沉默YAP逆转肺癌PC9细胞多柔比星耐药性及其机制研究

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［摘要］ **背景与目的：**耐药性是导致肺癌患者化疗失败的主要原因。探讨YAP对人肺癌PC9细胞多柔比星耐药的逆转作用及其机制。**方法：**利用体外筛选方法从多柔比星敏感性肺癌细胞系PC9获得耐药细胞克隆，并检测YAP的表达水平；利用shRNA沉默细胞中YAP的表达，应用MTS法检测肿瘤细胞药物敏感性，流式细胞术检测细胞周期、凋亡及对Rh-123的吸收能力，蛋白［质］印迹法(Western blot)和实时定量聚合酶链式反应(quantitative real-time polymerase chain reaction，QRT-PCR)技术检测ABCB1、ABCC1、p53、Runx2、ITGB2和ErbB4的表达水平及丝氨酸/苏氨酸蛋白激酶(serine/threonine kinase，AKT)的磷酸化水平变化。**结果：**经体外诱导获得多柔比星耐药细胞克隆PC9/Adr，且YAP蛋白在其中高表达，利用shRNA得到不同YAP沉默程度的PC9/Adr。YAP沉默后，细胞生长速度降低，细胞对多柔比星的敏感性显著增加，细胞周期被阻滞在G_0/G_1期，多柔比星诱导的细胞凋亡增多，细胞吸收Rh-123也增多，并与YAP的沉默程度呈正相关。Western blot和QRT-PCR结果显示，YAP沉默后，ABCB1、ABCC1、Runx2、ITGB2和ErbB4蛋白表达下调，而p53的表达上调，AKT的磷酸化水平则下降。**结论：**YAP过表达与PC9/Adr的耐药性相关，沉默YAP可恢复PC9/Adr对多柔比星的敏感性。这一作用与调节耐药相关基因的表达、促进细胞凋亡有关。

［关键词］ YAP；肺癌；多柔比星耐药

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YAP silencing reverses doxorubicin resistance in lung cancer cell line PC9 and its mechanism

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［**Abstract**］ **Background and purpose:** Drug resistance is a major cause of failure in lung cancer chemotherapy. This study aimed to investigate the effect of YAP on doxorubicin resistance in lung cancer and its underlying mechanism. **Methods:** Doxorubicin resistant lung cancer cell clones were established from parental sensitive cancer PC9 cell line via *in vitro* induction, and the expression of YAP was analyzed. YAP was down-regulated via shRNA to different levels. MTS assay was employed to determine cell proliferation and drug sensitivity. Flow cytometry was used to determine cell cycle distribution, apoptosis and uptake of Rh-123. Western blot and quantitative real-time polymerase chain reaction (QRT-PCR) assay were used to determine the expression of ABCB1, ABCC1, p53, Runx2, ITGB2 and ErbB4. The phosphorylation of serine/threonine kinase (AKT) was determined as well. **Results:** Doxorubicin resistant PC9/Adr cell clone was obtained with over-expressed YAP. Expression of YAP in PC9/Adr cells was down-regulated to different levels via shRNA. After YAP silencing, cell proliferation was reduced, while sensitivity to doxorubicin was increased. The cell cycle was significantly halted by G_0/G_1 phase. Doxorubicin induced-apoptotic rate and cellular uptake of Rh-123 were increased,

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with positive correlation to YAP silencing level. Western blot and QRT-PCR results showed that after YAP silencing, ABCB1, ABCC1, Runx2, ITGB2, and ErbB4 proteins were down-regulated, while the expression of p53 was up-regulated. Phosphorylation of AKT was inhibited as well. **Conclusion:** Over-expression of YAP is involved in doxorubicin resistance in PC9/Adr cell line. Silencing of YAP could restore doxorubicin sensitivity. The mechanism involves regulation of drug resistance-related genes and promotion of apoptosis.

