·论 著·

结肠癌T-cadherin表达水平分析与5-Aza-CdR对其表达和结肠癌细胞增殖、侵袭及凋亡的影响

刘辉勇，陈 烽，姚庆智，郭俏楠，张中一，陈志耀，林建清

福建医科大学附属第二医院肿瘤外科，福建 泉州，362000

［摘要］ **背景与目的：**结肠癌是临床常见恶性肿瘤，探讨抑癌蛋白T-cadherin在结肠癌中的表达情况及其与患者临床病理学特征的关系，并分析5-Aza-CdR对T-cadherin表达和结肠癌细胞增殖、侵袭及凋亡的影响。**方法：**收集福建医科大学附属第二医院2015—2016年40例手术切除的结肠癌组织及癌旁组织新鲜样本，并经过病理学检查验证，分别提取总RNA和总蛋白质。采用实时荧光定量聚合酶链反应（real-time fluorescence quantitative polymerase chain reaction，RTFQ-PCR）分析T-cadherin mRNA的表达，采用蛋白质印迹法（Western blot）分析T-cadherin蛋白水平，并分析T-cadherin的mRNA表达变化与患者临床病理学特征的关系。进一步以人结肠癌细胞系HT-29为研究对象，采用甲基化抑制剂5-Aza-CdR处理HT-29细胞，分别采用RTFQ-PCR和Western blot分析T-cadherin表达变化，采用细胞计数试剂盒（cell counting kit-8，CCK-8）分析细胞增殖，采用Transwell实验验证细胞侵袭能力，采用流式细胞术分析细胞凋亡。**结果：**T-cadherin在结肠癌组织的蛋白水平和mRNA表达均明显低于癌旁组织，其mRNA表达与淋巴结转移（F=5.316，P=0.009 3）和分化程度（F=5.807，P=0.006 4）明显相关，而与其他病理变量（包括性别、年龄、肿瘤大小和肿瘤浸润深度）无明显相关。药物5-Aza-CdR可以显著上调HT-29细胞中T-cadherin的表达水平，抑制HT-29细胞增殖、侵袭并促进细胞凋亡。**结论：**T-cadherin表达可能与结肠癌恶性程度密切相关，药物5-Aza-CdR处理可上调结肠癌细胞T-cadherin的表达，并抑制结肠癌细胞的增殖、迁移，促进结肠癌细胞凋亡，提示T-cadherin可能是结肠癌患者使用甲基化抑制剂5-Aza-CdR治疗的靶点。

［关键词］T-cadherin；结肠癌；5-Aza-CdR；治疗靶点

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Analysis of T-cadherin expression in colon cancer and the effect of 5-Aza-CdR on T-cadherin expression and proliferation, invasion and apoptosis of colon cancer cells

LIU Huiyong, CHEN Feng, YAO Qingzhi, GUO Qiaonan, ZHANG Zhongyi, CHEN Zhiyao, LIN Jianqing (Department of Oncological Surgery, the Second Affiliated Hospital of Fujian Medical University, Quanzhou 362000, Fujian Province, China)

Correspondence to: LIN Jianqing E-mail: ljq13905977336@163.com

［**Abstract**］ **Background and purpose:** Colon cancer is a common clinical malignant tumor. This study aimed to investigate the expression of tumor suppressor gene expression product T-cadherin in colon cancer and its relationship with clinicopathological features, and to analyze the effect of 5-Aza-CdR on T-cadherin expression and proliferation, invasion and apoptosis of colon cancer cells. **Methods:** The fresh samples of colon cancer tissues and adjacent tissues from the Second Affiliated Hospital of Fujian Medical University were collected and verified by pathological diagnosis from 2015 to 2016, and total RNA and protein were extracted respectively. The expression of T-cadherin in tissues was analyzed by real-time fluorescence quantitative polymerase chain reaction (RTFQ-PCR) and Western blot, and the relationship between the expression of T-cadherin and clinicopathological features was also analyzed. Furthermore, colon cancer HT-29 cells were treated with methylation inhibitor 5-Aza-CdR. The expression of T-cadherin in cells was analyzed by RTFQ-PCR and Western blot. Cell proliferation, invasive ability and apoptosis were analyzed by cell counting

