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同期放化疗诱导人结直肠癌细胞发生上皮–间质转化

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[摘　要]　**目的：**探讨放化疗抵抗的结直肠癌细胞发生上皮–间质转化(epithelial-mesenchymal transition，EMT)的意义。**方法：**采用5-FU化疗同期进行放疗对人结直肠癌野生型细胞(HCT116)进行干预，诱导放化疗共同抵抗的细胞株(HCT116CRR)并采用克隆形成实验进行放化疗抵抗性的鉴定。高倍显微镜下观察细胞形态学变化。采用Real-time PCR和Western印迹，检测上皮表型标志物E-cadherin，间质表型标志物N-cadherin、波形蛋白(vimentin)、核转录因子(Snail)mRNA及其蛋白的表达。**结果：**放化疗抵抗的结直肠癌细胞发生与EMT相符的形态学改变，细胞呈纺锤体状，极性消失，并出现伪足；Real-time PCR和Western印迹结果显示E-cadherin mRNA及蛋白表达下调；N-cadherin，vimentin，Snail mRNA及蛋白表达上调，差异有统计学意义($P<0.05$)。**结论：**放化疗抵抗后的人结直肠癌细胞发生EMT，其与结直肠癌的治疗抵抗相关。

[关键词]　人结直肠癌；肿瘤细胞；放化疗抵抗；上皮–间质转化

Chemoradiotherapy can induce epithelial-mesenchymal transition of the human colorectal cancer cells

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Abstract　**Objective:** To investigate the significance of epithelial-mesenchymal transition (EMT) of human colorectal cancer cells with resistance to chemoradiotherapy. **Methods:** Colorectal cancer cells were exposed to 5-fluorouracil (5-FU) while radiotherapy was given at the same time. A small number of cells that survived from chemoradiotherapy were obtained, and their morphological changes were observed by microscopy. Our group then identified the resistance using colony formation assay. Real-time PCR and Western blot were used to detect the mRNA and its protein expression of epithelial marker E-cadherin, mesenchymal marker N-cadherin, vimentin, and nuclear transcription factor snail. **Results:** Colorectal cancer cells which were resistant to chemoradiotherapy showed phenotypic changes consistent with EMT: spindle-cell shape, loss of polarity, and pseudopodia formation.

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Real-time PCR and Western blot results: E-cadherin mRNA and protein expression level were lower, and N-cadherin, Snail, vimentin mRNAs and proteins expression level were higher, the difference was statistically significant (P<0.05). **Conclusion:** Colorectal cancer cells resistance to chemoradiotherapy can induce epithelial-mesenchymal transition (EMT), and EMT may be related to resistance of chemoradiotherapy of colorectal cancer.

Keywords colorectal cancer; neoplasm cells; radiosensitization; epithelial-mesenchymal transition (EMT)

在全世界范围内，结直肠癌是目前发病率很高的恶性肿瘤，在男女性中都排名前三，其病死率位居所有恶性肿瘤死因的第二位[1]。放疗是结直肠癌综合治疗的主要手段，新辅助放化疗–手术–术后化疗是最新的NCCN临床实践指南推荐的标准化治疗方案。放疗是直肠癌综合治疗的主要手段。然而，在新辅助同期放化疗运用日趋广泛的同时，同期放化疗抵抗及放射增敏性不高等问题出现，患者接受新辅助同期放化疗后的完全病理缓解率只有8%~29%[2-4]，尚有一部分患者无法在新辅助治疗中受益。

