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RNAi 沉默 Snail 增加结肠癌对 5 氟尿嘧啶敏感性研究 *

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摘要 目的:研究 Snail 的抑制是否能增加耐药结肠癌细胞对 5-FU 的敏感性,评估其可能的信号转导通路。**方法:**使用 5- 氟尿嘧啶耐药 HCT116 细胞(HCT116 / 5-FU),评估细胞形态及分子的变化。通过靶向人 Snail 基因小干扰 RNA(siRNA)抑制 Snail 的表达。Annexin V/PI 染色用于评估 5-FU 诱导的细胞凋亡。Western blot 检测 caspase 以及可能的丝裂原活化蛋白激酶(MAPK)和线粒体途径。**结果:**HCT116 细胞对 5-Fu 耐药性的获得诱导了与 EMT 一致的形态学变化。RNA 干扰沉默 Snail 逆转 HCT116 / 5-FU 细胞 EMT 并增加了 5-FU 耐药 HCT116 细胞对 5-FU 的敏感性。可能的机制涉及 JNK 与线粒体途径的激活。**结论:**EMT 样表型的改变与 HCT116 细胞对 5-FU 耐药相关;siRNA 介导的 Snail 下调可能是一个潜在的克服 5-FU 化疗耐药的治疗方法。

关键词:上皮间质转化;结肠癌;5 氟尿嘧啶;Snail

中图分类号:R-33; R735.35 **文献标识码:**A **文章编号:**1673-6273(2017)10-1818-04

Suppression of Snail by Short Interfering RNA Enhanced 5-fluorouracil-induced Cell Death in Colon Cancer Cells*

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ABSTRACT Objective: We tested whether Snail suppression could increase the sensitivity of 5-FU-resistant colon cancer cells to 5-FU and further assessed possible signaling transduction pathways. **Methods:** Using a 5-fluorouracil-resistant HCT116 cells (HCT116/5-FU), we assessed the cellular morphology and molecular changes consistent with EMT. Expression of Snail was suppressed using a small interfering RNA (siRNA) targeting human Snail mRNA. Annexin V/propidium iodide (PI) apoptosis assay was performed to assess the 5-FU -induced apoptosis. The Caspase as well as possible MAPKs and mitochondrial pathways were determined by Western blot. **Results:** Acquisition of 5-FU resistance induces morphologic changes consistent with EMT in HCT116 cells. Silencing of Snail by RNA interference reversed the EMT of HCT116/5-FU cells and increased the sensitivity of 5-FU-resistant HCT116 cells to 5-FU, the possible mechanism involve activation of JNK/mitochondrial pathway. **Conclusion:** EMT-like phenotypic changes is associated with 5-FU resistance in HCT116 cells. siRNA-mediated Snail knockdown could be a potential novel therapeutic approach to overcoming chemoresistance during 5-FU chemotherapy.

Key words: Epithelial-to-mesenchymal transition; Colon cancer; 5-fluorouracil; Snail

