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· 基础研究 ·

柚皮素对甲状腺癌细胞凋亡、自噬的影响及其与AMPK/mTORC1通路的关系

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摘要

背景与目的: 柚皮素(NAR)是天然的黄酮类化合物,能够抑制宫颈癌、胃癌、舌鳞癌及肝癌细胞的生长,但其对甲状腺癌细胞的作用尚不明确。本实验探讨NAR对甲状腺细胞的影响,并初步研究其作用机制,以期为甲状腺癌的药物研发提供理论基础。

方法: 不同浓度的NAR处理甲状腺癌ACT-1细胞不同时间后,用MTT法检测细胞存活率,以观察NAR对ACT-1细胞的时间、浓度效应并计算IC₅₀值。在以上实验基础上,选择适合的浓度的NAR与适合的时间处理ACT-1细胞,然后分别采用Annexin V-FITC/PI法检测细胞凋亡;绿色荧光蛋白(GFP)标记质粒转染检测自噬小体变化;Western blot法检测自噬相关蛋白(LC3 I、LC3 II、p62)以及AMPK/mTORC1通路相关蛋白表达的变化,以及AMPK抑制剂对NAR作用的影响。

结果: NAR能明显抑制ACT-1细胞的存活,呈时间与浓度依赖性(均P<0.05),其12、24和48 h的IC₅₀值分别为85.65、50.12、38.94 μg/mL。选用25、50、100 μg/mL的NAR处理ACT-1细胞24 h后,细胞凋亡率、自噬小体数量、LC3 II/LC3 I和p-AMPK/AMPK蛋白表达明显升高,p62和p-mTORC1/mTORC1蛋白表达明显降低,均呈浓度依赖性(均P<0.05)。用100 μg/mL NAR同时加入25 μmol/L AMPK抑制剂后处理ACT-1细胞后,NAR的以上作用以及升高凋亡效应蛋白caspase-3的作用被明显抑制(均P<0.05)。

结论: NAR能够抑制甲状腺癌ACT-1细胞增殖,诱导凋亡,这可能与调控AMPK/mTORC1信号通路,增强自噬相关。

关键词

甲状腺肿瘤; 柚皮素; 自噬; 细胞凋亡; AMP活化蛋白激酶类; 雷帕霉素复合体1机制性靶标
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Effects of naringenin on apoptosis and autophagy of thyroid cancer cells and its association with AMPK/mTORC1 pathway

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Abstract

Background and Aims: Naringenin (NAR) is a natural flavonoid compound, which can inhibit the growth of cells

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of cervical cancer, gastric cancer, tongue squamous cell carcinoma and liver cancer, but its effect on thyroid cancer cells is not clear. Therefore, this study was conducted to investigate the effects of NAR on thyroid cells, and to preliminarily analyze its mechanism, so as to provide a theoretical basis for research and development of drugs against thyroid cancer.

Methods: Thyroid cancer ACT-1 cells were cultured with different concentrations of NAR for different time periods, and then, the cell viability rates were determined by MTT assay to observe the time and concentration effects of NAR on ACT-1 cells, and calculate the IC_{50} values. Based on above experiment, the proper concentrations of NAR and proper time span were chosen to treat the ACT-1 cells. After that, the cell apoptosis was detected by Annexin V-FITC/PI staining, change in autophagic bodies was observed by green fluorescent protein (GFP)-labeled plasmid transfection, and the changes in expressions of the autophagy-associated proteins (LC3 I, LC3 II, p62) and proteins in the AMPK/mTORC1 pathway, as well as the influences of AMPK inhibitor on NAR actions were examined by Western blot analysis.

Results: The survival of ACT-1 cells was significantly suppressed in a time- and concentration dependent manner by NAR treatment (all $P < 0.05$), and the IC_{50} values for 12, 24 and 48 h were 85.65, 50.12 and 38.94 $\mu\text{g/mL}$, respectively. In ACT-1 cells after treatment with the selected concentrations of 25, 50 and 100 $\mu\text{g/mL}$ NAR, the apoptosis rates, numbers of autophagic bodies and the protein expression levels of LC3 II/LC3 I and p-AMPK/AMPK were significantly increased, and the protein expression levels of p62 and p-mTORC1/mTORC1 were significantly decreased, with a concentration-dependent manner (all $P < 0.05$). In ACT-1 cells after treatment with 100 $\mu\text{g/mL}$ NAR and simultaneous 25 $\mu\text{mol/L}$ AMPK inhibitor, the above effects as well as the apoptosis-functional protein caspase-3 increasing effect of NAR on ACT-1 cells were all significantly suppressed (all $P < 0.05$).