[**Key words**] YAP; Lung cancer; Doxorubicin resistance

肺癌是一种常见的恶性肿瘤，已成为当前社会的重大公共卫生问题。肺癌的病因是多方面的，其中之一是家族遗传因素，携带*BACR*1和*BACR*2基因突变的个体具有更大患病倾向[1]。肺是维持人体生命活动的重要器官，早期肺癌可通过手术切除进行治疗，但肺癌细胞往往容易转移，一旦其随血液或淋巴液扩散至全身并在肺、骨和脑等部位形成转移灶，则难以治疗，危及生命[2]。对于发生转移不能采用手术治疗的肺癌，需用药物治疗。多柔比星是常用的化疗药物之一，但肺癌往往出现化疗药物耐受性，导致化疗失败，因此寻找克服肺癌耐药的靶点非常关键。

YAP是一种转录共活化因子，是Hippo信号通路中的重要成员。YAP可结合多个转录因子，包括p73、Runx2和TEADs[3]。有研究显示，YAP过表达与前列腺癌、卵巢癌、肝癌和肺癌等多种实体瘤的预后不良相关[4-7]。目前，Hippo/YAP信号通路在癌症中作用的研究，主要集中于YAP可促进细胞生长，阻止细胞凋亡。最近有研究发现，YAP在逆转肿瘤细胞多柔比星等抗肿瘤药物耐药中同样发挥作用[8-9]。因此，本研究探索YAP在多柔比星耐药性肺癌中的作用，并对其分子机制做初步探讨和验证。

1 材料和方法

1.1 主要试剂与仪器

人肺癌PC9细胞系购自中国科学院上海生命科学研究院生物化学与细胞生物学研究所细胞库；shRNA介导沉默的病毒载体及相关试剂购自上海吉玛制药技术有限公司；MTS和实时定量聚合酶链式反应(quantitative real-time polymerase chain reaction，QRT-PCR)试剂盒购自美国Promega公司；流式细胞检测试剂盒和流式细胞仪购自美国BD公司；单克隆抗体购自美国Santa Cruz公司；ECL免疫印迹底物试剂盒购自美国Millipore公司；酶标仪和QRT-PCR仪购自美国Thermo公司。

1.2 细胞培养及高转移细胞系的诱导

人肺癌PC9细胞培养于37 ℃、CO_2体积分数为5%的饱和湿度培养箱中，培养基中添加10%胎牛血清。0.25%胰酶-EDTA消化传代，所有实验均采用对数生长期细胞。为了获得多柔比星耐药细胞系，将PC9细胞培养于含10 nmol/L多柔比星的培养基中。1 d后，更换为不含多柔比星的培养基中，恢复培养6 d。以7 d为1个周期，每次倍增多柔比星的浓度，重复上述步骤，直至细胞克隆能耐受2 μmol/L以上的多柔比星。

1.3 shRNA沉默YAP

采用2个不同的shRNA(shRNA1和shRNA2)以实现不同的YAP沉默效果。shRNA1和shRNA2[10]的序列分别为5'-CCCAGTTAAATGTTCACCAAT-3'和5'-GCCACCAAGCTAGATAAAGAA-3'，shControl购自Santa Cruz公司，整合于慢病毒转染载体LV-3/GFP+Puro(购自上海吉玛制药技术有限公司)中。按照试剂操作说明书进行转染并筛选出转染成功的细胞。

1.4 MTS法检测

取对数生长期的细胞，以1×10^5个/mL接种到96孔微孔板中，每孔100 μL，培养过夜使细胞贴壁，分别加入0、0.2、0.5、1、2、3和5 μmol/L多柔比星，继续培养72 h，吸去培养基，按照试剂说明书的要求加入MTS，继续培养4 h。最后，用酶标仪测定490 nm波长下的吸光度(*D*)值，以*D*值表示细胞的数量。计算药物对细胞的抑制率，抑制率 = (1-实验组*D*值/溶剂对照组*D*值) × 100%。

1.5 流式细胞检测

取对数生长期的细胞，胰酶消化收集后，用PBS重选至10^6个/mL。对于细胞周期试验，采用PI染色法，先将细胞用70%乙醇固定2 h，洗去乙醇后，用含有50 μg/mL RNaseA、1% Triton X-100和40 μg/mL PI(购自美国BD公司)的染液染色30 min，上机检测。对于细胞凋亡试验，将收集后的细胞直接采用Annexin V-FITC/PI染色，根据试剂盒说明书进行。对于细胞吸收Rh-123试验，将细胞用10 μmol/L的Rh-123(购自美国Sigma公司)染色1 h，然后上机检测。

