姜黄素联合西达本胺通过下调AKT磷酸化和上调p53表达调控SKM-1细胞增殖和凋亡

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摘要 该文探讨了姜黄素联合西达本胺对SKM-1细胞增殖和凋亡的影响及其作用机制。体外培养SKM-1细胞, 取对数生长期细胞用于后续实验。对照组予以常规培养, 实验组分别用不同浓度(1、5、10、20、40 μmol/L)姜黄素、不同浓度(0.5、1、2、4、8 μmol/L)西达本胺和不同浓度(5、10 μmol/L)姜黄素联合不同浓度(0.5、1、2、4、8 μmol/L)西达本胺处理细胞, 采用CCK-8法检测各组细胞增殖活性, CompuSyn软件计算联合指数(combination index, CI), 流式细胞术检测各组细胞周期分布和凋亡情况, Western blot检测各组细胞CDK2、p16、Caspase-3、AKT、p-AKT、p53和γH2A.X的蛋白表达水平。结果显示, 在检测浓度范围内, 姜黄素和西达本胺以时间浓度依赖性的方式抑制SKM-1细胞的生长。联合使用时, 5 μmol/L姜黄素与2 μmol/L西达本胺具有协同抑制细胞增殖的作用。流式细胞术结果显示, 5 μmol/L姜黄素联合2 μmol/L西达本胺组的细胞周期明显阻滞于G_0/G_1期, 细胞凋亡率显著高于对照组和单独用药组。Western blot结果显示, 与对照组相比, 联合用药组的CDK2蛋白表达水平和p-AKT/AKT比例显著下降, 而p16、Caspase-3、p53和γH2A.X的蛋白表达水平显著增高。综上, 姜黄素联合西达本胺可显著抑制SKM-1细胞增殖, 阻滞细胞周期, 并促进细胞凋亡, 其机制可能与抑制AKT磷酸化和上调p53表达有关。

关键词 姜黄素; 西达本胺; SKM-1细胞; AKT; p53; 细胞增殖

Curcumin Combined with Chidamide Regulates Proliferation and Apoptosis of SKM-1 Cells by Suppressing AKT Phosphorylation and Upregulating p53 Expression

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Abstract This study aimed to investigate the effect of curcumin combined with chidamide on SKM-1 cell proliferation and apoptosis and explore the underlying mechanism. SKM-1 cells were cultured *in vitro*, and the cells in the logarithmic growth phase were used for subsequent experiments. Cells in the control group were cultured routinely. Cells in the experimental groups were treated with different concentrations of curcumin (1, 5, 10, 20, 40 μmol/L),

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different concentrations of chidamide (0.5, 1, 2, 4, 8 μmol/L) and different concentrations of curcumin (5, 10 μmol/L) combined with different concentrations of chidamide (0.5, 1, 2, 4, 8 μmol/L), respectively. CCK-8 assay was used to detect the proliferation activity. CI (combination index) was calculated by Compusyn software. Cell cycle distribution and apoptosis were detected by flow cytometry. The protein expression levels of CDK2, p16, Caspase-3, AKT, p-AKT, p53 and γH2A.X in each group were detected by Western blot. The results showed that curcumin and chidamide inhibited the growth of SKM-1 cells in a time- and concentration-dependent manner. Curcumin (5 μmol/L) and chidamide (2 μmol/L) had a synergistic inhibitory effect on cell proliferation. The results of flow cytometry showed that 5 μmol/L curcumin combined with 2 μmol/L chidamide arrested the cell cycle at G_0/G_1 phase, and the apoptotic rate in the combination treatment group was significantly higher than that in the control and the single drug groups. The results of Western blot showed that compared with the control group, in the combination treatment group, the protein expression level of CDK2 and p-AKT/AKT ratio were significantly decreased while the protein expression levels of p16, caspase-3, p53 and γH2A.X were significantly increased. Collectively, curcumin combined with chidamide could significantly inhibit the proliferation of SKM-1 cells, block cell cycle and promote cell apoptosis, whose mechanism might be related to the inhibition of AKT phosphorylation and upregulation of p53 expression.