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通信作者：林建清 E-mail: ljq13905977336@163.com

kit-8 (CCK-8), transwell assay and flow cytometry respectively. **Results:** The T-cadherin expression in colon cancer tissues was significantly lower than that in paracancerous tissues. The expression level of T-cadherin was significantly correlated with lymph node metastasis (F=5.316, P=0.009 3) and the degree of differentiation (F=5.807, P=0.006 4). Other pathologic parameters, including gender, age, tumor size and depth of tumor invasion, were not significantly correlated with T-cadherin expression. The 5-Aza-CdR treatment significantly up-regulated the expression of T-cadherin, inhibited the proliferation and invasion, and promoted the apoptosis of HT-29 cells. **Conclusion:** The expression of T-cadherin may be closely related to the malignancy of colon cancer. The 5-Aza-CdR treatment up-regulates the expression of T-cadherin, inhibits the cell proliferation and invasion, and promotes the apoptosis of HT-29 cells. It is suggested that T-cadherin could be an important target for 5-Aza-CdR drug therapy in patients with colon cancer.

［**Key words**］ T-cadherin; Colon cancer; 5-Aza-CdR; Therapeutic target

结肠癌是临床常见恶性肿瘤之一，2018年全球约有110万新发病例，死亡人数约为55万多人[1]。而在中国结肠癌也是最常见的胃肠道恶性肿瘤[2]。尽管结肠癌的总体疗效有所改善，但预后仍然不能令人满意。近年来，随着全基因组及全外显子测序技术的发展，新的癌症基因或抑癌基因不断被发现，并由此衍生出新的基因治疗方案，改善了患者的疗效及预后。但由于基因组学的复杂性及多样性，目前仍无法完全揭示肿瘤的发生、发展机制。因此发现新的肿瘤调节因子并揭示其内在的机制，在肿瘤治疗的研究中仍尤为重要。

T钙黏蛋白（T-cadherin），也称钙黏蛋白13（CDH13），是cadherin超家族的一个非典型成员，它通过糖基磷脂酰肌醇锚定在细胞膜上[3]。其常在各种类型的癌症中低表达，包括胃癌[4]、乳腺癌[5]、肺癌[6]、结肠癌[7]、皮肤鳞状细胞癌[8]和其他类型的癌症[9]。大量研究提示，T-cadherin作为一种抑癌蛋白具有潜在的意义，因此揭示其在结肠癌中的作用，有助于发现结肠癌治疗的新靶点。故本研究通过收集临床样本，在mRNA表达和蛋白水平上，分析T-cadherin的表达变化及其与患者临床病理学特征的关系，进而利用药物5-Aza-CdR处理人结肠癌细胞系HT-29，分析其对T-cadherin表达和HT-29细胞生物学行为的影响，初步阐明T-cadherin在结肠癌中的调控机制。

1 材料和方法

1.1 临床样本和细胞系

收集福建医科大学附属第二医院2015—2016年40例手术切除的结肠癌组织及癌旁组织新鲜样本，并经过病理学检查验证。患者包括例31例男性和9例女性，手术前均未接受放疗或者化疗。所有患者均被告知样本的用途并同意。取下的组织用PBS缓冲液冲洗2遍，立刻放置于液氮中保存及运输，用于总RNA提取及蛋白提取。HT-29细胞系购买自美国典型培养物保藏中心（American Type Culture Collection，ATCC）。人结肠癌HT-29细胞培养基为DMEM完全培养基（含10%胎牛血清和1%双抗）（美国Thermo公司），在37 ℃、CO_2体积分数为5%的条件下培养。细胞实验分为未处理组和5-Aza-CdR药物处理组。细胞药物处理：采用0.25%的胰酶消化HT-29细胞，结合移液器吸嘴吹打，使细胞充分悬浮，加入含血清培养基终止消化，将细胞分装至6个培养瓶中，加入DMEM完全培养基，置于细胞培养箱中培养24 h，换液，向其中3瓶加入5 μmol/L 5-Aza-CdR（美国Sigma公司），以不加药物者为对照组，每天换液，药物处理3 d后收集细胞。