Conclusion: NAR can inhibit the proliferation and induce the apoptosis in thyroid cancer ACT-1 cells, which may be related to its regulating AMPK/mTORC1 signaling pathway and the enhancing autophagy.

Key words

Thyroid Neoplasms; Naringenin; Autophagy; Apoptosis; AMP-Activated Protein Kinases; Mechanistic Target of Rapamycin Complex 1

CLC number: R736.1

甲状腺癌是全世界最普遍的内分泌实体瘤类型之一，其发病率呈逐年增加趋势^[1-2]。在大多数患者中，手术后进行放射性碘或观察治疗是有效的，但是其他罕见的甲状腺癌亚型患者的靶向治疗只能延长生存期，因此迫切需要寻找新型有效的治愈性药物用于甲状腺癌的临床治疗^[3]。柚皮素 (naringenin, NAR) 是从柑橘类植物中提取得到的黄酮类化合物，具有抗炎、抗病毒和抗肿瘤等作用^[4-5]。在急性白血病HL-60细胞中，NAR能够激活腺苷酸活化蛋白激酶 (AMP-activated protein kinase, AMPK) 诱导细胞自噬和凋亡^[6]。此外，AMPK/哺乳动物雷帕霉素靶蛋白复合物1 (mammalian target of rapamycin complex 1, mTORC1) 信号通路在调控细胞自噬和凋亡过程中至关重要^[7-8]，但NAR对甲状腺癌细胞增殖、凋亡和自噬的影响及其作用机制是否与AMPK/mTORC1途

径相关尚不清楚。本实验旨在探索NAR对甲状腺癌细胞凋亡和自噬的影响，并初步探索其作用机制，以期将NAR应用于甲状腺癌的临床治疗提供参考。

1 材料与方法

1.1 实验材料

1.1.1 实验细胞 甲状腺癌细胞株 ACT-1 购自上海冠导生物工程有限公司，批号 GD-C521997。

1.1.2 主要试剂和仪器 AMPK抑制剂 (compound C) (杭州昊鑫生物科技股份有限公司，批号 HY13418-1)；DMEM培养基、噻唑蓝 (MTT) 试剂、膜联蛋白 V-异硫氰酸荧光素/碘化丙啉 (Annexin V-FITC/PI) 细胞凋亡检测试剂盒、RIPA裂解液、ECL发光液 (北京 Solarbio 公司，批号分别为 11995、M8180、CA1020、R0010、

PE0010); 绿色荧光蛋白-微管相关蛋白1轻链3(GFP-LC3)质粒试剂盒(上海远慕生物科技有限公司,批号AT-V192); 兔抗人AMPK、磷酸化AMPK(p-AMPK)、核孔蛋白(p62)、微管相关蛋白1轻链3(LC3)I、LC3II、mTORC1、磷酸化mTORC1(p-mTORC1)、半胱氨酸蛋白酶-3(caspase-3)、甘油醛-3-磷酸脱氢酶(GAPDH)单克隆抗体、山羊抗人IgG二抗(美国Thermo Fisher Scientific公司,批号分别为MA5-32122、PA5-104982、PA1-16777、PA5-27357、PA5-27247、PA3-10054、PA3-11775、PA2-30472、A109226、A32731); CO₂细胞培养箱(上海Heal Force公司,型号HF160W); 酶标仪、凝胶成像仪(美国Bio-Rad公司,型号ELX081U、Gel Doc™XR+); 流式细胞仪(美国贝克曼公司,型号CytoFLEX); 荧光显微镜(德国蔡司公司,型号Axioplan 2 imaging E)。

1.2 实验方法

1.2.1 细胞培养 将ACT-1细胞用含有10%胎牛血清的DMEM培养基培养,置于37℃、5%CO₂培养箱中孵育,每2天更换1次培养液,待细胞融合度达80%左右,胰酶消化传代,收集细胞用于后续实验。