Keywords curcumin; chidamide; SKM-1 cells; AKT; p53; cell proliferation

骨髓增生异常综合征(myelodysplastic syndromes, MDS)是一种起源于造血干细胞的异质克隆性肿瘤, 其特征是骨髓病态造血, 并具有转化为急性髓系白血病(acute myeloid leukemia, AML)的高风险[1]。由MDS转化而来的AML(AML-MDS)患者对化疗药物不敏感, 治疗效果差, 且预后不良[2]。目前唯一可能治愈AML-MDS的方法是行异基因造血干细胞移植, 但由于年龄和配型等原因, 此方法并不适用于大部分患者[3-4]。因此, 探索新的治疗方案, 对于提高AML-MDS患者生存率, 改善患者生活质量具有重要意义。

姜黄素(curcumin)是一类从姜科植物的根茎提取得到的多酚类物质, 具有抗癌、抗炎和抗氧化等作用[5]。西达本胺(chidamide)是一种新型组蛋白去乙酰化酶(histone deacetylase, HDAC)抑制剂, 在临床上主要用于治疗复发及难治外周T细胞淋巴瘤[6]。有研究显示, 姜黄素与HDAC抑制剂(曲古霉素A)联合使用可显著增强曲古霉素A对肝癌细胞的增殖抑制作用[7], 姜黄素与伏立诺他联用时, 可协同增强人类癌细胞中的组蛋白乙酰化, 但在发挥细胞毒性作用时两者具有拮抗作用[8]。目前, 姜黄素联合西达本胺在AML-MDS中的作用和机制尚不清楚。故本研究旨在探索姜黄素联合西达本胺对AML-MDS细胞株SKM-1增殖的影响及可能的作用机制, 为AML-MDS治疗提供新的方案。

1 材料和方法

1.1 材料

姜黄素购自美国Sigma公司; 西达本胺由深圳微芯生物科技股份有限公司赠送; CCK-8试剂购自上海MedChemExpress公司; 逆转录试剂盒、RT-qPCR试剂盒购自日本TaKaRa公司; 胎牛血清(fetal bovine serum, FBS)购自德国PAN-Seratech公司; RPMI-1640购自美国Gibco公司; 兔抗人CDK2、p16、γH2A.X抗体购自沈阳万类生物科技有限公司; 兔抗人p53、p-AKT、AKT购自美国CST公司; Caspase-3抗体购自美国Immunoway公司; BCA试剂盒、青–链霉素、Trizol试剂、小鼠抗人β-actin抗体、辣根过氧化物酶(horseradish peroxidase, HRP)标记山羊抗兔IgG、HRP标记山羊抗小鼠IgG购自上海碧云天生物技术有限公司; 酶标仪、NANO DROP 2000为美国Thermo scientific公司产品; 流式细胞仪为美国Beckman Coulter公司产品; 荧光定量PCR仪为美国Bio-Rad公司产品; 凝胶成像仪为法国Vilber Lourmat公司产品。

1.2 方法

1.2.1 细胞培养 人AML-MDS细胞株SKM-1由华中科技大学附属同济医院周剑锋教授馈赠, 用含10% FBS和1%青–链霉素的RPMI-1640培养基, 置于37 ℃、5% CO_2、饱和湿度的孵箱中培养, 每2~3天传代1次。

1.2.2 CCK-8检测细胞增殖 取对数生长期细胞, 以5×10^3个/孔的密度接种于96孔板中, 西达本胺浓

度为0.5、1、2、4、8 μmol/L, 姜黄素浓度为1、5、10、20、40 μmol/L, 对照组为不含药物孔, 空白组为不含细胞和药物孔, 每组设3个复孔。培养24 h、48 h、72 h后, 每孔分别加入10 μL CCK-8工作液, 继续孵育3 h, 用酶标仪检测450 nm处的光密度(*D*)值, 按下列公式计算细胞增殖抑制率: 细胞增殖抑制率(%)=(对照组*D*值–实验组*D*值)/(对照组*D*值–空白组*D*值)×100%。

1.2.3 流式细胞术检测细胞周期 取对数生长期细胞, 以1×10^{6}个/孔的密度接种于6孔板中, 药物处理48 h后, 收集细胞, PBS清洗2次后加入预冷的75%乙醇, 4 °C固定过夜后加入含RNA酶(100 μg/mL)的碘化丙啶(propidium iodide, PI), 室温避光孵育30 min后用流式细胞仪检测细胞周期分布情况。