1.2 实时荧光定量聚合酶链反应（real-time fluorescence quantitative polymerase chain reaction，RTFQ-PCR）

TRIzol试剂购自宝生物工程（大连）有限公司，参照其说明书，提取组织及细胞的总RNA。通过ND-1000分光光度计（美国Thermo公司）检测RNA的浓度和纯度，确保$D_{260\ nm}/D_{280\ nm}$>2.0，$D_{230\ nm}/D_{260\ nm}$>1.8。采用RevertAid逆转录酶（美国Thermo公司）将RNA逆转录为cDNA。按照2×SGExcel FastSYBR Mixture［生工生物工程（上海）有限公司］说明书，配制

RTFQ-PCR反应体系（25 μL 2×SGExcel FastSYBR Mixture+1 μL上游引物+1 μL下游引物+1 μL cDNA，加入超纯水补齐至50 μL），上机检测（StepOnePlus™RTFQ-PCR仪，美国ABI公司），反应程序为：预变性95 ℃，20 s；变性95 ℃，3 s；退火/延伸60 ℃，30 s；35个循环。引物由生工生物工程（上海）有限公司合成。*T-cadherin*上游引物为5'-GATGTTGGCAAGGTAGTCGAT-3'，下游引物为5'-GCTCCCTGTGTTCTCATTGAT-3'。以GAPDH作为内参。*GAPDH*上游引物为5'-ACGGGAAGCTTGTCATCAATGG-3'，下游引物为5'-ATGGTGGTGAAGACGCCAGTGG-3'。根据$2^{-\Delta\Delta CT}$计算T-cadherin的相对表达量。

1.3 蛋白质印迹法（Western blot）检测

离心收集细胞，采用1×RIPA裂解液（美国Sigma公司）裂解细胞，利用BCA蛋白浓度测定试剂盒［生工生物工程（上海）有限公司］对蛋白定量。取50 μg蛋白样品与凝胶缓冲液混合，煮沸10 min，之后进行SDS-PAGE电泳。将蛋白转移至硝酸纤维素膜（美国Millipore公司）上。非特异性抗体采用含有5%脱脂牛奶的1×Tris缓冲液封闭，室温下温育2 h。在4 ℃下，膜与T-cadherin抗体或GAPDH抗体（美国OmnimAbs公司）在含有5%脱脂牛奶的1×TBS缓冲液中温育过夜。采用1×TBS洗涤3次后，膜与辣根过氧化物酶标记的兔IgG抗体（美国OmnimAbs公司）在室温下温育2 h。在Fluor Chem Systems下（美国ProteinSimple公司）观察结果，采用系统自带的软件（Alpha View Software）分析目标蛋白的相对水平。

1.4 细胞计数试剂盒（cell counting kit-8，CCK-8）分析细胞增殖

采用CCK-8试剂盒［东仁化学科技（上海）有限公司］分析细胞的增殖。在96孔板中，在每孔中接种5×10^3个细胞，总培养液体积为100 μL，每组设置5个复孔。在铺板后的1、2、3和4 d共4个时间点，分别向细胞中加入10%的CCK-8试剂，放置于37 ℃中温育2 h。将96孔板放在ELx808酶标仪（无锡百泰克生物技术有限公司）中进行检测。读取并记录每孔在450 nm的吸光度（*D*）值。