目前放化疗抵抗机制受到很多学者的广泛关注。Li等[5]的研究证实放射治疗后残余肝癌发生了上皮–间质转化(epithelial-mesenchymal transition，EMT)现象并且侵袭转移能力显著增强。臧春宝等[6]研究证实了X线照射后Eca109和Te1细胞呈EMT典型的形态学改变；体内外实验证实X射线照射后的食管癌鳞癌细胞和移植瘤下调上皮标志物E-cadherin表达，上调间质细胞标志蛋白N-cadherin和vimentin表达，这也符合EMT标志蛋白的变化，提示放射诱导食管鳞癌细胞发生EMT，继而增加放疗抗性。Gomez-Casal等[7]研究了放疗抵抗的非小细胞肺癌A549和H460细胞株的分子表型，表明放疗抵抗的非小细胞肺癌细胞中Snail，vimentin和N-cadherin表达升高，进一步说明了EMT与放疗抵抗的关系。综上，大量实验证实了放疗后的肿瘤细胞发生了EMT的改变。寻找一种有效的放射增敏剂显得尤为迫切。本实验拟检测放化疗抵抗结直肠癌细胞系EMT相关特征的改变及其意义。

1 对象与方法

1.1 对象

1.1.1 细胞株

人结直肠癌细胞株HCT116，购自中国科学院昆明动物所细胞库，保存于液氮；放化疗抵抗人结直肠癌细胞株HCT116CRR，按照课题组研究计划诱导成功且其放化疗抵抗性经鉴定，保存于液氮。

1.1.2 药品与试剂

胎牛血清，RPMI-1640，PBS以及青霉素和链霉素混合液，0.25%胰蛋白酶购自美国Hyclone公司；5-FU由昆明制药厂惠赠；细胞及组织总蛋白抽提试剂盒(KangChen，KC-415)，BCA蛋白质定量试剂盒(KangChen，KC-430)，KC^{TM}化学发光试剂盒(KangChen，KC-420)购自上海康成生物有限公司；E-cadherin，N-cadherin，Snail，vimentin Rabbit Monoclonal Antibody购自英国Abcam公司。

1.2 细胞培养

人结直肠癌细胞株HCT116和放化疗抵抗人结直肠癌细胞株HCT116CRR细胞培养于37 ℃、5% CO_2培养箱中，培养液为含10%胎牛血清和1%青链霉素混合液的RPMI-1640。

1.3 结直肠癌放化疗抵抗细胞株的诱导及鉴定

本课题组前期实验模拟临床治疗模式的大剂量同期放化疗连续冲击法，构建大肠癌细胞体外同期放化疗模型，已诱导出放化疗抵抗结直肠癌细胞(HCT116CRR)并对其放化疗抵抗性进行了鉴定[1]。

1.3.1 结直肠癌放化疗抵抗细胞株的诱导

选取HCT116细胞株体外培养，根据已有参考文献[8]和前期实验基础，分别选择10 μmol/L的5-FU作为化疗浓度和4 Gy的X射线作为放疗剂量，待细胞生长至约80%融合时将其暴露于10 μmol/L的5-FU中，同时在室温下给予4 Gy的6 mV X射线照射，继续将细胞暴露于5-FU培养至第24 h(从开始暴露于5-FU中开始计算)，更换新鲜培养液，待残余细胞恢复生长。

1.3.2 结直肠癌放化疗抵抗细胞株的鉴定

再用相同方法处理细胞9次，得到HCT116残余细胞株。用克隆形成实验鉴定残癌细胞株是否为放化疗抵抗细胞株。

1.4 Real-time PCR

实验分两个组：1)野生型人结直肠癌HCT116细胞；2)放化疗抵抗人结直肠癌HCT116CRR细

胞。两组细胞分别取对数生长期细胞，用胰酶消化得到细胞沉淀，待用。用Real-time PCR检测E-cadherin，N-cadherin，vimentin和Snail mRNA的表达情况。

