced the proliferation and clone formation ability of HNE-1/DDP cells, and increased the rate of apoptosis, promote the expression of C-caspase-3 and C-caspase-9 protein in cells, increase the level of cytochrome C protein in the cytoplasm, and reduced the level of cytochrome C protein in mitochondria. DDP treatment of HNE-1/DDP cells transfected with ERCC1 siRNA, the proliferation and cloning of cells decreased more, the rate of cell apoptosis increased more, the levels of C-caspase-3 and C-caspase-9 protein were higher in cells, the level of cytochrome C protein in cytoplasm was also higher, the level of cytochrome C protein in mitochondria decreased more. **Conclusion:** ERCC1 siRNA can reverse the resistance of nasopharyngeal carcinoma cell HNE-1/DDP to cisplatin, the mechanism is related to activation of mitochondrial apoptotic pathway.

Keywords nasopharyngeal carcinoma cell; cisplatin resistance; excision repair cross complement 1; apoptosis

肿瘤治疗以放疗、化疗、外科手术等为主，随着治疗技术的不断进步，肿瘤患者的生存质量明显提高，但是肿瘤的致死率仍然居高不下[1]。化疗是常用的肿瘤治疗的辅助方法，肿瘤耐药是影响化疗治疗效果的重要原因[2]。顺铂(cisplatin，DDP)是治疗鼻咽癌常用的药物之一，研究鼻咽癌DDP耐药机制对于提高鼻咽癌化疗敏感性具有重要意义[3]。切除修复交叉互补基因1(excision repair cross complement 1，ERCC1)是核苷酸切除修复过程的关键调控因子，参与宫颈癌、膀胱癌、非小细胞肺癌、卵巢癌等肿瘤DDP耐药发生，敲低卵巢癌DDP耐药细胞株SKOV3/DDP细胞中ERCC1的表达后，卵巢癌细胞的增殖能力降低，同时细胞对DDP的敏感性增加[4-7]。本研究旨在探究敲低ERCC1对鼻咽癌DDP耐药细胞株生长、凋亡的影响，为研究鼻咽癌DDP耐药机制提供参考。

1 材料与方法

1.1 材料

人鼻咽癌细胞HNE-1/DDP和HNE-1细胞培养于含有10%胎牛血清的RPMI1640培养液中，细胞购自于广州华韵(然科)生物科技有限公司；细胞质蛋白提取试剂盒、线粒体蛋白提取试剂盒购自美国Ambion公司；BeyoRT cDNA合成试剂盒购自北京华夏远洋科技有限公司；Real-time PCR试剂盒购自德国QIAGEN公司；ERCC1 siRNA重组慢病毒载体由汉恒生物科技(上海)有限公司构建；激活型caspase-3(C-caspase-3)抗体、细胞色素C(cytochrome C)抗体和激活型caspase-9(C-caspase-9)抗体购自美国Proteintech公司；DDP购自美国Sigma公司。

1.2 鼻咽癌 HNE-1/DDP 和 HNE-1 细胞 ERCC1 表达水平检测

1.2.1 qRT-PCR

人鼻咽癌细胞HNE-1/DDP和HNE-1细胞以PBS洗涤以后，离心，添加TRIzol(每10^7个细胞加1 mL)，吹打混合后，按照RNA提取试剂盒提取细胞中的总RNA，检测A_{260}/A_{280}为1.8~2.0，用BeyoRT cDNA合成试剂盒合成cDNA，引物由生工生物工程(上海)股份有限公司合成，序列为ERCC1：F 5'-GGCGACGTAATTCCCGACTA-3'，R 5'-AGTTCTTCCCCAGGCTCTGC-3'。β-actin：F 5'-AGAGCCTCGCCTTTGCCGAT-3'，R 5'-TGCCAGATTTTCTCCATGTCG-

T-3'。用qRT-PCR试剂盒进行检测，得到每个孔的Ct值，根据$2^{-\triangle\triangle Ct}$方法对ERCC1表达水平进行相对定量分析。