1.5 Transwell检测细胞侵袭能力

采用Transwell小室（美国Corning公司）分析细胞的侵袭，首先采用Matrigel基质胶（美国BD公司）包被小室。采用OPTI-MEM无血清培养基悬浮细胞并计数，取1×10^5个细胞加入上室，补齐OPTI-MEM无血清培养基至终体积为200 μL，下室加入700 μL含20%小牛血清的DMEM培养基。培养板放在37 ℃、CO_2体积分数为5%的细胞培养箱中培养24 h。次日，取出上室，去除培养基，用PBS洗涤2次，移入含800 μL甲醇的孔中固定30 min。去除固定液，加入0.1%结晶紫染色20 min。PBS冲洗小室后将其放在显微镜下观察，随机取9个视野，拍照。

1.6 细胞凋亡

采用购自江苏凯基生物技术股份有限公司的Annexin Ⅴ-FITC/PI细胞凋亡检测试剂盒分析细胞凋亡。胰酶消化悬浮不同处理组的细胞，300×*g*离心5 min收集细胞。将细胞与PI与Annexin Ⅴ试剂在室温下避光温育15 min，上流式细胞仪检测（Guava easyCyte™，美国Millipore公司）。

1.7 统计学处理

采用GraphPad Prism 5软件进行统计分析。通过*t*检验分析不同组别之间的差异。所有实验至少重复3遍。结果以$\bar{x}\pm s$表示。用*t*检验和单因素方差分析评估T-cadherin表达与结肠癌患者临床病理参数之间的关系。$P<0.05$为差异有统计学意义。

2 结 果

2.1 T-cadherin在结肠癌组织中的表达及其与临床病理学特征的关系

采用RTFQ-PCR技术分析T-cadherin在结肠癌组织中的mRNA表达水平。如图1和表1所示，T-cadherin在结肠癌组织中mRNA的表达水平显著低于癌旁组织（$t=12.61$，$P<0.000\ 1$）。Western blot检测结果进一步表明，T-cadherin蛋白在结肠癌组织中的水平也明显下降（$t=4.602$，$P<0.05$，图2）。T-cadherin的mRNA表达和结肠癌患者临床病理学特征关系分析显示，其mRNA表达水平

与淋巴结转移（F=5.316，P=0.009 3）及分化程度（F=5.807，P=0.006 4）明显相关，而与其余病理变量（包括性别、年龄、肿瘤大小和肿瘤浸润深度）无明显相关（表2）。

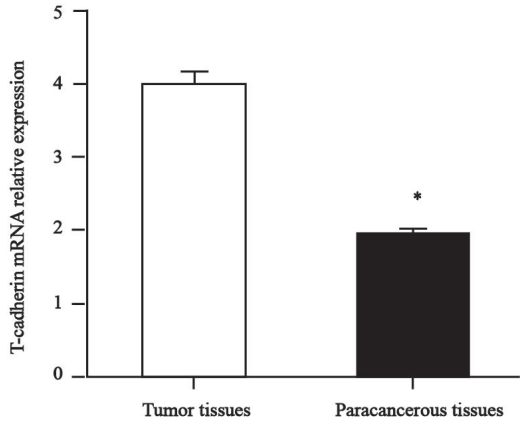

图 1　结肠癌组织及癌旁组织中T–cadherin mRNA的表达分析

Fig. 1　Analysis of T-cadherin mRNA expression in colon cancer tissues and paracancerous tissues

Compared with the paracancerous tissues, the expression of T-cadherin mRNA in colon cancer tissues was significantly down-regulated (P<0.000 1)

表 1　T–cadherin在结肠癌组织和癌旁组织的mRNA表达

Tab. 1　The mRNA expression of T-cadherin in colon cancer tissues and paracancerous tissues

Tissue	T-cadherin(mRNA) (N=40)	P value
Tumor	1.94±0.53	t=12.61 P<0.000 1
Paracancerous	4.04±0.77	