Chinese Library Classification(CLC): R-33; R735.35 Document code: A

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前言

耐放化疗与癌细胞上皮间充质转化(EMT)样表型的进展之间的分子与表型的关联密切。EMT 特征获得的过程中,上皮癌细胞失去促进细胞间接触的蛋白的表达, 如 E-cadherin、β -catenin,而获得间质细胞标记物的表达,如纤连蛋白,波形蛋白,和 N-cadherin,导致细胞骨架的重构及癌细胞迁移和侵袭的增强[1]。EMT 表型已在各种耐药的肿瘤中被发现,如吉西他滨耐药的胰腺癌[2],紫杉醇耐药卵巢癌、奥沙利铂耐药的结肠癌,和他莫昔芬耐药的乳腺癌[3-5]。这些研究表明,肿瘤的病程进展与 EMT,转移和耐药有密切相关。锌指转录因子 Snail 作为 E-cadherin 的抑制剂, 在 EMT 的触发过程发挥着不可替代的作用。Snail 的异位表达导致间质细胞标记物的活化、上皮细胞标志物的抑制并且与人类上皮肿瘤细胞系的转移相关[6,7]。通过 RNA 干扰抑制 Snail,从而降低转移性肿瘤细胞的转移能力[8]。也有一些证据表明,Snail 在卵巢癌细胞中通过抑制 p53 介导的细胞凋亡和触发干细胞样特性的获得来影响放疗和紫杉醇治疗的耐受[9]。最近的一份报告表明,Snail 可能提高胰腺癌细胞对 5- 氟尿嘧啶或吉西他滨化疗耐药[10]。本文通过 5 氟尿嘧啶筛选出 HCT116 细胞耐药株 (HCT116 / 5-FU), 并采用靶向 Snail 基因的 siRNA 并联合 5 氟尿嘧啶处理该细胞株, 研究 Snail 的抑制是否能增加耐药结肠癌细胞对 5-FU 的敏感性,评

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估其可能的信号转导通路。

1 材料与方法

1.1 材料

兔抗人多克隆抗体 Snail、p-JNK、p-ERK、p-p38、Bcl-2、Bax、caspase-3、PARP、DRP1、β -actin 单克隆抗体（Sigma 公司）；北京中山生物公司生产的羊抗兔 IgG(H+L)；American Type Culture Collection 生产的人结肠癌细胞株 HCT116；Life Technology 公司生产的细胞培养 DMEM、胰蛋白酶、小牛血清（FCS）；BD 公司生产的 AnnexinV-PI 双标凋亡试剂盒；Santa Cruz Biotechnology 生产的 control siRNA、Snail siRNA。

1.2 细胞的培养与处理

人结肠癌细胞 HCT116 / 5-FU(American Type Culture Collection，ATCC)，细胞置于的 DMEM 培养液中（含 10%胎牛血清、青霉素和链霉素各 100 U·mL^{-1}），于培养箱中常规培养。并接种于六孔板培养，当细胞密度达 50%～60%融合时，作为种子细胞，给 6 孔板每孔内置入 2× 10^5·mL^{-1} 的细胞悬液 1 mL，待细胞贴壁 24 h 后对细胞进行处理。处理细胞分生理盐水组、5-FU 组、Snail siRNA+5-FU 组、control siRNA 组、Snail siRNA 组，分别处理 36 h。

1.3 Western blot 分析

采用细胞蛋白提取试剂盒步骤进行提取蛋白质后备用。蛋白变性：将 2 μL loading buffer 加入 10 μL 标本内，100℃ 变性 3 min；上样后电泳：分离胶 120 V、2. 5 h，积层胶 80 V、30 min；于转移缓冲液中放入电泳后的聚丙烯酰胺凝胶平衡 20~ 60 min，将滤纸和硝酸纤维素膜切出，切出大小与凝胶大小一致，之后的 5-10 min 将其置入转移缓冲液中湿润，随后转膜，显影。

1.4 细胞凋亡率检测

各组细胞先用 0.25%胰蛋白酶消化，制成密度为 5 × 10^5～1 × 10^6 个 /mL 单个细胞悬液。用 PBS 离心洗涤 1 mL 细胞，1000 rpm，4 ℃离心弃上清液。将细胞重悬浮于 200 μL 结合缓冲液。加入 5 μL P I+10 μLAnnexin V-FITC，混匀后在之后的 15 min 内进行避光室温反应。将 300 μL 结合缓冲液加入，立即上流式细胞仪检测。结合 WinMD I219 Version 软件，用流式细胞仪 CELLQest 检测每个样品 1 × 10^6 个细胞。每个样本 1 × 10^6 个细胞中正常细胞、晚期凋亡、早期凋亡、死亡细胞比例均采用双染 Annexin V-F ITC /PI 法测定。

