具核梭杆菌通过调节肠道代谢产物丁酸钠上调Cdk1促进结直肠癌发展

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摘要 该文探讨了肠道微生物具核梭杆菌(*Fusobacterium nucleatum*, Fn)通过调节代谢产物丁酸钠(NaB)对结直肠癌(CRC)发生发展的影响及其分子机制。提取临床组织RNA和蛋白, RT-qPCR和Western blot检测肿瘤组织与正常/癌旁组织Cdk1的mRNA及蛋白表达, 同时检测具核梭杆菌的mRNA的相对水平, 并分析其与Cdk1的相关性。具核梭杆菌处理结直肠癌细胞24 h后检测周期相关蛋白Cdk1和P21的表达水平, 核磁共振检测培养基上清差异代谢物。不同浓度差异代谢物NaB处理结直肠癌细胞DLD-1、SW480、HCT116, 使用MTT和克隆形成实验检测DLD-1、SW480、HCT116增殖能力。流式细胞术检测NaB对结直肠癌细胞SW480周期阻滞位点, Western blot检测周期相关蛋白Cdk1、P21、C-myc的表达水平。流式细胞术检测NaB处理后结直肠癌细胞SW480凋亡率变化情况, Western blot检测凋亡相关蛋白Cleaved-Casepase3、Cleaved-PARP、Bcl-2的表达水平。采用MTT实验检测不同MOI具核梭杆菌作用结直肠癌细胞4、8、24 h后对其增殖能力的影响。将结直肠癌细胞与具核梭杆菌共培养24 h, Western blot检测相关蛋白Cdk1、c-myc、Cleaved-Caspase3的表达情况。结果显示, 在肿瘤组织中Cdk1蛋白水平和mRNA水平均明显高于正常/癌旁组织(P<0.05), 并且Cdk1与具核梭杆菌mRNA表达水平存在一定相关性。具核梭杆菌处理结直肠癌细胞DLD-1和HCT116后, Western blot结果显示Cdk1和P21蛋白水平上升。核磁共振结果表明, 菌处理组代谢模式与未处理组存在明显差异, 主要代谢物NaB相对含量明显低于未处理组(P<0.01)。使用1 mmol/L NaB处理结直肠癌细胞系DLD-1、SW480、HCT116 24 h后, 细胞生存率分别为(89.18±1.92)%、(85.07±0.61)%、(83.59±2.18)%, 且随着药物浓度升高, 药物对细胞活性的抑制率逐步上升。同时, 经1 mmol/L NaB处理后, DLD-1、HCT116和SW480细胞的克隆形成率相比于对照分别下降了(20.07±4.85)%、(36.47±5.31)%、(31.13±5.22)%。流式检测细胞周期显示, NaB引起结直肠癌细胞S期阻滞。NaB处理结直肠癌细胞24 h后, 周期相关蛋白P21表达水平上升, Cdk1、C-myc蛋白表达水平下降(P<0.05)。流式检测细胞凋亡显示, NaB引起结直肠癌细胞SW480凋亡增加。NaB处理结直肠癌细胞24 h后, 凋亡相关蛋白Cleaved-Casepase3、Cleaved-PARP表达水平上升, 抗凋亡蛋白Bcl-2表达水平下降。MOI=50的具核梭杆菌分别处理结直肠癌细胞SW480、HCT116 4 h后, 细胞增殖率分别增加了(4.45±0.25)%、(2.61±0.75)%; 并且随着具核梭杆菌MOI的增加和处理时间的延长, 结直肠癌细胞的增殖率逐渐上升。将SW480细胞与HCT116细胞与具核梭杆菌共培养24 h, Western blot结果显示, 具核梭杆菌感染促进了周期相关蛋白Cdk1、C-myc的表达, 而其与NaB共同处理

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时则大大减弱了这一作用; NaB诱导Caspase3的剪切, 导致Cleaved-Caspase3表达增加, 而具核梭杆菌感染则减弱了这一作用。综上所述, 肠道微生物具核梭杆菌通过调节肠道代谢物NaB上调Cdk1, 促进结直肠癌细胞增殖, 抑制结直肠癌细胞凋亡, 进而影响结直肠癌的发生发展。

关键词 具核梭杆菌; 结直肠癌; Cdk1; 增殖; 凋亡

Fusobacterium nucleatum Promotes Colorectal Cancer by Up-Regulating Cdk1 through Intestinal Metabolite Sodium Butyrate

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Abstract This study aimed to investigate the effects of intestinal microorganism *Fusobacterium nucleatum* on the occurrence and development of CRC (colorectal cancer) by regulating the metabolite NaB (sodium butyrate) and its molecular mechanism. RNA and protein were extracted from clinical tissue. RT-qPCR and Western blot were used to detect the expression of mRNA and protein of Cdk1 in tumor tissue and normal/paracancerous tissues. At the same time, the relative level of mRNA of *Fusobacterium nucleatum* was detected by RT-qPCR, and the correlation between mRNA and Cdk1 was analyzed. After the colorectal cancer cells were treated with *Fusobacterium nucleatum* for 24 hours, the expression levels of cycle-related proteins Cdk1 and P21 were detected. The differential metabolites in the culture medium were detected by NMR (nuclear magnetic resonance). Different concentrations of metabolite NaB were used to treat colorectal cancer cells DLD-1, SW480 and HCT116. MTT and colony formation test were used to detect the proliferation ability of DLD-1, SW480 and HCT116. The cycle arrest sites of SW480 induced by NaB were detected by flow cytometry, and the cycle-related proteins Cdk1, P21 and C-myc were detected by Western blot. Flow cytometry was used to detect the apoptosis rate of SW480 after NaB treatment, and the apoptosis-related proteins Cleaved-Casepase3, Cleaved-PARP and Bcl-2 were detected by Western blot. MTT assay was used to detect the effect of *Fusobacterium nucleatum* with different MOI on the proliferation of colorectal cancer cells after 4, 8, 24 h. Colorectal cancer cells were co-cultured *Fusobacterium nucleatum* for 24 h and the related protein Cdk1, c-myc, Cleaved-Caspase3 was detected by Western blot. The results showed that the levels of Cdk1 protein and mRNA in tumor tissues were significantly higher than those in normal/paracancerous tissues, and there was a certain correlation between Cdk1 and mRNA expression of *Fusobacterium nucleatum* ($P<0.05$). The results of Western blot showed that the levels of Cdk1 and P21 protein increased after treatment of colorectal cancer cells DLD-1 and HCT116 with *Fusobacterium nucleatum*. The results of NMR showed that there was a significant difference in metabolic pattern between the *Fusobacterium nucleatum* treated group and the untreated group, and the relative content of metabolite NaB was significantly lower than that in the untreated group ($P<0.01$). The cell viability of colorectal cancer cell lines DLD-1, SW480 and HCT116 treated with 1 mmol/L NaB for 24 h were (89.18±1.92)%, (85.07±0.61)% and (83.59±2.18)%, respectively. The inhibition rate of NaB on cell activity increased gradually with the increase of NaB concentration. At the same time, after 1 mmol/L NaB treatment, the clone formation rate of DLD-1, HCT116 and SW480 cells decreased by (20.07±4.85)%, (36.47±5.31)% and (31.13±5.22)%, respectively. Flow cytometry showed that NaB caused S-phase arrest of colorectal cancer cells. After colorectal cancer cells were treated with NaB for 24 h, the expression of cycle-related protein P21 increased, while the expression of