1.4.1 细胞总 RNA 抽提

每5×10^{6}个细胞加入1 mL TRIzol RNA提取试剂，提取实验组和对照组细胞的总RNA，液氮中保存。

1.4.2 PCR 扩增

反转录合成cDNA，再以cDNA为模板进行PCR扩增。引物设计软件：Primer 5.0(表1)。

所有的指标均按以下程序进行：

95 ℃，10 min；40个PCR循环[95 ℃，10 s；60 ℃，60 s(收集荧光)]。

取5 μL PCR产物于1.5%琼脂糖凝胶电泳后拍照并于凝胶图像分析系统进行扫描分析，计算E-cadherin，N-cadherin，vimentin，Snail mRNA与GAPDH mRNA的比值，实验重复3次。

1.5 Western 印迹

实验分两个组：1)野生型人结直肠癌HCT116细胞(N组)；2)放化疗抵抗人结直肠癌HCT116CRR细胞(CRR组)。两组细胞分别取对数生长期细胞，用胰酶消化得到细胞沉淀，待用。用Western印迹检测E-cadherin，N-cadherin，vimentin和Snail蛋白的表达情况。

表1 Real-time PCR引物

Table 1 Real-time PCR primers

基因名	双向引物序列	退火温度/℃	产物长度/bp
GAPDH (HUMAN)	F：5'-GGGAAACTGTGGCGTGAT-3' R：5'-GAGTGGGTGTCGCTGTTGA-3'	60	299
E-cadherin	F：5'-GAAACAGGATGGCTGAAGGTGAC-3' R：5'-TAAGCGATGGCGGCATTGTA-3'	60	278
N-cadherin	F：5'-AACGCCAGGCCAAACAACTT-3' R：5'-ATTCGTCGGATTCCCACAGG-3'	60	172
Vimenrin	F：5'-TCTGGATTCACTCCCTCTGGT-3' R：5'-CGTGATGCTGAGAAGTTTCGT-3'	60	107
Snail	F：5'-GCCTGGGTGCCCTCAAGAT-3' R：5'-TTGTGGAGCAGGGACATTCG-3'	60	265

1.5.1 电泳样品制备

用PBS洗涤贴壁生长的培养细胞2次，加入胰酶消化离心，再用PBS洗涤沉淀1次，加入细胞裂解液，离心，收集上清入EP管中。

1.5.2 SDS-聚丙烯酰胺凝胶电泳及电转移

取出上述蛋白样品和蛋白Marker，置100 ℃水浴5 min。灌制分离胶和浓缩胶，吸取样品及蛋白质Marker各15 μL，加入加样孔中。接通电源，取电压80 V，电泳约2 h。把滤纸、凝胶、硝酸纤维素薄膜(NC)按规定顺序放好，妥善固定后在转移缓冲液中进行电转移。

1.5.3 免疫学检测

将NC条放入封闭缓冲液中封闭1 h。用抗E-cadherin，N-cadherin，vimentin，Snail的稀释液浸泡NC条，4 ℃放置过夜。孵二抗1 h。TBST洗膜3次。清洗后置于反应液(KC^{TM}化学发光试剂盒中两种试剂等比例混合为反应液)中，室温孵育3 min，去除过量的溶液，将膜夹在两塑料薄膜之间，以X线胶片曝光。图片扫描保存为电脑文件，并用Image J分析软件将图片上每个特异条带灰度值的数字化。

1.6 统计学处理

采用SPSS 11.5软件进行统计学分析。采用t检验。以均数±标准差($\bar{x}\pm s$)表示。每组实验重复3次，以P<0.05为差异有统计学意义。

2 结果

2.1 细胞生物学形态的改变

根据前期实验结果[1]，放化疗抵抗的结直肠癌细胞形态发生与EMT相符的形态学改变：细胞呈纺锤体状，极性消失，并出现伪足(图1)；随后课题组运用克隆形成实验，对比研究了野生型