1.2.2 MTT 实验 将ACT-1细胞铺于96孔板(密度 4.5×10^4 个/孔),用浓度为0、6.25、12.50、25.00、50.00、100.00 μg/mL的NAR处理12、24、48 h,每个浓度设置6个复孔,每孔加入100 μL MTT,孵育4 h,加入二甲基亚砜(100 μL/孔)充分溶解,同时设置空白组(含有培养液、MTT、二甲基亚砜),使用酶标仪测量490 nm处的OD值,计算细胞存活率以及半数抑制率IC₅₀值。然后根据IC₅₀值,将实验分为对照组、NAR低、中、高浓度组,分别使用含有NAR浓度为0、25、50、100 μg/mL^[9]的无血清DMEM培养基培养24 h后,用于后续实验。细胞存活率(%) = (实验组OD值 - 空白组OD值) / (对照组OD值 - 空白组OD值) × 100%。

1.2.3 Annexin VP-FITC/PI 实验 调整ACT-1细胞密度为 1.2×10^6 个/孔,铺于6孔板,按1.2.2中分组及干预方法处理后,将各组细胞在无血清培养液培养24 h,进行Annexin V-FITC和PI染色,于流式细胞仪上检测各组凋亡情况。

1.2.4 GFP-LC3 转染检测自噬小体 根据转染试剂盒说明书步骤转染GFP-LC3质粒至细胞中,更

换培养基孵育24 h,参照1.2.2中分组及干预方法处理各组细胞24 h,荧光显微镜下观察细胞自噬活性情况(绿色点状聚集为自噬小体)。

1.2.5 Western blot 实验 向对照组、3个NAR浓度组细胞中加入RIPA裂解液,提取总蛋白,并采用BCA法测量蛋白含量。取25 μg蛋白,经凝胶电泳、转膜、封闭、清洗后,加入一抗LC3 I、LC3 II、p62、p-AMPK、AMPK、p-mTORC1、mTORC1和GAPDH(均为1:1 000),孵育过夜,加入二抗(1:5 000),曝光显影,分析条带。

1.2.6 AMPK 抑制剂干预实验 将ACT-1细胞随机分为空白对照组、NAR组、AMPK抑制剂组和NAR+AMPK抑制剂组,分别用无血清培养基、100 μg/mL NAR无血清培养基稀释液、25 μmol/L AMPK抑制剂无血清培养基、100 μg/mL NAR+25 μmol/L AMPK无血清培养基干预24 h。采用1.2.5中方法检测各组细胞LC3 I、LC3 II、p62、caspase-3、p-AMPK、AMPK、p-mTORC1、mTORC1和GAPDH蛋白表达情况。

1.3 统计学处理

用统计学软件SPSS 22.0分析实验数据,计量资料以平均数±标准差($\bar{x} \pm s$)描述,多组间比较使用单因素方差分析,进一步两组间比较行SNK-*q*检验, $P < 0.05$ 为差异有统计学意义。

2 结果

2.1 NAR 对 ACT-1 细胞存活率的影响

用NAR处理后的ACT-1细胞存活率随浓度增加呈下降趋势,同时随NAR作用时间的延长,ACT-1细胞存活率逐渐下降(均 $P < 0.05$)(图1)。经软件分析算得NAR处理ACT-1细胞12、24、48 h的IC₅₀值分别为85.65、50.12、38.94 μg/mL。后续实验选择NAR干预时间为24 h,药物浓度为25、50、100 μg/mL。