Cdk1 and C-myc decreased. Flow cytometry showed that NaB increased apoptosis of colorectal cancer cell SW480. After colorectal cancer cells were treated with NaB for 24 h, the expression of apoptosis-related proteins Cleaved-Casepase3 and Cleaved-PARP increased, while the expression of anti-apoptosis protein Bcl-2 decreased (P<0.05). After SW480, HCT116 cells were treated with *Fusobacterium nucleatum* (MOI=50) for 4 h, the proliferation rate of colorectal cancer cells increased by (4.45±0.25)% and (2.61±0.75)%, respectively. With the increase of the MOI and the prolongation of infection time, the proliferation rate of colorectal cancer cells increased gradually (P<0.05). SW480 and HCT116 were cultured with or without *Fusobacterium nucleatum* for 24 h. Western blot results showed that *Fusobacterium nucleatum* infection promoted the expression of cycle-related protein Cdk1, C-myc, which was greatly weakened by its co-treatment with NaB; NaB induced Caspase3 cleavage, resulting in an increase in Cleaved-Caspase3 expression, while *Fusobacterium nucleatum* infection weakened this effect. In conclusion, intestinal microorganism *Fusobacterium nucleatum* up-regulates Cdk1 by regulating intestinal metabolite NaB to promote the proliferation of colorectal cancer cells and inhibit the apoptosis of colorectal cancer cells, thus affecting the occurrence and development of colorectal cancer.

Keywords *Fusobacterium nucleatum*; colorectal cancer; Cdk1; proliferation; apoptosis

根据国际癌症研究机构(International Agency for Research on Cancer)对2018年GLOBOCAN癌症发病率和死亡率统计, 结直肠癌(colorectal cancer, CRC)是全球第三种最常见的癌症, 在癌症相关死亡率方面排名第二[1]。结直肠癌患病风险不仅与吸烟、肥胖、不健康的饮食习惯以及缺乏体育锻炼有关[2]还与家族史、多发性遗传性癌症综合征和某些内科疾病(如炎症性肠病)有关[3-6]。最近的筛查计划报告了CRC的发病率和死亡率都有所下降, 新的治疗方法使晚期CRC患者的总体生存率(overall survival, OS)翻了一番[7]。然而, CRC患者的生存率仍然很低, 主要原因是患者通常是在晚期被诊断出来的[8]。因此, 探究结直肠癌发生发展的机制并确定新的治疗靶点尤为重要。

肠道微生物菌群, 特别是具梭核杆菌(*Fusobacterium nucleatum*, Fn), 已被报道在结直肠癌的发生发展和患者预后中起作用。本实验室已初步证明, 具梭核杆菌在肿瘤组织中含量增加并能促进结直肠癌细胞的迁移[9]。已有证据表明, 具核梭杆菌通过创造适宜肿瘤生长的环境促进结直肠癌的形成与发展[10-12]。利用代谢组学对肠道代谢物进行检测能对微生物多样性分析数据进行补充, 从而进一步揭示肠道微生物与疾病之间的相互关系。短链脂肪酸(short-chain fatty acid, SCFA; 包括丁酸盐、丙酸盐和乙酸盐)是通过膳食纤维发酵从肠道菌群衍生的代谢产物。近年来, 越来越多的证据表明, 丁酸钠(sodium butyrate, NaB)在抑制结直肠癌发展进程中起到了重要的作用[12]。

细胞周期蛋白依赖性激酶1(cyclin dependent kinase 1, Cdk1)属于小丝氨酸/苏氨酸周期素依赖性蛋白激酶家族成员, 是一种高度保守的蛋白, 并且在细胞周期调控中起关键作用, 对于真核细胞周期的G_1/S和G_2/M相变至关重要。最近研究证明, Cdk1与多种癌症例如胃癌、肝癌等[13-14]相关。LI等[15]已发现, Cdk1过表达与结直肠癌患者预后不良有关。 但关于Cdk1促进结直肠癌发生发展的机制还有待进一步研究。本研究利用Western blot、细胞增殖实验(MTT比色法)、克隆形成实验、流式细胞术及实时定量基因扩增荧光检测技术尝试阐明具核梭杆菌通过短链脂肪酸丁酸钠调控结直肠癌发生发展的机制。

1 材料与方法

1.1 材料

1.1.1 细胞株 人结肠癌SW480、DLD-1、HCT116细胞株购于中国科学院典型培养物保藏委员会细胞库。

1.1.2 细菌 具核梭杆菌标准菌株25586购于上海复祥生物有限公司。

1.1.3 主要试剂及仪器 本实验所用所有抗体均购于Abcam公司; 培养细胞用血清购于Gibco公司; 培养细菌用固体培养基购于江苏海博生物科技有限公司; 流式凋亡检测试剂盒购于BD公司; 流式周期检测试剂盒购于北京四正柏生物科技有限公司;

Centrifuge 5417R台式高速冷冻离心机购自德国Eppendorf公司; Varioskan flash全波长酶标仪购自美国Thermo scientific公司; 凝胶成像系统购自美国Bio-Rad公司。

1.2 实验方法

1.2.1 细胞培养 将SW480、DLD-1细胞培养于含10%胎牛血清的DMEM培养基, HCT116培养于含10%胎牛血清的McCoys-5A培养基, 再将其置于5% CO_2、37 ℃的条件下培养, 当细胞融合达90%时即可传代。

1.2.2 核磁共振检测代谢物 具核梭杆菌(MOI=100)处理结直肠癌细胞DLD-1, 24 h后取1 mL培养基至15 mL离心管中, 加入冰甲醇、氯仿涡旋混匀, 再加入冰氯仿1 mL, 涡旋混匀, 置于冰上静置15 min; 8 000 r/min离心20 min后取上清液。冻干后将冻干粉末重新溶解于含0.1 mmol/L TSP的重水(D_2O)中, 12 000 r/min离心10 min, 将上清液转移至核磁管中进行核磁共振(nuclear magnetic resonance, NMR)检测。

1.2.3 Western blot检测蛋白表达水平 提取细胞或组织蛋白, 采用BCA法进行定量, 经SDS-PAGE, 再转移至PVDF膜, TBST封闭液室温下封闭2 h, 加一抗(工作浓度为1:1 000) 4 ℃ 孵育过夜, 二抗(工作浓度为1:1 000)室温孵育30 min, ECL显影, Bio-Rad凝胶成像系统摄像, 再用ImageJ软件处理系统分析。