细胞株和放化疗后的细胞株对放化疗的敏感性。结果证实，放化疗后的细胞株具有明显的放化疗抵抗性。

2.2 E-cadherin，N-cadherin，vimentin，Snail mRNA 在各组的表达水平

用RT-PCR检测出四组细胞中E-cadherin，N-cadherin，vimentin，Snail mRNA的相对表达量(表2和图2~3)。结果显示E-cadherin mRNA的表达情况，野生型结直肠癌HCT116细胞组高于放化疗抵抗结直肠癌HCT116CRR细胞组；N-cadherin，vimentin，Snail mRNA的表达情况，放化疗抵抗结直肠癌HCT116CRR细胞组高于野生型结直肠癌HCT116细胞组，差异有统计学意义(P<0.05)。

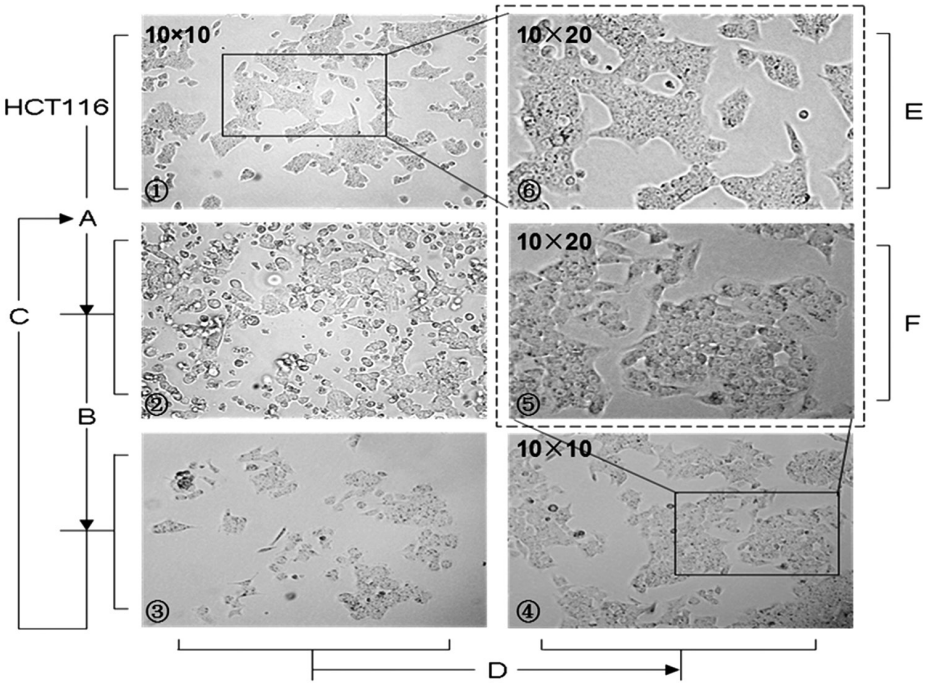

图1 放化疗抵抗体外模型的建立

Figure 1 Establishment of 5-FU-based chemoradiation resistance in vitro model

(A)HCT116细胞暴露于10 μmol/L 5-FU并在室温下给予4 Gy，6 mV X线照射。继续暴露于5-FU中培养至第24 h，会有一些细胞发生凋亡；(B)给存活的肿瘤细胞更换新鲜培养液；(C)继续同期放化疗，重复9次；(D)最终存活的细胞即为体外同期放化疗模型；(E)野生型HCT116细胞株；(F)放化疗抵抗HCT116CRR细胞株。

(A) HCT116 cells were exposed to 10 μmol/L 5-FU and a single dose of 4 Gy of 6 mV X-ray. After radiation treatment, the tumor cells were incubated in 5-FU for additional 24 h, and a number of them underwent apoptosis; (B) the remaining tumor cells were transferred to fresh culture medium for recovery; (C) tumor cells were subjected to 5-FU and X-ray again. This performance was repeated for 9 times; (D) the subcultured remaining tumor cells were collected to construct the 5-FU-based CRR in vitro model ; (E) parental HCT116 cell line; (F) colorectal cancer cell line resistance to chemoradiotherapy.