2.2 NAR 对 ACT-1 细胞凋亡的影响

与对照组相比,3个浓度的NAR处理组ACT-1细胞凋亡率均明显增加,且呈浓度依赖性(均 $P < 0.05$)(图2)。

2.3 NAR 对 ACT-1 细胞自噬的影响

转染GFP-LC3质粒后,与对照组相比,ACT-1细胞株经不同浓度NAR作用后,各组细胞内均出现自噬小体,且自噬小体数量随浓度增加呈逐渐增加趋势(图3)。

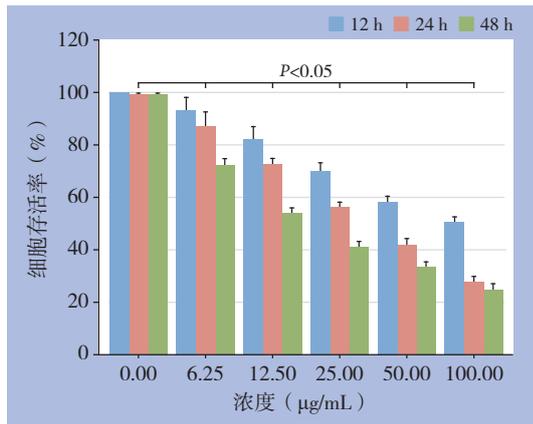


图1 NAR对ACT-1细胞存活率的影响

Figure 1 The effects of NAR on the survival rate of ACT-1 cells

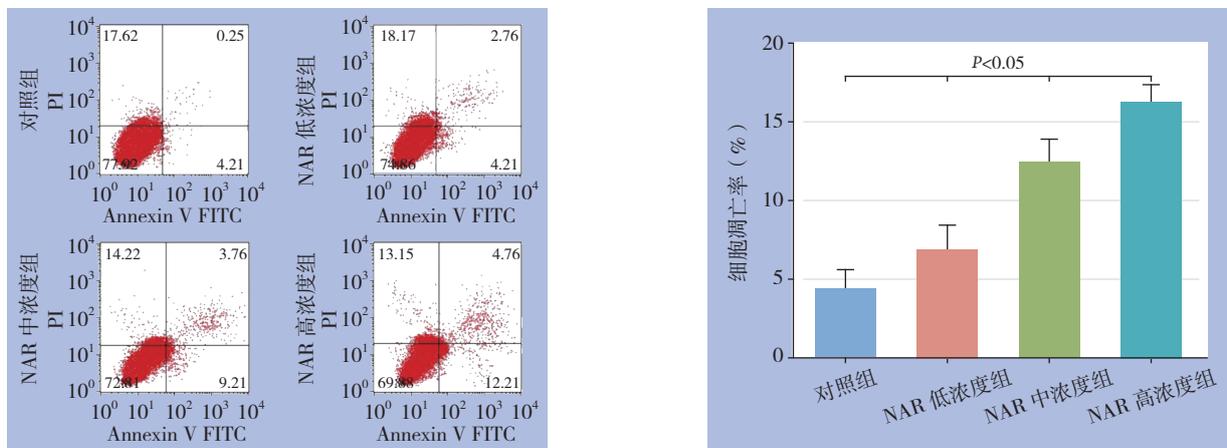


图2 NAR对ACT-1细胞凋亡的影响

Figure 2 Effect of NAR on the apoptosis of ACT-1 cells

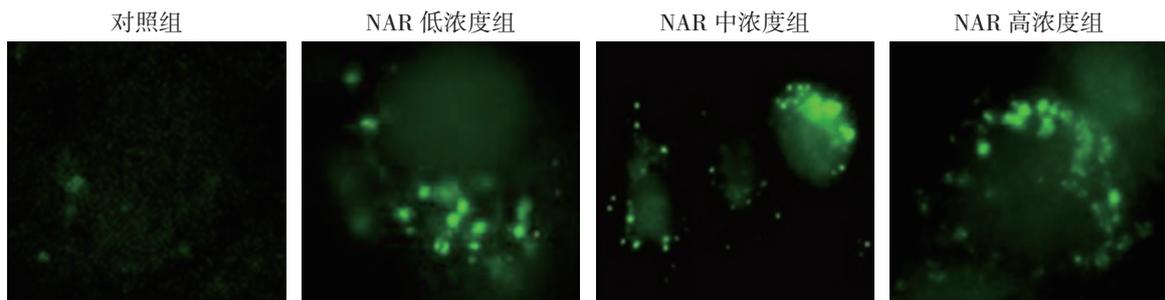


图3 NAR对ACT-1细胞自噬的影响(绿色荧光代表自噬小体)(×1000)

Figure 3 Effect of NAR on autophagy of ACT-1 cells (green fluorescence showing hte autophagosomes) (×1000)

2.4 NAR对自噬相关蛋白LC3II、LC3I和p62蛋白表达的影响

与对照组比较, 3个NAR处理组ACT-1细胞LC3II/LC3I蛋白表达水平均明显升高, 而p62蛋白表达水平均明显降低, 均呈浓度依赖性(均 $P < 0.05$) (图4)。