1.2.4 RT-qPCR检测mRNA表达水平 采用Trizol法提组织或细胞总RNA后将RNA逆转录为cDNA, 配制PCR反应液于8连管, 加入cDNA后把各排8连管放在掌上离心机上离心数秒后进行RT-qPCR反应, 设置反应程序为: 95 ℃ 30 s; 95 ℃ 5 s, 60 ℃ 60 s, 循环40次; 熔解曲线为: 95 ℃ 15 s , 60 ℃ 15 s , 95 ℃ 15 s。用GraphPad Prism软件处理结果进行分析。

1.2.5 MTT检测细胞增殖能力 选取处于对数生长期的肿瘤细胞, 经过消化吹打, 配制成单细胞悬液。计数后以8 000个/孔接种到96孔板中于5% CO_2、37 ℃培养箱内培养过夜。吸去旧培养基, 重新加入培养基并且以浓度为0、1、2、5 mmol/L丁酸钠处理细胞。在24、48、72 h后, 弃掉混合液, 每孔加入10 μL MTT溶液(5 mg/mL)和90 μL培养基, 于37 ℃、5% CO_2培养箱内继续培养4 h。弃上清, 每孔加入100 mL DMSO, 黑暗环境下置于摇床摇至结晶充分溶解, 酶联免疫检测仪检测波长为570 nm处各孔的吸光度(*D*)值, 记录结果。以细胞存活率为纵坐标, 以时间为横坐标作图。

1.2.6 克隆形成检测细胞增殖能力 选取处于对数生长期的肿瘤细胞, 经过消化吹打, 配制成单细胞悬液。以800个/孔密度接种到6孔板中, 置于37 ℃、5% CO_2培养箱中培养10天。 当观察到肉眼可见的细胞克隆后, 弃去培养液, 多聚甲醛固定30 min, 弃固定液, 适量的结晶紫染色20 min。流水冲洗, 空气中干燥。肉眼计算细胞克隆数, 计算克隆形成率。克隆形成率(%)＝形成克隆数/接种细胞数×100%。实验重复3次求平均值。

1.2.7 流式细胞术检测细胞凋亡率 将结直肠癌细胞接种于6孔板中, 每孔接种1×10^5个细胞, 待细胞贴壁后用不同浓度丁酸钠处理24 h后, 不含EDTA胰酶消化收集各组细胞, 加入500 mL Binding Buffer重悬细胞, 随后加入AnnexinV-PE和7-AAD染液各5 μL, 避光室温孵育15 min, 用流式细胞仪检测, 结果采用FlowJo进行分析。

1.2.8 流式细胞术检测细胞周期 将结直肠癌细胞接种于6孔板中, 每孔接种1×10^5个细胞, 待细胞贴壁后用不同浓度丁酸钠处理24 h后, 胰酶消化收集各组细胞, 冰浴的PBS重悬细胞2次。乙醇固定1~24 h, 离心沉淀细胞, PBS重悬细胞后弃上清, PI染色30 min, 用流式细胞仪检测细胞周期。采用CytExpert软件进行细胞DNA含量分析和光散射分析。

1.2.9 统计方法 采用SPSS 19.0软件进行统计学分析。正态分布的连续变量以均值±标准差($\bar{x}\pm s$)表示, 两组之间比较采用*t*检验; 非正态分布的连续变量以中位数(四分位数)表示, 两组之间比较采用非参数检验。计数资料以百分率(%)表示, 两组之间比较采用χ^2检验。$P<0.05$表示差异有统计学意义。

2 结果

2.1 结直肠癌组织与正常/癌旁组织Cdk1 mRNA及蛋白表达情况

本实验所用40例临床样本收集自温州医科大学结直肠癌临床研究中心, 病人信息如表1所示。提取组织RNA进行RT-qPCR, 检测*Cdk1*在不同结直肠组织中mRNA的表达水平, 肿瘤组织mRNA表达水平显著上升(图1A); 同时, 通过Western blot方法检测Cdk1蛋白表达水平, 并用ImageJ软件分析Cdk1蛋白表达情况。如图1B和图1C所示: 在肿瘤组织中, Cdk1蛋白水平明显高于正常/癌旁组织。说明Cdk1

在结直肠癌发生、发展过程中具有一定的作用。

2.2 具核梭杆菌(ATCC25586)促进结直肠癌细胞Cdk1的表达并抑制丁酸钠的代谢

从临床收集新鲜组织样本, 提取组织RNA进行RT-qPCR, 检测*Cdk1*以及具核梭杆菌在不同结直肠组织中mRNA的表达水平。如图2A所示, 具核梭杆菌相对表达量与*Cdk1* mRNA表达量之间具有一定相关性。

具核梭杆菌处理结直肠癌细胞24 h后提取蛋白, Western blot结果显示, 经具核梭杆菌处理后Cdk1蛋白表达量上升, 细胞周期蛋白依赖性激酶抑制剂P21蛋白表达量下降(图2B)。具核梭杆菌处理DLD-1结直肠癌细胞24 h后, 收集培养基上清, 冻干后核磁共振分析培养基代谢产物差异。PCA模式分析图显示, 菌处理组培养基与对照组培养基代谢物在PC1方向上有明显的区分, 表明两者的代谢模式具有明显差异(图2C)。丁酸作为肠道微生物的重要代谢产物, 已有大量体外和体内研究表明, 丁酸在调节免疫和炎症反应及肠屏障功能中起着重要作用。核磁共振结果表明, 经具核梭杆菌处理结直肠癌细胞后, 丁酸盐胞外浓度明显降低, 同时我们也发现乳酸盐及醋酸盐胞外相对含量相较于对照组明显上升(图2D)。

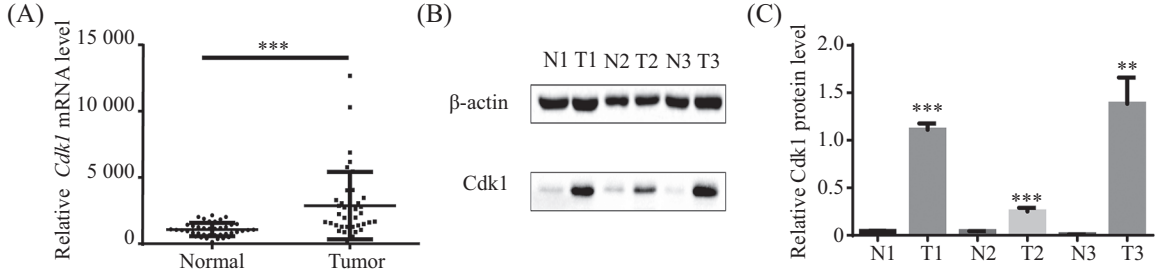
2.3 不同浓度丁酸钠对结直肠癌细胞增殖与克隆形成能力的影响

使用不同浓度NaB(0、1、2、5 mmol/L)分别处理结直肠癌细胞DLD-1、HCT116和SW480, 采用MTT增殖实验检测NaB作用结直肠癌细胞24、48、72 h后对其增殖能力的影响。如图3A~图3C所示: 浓度为1 mmol/L的NaB处理DLD-1、HCT116、SW480细胞24 h后, 细胞生存率分别为(89.18±1.92)%、(85.07±0.61)%、(83.59±2.18)%; 而且随着药物浓度升高, 药物对细胞活性的抑制率逐步上升。不