1.6 蛋白［质］印迹法(Western blot)

收集细胞裂解提取蛋白，以12%SDS-PAGE法分离蛋白质，然后转移至PVDF膜上，以不同的抗体检测目标蛋白(4 ℃过夜)。洗去一抗，以HRP连接的二抗温育1 h，洗涤，以ECL试剂盒显示免疫反应条带。β-actin作为内参，检测细胞中YAP、ABCB1、ABCC1、p53、Runx2、ITGB2和ErbB4的表达水平及丝氨酸/苏氨酸蛋白激酶(serine/threonine kinase，AKT)的磷酸化水平。

1.7 QRT-PCR

用Trizol法提取各组总RNA，用QRT-PCR试剂盒进行反转录得到cDNA，然后检测其中YAP、ABCB1、ABCC1、p53、Runx2、ITGB2和ErbB4的mRNA水平。YAP上游引物序列：5'-TGAACAAACGTCCAGCAAGATAC-3'，下游引物序列：5'-CAGCCCCCAAAATGAACAGTAG-3'；ABCB1上游引物序列：5'-AAAAAGATCAACTCGTACCACTC-3'，下游引物序列：5'-GCACAAAATACACCAACAA-3'；ABCC1上游引物序列：5'-CTGGGAACATGATTAGGAAGC-3'，下游引物序列：5'-GAGGATTTCCCAGAGCCGAC-3'；p53上游引物序列：5'-GCCCAACAACACCAGCTCC-3'，下游引物序列：5'-CCTGGGCATCCTTGAGTTCC-3'；Runx2上游引物序列：5'-TTACTTACACCCCGCCAGTC-3'，下游引物序列：5'-TATGGAGTGCTGCTGGTCTG-3'；ITGB2上游引物序列：5'-CAGGTGTGACACTGGCTACAT-3'，下游引物序列：5'-CTGCCCGTATATCAGCTTGCC-3'；ErbB4上游引物序列：5'-GCAAGAATTGACTCGAATA-3'，下游引物序列：5'-CTGGAATTGTGCTAGTTG-3'；GAPDH上游引物序列：5'-CTTAGATTTGGTCGTATTGG-3'，下游引物序列：5'-GAAGATGGTGATGGGATT-3'。

1.8 统计学处理

实验数据以$\bar{x}\pm s$表示，使用SPSS 13.0软件进行分析。采用单因素方差分析(One-way ANOVA)进行比较，$P<0.05$为差异有统计学意义。

2 结 果

2.1 构建多柔比星耐受PC9/Adr细胞，检测细胞YAP表达

经过体外筛选后，成功获得耐受多柔比星且高表达YAP的细胞系，命名为PC9/Adr。MTS试验显示，多柔比星抑制PC9和PC9/Adr的半抑制浓度(IC_{50})值分别为0.15和2.3 μmol/L。Western blot结果显示，PC9/Adr细胞中YAP表达高于PC9细胞(图1)。

2.2 ShRNA沉默后YAP在多柔比星耐受PC9/Adr细胞中的表达

成功获得shRNA转染细胞克隆，Western blot和QRT-PCR技术检测显示，经shRNA1和shRNA2沉默后，PC9/Adr细胞中的YAP蛋白和mRNA表达水平显著下降，而shControl组与PC9/Adr母细胞组相比差异无统计学意义(图2)。

2.3 YAP沉默显著增加肿瘤细胞多柔比星敏感性

MTS细胞增殖实验显示，shRNA1和shRNA2下调PC9/Adr细胞中YAP表达水平的同时，细胞对多柔比星的敏感性显著提高，IC_{50}值分别为0.53和0.32 μmol/L(图3)。

