桦木酸通过调节 TGF- β /Smad 信号通路抑制结肠癌细胞转移的研究

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摘要 目的:探讨桦木酸(BA)对人结肠癌细胞株 HCT116 生长和转移的影响及其可能的作用机制。**方法:**将 HCT116 培养于含 10% 胎牛血清和含 1% 青霉素/链霉素混合液的 McCoy's 5A 培养基中, 于 37 °C、5% CO₂ 培养箱中常规培养。将 HCT116 细胞以 0.2 × 10⁵ 个/mL 的密度接种于 96 孔板培养过夜后, 分别用不同浓度的 BA(0、10、20 和 40 μmol/L) 干预细胞 24、48、72 h, 然后加入 CCK8 检测其在 450 nm 波长处的吸光值(A 值)。将 HCT116 细胞以 0.4 × 10⁵ 个/mL 的密度接种于 12 孔板培养过夜后, 分别用不同浓度的 BA(0、10、20、40 μmol/L) 干预细胞 48 h, 消化离心收集细胞并用台盼蓝染色, 采用 Countstar BioMed 自动细胞计数仪计算细胞总数。将 HCT116 细胞以 0.4 × 10⁵ 个/mL 的密度接种于 12 孔板培养过夜后, 分别用不同浓度的 BA(0、10、20、40 μmol/L) 干预细胞 48 h, 然后消化离心收集细胞并分别用 1.0 × 10⁵ 个/mL 的细胞悬液进行细胞迁移和侵袭的 Transwell 实验, 检测不同浓度 BA 干预 48 h 后对 HCT116 迁移和侵袭能力的影响。采用 Western blot 法检测 BA 干预 48 h 后对 HCT116 细胞转移相关蛋白 TGF- β 和 Smad2/3 的影响。**结果:**① CCK8 检测结果显示, 与 0 μmol/L 组比较, 经不同浓度的 BA 干预 24、48、72 h 后, 各组细胞的活力均显著降低, 差异均有统计学意义($P < 0.05$); 细胞计数结果显示, 与 0 μmol/L 组比较, 经不同浓度的 BA 干预 48 h 后, 各组的细胞数明显减少, 差异具有统计学意义($P < 0.05$)。② Transwell 检测细胞迁移和侵袭结果显示, 与 0 μmol/L 组比较, 经不同浓度的 BA 干预 48 h 后迁移或侵袭到小室外膜的细胞数目受到不同程度的明显抑制, 差异具有统计学意义($P < 0.05$)。③ Western blot 检测结果显示, 与 0 μmol/L 组比较, 经不同浓度的 BA 干预 48 h 后 TGF- β 和 Smad2/3 蛋白表达水平明显下降。**结论:**桦木酸具有抑制结肠癌细胞生长和转移的作用, 其作用机制可能通过调控 TGF- β /Smad 信号通路来实现。

关键词 结肠癌; 桦木酸; 转移; TGF- β /Smad 信号通路

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据美国癌症协会 2019 年统计数据显示,预计大肠癌(colorectal cancer,CRC)新增病例 145 600 例,死亡病例 51 020 例,其发病率无论在男性还是女性当中均位列前三^[1],严重威胁患者生命,降低患者的生活质量。目前大肠癌采取的主要治疗手段是外科手术和化疗,在治疗过程中的癌细胞转移是导致化疗失败和患者死亡的主要原因之一。因此,抑制大肠癌的转移是降低患者死亡率的关键所在。上皮间质转化(epithelial-mesenchymal transition,EMT)指的是上皮细胞向具有间质细胞表型转化的过程,该过程可使肿瘤细胞获得更高的迁移和侵袭能力^[2-3]。现有的研究已经证实,TGF- β /Smad 通路是介导 EMT 发生的重要通路之一,TGF- β /Smad 通路在肿瘤的转移过程中起着重要的调控作用^[4]。天然药物因其毒副作用小,具有调控多靶点和多通路的特点,在抗肿瘤治疗中的作用与日俱增。目前已有中药联合外科手术、中药联合化疗均可降低肿瘤的转移和复发以及术后不良反应,从而降低患者死亡率和提高生活质量的诸多报道^[5]。桦木酸(betulinic acid,BA)是一种天然的五环三萜类化合物,广泛存在于多种天然中药中。现有的多项研究已经表明,BA 不仅具有抗肿瘤、抗炎、抗血管生成和免疫调节等方面的特性,而且可以通过促进细胞凋亡和抑制细胞 EMT 达到其抗肿瘤作用^[6-7]。为了进一步探讨 BA 对结肠癌转移的作用机制,本研究通过体外培养结肠癌细胞株 HCT116,检测 BA 对其生长和迁移、侵袭的影响,探讨其可能的分子机制,为其开发成治疗结肠癌的潜在天然药物提供进一步的实验依据。