表1 40例结直肠组织临床样本信息

Table 1 Clinical sample information of 40 cases of colorectal tissue

临床特征 Clinical features	类型 Types	病例数 Number of cases
Age	≥60	27
	<60	13
Gender	Male	27
	Femal	13
Tumor size	≥4 cm	25
	<4 cm	15
TNM stage	I	10
	II	14
	III	12
	IV	4

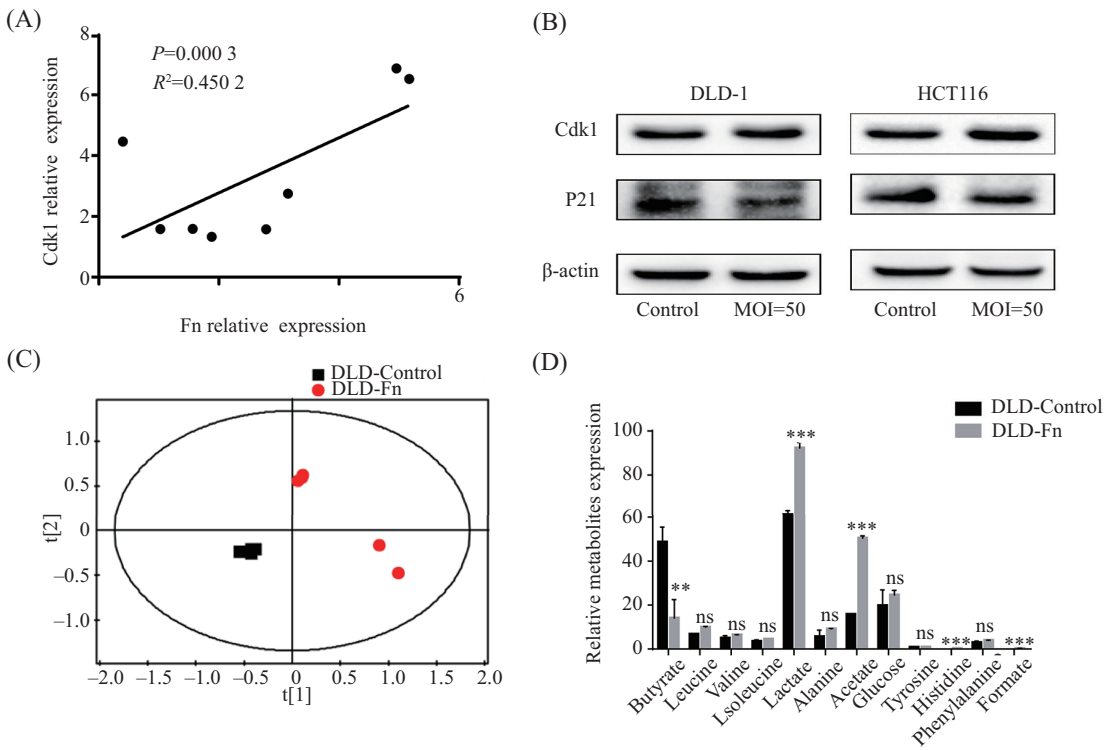


A: RT-qPCR检测结果显示, *Cdk1* mRNA表达量在肿瘤组织标本中明显高于正常/癌旁组织; B、C: Western blot检测结果显示, Cdk1蛋白表达量在肿瘤组织标本中明显高于正常/癌旁组织。N1~N3: 正常/癌旁组织; T1~T3: 结直肠癌肿瘤组织。**P<0.01, ***P<0.001, 与正常/癌旁组相比。

A: the results of RT-qPCR showed that the expression of *Cdk1* mRNA in tumor tissues was significantly higher than that in normal/paracancerous tissues; B,C: the results of Western blot showed that the expression of Cdk1 protein in tumor tissues was significantly higher than that in normal/paracancerous tissues. N1~N3: normal/paracancerous tissues; T1~T3: colorectal cancer tissues. **P<0.01, ***P<0.001 *vs* the normal/paracancerous group.

图1 结直肠癌组织与正常/癌旁组织Cdk1蛋白及mRNA表达情况

Fig.1 Expression of Cdk1 protein and mRNA in colorectal cancer tissues and normal/paracancerous tissues



A: 结直肠癌组织中Cdk1与具核梭杆菌相关性; B: 具核梭杆菌处理结直肠癌细胞后Cdk1、P21蛋白表达情况; C: PCA模式分析图显示具核梭杆菌处理DLD-1细胞24 h后, 胞外培养基代谢模式改变; D: 具核梭杆菌处理DLD-1后, 培养基内代谢物发生明显变化, 丁酸盐含量显著下降。Control: 对照组; MOI=50: 实验组(具核梭杆菌:细胞=50:1); DLD-Control: DLD-1细胞未处理组; DLD-Fn: DLD-1细胞具核梭杆菌处理组。ns: P>0.05, **P<0.01, ***P<0.001, 与对照组相比。

A: correlation between Cdk1 and *Fusobacterium nucleatum* in colorectal cancer; B: Cdk1 and P21 protein expression of colorectal cancer cells treated with *Fusobacterium nucleatum*; C: PCA pattern analysis showed that the metabolic pattern of extracellular medium changed after DLD-1 cells were treated with *Fusobacterium nucleatum* for 24 hours; D: after DLD-1 was treated with *Fusobacterium nucleatum*, the metabolites in the culture medium changed significantly and the content of butyrate decreased significantly. Control: control group; MOI=50: treated group (*Fusobacterium nucleatum*:cells=50:1); DLD-Control: DLD-1 cells untreated group; DLD-Fn: DLD-1 cells treated with *Fusobacterium nucleatum*. ns: P>0.05,**P<0.01, ***P<0.001 *vs* control group.

图2 具核梭杆菌影响结直肠癌细胞周期相关蛋白表达与代谢产物

Fig.2 Effects of *Fusobacterium nucleatum* on the expression of cycle-related proteins and metabolites in colorectal cancer cells