2 结果

2.1 HCT116 获得 5-FU 耐药性诱导 EMT

HCT116 与 HCT116 耐药细胞株的形态学结果显示：HCT116 细胞显示鹅卵石样外观并有致密的细胞 - 细胞连接，细胞边缘的形状呈圆形。相反，HCT116 / 5-FU 细胞有梭形形态，细胞间的分离表明细胞粘附的缺失，并有伪足形成的增多。这种变化是典型的 EMT 表型（图 1）。无 5-FU 培养 HCT116 / 5-FU 细胞 6 月后 EMT 形态学无改变，表明这一过程具不可逆性。为确定 5-FU 耐药的获得是否引起与 EMT 一致的特定分子的变化，我们检测上皮和间充质细胞表型标记的表达。Western blot 显示 E-cadherin 与 Claudin-1 表达显著降低，而波形蛋白、纤维连接蛋白与 N-cadherin 的表达则显著升高，同时 Snail 的表达也显著升高（图 2）。

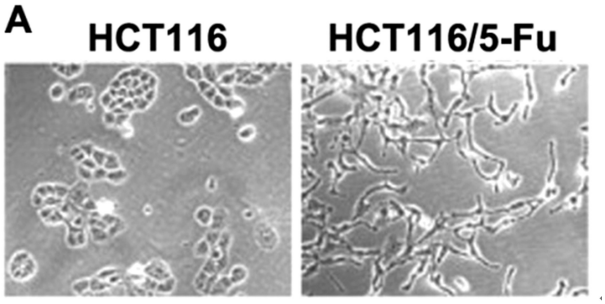

图 1 HCT116 与 HCT116 耐药细胞株的形态学结果

Fig. 1 Morphologic changes of the HCT116 cells and 5-FU-resistant HCT116 cells

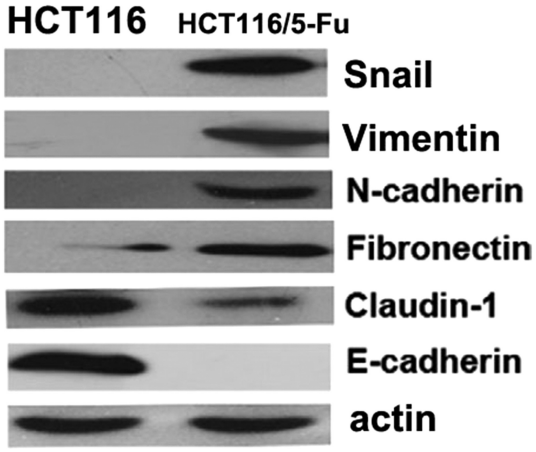

图 2 HCT116 与 HCT116 耐药细胞株的上皮和间充质细胞表型标记物的表达变化

Fig. 2 EMT- phenotypic changes in HCT116 cells and 5-FU-resistant HCT116 cells

2.2 Snail siRNA 逆转 HCT116 / 5-FU 细胞 EMT

HCT116 / 5-FU 细胞经 Snail siRNA 处理 24h 后，Snail 蛋白显著下调（图 3），同时，细胞形态失去了 EMT 特征，恢复到鹅卵石样外观并有致密的细胞 - 细胞连接（图 4）。