1 材料与方法

1.1 主要试剂

McCoy's 5A 培养液、0.25%胰蛋白酶(Trypsin)和胎牛血清(美国 Thermo Fisher 公司);青霉素、链霉素混合液和磷酸盐缓冲液(PBS)(美国 Hyclone 公司);Smad2/3、TGF- β 和 GAPDH 抗体(美国 CST 公司);BA(美国 SIGMA 公司);CCK8 试剂盒(Abbkine 公司);结晶紫、台盼蓝(北京索莱宝科技有限公司);SDS-PAGE 蛋白上样缓冲液(5 $\times$)(Beyotime 公司)。

1.2 主要仪器

37 $^{\circ}\text{C}$ CO_2 培养箱(美国 Thermo Forma 公司);超净工作台(苏州净化设备公司);Countstar BioMed 自动细胞计数仪(上海艾力特生命科学有限公司);倒置显微镜系统(德国 Leica 仪器有限公司)。

1.3 细胞株

人结肠癌细胞株 HCT116 购自中国科学院细胞

库,培养于含 10%胎牛血清和含 1%双抗(青霉素、链霉素各 100 U/mL)的 McCoy's 5A 培养基中,于 37 $^{\circ}\text{C}$ 、5% CO_2 培养箱中常规培养。

1.4 药物配置

将 BA 溶解于二甲基亚砜(DMSO)溶液中配置成 25 mmol/L 的母液,使用超声波助溶 30 min,溶液呈无色透明状态后进行分装并放置-20 $^{\circ}\text{C}$ 冰箱保存。在细胞加药时用 McCoy's 5A 培养液稀释成工作浓度为 10、20、40 $\mu\text{mol/L}$ 。

1.5 CCK8 法检测细胞活力

人结肠癌细胞株 HCT116 在 T25 培养瓶中培养至对数生长期,以 0.2×10^5 个/mL 细胞密度接种于 96 孔板中,每孔细胞悬液 100 μL ,每个浓度做 6 个复孔,待第 2 天细胞贴壁后分别给予浓度为 0、10、20、40 $\mu\text{mol/L}$ 的 BA 溶液干预。在加药后 24 h、48 h、72 h 加入 CCK8 并检测其在 450 nm 波长处的吸光度(A 值)。

细胞活力 = 各浓度组 A 值 / 0 $\mu\text{mol/L}$ 组 A 值

1.6 细胞计数检测细胞生长能力

人结肠癌细胞株 HCT116 以 0.4×10^5 个/mL 密度接种于 12 孔板中,待第 2 天细胞贴壁后给予浓度为 0、10、20、40 $\mu\text{mol/L}$ 的 BA 溶液干预。药物干预 48 h 后,消化离心收集细胞,以完全培养基重悬混匀细胞,与 0.2%台盼蓝 1:1 混合后加入计数板中,Countstar BioMed 自动细胞计数仪计算细胞总数。

1.7 Transwell 检测细胞转移能力

按照 0.4×10^5 个/mL 细胞密度接种 HCT116 细胞于 12 孔板中,待第 2 天细胞贴壁后给予浓度为 0、10、20、40 $\mu\text{mol/L}$ 的 BA 溶液干预。药物干预 48 h 后,消化离心收集细胞,用空白培养基配置密度为 1.0×10^5 个/mL 的细胞悬液,分别进行细胞迁移和侵袭实验。① 细胞侵袭实验:将重悬后的细胞接种 200 μL 于铺有 30 μL Matrigel 基质胶的 Transwell 板上室,下室中加入 700 μL 的 M5A 完全培养基,48 h 后小心弃去培养基,PBS 缓冲液洗一遍,用含有 PBS 缓冲液的棉签轻轻擦拭小室膜上侧的细胞,在上室和下室中分别加入适量 4%多聚甲醛溶液固定 20 min,结晶紫染色 20 min,PBS 缓冲液洗涤后于倒置显微镜下拍照。② 细胞迁移实验:细胞接种于不含基质胶的小室内,24 h 后用多聚甲醛固定、结晶紫染色后在倒置显微镜下拍照。