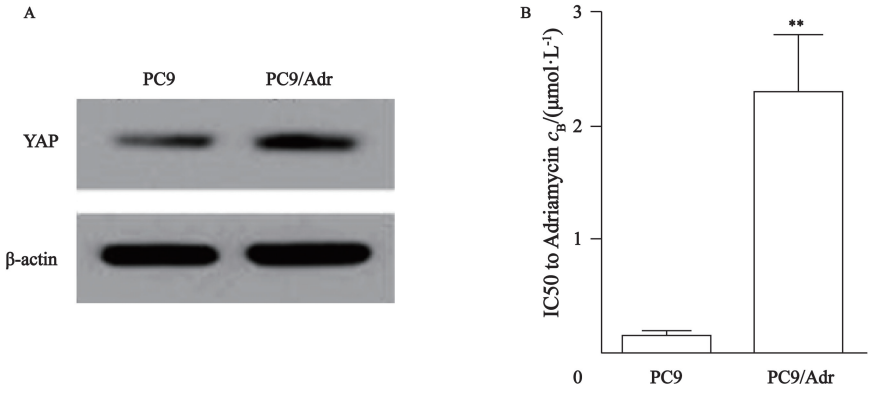

图 1　YAP在多柔比星耐受PC9/Adr细胞中的表达

Fig. 1　The expression of YAP in doxorubicin resistance PC9/Adr cells

A: The protein expression of YAP in doxorubicin resistance PC9/Adr cells; β-actin was used as an internal control. B: The IC_{50} of PC9 cells and PC9/Adr cells for doxorubicin; **: P<0.01 as compared with PC9 cells

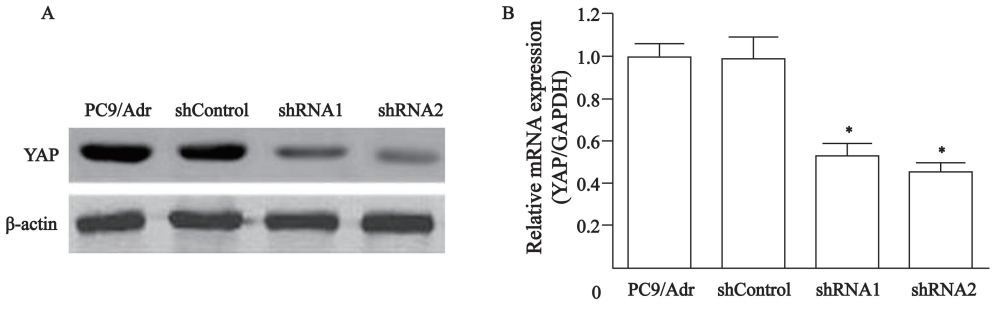

图 2　ShRNA沉默后YAP在多柔比星耐受PC9/Adr细胞中的表达

Fig. 2　The expression of YAP in doxorubicin resistance PC9/Adr cells after shRNA silencing

A: The protein expression of YAP in doxorubicin resistance PC9/Adr cells after shRNA silencing; β-actin was used as an internal control. B: The mRNA expression of YAP in doxorubicin resistance PC9/Adr cells after shRNA silencing; GAPDH was used as an internal control; *: P<0.05, as compared with PC9/Adr cells

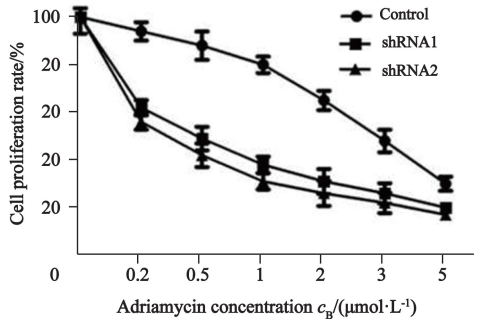

图 3　YAP沉默显著增加肿瘤细胞多柔比星敏感性

Fig. 3　YAP silencing improves doxorubicin sensitivity to tumor cells significantly

2.4　YAP沉默阻滞细胞周期，促进多柔比星诱导的细胞凋亡，增加细胞内Rh-123含量

YAP沉默后，相比对照组，可见G_0/G_1期细胞比例升高。而用0.5 μmol/L多柔比星处理细胞24 h后，相比对照组，shRNA1和shRNA2组细胞凋亡比例显著升高，同时，shRNA1和shRNA2组细胞内Rh-123荧光强度显著增强，说明细胞对Rh-123的吸收增加(图4)。