1.2.4 流式细胞术检测细胞凋亡 取对数生长期细胞, 以1×10^{6}个/孔的密度接种于6孔板中, 药物处理48 h后, 收集细胞, PBS清洗2次后, 重悬细胞, 加入5 μL 50 mg/L Annexin V-FITC和10 μL PI, 避光孵育15 min, 用流式细胞仪检测细胞凋亡情况。

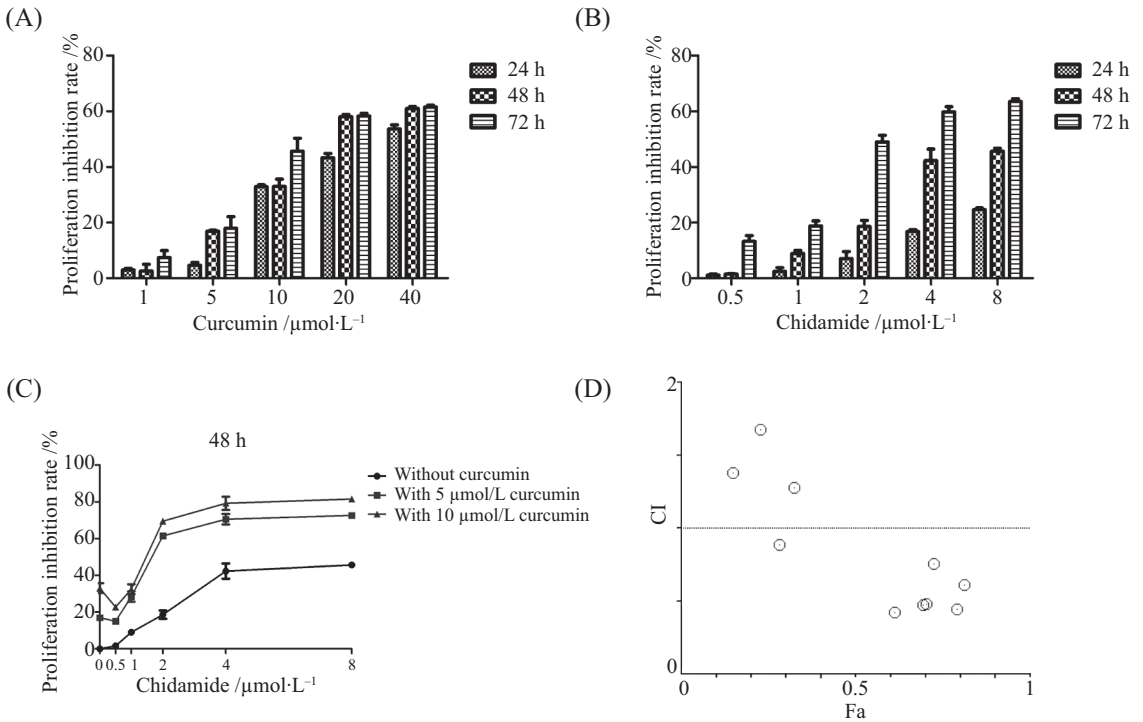
1.2.5 Western blot检测相关蛋白表达水平 收集处理48 h的各组细胞, 提取细胞总蛋白, 用BCA法测定蛋白浓度。取30 μg总蛋白上样, 经SDS-PAGE电泳后转至PVDF膜上, 5%脱脂奶粉室温封闭2 h后, 分别加入相应一抗(1:1 000), 4 °C孵育过夜。次日用TBS-T清洗3次, 加入相应二抗(1:1 000), 室温孵育1 h, 用ECL化学发光试剂盒成像, Fusion软件分析条带灰度值。

1.2.6 统计学方法 采用SPSS 22.0和GraphPad Prism 5.01软件进行统计学分析, 采用CompuSyn软件计算联合指数(combination index, CI), 定量数据以均值±标准差($\bar{x}\pm s$)表示, 两组间比较采用独立样本*t*检验, 多组间比较采用单因素分析。$P<0.05$表示差异具有统计学意义。