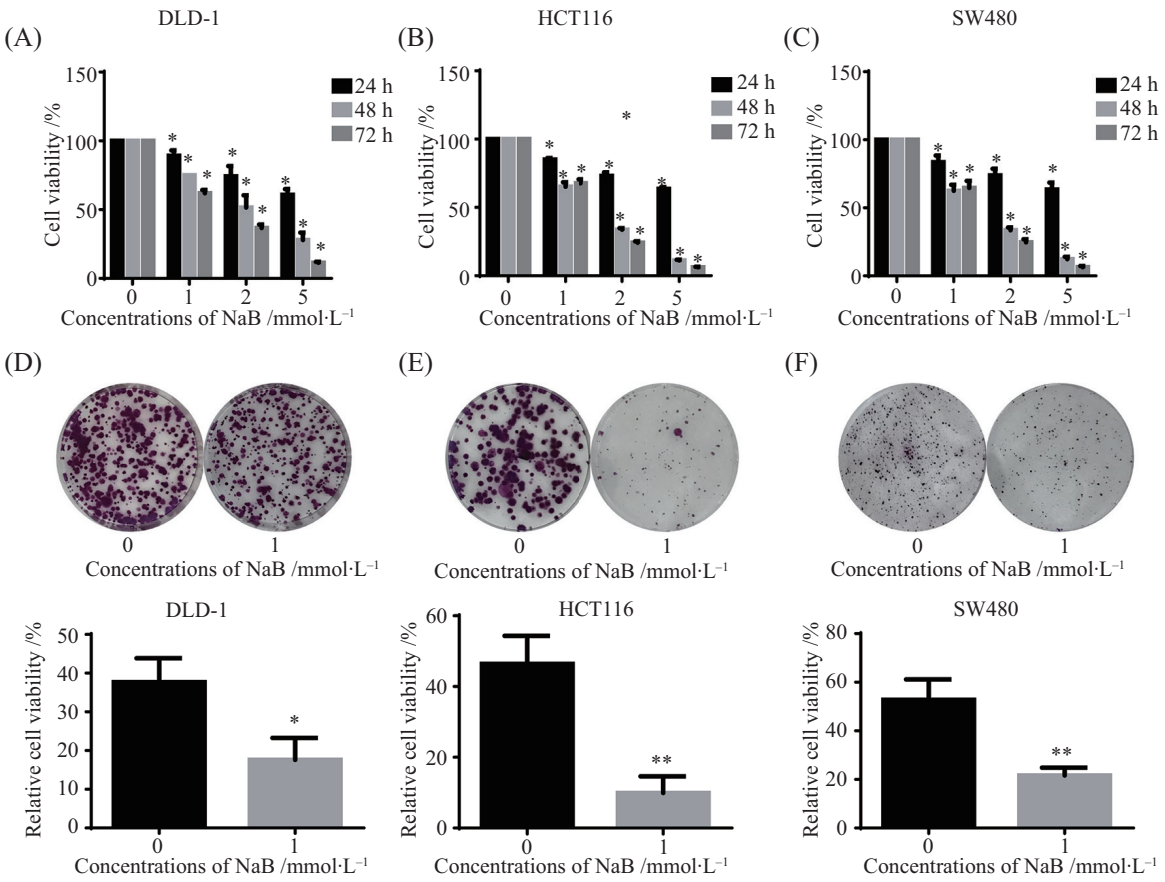
同浓度作用下, 随着丁酸钠作用时间增加, DLD-1、HCT116、SW480细胞生存率显著下降(P<0.05)。这说明丁酸钠对结直肠癌细胞增殖的抑制呈现浓度和时间依赖性。

同时, 我们也采用平板克隆形成实验检测NaB对结直肠癌细胞DLD-1、HCT116、SW480细胞系克隆形成能力的影响。结果如图3D~图3F所示: 经1 mmol/L NaB处理后, DLD-1、HCT116和SW480细胞的克隆形成率相比于对照分别下降了(20.07±4.85)%、(36.47±5.31)%、(31.13±5.22)%。结果说明, NaB可以有效抑制结直肠癌细胞克隆形成, 与细胞增殖实验结果一致。

2.4 丁酸钠通过周期相关蛋白影响结直肠癌细胞周期

用1 mmol/L NaB处理结直肠癌细胞SW480 24 h, 通过流式细胞仪分析SW480细胞周期变化。结果显示: NaB处理24 h后, SW480细胞S期细胞比例比对照上升5.38%。实验结果说明, NaB将结直肠癌细胞SW480阻滞于S期, 并抑制其增殖(图4G~图4I)。

为了进一步分析NaB引起结直肠癌细胞周期阻滞的原因, 我们通过Western blot对肿瘤周期信号通路中的相关因子进了检测。P21也被称为细胞周期蛋白依赖性激酶1抑制剂, 是细胞周期蛋白依赖性激酶抑制剂(cyclin-dependent kinase inhibitor, CKI), 其能够抑制所有细胞周期蛋白/CDK复合物。用不同浓度NaB(0、0.5、1、2、5 mmol/L)处理结直肠癌细胞DLD-1、HCT116、SW480, 24 h后检测P21、Cdk1、C-myc蛋白表达情况。如图4A~图4F所示: NaB处理显著提高了结直肠癌细胞P21蛋白的表达水平, 并且随着NaB浓度升高, P21蛋白表达水平上升, 差异有统



A~C: MTT实验结果显示, NaB抑制结直肠癌细胞增殖; D~F: 克隆形成实验结果显示, NaB抑制结直肠癌细胞克隆形成能力。*P<0.05, **P<0.01, 与NaB未处理组相比。

A-C: the results of MTT assay showed that NaB inhibited the proliferation of colorectal cancer cells; D-F: the results of colony formation assay showed that NaB could inhibit the colony formation of colorectal cancer cells. *P<0.05, **P<0.01 *vs* NaB untreated group.

图3 不同浓度NaB对结直肠癌细胞增殖与克隆形成能力的影响

Fig.3 Effects of different concentrations of NaB on proliferation and colony formation of colorectal cancer cells

计学意义(P<0.05)。同时, 结果也表明, NaB明显抑制了细胞Cdk1、C-myc的蛋白表达水平, 并且随着NaB浓度升高, Cdk1、C-myc蛋白表达水平下降, 差异具有统计学意义(P<0.05)。结果说明: 丁酸钠可通过促进P21蛋白表达, 进而抑制Cdk1和C-myc的表达, 从而阻滞了结直肠癌细胞周期的进行。