2.5　YAP沉默下调肿瘤细胞中ABCB1、ABCC1、Runx2、ITGB2和ErbB4的表达，上调p53的表达，抑制AKT磷酸化

Western blot结果显示，与对照组相比，在shRNA1和shRNA2组细胞中，与药物运输相关的蛋白ABCB1和ABCC1表达水平下降，促进细胞生长和抗凋亡的Runx2、ITGB2和ErbB4蛋白表达水平亦下降，抑癌基因*p*53的表达上调(图5A)。QRT-PCR结果显示，与对照组相比，在shRNA1和shRNA2组细胞中ABCB1、ABCC1、Runx2、ITGB2和ErbB4 mRNA表达显著下调，p53 mRNA表达显著上调(图5B)。Western blot结果显示，YAP沉默之后，AKT磷酸化水平显著下降，而总AKT的表达未受显著的影响(图5C)，说明AKT信号通路的活性受到抑制。

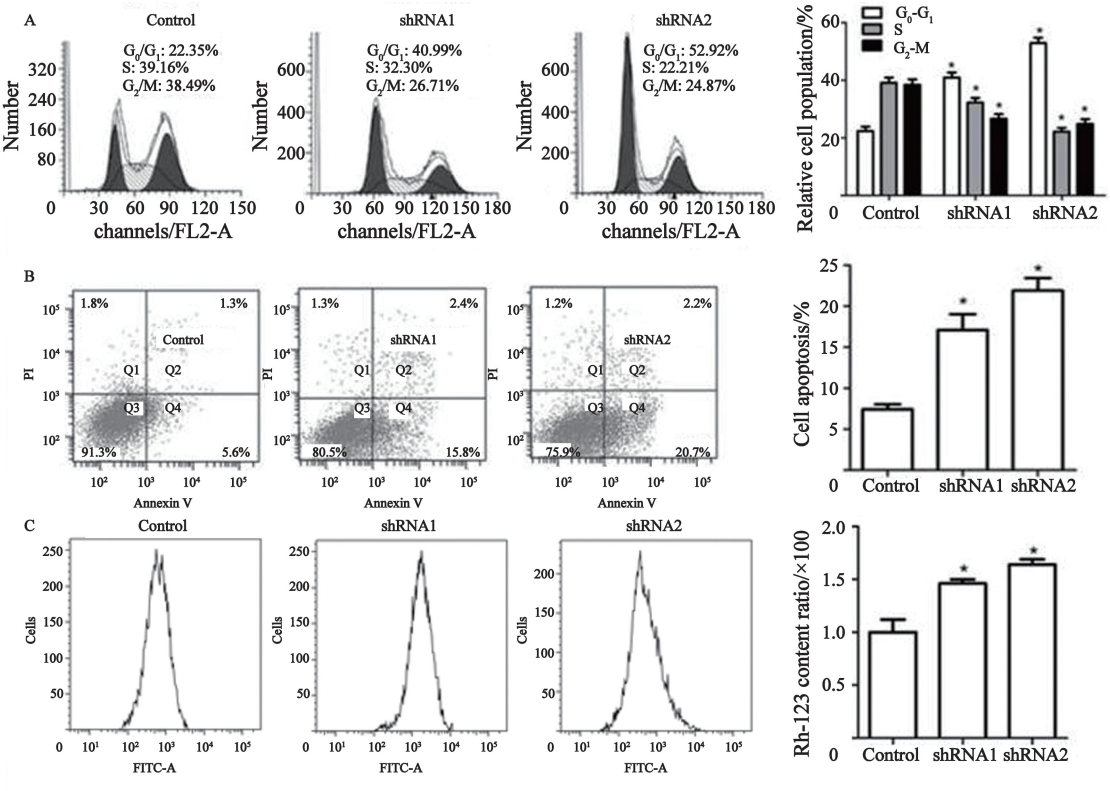

图 4 YAP沉默阻滞细胞周期，促进多柔比星诱导的细胞凋亡，增加细胞内Rh–123含量

Fig. 4 YAP silencing halts cell cycle, induces apoptosis and improves cellular content of Rh-123

A: YAP silencing halted cell cycle by G_0/G_1 phase; B: YAP silencing induced cell apoptosis by doxorubicin; C: YAP silencing improved cellular content of Rh-123; *: $P<0.05$, as compared with control group