表2 两组细胞中四个基因相对表达量(n=3，$\bar{x} \pm s$)

Table 2 Expressions of the four genes in their groups (n=3，$\bar{x} \pm s$)

基因	N组	CRR组	P
E-cadherin	1	0.54 ± 0.01*	<0.001
N-cadherin	1	3.50 ± 0.50*	0.01
Snail	1	2.64 ± 0.19*	<0.001
vimentin	1	2.58 ± 0.24*	0.008

与N组比较，*P<0.05。N组：野生型结直肠癌HCT116细胞组；CRR组：放化疗抵抗结直肠癌HCT116CRR细胞组。

Compared with group N, *P<0.05. N: parental cell line; CRR: chemoradiotherapy-resistant cell line.

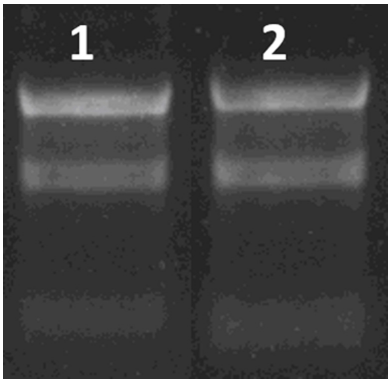

图2 样本的总RNA

Figure 2 Total RNA of samples

28S和18S核糖体RNA的带非常亮而浓(其大小决定于用于抽提RNA的物种类型)，上面一条带的密度大约是下面一条带的2倍。还有可能观察到一个更小稍微扩散的带，它由低分子量的RNA(tRNA和5S核糖体RNA)组成。在18S和28S核糖体带之间一般可以看到一片弥散的EB染色物质，可能是由mRNA和其他异型RNA组成。RNA制备过程中如果出现DNA污染，将会在28S核糖体RNA带的上面出现，即更高分子量的弥散迁移物质或者带。RNA的降解表现为核糖体RNA带的弥散。Lane 1：N组；Lane 2：CRR组。

The 28S and 18S ribosomal RNA bands should be fairly sharp, intense bands. The intensity of the upper band should be about twice of the lower band. Smaller, more diffuse bands representing low molecular weight RNAs (tRNA and 5S ribosomal RNA) may be present. It is normal to see a diffuse smear of ethidium bromide staining material migrating between the 18S and 28S ribosomal bands, probably comprised of mRNA and other heterogeneous RNA species. DNA contamination of the RNA preparation will be evident as a high molecular weight smear or band migrating above the 28S ribosomal RNA band. Degradation of the RNA will be reflected by smearing of ribosomal RNA bands. Lane 1: N group; Lane 2: CRR group.

2.3 E-cadherin，N-cadherin，vimentin，Snail 蛋白在各组的表达水平

E-cadherin，N-cadherin，vimentin，Snail蛋白在各组的表达水平(图4)，4种蛋白在两组中的表达灰度值量化比较(图5)。图5可见结果显示E-cadherin蛋白的表达情况，野生型结直肠癌HCT116细胞组高于放化疗抵抗结直肠癌HCT116CRR细胞组；N-cadherin，vimentin，Snail蛋白的表达情况，放化疗抵抗结直肠癌HCT116CRR细胞组高于野生型结直肠癌HCT116细胞组，差异有统计学意义(P<0.05)。

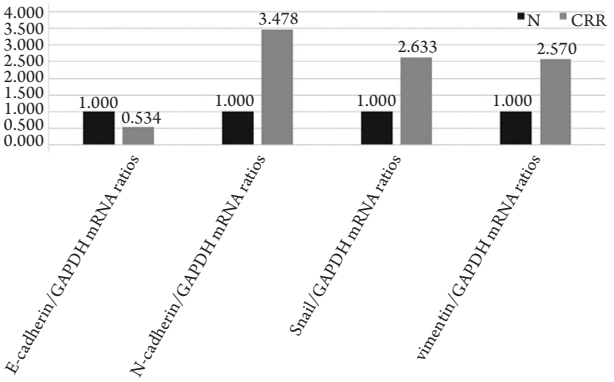

图3 放化疗抵抗结直肠癌HCT116CRR细胞与野生型结直肠癌HCT116细胞中E-cadherin，N-cadherin，vimentin，Snail mRNA表达量的区别