1.2.2 Western 印迹法

人鼻咽癌细胞HNE-1/DDP和HNE-1细胞中添加蛋白裂解液(RIPA按照1:100的比例加入PMSF中)，放在冰上裂解30 min以后，12 000 r/min离心10 min，取上清，保存在−80 ℃。蛋白定量检测用BCA法，步骤同试剂盒操作说明。配制SDS-PAGE凝胶，设置电泳仪电压为70 V，400 mA电泳30 min后，再设置电泳仪电压为90 V，400 mA继续电泳。裁剪PVDF膜，常规方法转膜，转膜仪电压设置为50 V，250 mA，电转移时间为2.5 h。转膜后的PVDF膜放在摇床上，置于封闭液中孵育2 h，加入一抗在室温孵育2 h后(一抗以1:400稀释)，再加入二抗(二抗以1:2 000稀释)孵育2 h，经ECL发光以后，放在Bio-rad成像系统中采集图像，分析条带的光密度值，以β-actin作为参照，分析目的蛋白相对表达量。

1.3 细胞分组转染

HNE-1/DDP细胞在慢病毒感染前24 h，种植到6孔细胞培养内，每孔加入10^5个细胞，培养24 h以后，在细胞中加入稀释的慢病毒液(MOI =10)，培养12 h以后，更换细胞培养液，培养3 d后，观察细胞荧光表达情况，干扰效率高于90%用于后续实验。HNE-1/DDP细胞分成Control组、DDP组、ERCC1 siRNA组和ERCC1 siRNA+DDP组。Control组和DDP组为感染阴性对照慢病毒的HNE-1/DDP细胞，ERCC1 siRNA组和ERCC1 siRNA+DDP组为感染ERCC1 siRNA慢病毒的HNE-1/DDP细胞，DDP组和ERCC1 siRNA+DDP组细胞在实验0 h时在培养液中添加2 μg/mL的DDP。以qRT-PCR和Western印迹法检测Control组、ERCC1 siRNA组细胞中ERCC1表达水平，确定干扰效果，步骤同1.2。

1.4 MTT 测定细胞增殖

HNE-1/DDP细胞接种到96孔板，每个孔中加6 000个细胞，分组处理以后，置于37 ℃培养箱内孵育48 h以后，添加MTT反应液(每孔20 μL)，在37 ℃的培养箱内孵育3.5 h后，添加DMSO溶液，继续培养10 min，置于酶标仪上检测$A_{490\ nm}$，设置空白孔调零。计算细胞存活率变化(Control组细胞存活率为100%)。

1.5 细胞克隆实验测定细胞克隆能力

按组别把细胞种植到60 mm的培养皿内，每个培养皿中添加2 mL细胞悬浮液(含100个细胞)，培养12 d以后，用95%乙醇固定，苏木精染色后，观察>50个细胞的克隆数目，结果以Control组细胞克隆形成率为100%，分析DDP组，ERCC1 siRNA组和ERCC1 siRNA+DDP组克隆形成率变化。

1.6 流式细胞术检测细胞凋亡

按各组方法培养48 h后，用PBS将细胞洗涤3次，添加0.25%胰蛋白酶将细胞制成单细胞悬浮液，离心后，收集细胞，用Binding Buffer 500 μL悬浮以后，继续添加PI和Annexin V-FITC染液，在避光环境反应20 min，使用流式细胞仪检测细胞凋亡变化。

1.7 Western 印迹法检测 C-caspase-3，C-caspase-9 和 cytochrome C 蛋白表达变化

各组培养48 h以后，用Western印迹法检测细胞总蛋白中C-caspase-3，C-caspase-9水平，用Western印迹法检测细胞线粒体和细胞质蛋白中cytochrome C水平，Western印迹法步骤同上，线粒体和细胞质蛋白提取参照试剂盒说明。

1.8 统计学处理

采用SPSS 21.0软件进行数据分析。数据以均数±标准差($\bar{x}\pm s$)表示，两组数据采用独立样本t检验，多组差异比较用单因素方差分析，组间比较用SNK-q检验。P<0.05为差异有统计学意义。

2 结果

2.1 ERCC1 在鼻咽癌细胞中的表达水平

HNE-1/DDP细胞和HNE-1细胞中ERCC1 mRNA水平依次为1.00，1.89±0.23，蛋白水平为0.35±0.06和0.74±0.09。HNE-1/DDP细胞中ERCC1 mRNA和蛋白水平高于HNE-1细胞(P<0.05，图1)。