2.5 NAR对ACT-1细胞p-AMPK、AMPK、p-mTORC1和mTORC1蛋白表达的影响

与对照组比较, 3个NAR处理组ACT-1细胞

p-AMPK/AMPK蛋白表达水平均明显升高, 而p-mTORC1/mTORC1蛋白表达水平均明显降低, 均呈浓度依赖性(均 $P < 0.05$) (图5)。

2.6 NAR与AMPK抑制剂共处理对各组细胞凋亡和自噬蛋白的影响

与空白对照组比较, NAR组p-AMPK/AMPK、LC3II/LC3I、caspase-3蛋白表达明显升高, p-mTORC1/mTORC1和p62蛋白表达明显降低(均 $P < 0.05$) ; AMPK抑制剂组p-AMPK/AMPK、

LC3II/LC3I、caspase-3 蛋白表达明显降低, p-mTORC1/mTORC1 和 p62 蛋白表达明显升高 (均 $P < 0.05$)。与 NAR 组比较, NAR+AMPK 抑制剂组 p-AMPK/AMPK、LC3II/LC3I、caspase-3 蛋白表达明显降低, p-mTORC1/mTORC1 和 p62 蛋白

表达明显升高 (均 $P < 0.05$)。与 AMPK 抑制剂组比较, NAR+AMPK 抑制剂组 ACT-1 细胞 p-AMPK/AMPK、LC3II/LC3I、caspase-3 蛋白表达明显升高; p-mTORC1/mTORC1 和 p62 蛋白表达明显降低 (均 $P < 0.05$) (图6)。

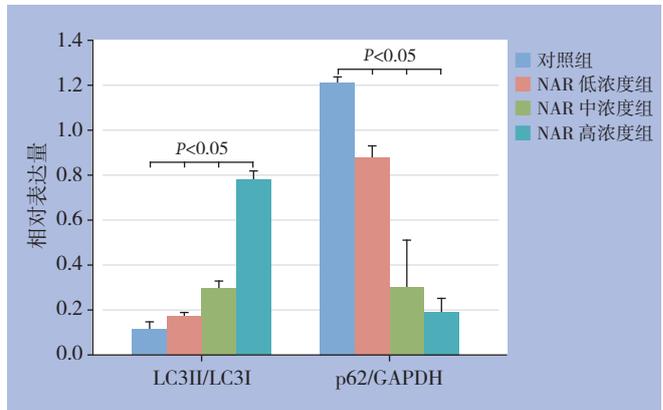
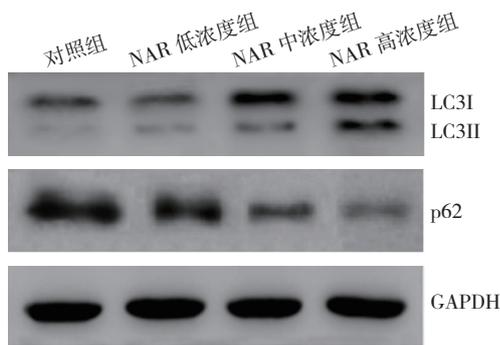


图4 各组 ACT-1 细胞 LC3II/LC3I 和 p62 蛋白表达情况

Figure 4 The expressions of LC3II/LC3I and p62 protein in each group of ACT-1 cells

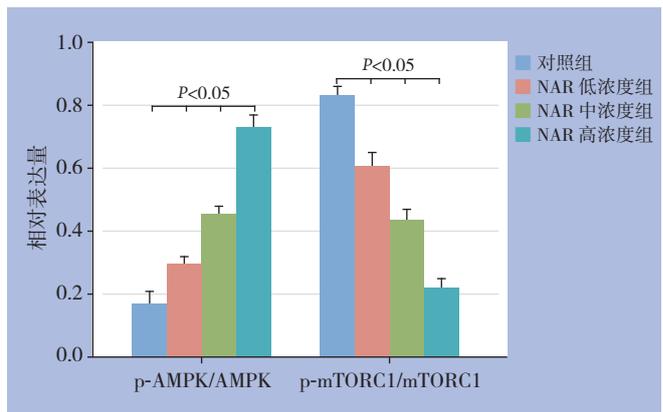
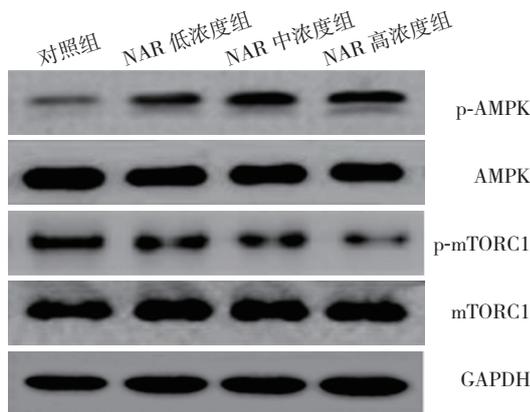