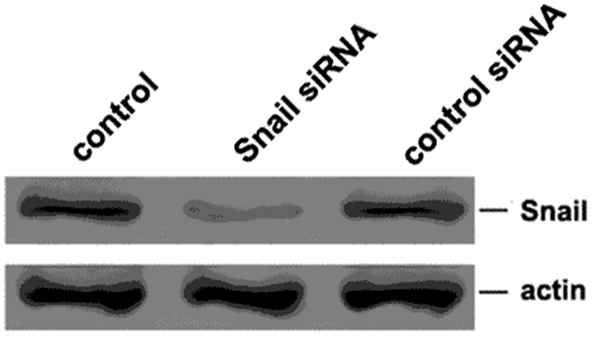

图 3 HCT116 / 5-FU 细胞经 Snail siRNA 处理前后 Snail 蛋白的变化

Fig. 3 Changes of snail protein after HCT116 / 5-FU cells treated by Snail siRNA

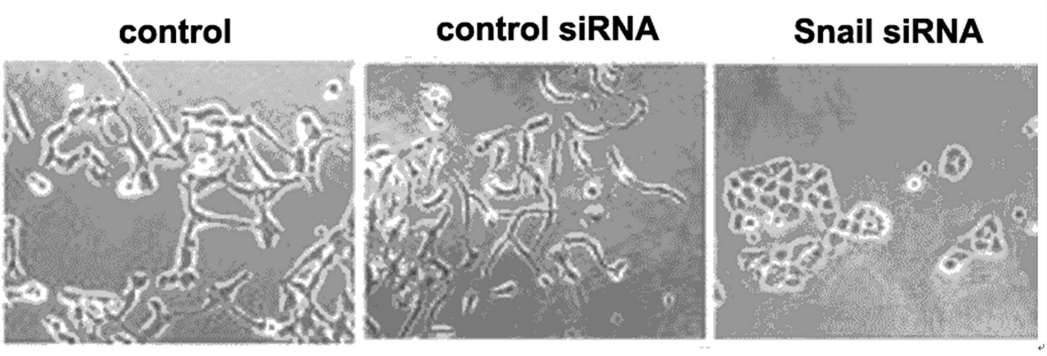

图 4 HCT116 / 5-FU 细胞经 Snail siRNA 处理前后形态学变化

Fig. 4 Morphologic changes of the 5-FU-resistant HCT116 cells after silencing of Snail by RNA interference