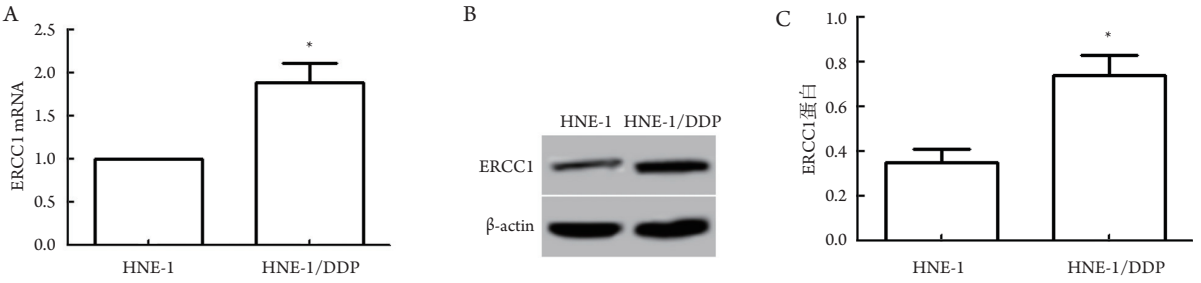

图1 ERCC1在鼻咽癌HNE-1/DDP和HNE-1细胞中的表达水平

Figure 1 Expression level of ERCC1 in nasopharyngeal carcinoma HNE-1/DDP and HNE-1 cells

(A)鼻咽癌HNE-1/DDP和HNE-1细胞中ERCC1 mRNA表达水平；(B)Western印迹法检测ERCC1在鼻咽癌HNE-1/DDP和HNE-1细胞中的表达水平；(C)鼻咽癌HNE-1/DDP和HNE-1细胞中ERCC1蛋白表达水平。与HNE-1比较，*P<0.05；ERCC1 mRNA：t=6.702，P=0.003；ERCC1蛋白：t=6.245，P=0.003。

(A) Expression level of ERCC1 mRNA in HNE-1/DDP and HNE-1 cells of nasopharyngeal carcinoma; (B) Western blot was used to detect the expression of ERCC1 in HNE-1/DDP and HNE-1 cells; (C) Expression of ERCC1 in HNE-1/DDP and HNE-1 cells. Compared with HNE-1, *P<0.05; ERCC1 mRNA: t=6.702, P=0.003; ERCC1 protein: t=6.245, P=0.003.

2.2 ERCC1 siRNA 降低鼻咽癌 HNE-1/DDP 细胞中 ERCC1 表达水平

HNE-1/DDP细胞转染ERCC1 siRNA后，细胞中的ERCC1 mRNA和蛋白水平明显降低，ERCC1 siRNA可降低HNE-1/DDP细胞中ERCC1的表达水平(图2)。

2.3 ERCC1 siRNA 增强 DDP 对 HNE-1/DDP 细胞生长的抑制作用

ERCC1 siRNA转染后的HNE-1/DDP细胞存活率和克隆形成率均降低，DDP处理也可以降低HNE-1/DDP细胞存活率和克隆形成率，并且ERCC1 siRNA和DDP联合对细胞增殖和克隆形成能力的抑制作用更强(图3)。

2.4 ERCC1 siRNA 增强 DDP 对 HNE-1/DDP 细胞凋亡促进作用

ERCC1 siRNA转染后的HNE-1/DDP细胞凋亡率升高，DDP处理也可以促进HNE-1/DDP细胞凋亡，并且ERCC1 siRNA和DDP联合对细胞凋亡促进作用更强(图4)。

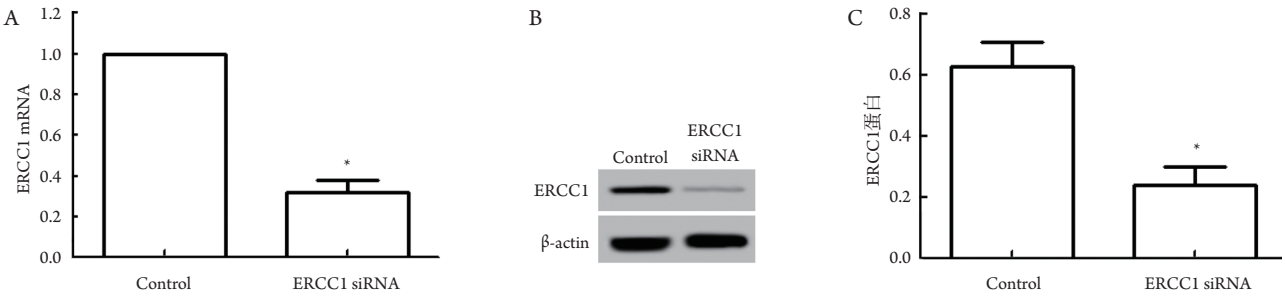

图2 ERCC1 siRNA转染以后鼻咽癌HNE-1/DDP细胞中ERCC1表达变化

Figure 2 Changes of ERCC1 expression in nasopharyngeal carcinoma HNE-1/DDP cells after transfection of ERCC1 siRNA