图5 各组 ACT-1 细胞 p-AMPK、AMPK、p-mTORC1 和 mTORC1 表达情况

Figure 5 Expressions of p-AMPK, AMPK, p-mTORC1 and mTORC1 in each group of ACT-1 cells

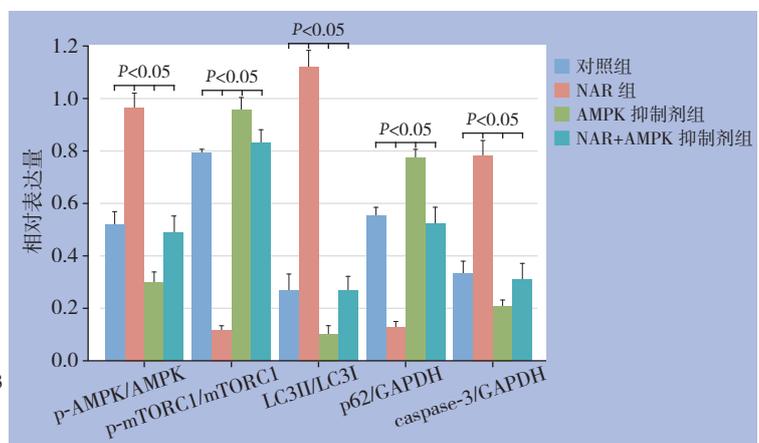
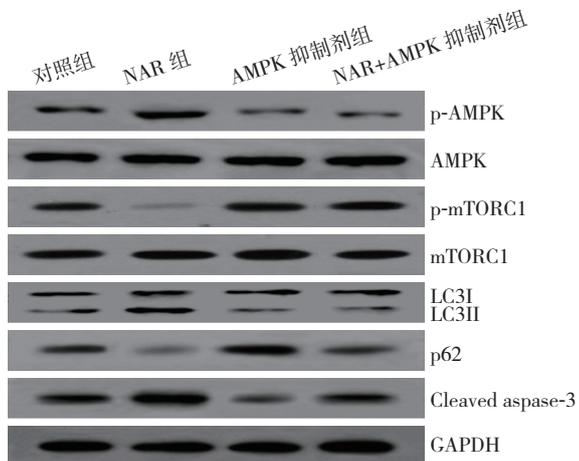


图6 各组 ACT-1 细胞 p-AMPK/AMPK、p-mTORC1/mTORC1、LC3II/LC3I、p62 和 caspase-3 蛋白表达情况

Figure 6 Expressions of p-AMPK/AMPK, p-mTORC1/mTORC1, LC3II/LC3I, p62 and caspase-3 in each group of ACT-1 cells

3 讨论

目前,手术和外科手术加放射性碘治疗(标准治疗)甲状腺癌会产生永久并发症,严重影响患者的生活质量^[10]。此外,甲状腺后遗症可能在数年至数十年随访后出现,且它们产生的临床症状可能是惰性或非特异性的,给临床治疗带来挑战^[11-12]。因此积极探索更有效的治疗药物以代替放射治疗可能有助于改善甲状腺癌的治疗现状。NAR作为一种柚皮苷衍生物,由于其广泛的生物活性,而成为抗肿瘤方面研究的热点^[13]。曾德贵等^[14]发现,NAR能够抑制人肺鳞癌细胞增殖。本研究结果显示,ACT-1细胞经不同浓度NAR处理后,细胞存活率呈浓度依赖性下降,提示NAR对甲状腺癌细胞增殖具有抑制作用。