1.8 Western blot 检测 TGF- β 和 Smad2/3 的表达

以 0.4×10^5 个/mL 细胞密度接种 HCT116 细胞,给药干预 48 h 后收集细胞,RIPA 裂解液提取总

蛋白,BCA 定量法测定蛋白浓度;各蛋白样品均加入 1/4 体积的 SDS-PAGE 蛋白上样缓冲液(5×),在 100 ℃金属锅中变性 5 min 后,使用 SDS-PAGE 电泳法进行凝胶电泳,每个孔上样量 50 μg,电泳结束后将蛋白转移到 PVDF 膜,用 5%脱脂奶粉封闭 2 h 后加入一抗 Smad2/3(1:1 000)、TGF-β(1:1 000)和 GAPDH(1:5 000)4 ℃孵育过夜。采用辣根过氧化物酶标记的二抗(1:5 000)常温孵育 1~2 h 后,用 Bio-Red 凝胶成像系统进行图像分析。

1.9 统计学方法

采用 SPSS 22.0 软件进行统计分析。计量资料服从正态分布者采用($\bar{x}\pm s$)表示,2 组间比较采用 *t* 检验,3 组以上采用单因素方差分析;非正态分布采用秩和检验。以 *P*<0.05 为差异具有统计学意义。

2 结 果

2.1 BA 对 HCT116 细胞生长的影响

与 0 μmol/L 组比较,不同浓度的 BA 干预后均能显著抑制 HCT116 细胞的活力(*P*<0.05),且随着 BA 浓度的增加,对 HCT116 细胞活力的抑制作用也逐渐加强,见图 1。与 0 μmol/L 组比较,不同浓度的 BA 干预后均能显著降低细胞计数(*P*<0.05),且随着 BA 浓度的升高,HCT116 细胞数逐渐减少,见图 2。由此可见,BA 可以抑制 HCT116 细胞的生长。

2.2 BA 对 HCT116 细胞迁移和侵袭的影响

与 0 μmol/L 组比较,不同浓度的 BA 干预后均能导致迁移或侵袭到小室外膜的 HCT116 细胞数目明显减少(*P*<0.05),且随着浓度的增加细胞数目逐渐减少,提示不同浓度的 BA 均能抑制 HCT116 细

注:与 0 μmol/L 组比较,1) *P*<0.05。
Note: Compared with the 0 μmol/L group, 1) *P*<0.05.

图 1 4 组 HCT116 细胞活力的比较
Figure 1 Comparison of cell viability of HCT116 cells in four groups

注:与 0 μmol/L 组比较,1) *P*<0.05。
Note: Compared with the 0 μmol/L group, 1) *P*<0.05.

图 2 4 组 HCT116 细胞数的比较
Figure 2 Comparison of cell number of HCT116 cells in four groups

胞的迁移和侵袭能力,说明 BA 可以抑制 HCT116 细胞的转移。见图 3 和图 4。

注:与 0 μmol/L 组比较,1) *P*<0.05。
Note: Compared with the 0 μmol/L group, 1) *P*<0.05.

图 3 4 组 HCT116 细胞迁移能力的比较(×400)

Figure 3 Comparison of migration capacity of HCT116 cells in four groups (×400)

注:与 0 $\mu\text{mol/L}$ 组比较,1) $P < 0.05$ 。

Note: Compared with 0 $\mu\text{mol/L}$ group, 1) $P < 0.05$.