Figure 3 Expressions of E-cadherin, N-cadherin, vimentin and Snail mRNA in parental cell line and chemoradiotherapy-resistant cell line of colorectal cancer HCT116 cell line

N：野生型结直肠癌HCT116细胞组；CRR：放化疗抵抗结直肠癌HCT116CRR细胞组。

N: Parental cell line; CRR: Chemoradiotherapy-resistant cell line.

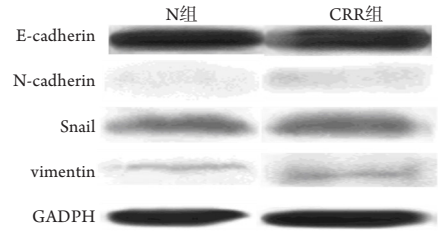

图4 两组E-cadherin，N-cadherin，Snail，vimentin及内参GADPH的表达水平

Figure 4 Expressions of E-cadherin, N-cadherin, Snail, vimentin and GADPH proteins in each group

N：野生型结直肠癌HCT116细胞组；CRR：放化疗抵抗结直肠癌HCT116CRR细胞组。

N: Parental cell line; CRR: Chemoradiotherapy-resistant cell line.

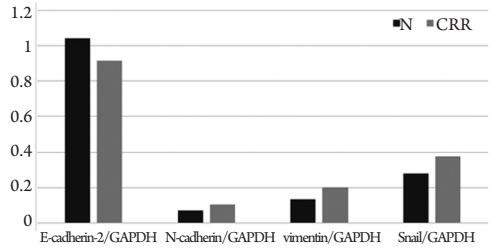

图5 放化疗抵抗结直肠癌HCT116CRR细胞与野生型结直肠癌HCT116细胞中E-cadherin，N-cadherin，vimentin，Snail蛋白表达的影响

Figure 5 Expressions of E-cadherin, N-cadherin, vimentin and Snail proteins in parental cell line and chemoradiotherapy-resistant cell line of colorectal cancer HCT116 cell line

N：野生型结直肠癌HCT116细胞组；CRR：放化疗抵抗结直肠癌HCT116CRR细胞组。

N: parental cell line; CRR: chemoradiotherapy-resistant cell line.

3 讨论

在EMT中，上皮细胞获得成纤维细胞样特性，细胞常呈纺锤体状，并出现伪足；细胞极性丧失，细胞间黏附减少，迁移和运动能力增强；同时细胞表型发生改变，即上皮表型标志物如E-cadherin等逐渐丧失，而波形蛋白(vimentin)和N-cadherin等间充质表型特征分子表达上调，核转录因子Snail表达上调。本实验结果表现了以上特性，足以说明放化疗抵抗的结直肠癌细胞发生了EMT。

在直肠癌的治疗当中，放射治疗占据很重要的地位，由于放射敏感性不高以及放疗抵抗等问题，放射治疗的临床疗效并不理想。大量学者证实了放疗或化疗后的肿瘤细胞发生了EMT且其机制可能与肿瘤干细胞相关，但目前同时具有放疗抵抗和化疗耐药的细胞株还未见其他报道。

总之，本研究结果显示：结直肠癌放化疗抵抗诱导了EMT的产生，并且其可能与结直肠癌肿瘤干细胞相关。深入探讨结直肠癌放化疗抵抗诱导EMT的机制，并与结直肠癌肿瘤干细胞结合进行研究终将揭示其放化疗耐受及转移的机制，为结直肠癌的辅助治疗提供依据。

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