The difference was statistically significant

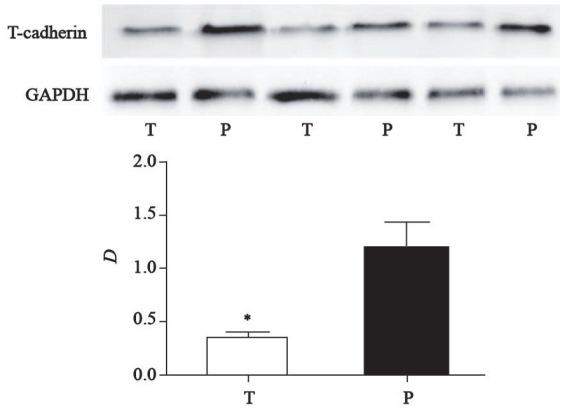

图 2　结肠癌组织及癌旁组织中T–cadherin 蛋白水平的分析

Fig. 2　Analysis of T-cadherin protein level in colon cancer tissues and paracancerous tissues

T: Tumor tissues; P: Paracancerous tissues; N=40, t=4.602; *: P<0.05, compared with each other

表 2　T–cadherin mRNA表达与结肠癌患者临床病理特征的关系

Tab. 2　Relationship between T-cadherin mRNA expression and clinicopathological features of patients with colon cancer

Variant	Total	T-cadherin (mRNA)	t or F	P value
Age/year			0.023	0.981 2
< 60	24	1.94±0.57		
⩾60	16	1.94±0.47		
Gender			0.742	0.462 6
Male	31	1.97±0.52		
Female	9	1.82±0.54		
Tumor size d/cm			1.098	0.284 5
< 5	27	2.00±0.50		
⩾5	13	1.80±0.56		
Depth of tumor invasion			0.378	0.707 5
T_1-T_2	8	1.88±0.40		
T_3-T_4	32	1.96±0.56		
Lymph node metastasis			5.316	0.009 3
N_0	6	2.33±0.48		
N_1	13	2.13±0.50		
N_2	21	1.71±0.45		
Differentiation			5.807	0.006 4
High	6	2.47±0.39		
Middle	4	2.25±0.50		
Low/undifferentiated	30	1.79±0.47		

2.2　5–Aza–CdR对T–cadherin表达的影响

采用甲基化抑制剂处理细胞后，分别通过RTFQ-PCR和Western blot检测T-cadherin在细胞中的表达变化。结果发现，抑制甲基化后，T-cadherin在转录水平（图3）和翻译水平上（图4）的表达均发生明显上调。

2.3　T–cadherin与HT–29细胞增殖、侵袭和凋亡之间的关系

CCK-8实验表明，与空白对照组相比，药物处理后，HT-29细胞增殖受到明显的抑制（图5）。Transwell实验结果表明，5-Aza-CdR干预组的细胞侵袭能力明显下降（图6）。流式细胞术分析结果发现，5-Aza-CdR处理可以明显提高肿瘤细胞的凋亡，凋亡率可以达到30%（图7）。

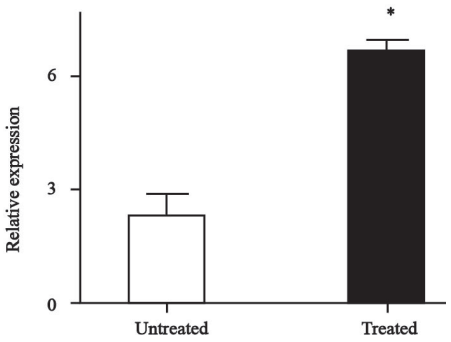

图 3 5–Aza–CdR处理后细胞T–cadherin mRNA表达变化

Fig. 3 Changes of T-cadherin mRNA expression in cells treated with 5-Aza-CdR

After methylation inhibitor treatment, the expression of T-cadherin mRNA in cells was significantly up-regulated.*: P<0.05, compared with each other