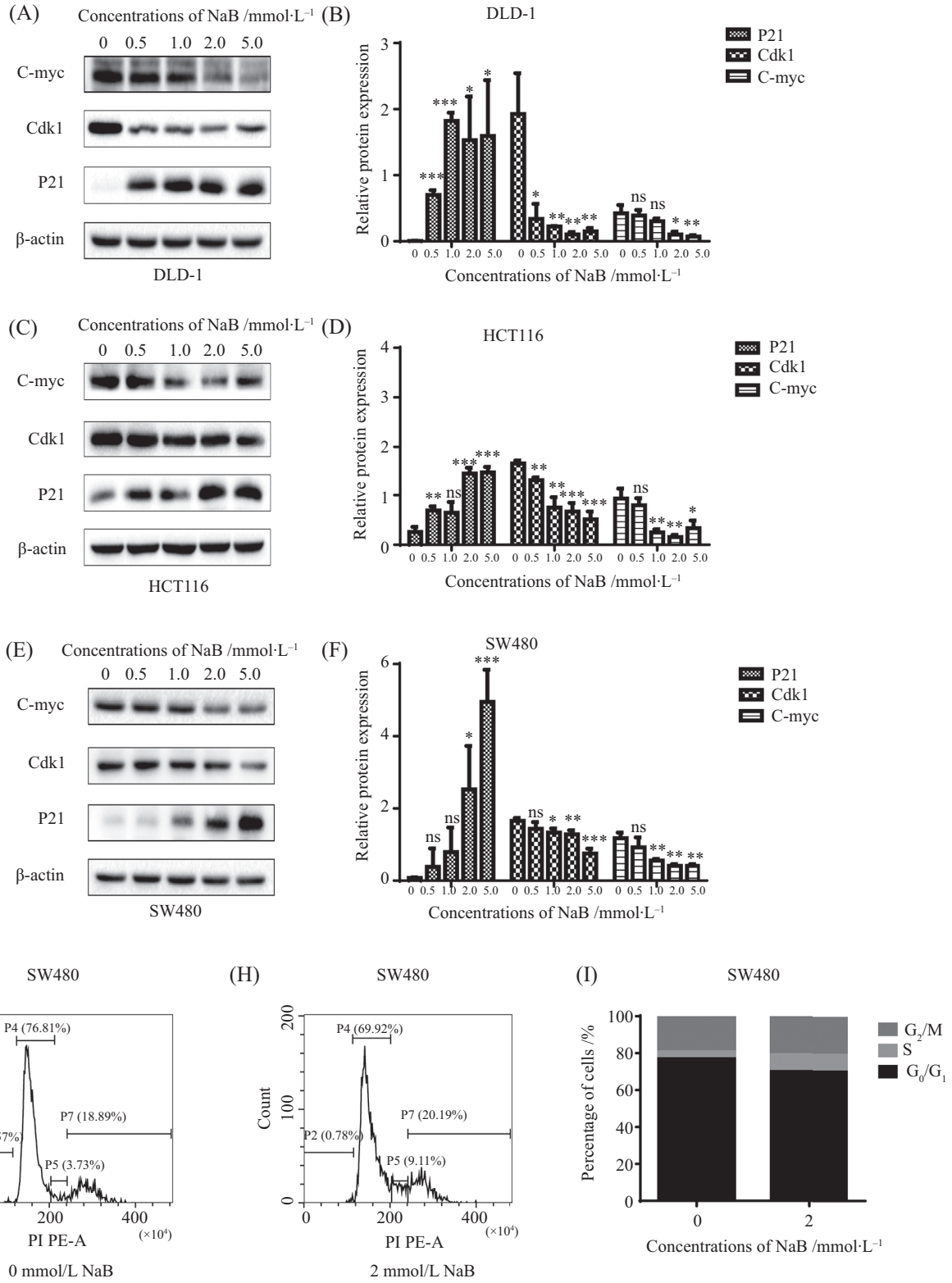
2.5 丁酸钠通过凋亡相关蛋白影响结直肠癌细胞凋亡

用不同浓度NaB处理结直肠癌细胞SW480 24 h, 通过流式细胞仪检测细胞凋亡情况。结果显示: 随着丁酸钠浓度的升高, 结直肠癌细胞凋亡率逐渐升高, 2、5 mmol/L NaB处理24 h后, 凋亡率分别为5.26%和13.28%(图5G)。结果表明: 随着NaB浓度增加, 结直肠癌细胞SW480凋亡率增加, 证明结直肠癌细胞凋亡对NaB浓度具有浓度依赖性。

为了进一步分析NaB促进结直肠癌细胞凋亡的分子机理, 我们用不同浓度NaB分别处理结直肠癌细胞DLD-1、HCT116和SW480, 24 h后Western blot检测凋亡相关蛋白Cleaved-Casepase3、Cleaved-PARP、Bcl-2等表达情况。如图5A~图5F所示: 随着NaB浓度升高, Cleaved-Casepase3、Cleaved-PARP蛋白表达水平明显增加升高, 而抗凋亡蛋白Bcl-2表达水平显著下降。实验结果说明: NaB能通过Casepase3的活化有效促进结直肠癌细胞的凋亡, 并且这种促凋亡作用呈现明显的浓度依赖性。

2.6 具核梭杆菌通过调节肠道代谢产物丁酸盐促进结直肠癌发展

使用不同MOI(=50、100)具核梭杆菌分别处理结直肠癌细胞SW480和HCT116, 采用MTT增殖实验检测具核梭杆菌作用结直肠癌细胞4、8、24 h后对其增殖能力的影响。如图6A和图6B所示: 使用MOI=50的具核梭杆菌处理细胞SW480、HCT116