(A)鼻咽癌细胞中ERCC1 mRNA表达水平；(B)Western印迹法测定鼻咽癌细胞中ERCC1蛋白表达；(C)鼻咽癌细胞中ERCC1蛋白表达水平。与Control组比较，*P<0.05；ERCC1 mRNA：t=19.630，P<0.001；ERCC1蛋白：t=6.755，P=0.003。

(A) Expression level of ERCC1 mRNA in nasopharyngeal carcinoma cells; (B) Western blot was used to detect the expression of ERCC1 protein in nasopharyngeal carcinoma cells; (C) Expression level of ERCC1 protein in NPC cells. Compared with Control group, *P<0.05; ERCC1 mRNA: t=19.630, P<0.001; ERCC1 protein: t=6.755, P=0.003.

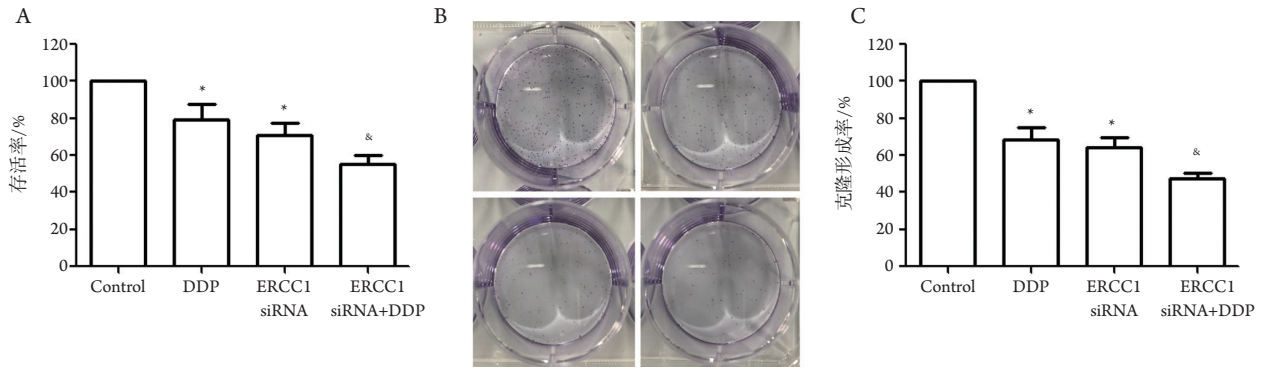

图3 ERCC1 siRNA和DDP对鼻咽癌HNE-1/DDP细胞增殖和克隆能力影响

Figure 3 Effect of ERCC1 siRNA and DDP on proliferation and clone ability of nasopharyngeal carcinoma HNE-1/DDP cells

(A)各组鼻咽癌细胞存活率；(B)平板克隆形成实验检测鼻咽癌细胞克隆形成能力；(C)各组鼻咽癌HNE-1/DDP细胞克隆形成率。与Control组比较，*P<0.05；与ERCC1 siRNA，DDP组比较，$^{\&}P$<0.05。存活率：F=29.393，P<0.001；克隆形成率：F=59.6677，P<0.001。

(A) Survival rate of NPC cells in each group; (B) Colony forming ability of nasopharyngeal carcinoma cells was detected by plate clone assay; (C) Colony formation rate of HNE-1/DDP cells in each group. Compared with the Control group, *P<0.05; compared with the ERCC1 siRNA and DDP group, $^{\&}P$<0.05. Survival rate: F=29.393, P<0.001; colony formation rate: F=59.6677, P<0.001.