图 4 4 组 HCT116 细胞侵袭能力的比较($\times 400$)

Figure 4 Comparison of invasion capacity of HCT116 cells in four groups ($\times 400$)

2.3 BA 对 TGF- β 和 Smad2/3 蛋白表达的影响

与 0 $\mu\text{mol/L}$ 组比较,不同浓度的 BA 干预 48 h 后均能够显著下调 HCT116 细胞 Smad2/3 和 TGF- β 的蛋白表达,且抑制作用随着 BA 浓度的增加而加强。由此可见,BA 对 HCT116 细胞转移的抑制作用可能是通过抑制 TGF- β /Smad 通路来实现的。见图 5。

图 5 4 组 HCT116 细胞 TGF- β 和 Smad2/3 蛋白表达的比较

Figure 5 Comparison of TGF- β and Smad2/3 protein expression of HCT116 cells in four groups

3 讨论

国家综合癌症网络(NCCN)指南建议晚期结肠癌患者采取手术和化疗为主的联合治疗^[8]。现有临床研究已经表明,大多数的结肠癌患者在发现肿瘤时已经处于中晚期,癌细胞发生不同程度的转

移^[9],化疗是其主要治疗手段,然而化疗带来的毒副作用导致患者生活质量大幅下降,患者依从性差,最终导致化疗失败,以致患者死亡。

TGF- β 属于转化生长因子超家族成员,包含多种生长因子、生长分化因子(GDFs)和骨形成蛋白(BMP)^[10],TGF- β R I 和 TGF- β R II 组成受体二聚体,可引起 Smads 蛋白受体磷酸化(Smad 1、2、3、5、8),然后和 Smad 4 一起进入细胞核,导致下游基因的转录^[11]。近年来的研究表明,TGF- β 的异常表达可导致 EMT,是介导多种肿瘤转移的主要诱因^[12-15]。

BA 广泛存在于桉树皮、桑白皮、桦树皮等多种植物中,尤以白桦树皮含量最高^[16-17]。现有的研究已经证明 BA 具有抗 HIV^[18]、免疫调节^[19-20]、抗氧化刺激^[21-22]、抗菌^[23]等广泛的药理作用,而且 BA 可以通过抑制增殖,诱导凋亡,阻滞细胞周期,促进迁移和侵袭以及诱导肿瘤细胞自噬等方面抑制大肠癌、卵巢癌、乳腺癌、黑色素瘤等的发展。BA 作为一种天然药物,因其广谱抗肿瘤活性以及护肝特性在抗肿瘤中受到广泛的青睐,且与许多化疗药联用后还能起到增效减毒的作用。但迄今为止,BA 抗肿瘤的作用靶点和机制尚未完全阐明。因此,本研究通过体外培养的人结肠癌细胞株 HCT116,观察 BA 对其生长和迁移、侵袭的影响,并探讨其抑制转移的分子机制。本研究结果不仅进一步表明 BA 能够抑制结肠癌细胞的生长、迁移和侵袭,而且首次发现其抑制转移的作用机制可能是通过调控 TGF- β /Smad

信号通路来实现的。本研究结果有望为BA开发成治疗结肠癌的天然药物提供进一步的实验依据。

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Betulinic Acid Inhibits the Metastasis of Colon Cancer Cells through Regulating TGF- β /Smad Signaling Pathway

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ABSTRACT Objective: To explore the effect of betulinic acid (BA) on the growth and metastasis of colon cancer cell line HCT116 and the underlying possible mechanism. **Methods:** HCT116 cells were cultured with McCoy's 5A medium containing 10% fetal bovine serum and 1% penicillin/streptomycin mixed solution in a 37 °C, 5% CO₂ incubator. HCT116 cells were seeded at a density of 0.2×10^5 cells/mL in a 96-well plate and cultured overnight. Then the cells were treated with different concentrations (0, 10, 20, and 40 $\mu\text{mol/L}$) of BA for 24, 48 and 72 h, respectively. And then CCK8 was added to detect its absorbance (*A* value) at 450 nm. HCT116 cells were seeded at a density of 0.4×10^5 cells/mL in a 12-well plate and cultured overnight. The cells were then collected by digestion and centrifugation, and stained with trypan blue after treating with different concentrations (0, 10, 20, and 40 $\mu\text{mol/L}$) of BA for 48 h. The total number of cells was calculated by using a Countstar BioMed automatic cell counter. HCT116 cells were seeded at a density of 0.4×10^5 cells/mL in a 12-well plate and cultured overnight. Then the cells were collected by digestion and centrifugation after treating with different concentrations (0, 10, 20, and 40 $\mu\text{mol/L}$) of BA for 48 h. 1.0×10^5 cells/mL cell suspension was used for the Transwell experiment of cell migration and invasion, respectively. The effects of different concentrations of BA intervention for 48 h on the migration and invasion ability of HCT116 were analyzed. Western blot was used to detect the effect of BA on the protein expression of TGF- β and Smad2/3 in HCT116 cells after treated with BA for 48 h. **Results:** (1) CCK8 assay showed that the cell viability in each group was significantly reduced after treatment with different concentrations of BA for 24, 48 and 72 h, and the differences were statistically significant compared with the 0 $\mu\text{mol/L}$ group respectively ($P < 0.05$); The cell count results showed the number of cells in each group was remarkably decreased, and the differences were statistically significant compared with the 0 $\mu\text{mol/L}$ group respectively ($P < 0.05$). (2) Transwell assay showed that the number of cells that migrated or invaded into the membrane was profoundly inhibited in each group after treatment with different concentrations of BA for 48 h, and the differences were statistically significant compared with the 0 $\mu\text{mol/L}$ group respectively ($P < 0.05$). (3) Western blot assay showed that the protein expression of TGF- β and Smad2/3 was obviously decreased in each group after treated with different concentrations of BA for 48 h compared with the 0 $\mu\text{mol/L}$ group. **Conclusion:** BA can inhibit the growth and metastasis of colon cancer cells, and its mechanism may be achieved by regulating the TGF- β /Smad signaling pathway.