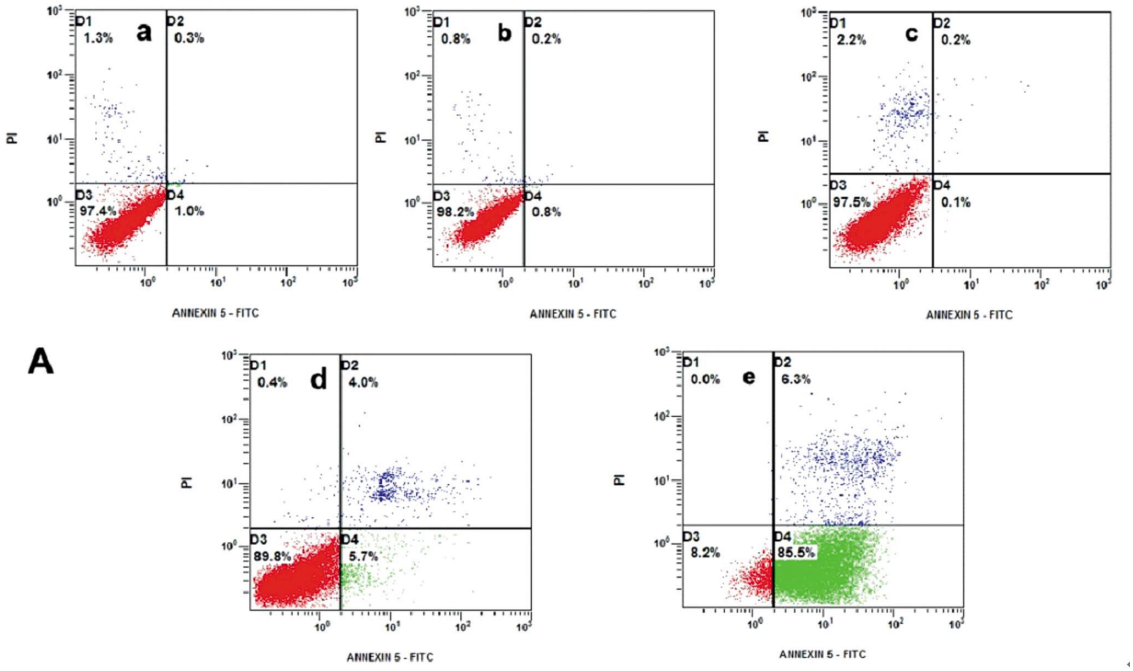

图 5 不同处理方式对细胞凋亡的影响

Fig. 5 Effects of different treating ways on the apoptosis of cells

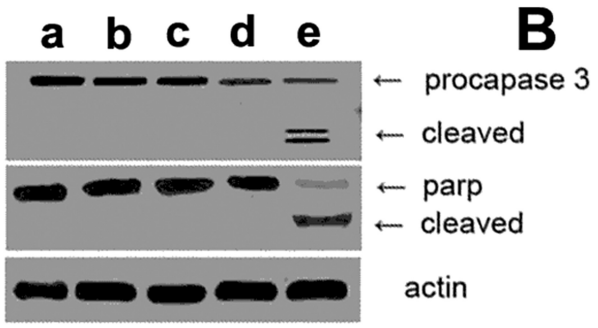

图 6 不同处理方式对细胞凋亡分子表达变化

Fig. 6 Expression changes of the apoptosis cells after different treating ways

2.3 Snail siRNA 增加 HCT116 / 5-FU 细胞对 5-FU 敏感性

单独 Snail siRNA 处理对 HCT116 / 5-FU 细胞无影响，Snail siRNA+5-FU 联合处理 HCT116 / 5-FU 细胞 36 h 后，凋亡细胞的比例较其他各组显著增加(图 5)。Western Blot 显示：caspase 3 与 PARP 在联合处理组中有了明显的剪切，而在其他 4 组对照组中无发生(图 6)。

2.4 Snail 沉默激活线粒体途径

在激活分析中，仅有 JNK 的磷酸化在 3 种 MAPKs 中最为明显。由于 MAPK 可能参与调控细胞凋亡的线粒体途径的激活，我们分析了线粒体途径相关蛋白，Western Blot 显示：联合治疗促进了细胞色素 C 在胞浆的释放，BAX 的上调以及 Bcl-2 的下调，证明了线粒体途径在 Snail 沉默中的作用(图 7)。

3 讨论

尽管治疗手段有所改善，对化疗的耐受仍然是结肠癌治疗的一个重大的障碍。透彻了解化疗后残余肿瘤细胞生存的机制最终可能提供更有效的化疗策略。在这项研究中，我们使用 5-氟尿嘧啶耐受的结肠癌细胞 HCT116 观察 5-FU 耐药的分子机制和相关的细胞行为。结果表明，HCT116 / 5-FU 细胞发生上皮间质转化(EMT)。这可以由形态学改变证实：梭形细胞伪足的形成；分子标记蛋白的变化：E-cadherin 的表达减少，和波形

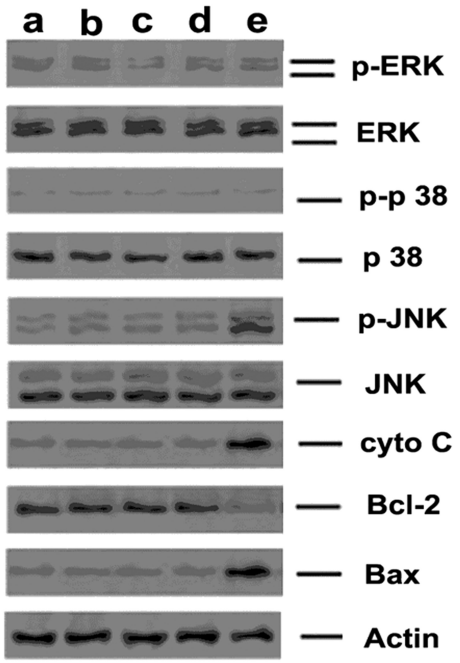

图 7 Snail 沉默后线粒体途径相关蛋白表达变化

Fig. 7 The expression changes of the mitochondrial pathway-related proteins after silencing of Snail