2 结果

2.1 姜黄素联合西达本胺显著抑制SKM-1细胞增殖活性

CCK-8结果显示, 单用姜黄素和西达本胺对SKM-1细胞增殖的抑制作用随着药物浓度和作用时间增加而增加, 呈时间浓度依赖性(图1)。当姜黄素(5、10 μmol/L)与不同浓度的西达本胺联合使用



A: 不同浓度姜黄素处理; B: 不同浓度西达本胺处理; C: 姜黄素联合西达本胺处理; D: 联合效应。Fa: 效应值, CI: 联合指数。

A: treated with different concentrations of curcumin; B: treated with different concentrations of chidamide; C: treated with curcumin combined with chidamide; D: the effect of the combined treatment. Fa: fraction affected; CI: combination index.

图1 CCK-8检测SKM-1细胞增殖抑制率

Fig.1 The proliferation inhibition rate of SKM-1 cells was detected by CCK-8 assay

时, 有三个组的联合方案具有拮抗作用, 其余七组均具有协同作用(图1和表1)。其中, 5 μmol/L姜黄素与2 μmol/L西达本胺联合使用的联合指数最小(CI=0.42), 故本研究选择此联合方案进行后续实验。

2.2 姜黄素联合西达本胺诱导细胞周期阻滞

流式细胞术检测结果显示, 与对照组相比, 2 μmol/L西达本胺作用于SKM-1细胞48 h后, SKM-1细胞周期分布没有显著变化(P>0.05), 而经5 μmol/L姜黄素处理后, G_0/G_1期细胞数目增多, S期细胞数目减少(P<0.001)。与对照组相比, 联合处理组的G_0/G_1期细胞数目明显增多, S期和G_2/M期数目明显减少(P<0.001)。此外, 联合处理组的G_2/M期细胞数目明显少于姜黄素处理组和西达本胺处理组(P<0.05)(图2)。