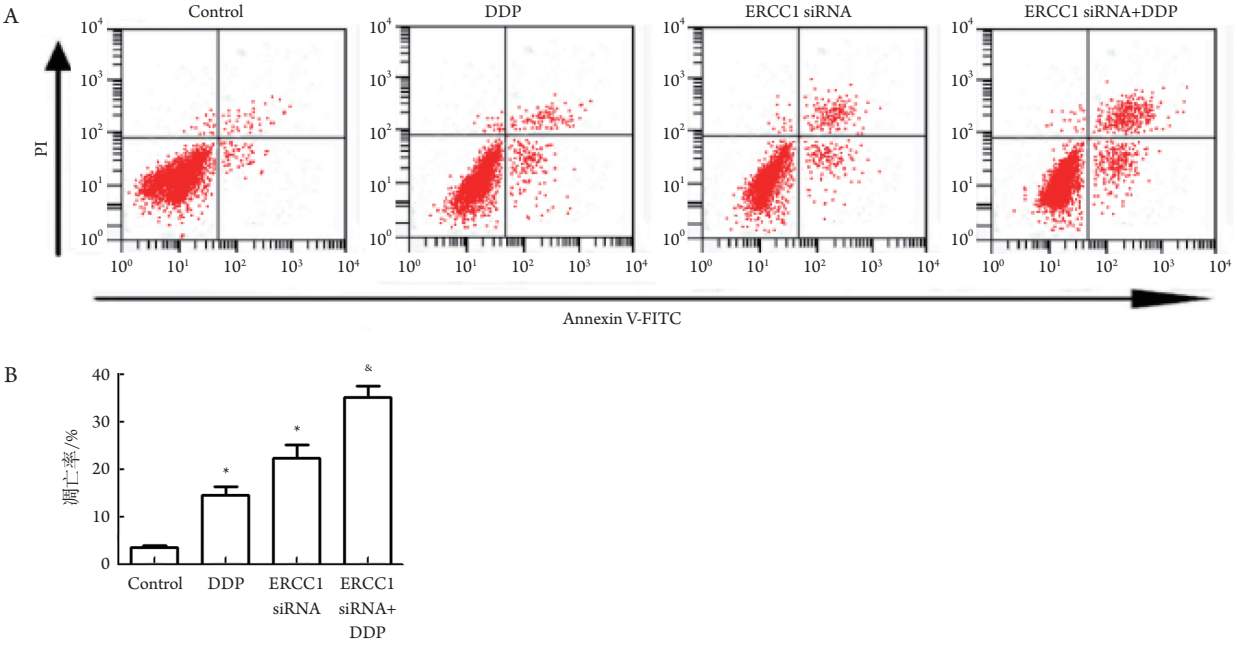

图4 ERCC1 siRNA和DDP对鼻咽癌细胞凋亡影响

Figure 4 Effects of ERCC1 siRNA and DDP on apoptosis of nasopharyngeal carcinoma cells

(A)流式细胞术检测细胞凋亡；(B)鼻咽癌细胞凋亡率。与Control比较，*P<0.05；与ERCC1 siRNA组，DDP组比较，$^{\&}P$<0.05；F=114.153，P<0.001。

(A) Flow cytometry was used to detect apoptosis; (B) Apoptosis rate of nasopharyngeal carcinoma. Compared with the Control group, *P<0.05; compared with the ERCC1 siRNA and DDP group, $^{\&}P$<0.05; F=114.153，P<0.001.

2.5 ERCC1 siRNA 增强 DDP 对 HNE-1/DDP 细胞中 C-caspase-3 和 C-caspase-9 蛋白表达诱导作用

ERCC1 siRNA转染后的HNE-1/DDP细胞中C-caspase-3和C-caspase-9蛋白水平升高，DDP处理也可以促进HNE-1/DDP细胞中C-caspase-3和C-caspase-9蛋白表达，并且ERCC1 siRNA和DDP联合对细胞中C-caspase-3和C-caspase-9蛋白表达促进作用更强(图5)。

2.6 ERCC1 siRNA 增强 DDP 对 HNE-1/DDP 细胞线粒体释放 cytochrome C 诱导作用

ERCC1 siRNA转染后的HNE-1/DDP细胞线粒体中cytochrome C蛋白水平降低，细胞质中cytochrome C蛋白水平升高，DDP处理也可以降低HNE-1/DDP细胞线粒体cytochrome C蛋白水平，提高细胞质中cytochrome C蛋白水平，并且ERCC1 siRNA和DDP联合对细胞质和线粒体中cytochrome C蛋白影响作用更强(图6)。