蛋白和 N-cadherin 水平增加;并与转录因子 Snail 的表达显著增加相关。与本研究结果一致的是 EMT 的诱导在其他化疗药物产生获得性耐药也在其他肿瘤中被报导[2-5,11]。EMT 可能赋予细胞抗凋亡的能力已得到公认。在大多数的癌症中,Snail 的上调伴随着 E-cadherin 基因表达的下调,表明粘附功能的损失[12]。因此,Snail 已被确定为 E-cadherin 的一个抑制剂和触发 EMT 的关键分子。研究表明 Snail 可能与肿瘤的发生、发展相关[13]。Espineda 等报道,在数种肿瘤中 Snail 通过抑制 Na, K-ATPase β 亚基诱导 EMT[14]。通过体外和体内实验,Miyoshi 等表明,Snail 通过调节 MMP 表达增强肝癌细胞侵袭和转移能力 [15]。在乳腺癌中,Snail 的过表达被证明抑制参与上皮细胞紧密连接的 Claudin-1 的表达从而导致肿瘤的进展[16]。因此,Snail 被认为既参与肿瘤的早期事件还包括了后期阶段。本研究表明,Snail 的上调可能是 5-FU 诱导的 EMT、药物耐受的关键因素。合理应用 5-FU 组合及 Snail 沉默可能是治疗结肠癌的一个潜在方法。首先,单独沉默 Snail 几乎没有引起 HCT116/5-FU 的自发凋亡。第二,结肠癌细胞耐药的增加会导致 5-FU 对结肠癌疗效不佳。因此,需要增加大 5-FU 剂量,这反过来对其他器官如耳蜗,肾脏的毒副作用将不可避免地增加。第三,Snail 沉默明显提高 HCT116 细胞对 5-FU 诱导细胞凋亡的响应。因此,5-FU 与 Snail 沉默联合应用可能是结肠癌治疗的一种有效的方法,从而有助于降低 5-FU 剂量,减少 5-FU 对多种正常器官和组织的毒副作用。

Snail 沉默使 HCT116 细胞增强对 5 氟尿嘧啶的敏感性的机理仍不清楚。丝裂原活化蛋白激酶(MAPK),被证明在细胞凋亡中扮演重要作用,主要包括 ERK,p38 及 JNK[17]。这三种蛋白中,JNK 被证明扮演了最主要的作用。据报道 Vav3 蛋白可能通过通过调节 JNK 信号转导通路抑制耐药基因 MDR1/P-gp 与 GSTπ 在胃癌耐药中发挥作用[16]。在结肠癌细胞中,白藜芦醇与 5-FU 联用通过调节 JNK 信号转导通路阻断细胞 S 期并增强 DNA 损伤从而诱导细胞凋亡[18]。本研究结果表明,伴随细胞凋亡增加的同时,磷酸化 JNK 在 5-FU 与 Snail siRNA 联合处理的细胞中明显上调。有证据表明,JNK 可能参与调控细胞凋亡的线粒体途径的激活[19]。Bcl-2 和 Bax 在线粒体途径介导的细胞凋亡中发挥重要作用。细胞凋亡或生存是由 Bcl-2 中的促凋亡和抗凋亡成员之间的平衡决定。Bax 能促进细胞色素 C 释放至胞浆,从而激活 Caspase-3。为进一步了解 Snail 沉默对 HCT116 细胞恢复 5-FU 药敏的确切机制,我们分析了线粒体途径相关蛋白的表达。结果显示 HCT116/5-FU 经 Snail 沉默后 5-FU 促进了细胞色素 C 在胞浆的释放,BAX 的上调以及 Bcl-2 的下调,提示线粒体途径在这一过程中的激活。

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