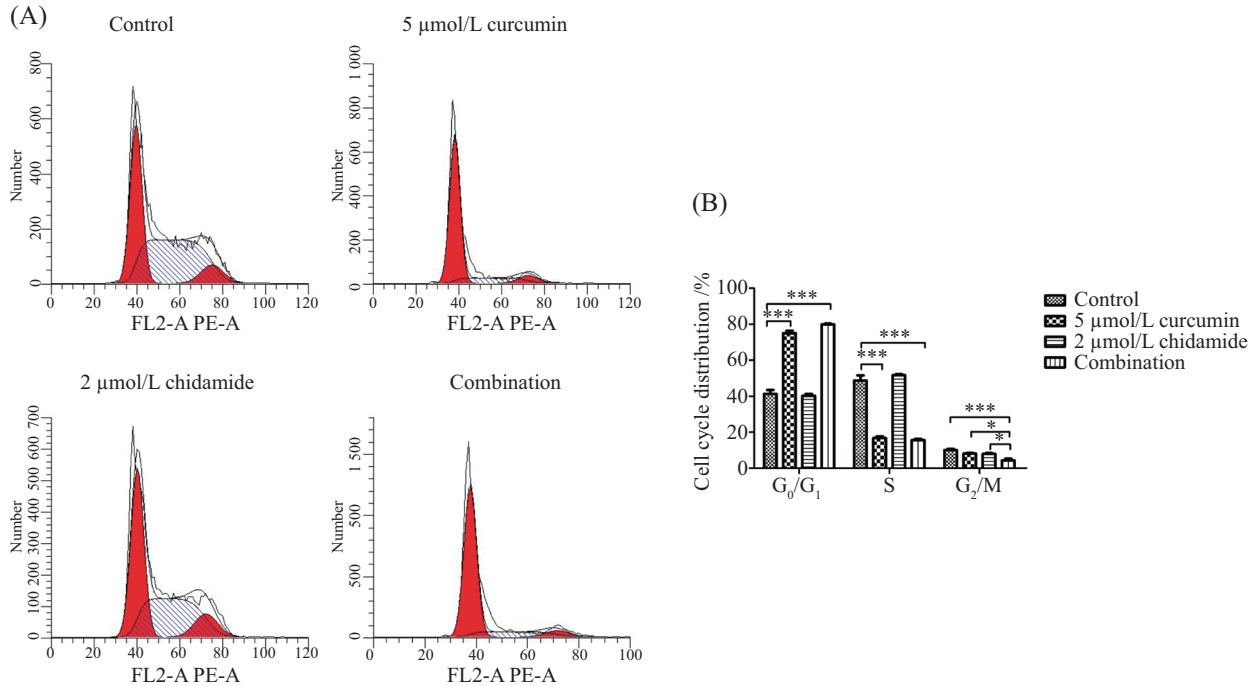
2.3 姜黄素联合西达本胺增加细胞凋亡率

流式细胞术结果如图3所示, 与对照组相比, 2 μmol/L西达本胺作用48 h后SKM-1细胞凋亡率明

表1 不同浓度药物联合下细胞的增殖抑制效应和联合指数

Table 1 Inhibitory effect and combined index of different concentrations of combined drugs on cell proliferation

西达本胺/μmol·L^{-1} Chidamide /μmol·L^{-1}	姜黄素/μmol·L^{-1} Curcumin /μmol·L^{-1}	效应值 Fraction affected	联合指数 Combination index
0.5	5	0.150 44	1.377 83
1	5	0.284 53	0.886 44
2	5	0.614 39	0.421 60
4	5	0.705 24	0.479 59
8	5	0.726 24	0.756 75
0.5	10	0.227 55	1.680 83
1	10	0.324 57	1.278 24
2	10	0.694 71	0.474 03
4	10	0.792 03	0.442 47
8	10	0.815 12	0.615 55



A: 流式细胞术检测细胞周期分布; B: 细胞周期分布统计分析, *P<0.05, ***P<0.001。

A: cell cycle distribution was detected by flow cytometry; B: statistical analysis of cell cycle distribution, *P<0.05, ***P<0.001.

图2 流式细胞术检测各组细胞周期分布

Fig.2 Cell cycle distribution of each group was detected by flow cytometry

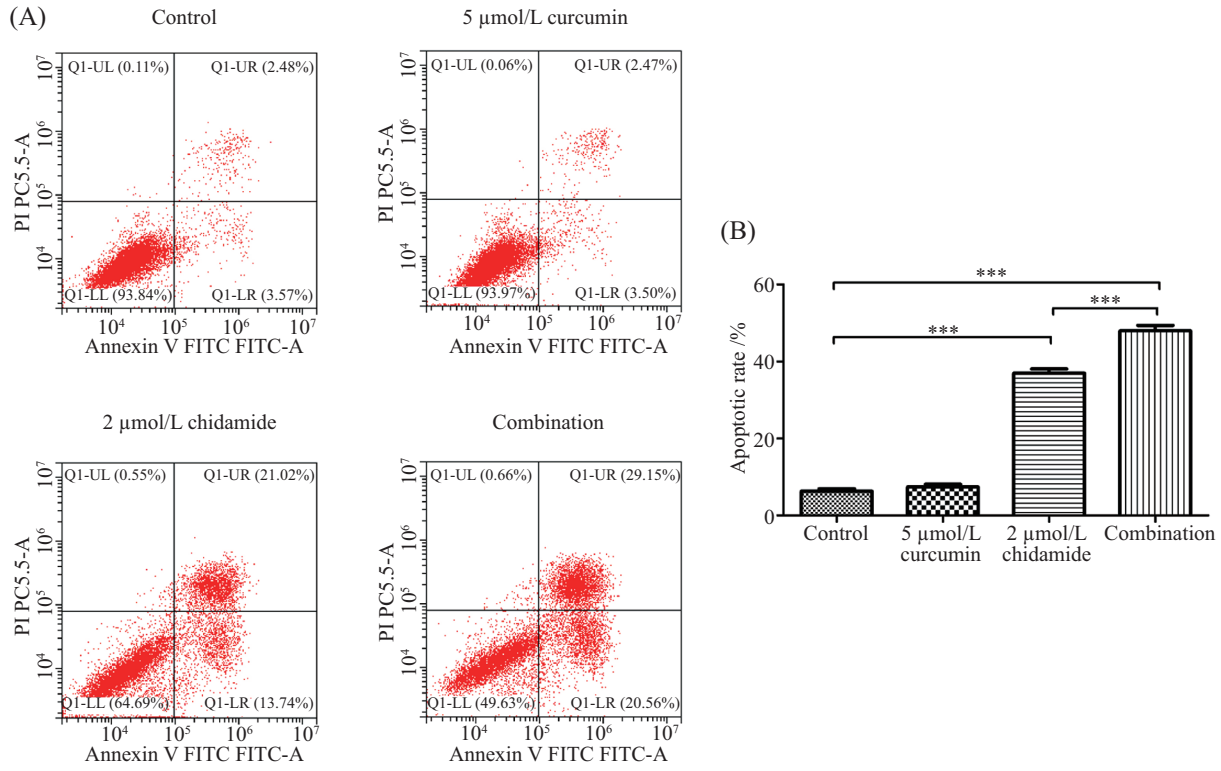
显增加(P<0.001), 而5 μmol/L姜黄素处理后没有显著变化(P>0.05), 但联合处理组的细胞凋亡率明显高于对照组、姜黄素处理组和西达本胺处理组(P<0.001)。