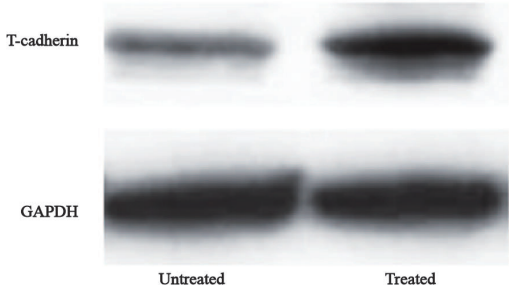

图 4 5–Aza–CdR处理后，细胞T–cadherin 蛋白表达变化甲基化抑制剂处理后，细胞中T–cadherin 蛋白水平明显上调

Fig. 4 Change of T-cadherin protein expression in cells treated with 5-Aza-CdR

After methylation inhibitor treatment, the expression of T-cadherin protein in cells was significantly up-regulated

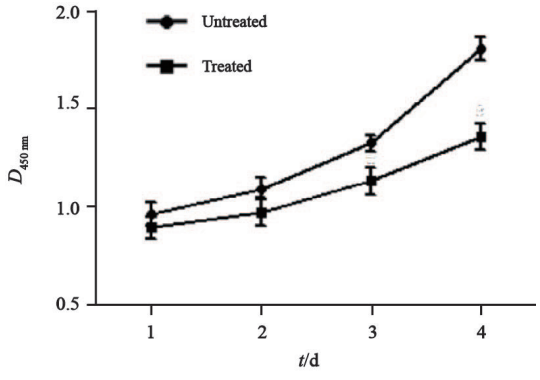

图 5 CCK–8分析5–Aza–CdR对HT–29细胞增殖的影响

Fig. 5 CCK-8 assay the effect of methylation inhibitor on the proliferation of HT-29 cells

After 5-Aza-CdR treatment, the ability of cell proliferation was significantly inhibited.*: P<0.05, compared with each other

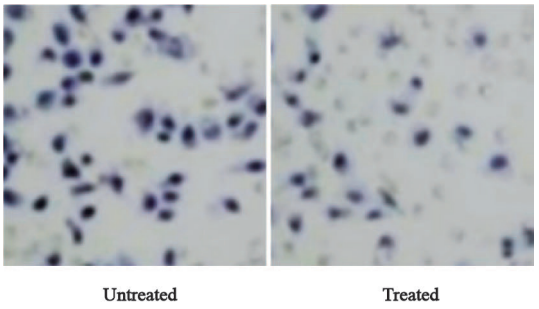

图 6 Tranwell分析5–Aza–CdR对HT–29细胞侵袭的影响

Fig. 6 Tranwell assay showed the effect of 5-Aza-CdR on the invasion of HT-29 cells

After 5-Aza-CdR treatment, the ability of cell invasion was significantly inhibited

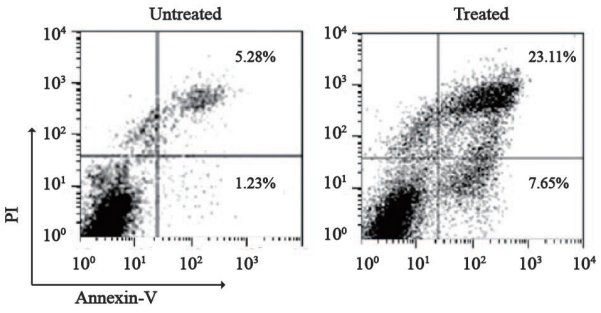

图 7 流式细胞术分析5–Aza–CdR对HT–29细胞凋亡的影响

Fig. 7 Flow cytometry was used to analyze the effect of 5-Aza-CdR on the apoptosis of HT-29 cells

5-Aza-CdR could significantly inhibit cell apoptosis

3 讨 论

结肠癌是常见恶性肿瘤之一，死亡率居所有癌症第五位[1]，揭示其内在的发生、发展机制对于结肠癌患者的治疗意义重大。近年来基因组学的快速发展，已经逐渐成为癌症研究的一个重要领域。因此发现结肠癌新突变基因，将有助于机制的探讨及治疗的改变。