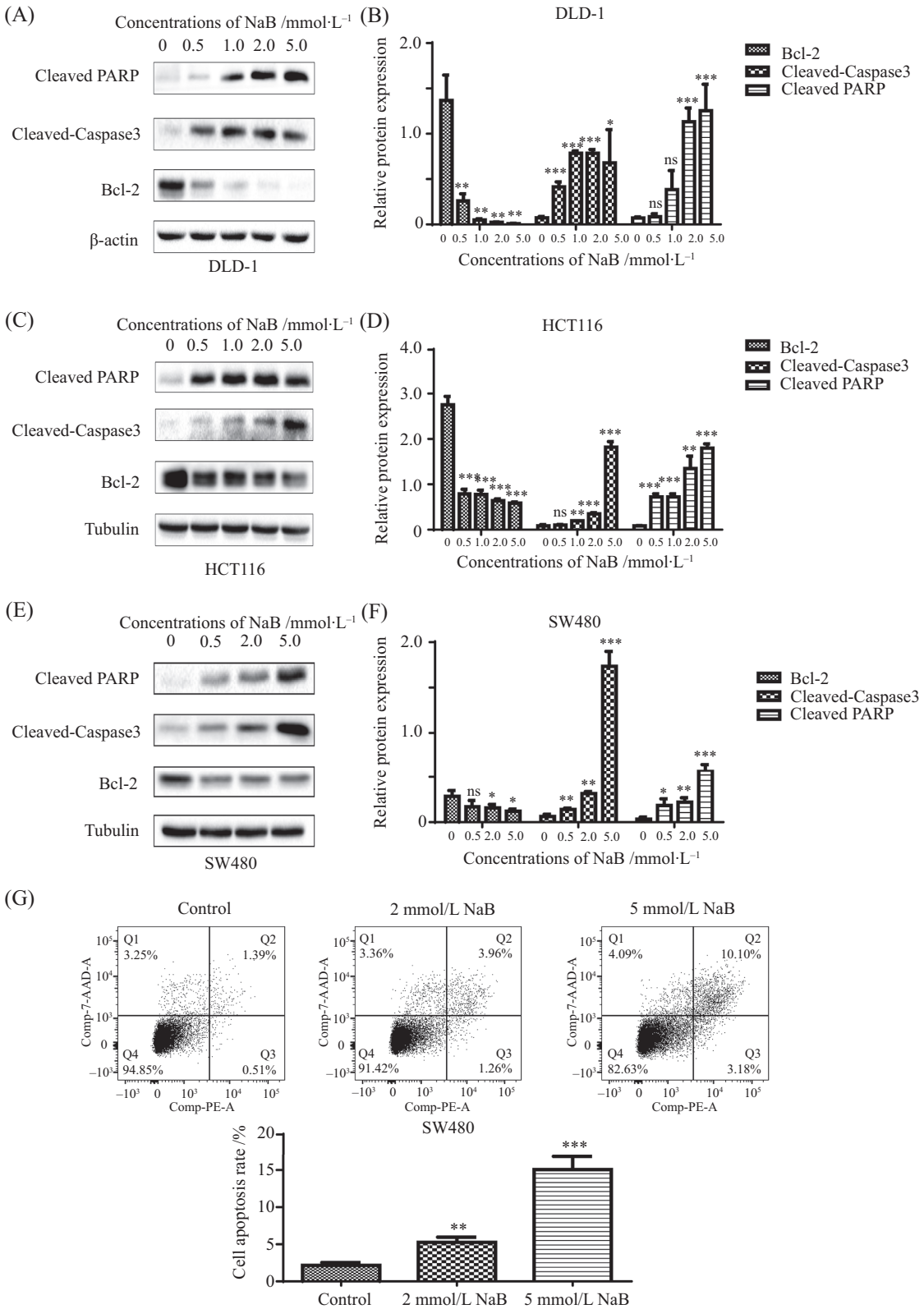


A~F: Western blot结果显示, NaB处理结直肠癌细胞24 h后, 周期相关蛋白P21表达水平上升, Cdk1、C-myc蛋白表达水平下降; G~I: 流式检测细胞周期显示, NaB引起结直肠癌细胞S期阻滞, 处理组S期比例上升。ns: P>0.05, *P<0.05, **P<0.01, ***P<0.001, 与NaB未处理组相比。

A-F: the results of Western blot showed that the expression of cycle-associated protein P21 increased and the expression of Cdk1 and C-myc decreased after 24 hours of NaB treatment in colorectal cancer cells; G-I: flow cytometry showed that NaB induced S-phase arrest of colorectal cancer cells, and the proportion of S-phase increased in the treatment group. ns: P>0.05, *P<0.05, **P<0.01, ***P<0.001 *vs* NaB untreated group.

图4 NaB通过周期相关蛋白影响结直肠癌细胞周期

Fig.4 NaB affects the cell cycle of colorectal cancer cells through cycle-related proteins

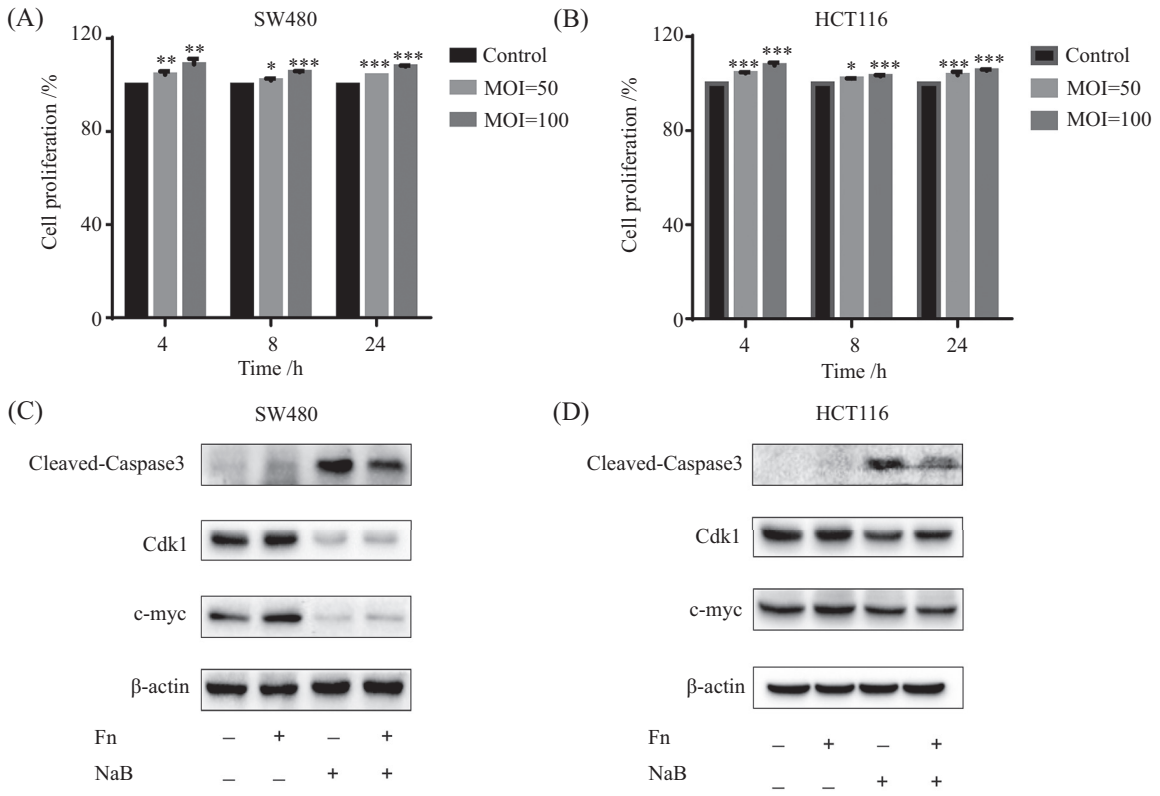


A~F: WB结果显示, NaB处理结直肠癌细胞24 h后, 凋亡相关蛋白Cleaved-Casepase3、Cleaved-PARP表达水平上升, 抗凋亡蛋白Bcl-2表达水平下降; G: 流式检测细胞凋亡显示, NaB引起结直肠癌细胞凋亡增加。ns: $P>0.05$, *$P<0.05$, **$P<0.01$, ***$P<0.001$, 与NaB未处理组相比。

A-F: the results of WB showed that the expression of apoptosis-related protein Cleaved-Casepase3, Cleaved-PARP increased and the expression of anti-apoptosis protein Bcl-2 decreased after colorectal cancer cells were treated with NaB for 24 hours; G: flow cytometry showed that NaB induced apoptosis in colorectal cancer cells. ns: $P>0.05$, *$P<0.05$, **$P<0.01$, ***$P<0.001$ *vs* NaB untreated group.

图5 NaB通过凋亡相关蛋白影响结直肠癌细胞凋亡

Fig.5 NaB affects apoptosis of colorectal cancer cells through apoptosis-related proteins



A、B: MTT结果显示, 具核梭杆菌处理促进结直肠癌细胞增殖; C、D: WB结果显示, 具核梭杆菌感染促进Cdk1、c-myc的表达。*P<0.05, **P<0.01, ***P<0.001, 与NaB未处理组相比。

A,B: the MTT results showed that the treatment with *Fusobacterium nucleatum* promoted the proliferation of colorectal cancer cells; C,D: the results of WB showed that *Fusobacterium nucleatum* infection promoted the expression of Cdk1, c-myc. *P<0.05, **P<0.01, ***P<0.001 *vs* NaB untreated group.