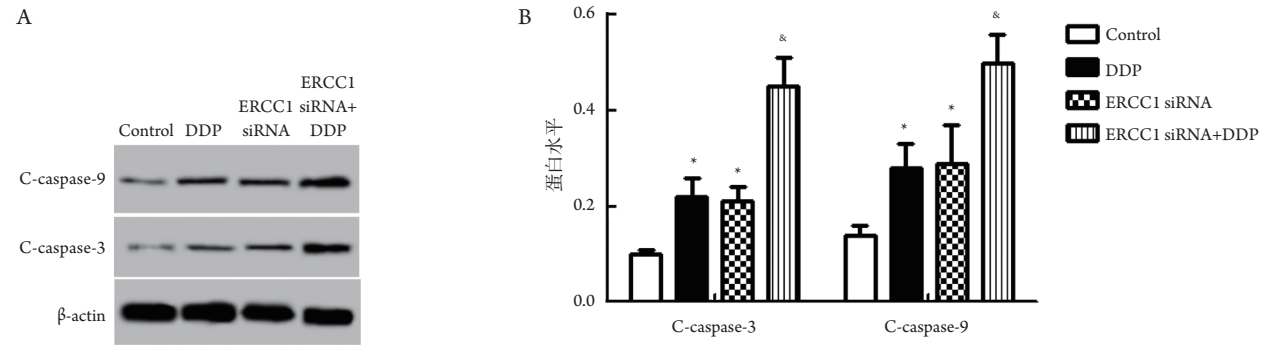

图5 ERCC1 siRNA和DDP对鼻咽癌HNE-1/DDP细胞中活化的caspase-3和caspase-9蛋白水平影响

Figure 5 Effect of ERCC1 siRNA and DDP on the level of activated caspase-3 and caspase-9 protein in HNE-1/DDP cells of nasopharyngeal carcinoma

(A)Western印迹法检测细胞中活化的caspase-3和caspase-9蛋白表达；(B)细胞中活化的caspase-3和caspase-9蛋白水平。与Control组比较，*P<0.05；与ERCC1 siRNA组和DDP组比较，$^{\&}P$<0.05。C-caspase-3：F=41.871，P<0.001；C-caspase-9：F=20.488，P<0.001。

(A) Western blot was used to detect the expression of caspase-3 and caspase-9 protein in cells; (B) Level of caspase-3 and caspase-9 protein activated in cells. Compared with the Control group, *P<0.05; compared with ERCC1 siRNA and DDP, $^{\&}P$<0.05; C-caspase-3: F=41.871, P<0.001; C-caspase-9: F=20.488, P<0.001.

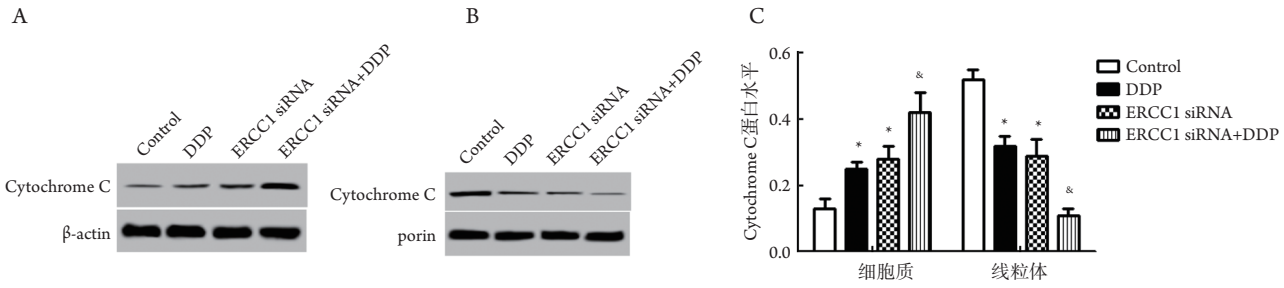

图6 Western印迹法检测ERCC1 siRNA和DDP对鼻咽癌HNE-1/DDP细胞线粒体和细胞质中cytochrome C蛋白水平影响

Figure 6 Western blot analysis of the effects of ERCC1 siRNA and DDP on the levels of cytochrome C protein in mitochondria and cytoplasm of HNE-1/DDP cells

(A)细胞质中cytochrome C蛋白水平；(B)线粒体中cytochrome C蛋白水平；(C)细胞质和线粒体中cytochrome C蛋白水平。与Control组比较，*P<0.05；与ERCC1 siRNA组和DDP组比较，$^{\&}P$<0.05；细胞质：F=26.215，P<0.001；线粒体：F=26.215，P<0.001。

(A) Cytochrome C protein level in cytoplasm; (B) Cytochrome C protein level in mitochondria; (C) Cytochrome C protein level in cytoplasm and mitochondria. Compared with the Control group, *P<0.05; compared with ERCC1 siRNA and DDP, $^{\&}P$<0.05. Cytoplasm: F=26.215, P<0.001; mitochondria: F=26.215, P<0.001.