自噬是一种自降解系统,在癌症治疗过程中,它可以保护癌细胞免受化学疗法的侵害,也可以杀死凋亡途径不活跃的癌细胞^[15]。此外,药物和饥饿等因素干预能够改变细胞自噬能力,以维持细胞稳态,参与组织发育、免疫调节和肿瘤发生发展等生理病理过程^[16]。LC3I和LC3II是LC3的2种不同表现形式,在自噬形成期间,LC3I可以转化LC3II,因此LC3II含量与自噬膜囊泡数量呈正相关,通常以LC3II/LC3I比值作为自噬小体标记物来评价自噬的形成^[17-18]。p62是细胞自噬的特异性底物,可用于检测自噬通量,其表达水平与自噬活性成反比^[19]。研究^[6]显示,NAR能够诱导白血病HL-60细胞自噬,抑制白血病的发展。本研究发现,NAR处理ACT-1细胞后,细胞凋亡率和LC3II/LC3I蛋白显著增加,p62蛋白显著降低,提示NAR干预后诱导了LC3I向LC3II转变。荧光显微镜下观察,NAR干预使绿色荧光富集,且绿色荧光强度随NAR浓度增加而增加,进一步表明NAR能够促进ACT-1细胞自噬体的形成。

细胞凋亡受细胞内外信号调节,其特征在于死亡靶细胞形态、线粒体膜通透性和凋亡小体等形成,该过程可维持细胞内环境的稳定,是细胞发育和程序性死亡的主要机制,在调控肿瘤发生发展中至关重要^[20-21]。近期研究显示^[9,13,22-23],NAR能够促进宫颈癌、胃癌、舌鳞癌及肝癌细胞凋亡。本研究用NAR处理ACT-1细胞后经流式分析发现,NAR可诱导细胞发生凋亡,且随NAR浓度的增加,凋亡率增加,提示NAR可诱导凋亡的发生。此外,自噬与凋亡之间存在的平衡过程在细

胞存活和死亡中至关重要^[24-25],因此推测NAR可能通过诱导ACT-1细胞自噬,从而促进其凋亡,发挥对甲状腺细胞的毒性作用,但其具体作用机制尚不清楚。

AMPK是一种重要的能量因子,可以协调细胞代谢途径,平衡营养供应和能量需求,在控制人类疾病中至关重要^[26]。mTOR是细胞生长调节剂,能够形成mTORC1和mTORC2复合物,且由于其在癌症中被高度激活,成为提高癌症治疗效率的靶标^[27]。此外,在胰腺癌细胞中,激活AMPK信号通路,能够降低mTOR磷酸化水平,诱导细胞自噬,抑制增殖^[28]。徐琛莹等^[29]发现,雌激素受体 β 能够通过激活AMPK/mTORC1轴,诱导自噬发生。本研究结果显示,ACT-1细胞经不同浓度NAR作用后,p-AMPK/AMPK蛋白表达显著升高,p-mTORC1/mTORC1蛋白表达显著降低,提示NAR可能通过调节AMPK/mTORC1信号通路,诱导甲状腺癌细胞自噬。caspase-3是细胞凋亡的效应蛋白,其受到凋亡刺激后会被激活,因而被称为“凋亡的执行官”^[30]。为进一步验证AMPK/mTORC1信号通路在NAR治疗过程中的作用,使用AMPK抑制剂和NAR共同干预甲状腺癌细胞后发现,AMPK抑制剂抑制了NAR作用下甲状腺癌细胞LC3II/LC3I和cleaved caspase-3蛋白表达,并促进p62蛋白表达水平,提示AMPK抑制剂可逆转NAR对甲状腺癌细胞自噬和凋亡的诱导作用,推测NAR能够诱导甲状腺癌细胞自噬和凋亡,可能与调控AMPK/mTORC1轴相关。

综上所述,NAR能够抑制甲状腺癌细胞增殖,促进自噬,并诱导细胞凋亡,可能与激活AMPK活化,抑制mTORC1蛋白表达相关,表明NAR在治疗甲状腺癌方面的潜在价值,可能为甲状腺癌治疗提供新思路。但临床甲状腺癌的发生涉及家族遗传等多方面因素,且具有转移和侵袭性,因此针对NAR对其治疗效果及作用机制,仍需联合动物模型和临床试验进行探索。

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