图6 具核梭杆菌通过调节肠道代谢产物丁酸盐促进结直肠癌发展

Fig.6 *Fusobacterium nucleatum* promotes the development of colorectal cancer by regulating intestinal metabolite butyrate

4 h后, 细胞增殖率分别增加了(4.45±0.25)%、(2.61±0.75)%; 并且随着菌量的增加和处理时间的延长, 结直肠癌细胞的增殖率逐渐上升(P均<0.05)。这说明, 具核梭杆菌对结直肠癌细胞的增殖具有明显的促进作用。

在前期核磁检测结果中, 我们发现经具核梭杆菌处理细胞后, 胞外的丁酸盐含量明显下降, 说明具核梭杆菌可能抑制细胞代谢产物丁酸盐的形成。为了进一步探讨具核梭杆菌是否通过调节代谢产物丁酸盐促进结直肠癌的发生发展, 我们将SW480细胞与HCT116细胞与具核梭杆菌共培养24 h。Western blot结果显示, 具核梭杆菌感染促进了周期相关蛋白Cdk1, c-myc的表达, 说明菌处理促进了结直肠癌细胞周期发展, 而其与NaB共同处理时则大大减弱了这一作用(图6和图6D), 这与前期NaB阻滞结直肠癌细胞周期结果相一致; NaB诱导Caspase3的切割, 导致Cleaved-Caspase3表达水平增加; 在此基础上用具核梭杆菌共同处理, 发现具核梭杆菌在一定程度上可以减弱NAB诱导凋亡的能力(图6C和图6D)。这些结果表明, 具核梭杆菌通过抑制结直肠癌细胞代谢产物丁酸钠的形成进而促进结直肠癌发生与发展。

3 讨论

肠道微生态与结直肠癌的发生发展存在密切联系。研究表明, 结直肠癌中健康的肠道菌群可促进肠道稳态, 并能发挥抗癌作用, 但是某些微生物群也会通过代谢产物对上皮细胞产生负面影响。在CRC患者中经常观察到微生物平衡的紊乱。与CRC相关的微生物物种包括脆弱拟杆菌、大肠杆菌、解链球菌、粪肠球菌和核梭杆菌等菌株[16]。

有研究表明, 摄入高纤维饮食降低了CRC的风险[17-19]。纤维的摄入可能与肠道中的糖酵解微生物活性有关, 尤其是与丁酸的原位合成有关。丁酸盐是由未消化的膳食纤维发酵产生的, 具有良好的抗

肿瘤活性, 但其与肠道微生物之间的关系以及抑制结直肠癌发生发展的作用机制尚不完全清楚。本实验通过核磁共振检测具核梭杆菌处理的结直肠癌细胞DLD-1培养基上清, 发现菌处理组代谢模式与对照组有明显差异, 并且这种代谢模式的差异主要由乳酸盐、醋酸盐、丁酸盐导致。值得一提的是, 经具核梭杆菌处理后结直肠癌细胞胞外丁酸盐含量明显低于对照组(图2C和图2D)。说明具核梭杆菌处理结直肠癌细胞后, 细胞代谢产物发生改变, 肿瘤细胞生长环境发生改变。于是我们使用差异代谢产物丁酸钠处理结直肠癌细胞, 随着丁酸钠浓度增加和处理时间延长, 结直肠癌细胞增殖被有效抑制(图3), 且随着浓度升高细胞存活率明显降低, 说明丁酸钠对结直肠癌细胞的细胞活性抑制率呈浓度和时间依赖性。

Cdk1在细胞周期调控中起着关键作用, 与多种癌症相关, 例如在乳腺癌、肝癌中呈过度表达[20-21]。通过对结直肠癌组织进行RT-qPCR, 我们发现具核梭杆菌与Cdk1之间存在一定相关性(图2A)。同时使用具核梭杆菌处理结直肠癌细胞, Cdk1蛋白表达量上升(图2B)证明了两者之间存在一定联系。本文的WB结果表明, Cdk1在结直肠癌组织中含量高于癌旁/正常组织(表1和图1A), 同时RT-qPCR的结果验证了Cdk1在结直肠癌组织中过表达(图1B和图1C), 表明Cdk1可能促进结直肠癌的发生。P21也被称为细胞周期蛋白依赖性激酶1抑制剂, 是CKI, 它能够抑制所有细胞周期蛋白/CDK复合物。也有研究表明, *C-myc*调控基因普遍参与细胞周期的进展和代谢, 包括Cdk、细胞周期蛋白和核糖体RNA[22]。*C-myc*靶基因在多种癌细胞类型中通过直接或间接途径影响多种细胞过程。当直接激活致癌途径时, C-myc影响包括代谢适应、细胞分裂和生存在内的生理过程[23], 已证实丁酸钠通过调控周期相关蛋白抑制结直肠癌进程[24]。 我们通过流式细胞术证明丁酸钠处理结直肠癌细胞SW480 24 h后, 将细胞周期阻滞在S期(图4G~图4I)。同时WB结果也表明, 丁酸钠促进了结直肠癌细胞P21蛋白的表达, 而Cdk1和c-myc的表达水平明显受到抑制(图4A~图4F)。这些结果证明, 丁酸钠可通过P21、c-myc和Cdk1等周期相关蛋白调控结直肠癌周期进程。

丁酸钠作为一种去乙酰化酶抑制剂, 已被证实可以诱导神经胶质瘤、结直肠癌等细胞的凋亡[25-26]。细胞凋亡过程由多种因素(包括细胞应激、DNA损伤和免疫监视)触发的几条信号通路(包括内源性和外源性)介导。凋亡途径与其他信号机制的相互作用也可以影响细胞死亡[27]。 我们通过流式细胞术发现丁酸钠处理结直肠癌细胞后, 结直肠癌的凋亡明显增加, 且凋亡率随着丁酸钠浓度增高而上升。WB实验证明, 凋亡相关蛋白Cleaved-Caspase3、Cleaved-PARP显著上升, Bcl-2蛋白表达明显降低, 表明丁酸钠通过激活Casepase3促进细胞凋亡, 且凋亡率的增加呈浓度依赖性。

在前期核磁共振结果显示, 具核梭杆菌处理结直肠癌细胞后, 胞外丁酸盐含量明显下降, 说明具核梭杆菌可能抑制代谢产物丁酸盐的形成。为进一步探讨具核梭杆菌是否通过调节代谢产物丁酸盐促进结直肠癌的发生发展, 我们将结直肠癌细胞与具核梭杆菌共培养24 h。Western blot结果显示, 具核梭杆菌感染促进了周期相关蛋白Cdk1、c-myc的表达, 说明菌处理促进了结直肠癌细胞周期发展, 而其与NaB共同处理时则大大减弱了这一作用(图6C和图6D), 这与前期NaB阻滞结直肠癌细胞周期结果相一致; NaB诱导Caspase3的剪切, 导致Cleaved-Caspase3表达水平增加; 在此基础上用具核梭杆菌共同处理, 发现菌处理在一定程度减弱了NAB诱导凋亡的能力(图6C和图6D)。这些结果表明, 具核梭杆菌通过抑制肠道代谢产物丁酸钠的形成进而促进结直肠癌发生与发展。综上所述, 具核梭杆菌通过改变肠道内代谢物NaB进而影响结直肠癌的形成与发展。但具核梭杆菌与肠道代谢物之间的联系及其与结直肠癌的发生发展之间的机制还有待进一步探究。

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