钙黏蛋白是细胞表面跨膜蛋白的一个超家族，传统上分为经典钙黏蛋白和非经典钙黏蛋白[10]。早有研究报道了钙黏蛋白在肿瘤中的作用，如E-cadherin，是肿瘤中潜在的抑制因子[11]。然而，某些钙黏蛋白也被认为是促癌蛋白，如P-cadherin[12]和N-cadherin[13]。T-cadherin是钙黏蛋白超家族的一个非典型成

员，是目前已知的唯一一种通过GPI锚定而非跨膜结构域膜锚定的钙黏蛋白[14]。人类T-cadherin的基因在几种类型的癌症中均发生沉默，长期以来被认为具有抗癌作用[15]。已有研究表明，T-cadherin可能抑制肿瘤的发展，包括肿瘤细胞增殖、侵袭和微血管生成，这一过程涉及多个信号转导通路，包括Akt和SET7/9-p53信号通路[16-17]。但T-cadherin在结肠癌发病机制中的作用研究尚少，因此，揭示T-cadherin在结肠癌中的作用具有重要意义。

钙黏蛋白已被鉴定为多种癌症中的肿瘤抑制基因。Wang等[18]研究发现来自前列腺癌转移的细胞系DU145中T-cadherin的外源性表达降低了致瘤性，而来自良性前列腺增生的BPH1中T-cadherin转录的敲低促进了肿瘤发生；Wei等[19]研究发现下调T-cadherin的表达可能有助于胃癌进展；Kong等[5]研究发现T-cadherin低表达的腋窝淋巴结阳性乳腺癌患者的预后较差。在本研究中，我们对40例结肠癌样本进行了分析，发现T-cadherin在结肠癌组织中的表达显著低于癌旁正常组织，其表达水平与淋巴结转移和分化程度明显相关，提示T-cadherin异常表达在结肠癌的发生、发展中可能发挥抑癌作用。

T-cadherin的表达变化也与*T-cadherin*基因启动子区域的甲基化水平有关。DNA甲基化是真核生物关键的表观遗传修饰之一，参与了microRNAs表达的调控[20]和基因选择性剪接[21]。异常DNA甲基化常使T-cadherin在癌症中呈现低表达，并且促进肿瘤的发生、发展，如肺癌[22]、膀胱癌[23]、鼻窦癌[24]、食管癌[25]。异常DNA甲基化在结直肠癌的发生过程中起着重要的作用[26]。最近一项Meta分析结果表明，*T*-cad*herin*基因甲基化在结肠癌的发生、发展中起重要作用[27]。5-Aza-CdR可在体内外重新上调因高甲基化状态而沉默的抑癌基因，导致细胞分化和凋亡，从而抑制肿瘤进展[28]。本研究采用甲基化抑制剂5-Aza-CdR成功上调T-cadherin的表达，通过细胞生物学行为观察发现5-Aza-CdR药物处理与结肠癌细胞的增殖和侵袭的抑制及诱导凋亡密切相关，提示5-Aza-CdR可通过抑制T-cadherin甲基化逆转T-cadherin表达下调，并抑制结肠癌细胞的增殖、侵袭和抗凋亡。相关研究已报道了T-cadherin水平上调对癌细胞行为的影响，如T-cadherin的重新表达减少了人类前列腺癌[18]、黑色素瘤[29]、乳腺癌[30]和肝细胞癌细胞系[31]的恶性特性等。但T-cadherin去甲基化影响结肠癌发展的作用机制仍需进一步研究。

本研究结果证实，T-cadherin在结肠癌组织中呈低表达，甲基化抑制剂处理可上调T-cadherin表达，并且抑制肿瘤细胞增殖和侵袭能力，促进细胞凋亡。本研究结果提示，T-cadherin在结肠癌中发挥抑癌基因作用，并且可能是结肠癌患者使用DNA甲基化抑制剂进行治疗的靶点。

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