3 讨论

DDP是肿瘤治疗中的常用药物，DDP可以同细胞内的DNA结合，诱导DNA损伤，肿瘤耐药是DDP治疗肿瘤中的难点，目前对于肿瘤细胞DDP耐药机制尚不明确。核苷酸切除修复是目前已知的DDP耐药发生的重要原因，是DNA修复的途径之一，具有促进DNA大块损伤修复的作用[8-11]。ERCC1是一个高度保守的核酸内切酶，其编码的蛋白质含有297个氨基酸，参与核苷酸切除修复过程，ERCC1对于生命维持具有至关重要的作用，敲低ERCC1的小鼠在断奶以后会立即死亡[12-13]。在卵巢癌等肿瘤细胞中的研究[14-15]表明：过表达ERCC1可以引起肿瘤细胞DDP耐药，ERCC1是肿瘤耐药的一个重要决定因子。本研究结果显示：敲低ERCC1表达后的鼻咽癌DDP耐药细胞株对DDP的敏感性增加，敲低ERCC1可以协同DDP诱导鼻咽癌细胞凋亡，抑制鼻咽癌细胞生长。

研究[16-17]表明：ERCC1不仅参与肿瘤细胞耐药过程，还参与肿瘤细胞的生长、凋亡过程，在肺癌等肿瘤细胞中，敲低ERCC1可以通过激活细胞内caspase凋亡反应诱导肿瘤细胞凋亡发生。Caspase是目前研究较为透彻的与细胞凋亡有关的蛋白家族，其几乎参与人体内所有细胞凋亡发生过程，caspase-3作为该凋亡反应的下游执行因子，其活化水平的高低是细胞凋亡发生的标志，caspase-9是凋亡反应的启动因子，其可以被线粒体凋亡途径激活[18-21]。本研究表明：敲低ERCC1和DDP都可以诱导鼻咽癌细胞中活化的caspase-9，caspase-3表达，并且二者联合后细胞中活化的caspase-9，caspase-3表达水平更高，敲低ERCC1可以通过促进细胞中caspase级联反应的激活协同DDP诱导鼻咽癌细胞凋亡。

线粒体途径是细胞凋亡发生的经典途径，细胞内线粒体释放cytochrome C是线粒体凋亡途径的关键，cytochrome C只有进入细胞质后才可以激活caspase-9，诱导caspase级联反应激活，细胞在受到细胞因子等刺激以后，线粒体膜通透性发生改变，线粒体中的cytochrome C进入细胞质中，诱导caspase-9激活，促进细胞凋亡发生[22-25]。本研究表明：敲低ERCC1和DDP都可以诱导鼻咽癌细胞线粒体中cytochrome C释放至细胞质中，并且二者联合对线粒体释放cytochrome C的促进作用更强，敲低ERCC1可能通过激活线粒体凋亡途径增加鼻咽癌细胞对DDP的敏感性。这为研究鼻咽癌DDP耐药机制奠定了基础，为靶向ERCC1提高鼻咽癌DDP敏感性的临床应用提供了试验依据。

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本文引用： 陆晓明, 王丹, 万欢. ERCC1 siRNA对鼻咽癌细胞HNE-1/DDP顺铂耐药的影响及机制[J]. 临床与病理杂志, 2018, 38(12): 2538-2545. doi: 10.3978/j.issn.2095-6959.2018.12.002

Cite this article as: LU Xiaoming, WANG Dan, WAN Huan. Effect of ERCC1 siRNA on cisplatin resistance in nasopharyngeal carcinoma cell line HNE-1/DDP and its mechanism[J]. Journal of Clinical and Pathological Research, 2018, 38(12): 2538-2545. doi: 10.3978/j.issn.2095-6959.2018.12.002