2.4 姜黄素联合西达本胺影响细胞周期和凋亡相关蛋白的表达水平

与对照组相比, 5 μmol/L姜黄素联合2 μmol/L西达本胺处理SKM-1细胞48 h后, 周期相关蛋白CDK2表达水平下降, p16蛋白水平上调, 凋亡相关因子Caspase-3蛋白表达水平上调(P<0.05)(图4)。

2.5 姜黄素联合西达本胺影响AKT、p-AKT、p53和p16蛋白表达水平

与对照组相比, 5 μmol/L姜黄素联合2 μmol/L西

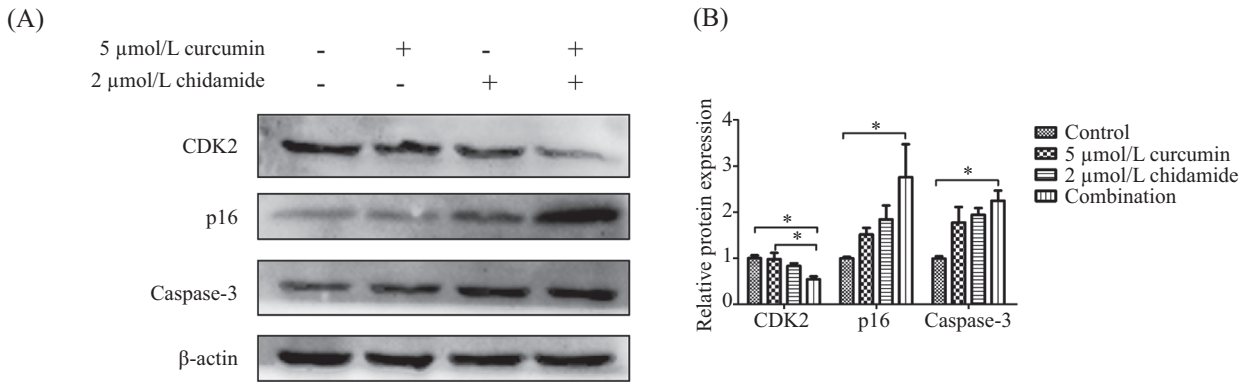


A: 流式细胞术检测细胞凋亡; B: 细胞凋亡率统计分析, ***P<0.001。

A: cell apoptosis was detected by flow cytometry; B: statistical analysis of cell apoptotic rate, ***P<0.001.