KEY WORDS colon cancer; betulinic acid; transfer; TGF- β /Smad signaling pathway

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Mechanism of Electroacupuncture Quchi (LI 11)-Yanglingquan (GB 34) in Rats with Spasticity of Cerebral Apoplexy

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ABSTRACT Objective: The purpose of this study is to analyze the effects of electroacupuncture Quchi (LI 11)-Yanglingquan (GB 34) in Sprague-Dawley (SD) rats with spasticity of cerebral apoplexy (SCA) on the nerve injury, ultrastructural changes in synapses, and brain derived neurotrophic factor (BDNF), tyrosine protein kinase B (TrkB) and receptor gene expression levels in hippocampus, so as to reveal the efficacy and mechanism of electroacupuncture. **Methods:** A total of thirty-six SD rats were randomly divided into electroacupuncture group, sham operation group, model group and blank group, with nine SD rats in each group. A rat model of stroke and limb paralysis was prepared by modified Zea-Longa suture method and internal capsule injection of NMDA receptor method. The behavioral scoring confirmation model was followed by electroacupuncture treatment for five days. The ultrastructural changes of synapses in hippocampus were observed by electron microscopy. The expressions of BDNF and TrkB related genes in hippocampus were detected by RT-PCR. The expression of BDNF and TrkB protein in hippocampus was detected by Western blot. **Results:** (1) The neurological scores and Ashworth assessment of muscle tension in the model group were significantly higher compared with those in the blank group and the sham operation group respectively ($P < 0.01$). Compared with the model group, the neurological scores and Ashworth assessment of muscle tension in the electroacupuncture group were statistically significant ($P < 0.05$). (2) The number of synapses in the model group was significantly reduced and the anterior and posterior membranes and gaps were blurred. Besides, the number of synaptic vesicles was significantly reduced, the density uneven, the terminal cytoplasm of the postsynaptic cells severely damaged, and the dense density of postsynaptic bands decreased; The electroacupuncture group was denser than the model group, and the synaptic shape was closer to the sham operation group. The number of synaptic vesicles, the concave synapses increased, while the post-synaptic cell terminal cytoplasmic degeneration was significantly reduced compared with the model group. In addition, the dense substance thickened and the gap was narrowed. (3) The expression of BDNF-TrkB protein and gene in the electroacupuncture group increased significantly compared with that in the model group ($P < 0.05$). The expression of BDNF-TrkB protein and gene in the model group decreased significantly compared with that in the blank group and the sham operation group respectively ($P < 0.01$). **Conclusion:** Electroacupuncture Quchi (LI 11)-Yanglingquan (GB 34) regulate the expression of BDNF-TrkB gene and protein in hippocampus of the brain. High expression of the neurotrophic factor in hippocampus may promote the remodeling of synapses and improve the motor function of the central nervous system, thus achieving the relief of spasticity.

KEY WORDS stroke; spasm; electroacupuncture; Quchi (LI 11); Yanglingquan (GB 34); brain derived neurotrophic factor; tyrosine protein kinase B

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