图3 流式细胞术检测各组细胞凋亡率

Fig.3 The apoptotic rate of each group was detected by flow cytometry

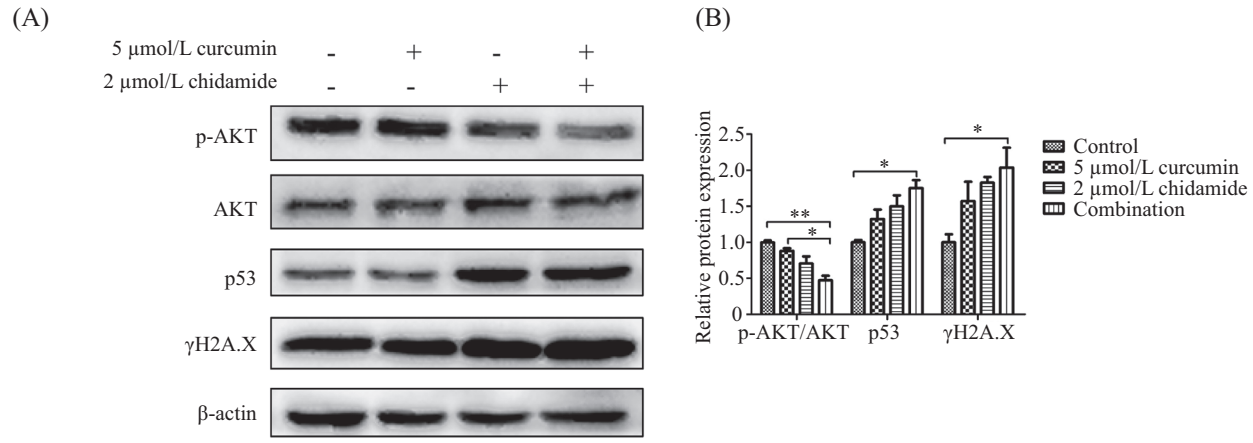


A: Western blot检测各组细胞CDK2、p16和Caspase-3的蛋白表达水平; B: 相对蛋白定量分析, *P<0.05。

A: the protein expression levels of CDK2, p16 and Caspase-3 in each group were detected by Western blot; B: statistical analysis of protein levels, *P<0.05.

图4 姜黄素联合西达本胺影响SKM-1细胞CDK2、p16和Caspase-3的蛋白表达水平

Fig.4 Curcumin combined with chidamide affected the protein expression levels of CDK2, p16 and Caspase-3 in SKM-1 cells



A: Western blot检测各组细胞p-AKT、AKT、p53和γH2A.X的蛋白表达水平; B: 相对蛋白定量分析, *P<0.05, **P<0.01。

A: the protein expression levels of p-AKT, AKT, p53 and γH2A.X in each group were detected by Western blot; B: statistical analysis of protein levels, *P<0.05, **P<0.01.

图5 姜黄素联合西达本胺影响SKM-1细胞p-AKT、AKT、p53和γH2A.X的蛋白表达水平

Fig.5 Curcumin combined with chidamide affected the protein expression levels of p-AKT, AKT, p53 and γH2A.X in SKM-1 cells

达本胺处理SKM-1细胞48 h后, p-AKT/AKT比例显著下降(P<0.01), p53和DNA双链损伤标志物γH2A.X蛋白表达水平上调(P<0.05)(图5)。

3 讨论

目前, 化疗是AML-MDS患者治疗的主要手段, AML-MDS细胞对化疗药物的不敏感性是影响患者生存和预后的重要因素[2,9]。近年来, 姜黄素被证实在多种癌症中具有抗肿瘤作用, 可参与调控肿瘤细胞的增殖、凋亡和侵袭等, 是一种潜在的抗肿瘤药物[10]。西达本胺是一种新型HDAC抑制剂, 可抑制白血病细胞增殖[11]。有研究显示, 姜黄素可增强HDAC抑制剂诱导的抗增殖和促凋亡作用[12], 且对正常细胞的毒性很小[13], 这为姜黄素联合西达本胺用于治疗AML-MDS提供了新视角。

在本研究中, CCK-8法检测结果证实, 姜黄素与西达本胺单药处理SKM-1细胞均可抑制细胞活性。以往研究结果也显示, 姜黄素和西达本胺分别以时间浓度依赖性的方式抑制白血病细胞, 包括SKM-1、KG-1α和HEL细胞等的活性[14-16]。此外, 我们还发现, 5 μmol/L姜黄素与2 μmol/L西达本胺联合作用时的协同作用最明显, 故我们选用此联合方案进行后续研究。流式细胞术结果显示, 姜黄素与西达本胺联合用药组的细胞周期明显阻滞于G_0/G_1期, 且SKM-1细胞凋亡率明显高于单药组。以上结果表明, 姜黄素联合西达本胺这一治疗方案具有可行性。本研究还发现, 当较低浓度的西达本胺与姜黄素联合使用时, 两者呈拮抗效应, 而当西达本胺浓度增大时, 联合效应由拮抗转为协同, 这可能与药物代谢途径和作用靶点有关。当两者的作用机制相互影响时, 可能会产生协同或拮抗作用。以上结果说明, 在一定浓度范围内, 调节两药的联合浓度可以提高药物疗效, 但体内疗效和具体的药物联合浓度还需进一步探索。

抗癌药物主要通过抑制细胞增殖和诱导细胞凋亡来发挥抗肿瘤作用。细胞周期失调可导致细胞异常增殖, 是癌症的一个标志性特征。细胞周期蛋白依赖性激酶(cyclin-dependent kinase, CDK)是细胞周期时相转化的关键调节酶[17], 其中, CDK2在调控G_1期-S期过渡中起着重要作用[18]。p16蛋白是调控细胞周期的核心因子, 能抑制CDK活性, 并阻止细胞从G_1期转向S期[19]。从流式细胞术分析结果可知, 姜黄素与西达本胺联合处理后的细胞主要被阻滞在G_0/G_1期, 而S期细胞数目减少。Western blot结果显示, 联合处理能下调CDK2的表达而上调p16的表达, 提示姜黄素联合西达本胺诱导细胞周期阻滞的机制可能与其调控CDK2和p16的表达水平有关。

细胞凋亡主要通过内源性和外源性途径引发, Caspase-3在这两条途径中均发挥作用, 是细胞凋亡的主要执行者[20]。此外, H2A.X是组蛋白家族H2A的一个分子, 参与DNA的损伤修复, DNA双链断裂后H2A.X发生磷酸化, 形成γH2A.X[21-22]。相关研究证明, H2A.X的磷酸化在细胞凋亡过程中起着关键调控作用[23-24]。流式细胞术结果显示, 姜黄素与西达本胺联合处理后的细胞凋亡率明显增加, 同时,

Western blot结果显示联合处理可上调Caspase-3和γH2A.X的表达, 表明姜黄素联合西达本胺诱导的细胞凋亡与调控Caspase-3和γH2A.X的表达水平有关。以上结果表明, 与单用姜黄素或单用西达本胺相比, 联合用药显著下调CDK2的蛋白表达水平, 同时上调p16、Caspase-3和γH2A.X的蛋白表达水平, 从而阻滞SKM-1细胞于G_0/G_1期, 并诱导细胞凋亡。

此外, 姜黄素和西达本胺的抗增殖效应均可通过抑制AKT的激活从而发挥作用[25-26]。AKT是丝氨酸/苏氨酸蛋白激酶家族成员, 主要调控细胞存活、增殖、分化、葡萄糖代谢和蛋白质合成等细胞进程, 可直接参与和影响与肿瘤发生相关的生物学现象[27-29]。有研究发现, 姜黄素可通过抑制AKT的磷酸化, 从而激活p53的表达, 抑制细胞增殖[30]。此外, p53可在DNA损伤的状态下被激活, 影响下游基因表达, 从而促进细胞周期阻滞和凋亡[31-32]。GABAI等[33]报道, p53信号通路被激活后会提高γH2A.X在细胞内的表达, 增强DNA损伤, 并进一步激活p53通路, 形成一个正反馈回路, 从而抑制肿瘤细胞增殖。我们的结果发现, 姜黄素联合西达本胺可显著抑制AKT的磷酸化水平, 上调p53的蛋白表达水平。另有研究显示, AKT磷酸化水平下调可使p53表达水平增高, 导致CDK2表达水平下调[34], p16[35]和Caspase-3[36]表达水平上调, 从而抑制细胞增殖, 我们的结果与之一致。由此推测, 姜黄素联合西达本胺可能通过抑制AKT磷酸化和上调p53表达, 从而诱导细胞周期阻滞并促进细胞凋亡。

综上所述, 姜黄素与西达本胺联用对SKM-1细胞增殖具有协同抑制作用, 其作用机制可能与抑制AKT磷酸化和上调p53表达有关。总之, 本研究为姜黄素联合西达本胺用于治疗AML-MDS提供了研究思路和实验依据, 但其具体机制和治疗效果还需进一步的研究。

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