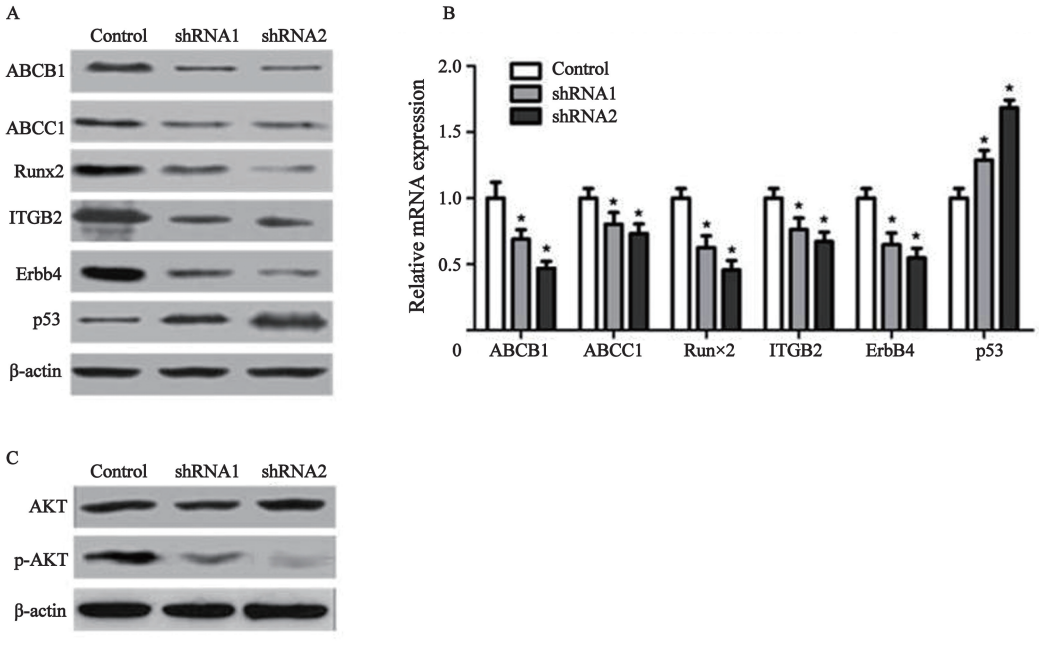

图 5 YAP沉默对细胞增殖及细胞凋亡相关基因的影响

Fig. 5 The effect of YAP silencing on the tumor proliferation and apoptosis related gene

A: YAP silencing down-regulated the protein expression of ABCB1, ABCC1, Runx2, ITGB2 and ErbB4, up-regulated the protein expression of p53 in tumor cells; β-actin was used as an internal control. B: YAP silencing down-regulated the mRNA expression of ABCB1, ABCC1, Runx2, ITGB2 and ErbB4, up-regulated the mRNA expression of p53 in tumor cells; GAPDH was used as an internal control; *: $P<0.05$, as compared with control group. C: YAP silencing suppressed the phosphorylation of AKT in tumor cells; β-actin was used as an internal control

3 讨 论

在本研究中，我们首先获得了肺癌耐受多柔比星且过表达YAP2的细胞系PC9/Adr，进而采用shRNA转染，得到YAP沉默细胞克隆。MTS显示，YAP沉默后，肺癌细胞对多柔比星的敏感性提高。结果表明，YAP在PC9/Adr细胞多柔比星耐受中发挥重要作用。因此，进一步探讨和验证沉默YAP逆转PC9/Adr细胞耐药性的机制。首先对YAP沉默后细胞的周期分布进行分析，流式细胞分析结果显示，YAP沉默后G_0/G_1期的比例升高。Western blot结果显示，在沉默YAP之后，p53蛋白的表达水平升高。*p*53是抑癌基因，可将细胞周期阻滞在G_1期，同时也可诱导细胞凋亡[11-12]。然而，将细胞阻滞在G_0/G_1期可能并不是沉默YAP逆转耐药性的直接原因。肿瘤细胞耐药的另一种常见机制是在膜上过表达药物转运蛋白，如ABCB1和ABCC2，将药物泵出细胞外，减少胞内药物浓度[13]。先利用流式细胞术检测细胞对Rh-123的吸收能力，结果显示，耐药细胞中Rh-123的吸收下降，而YAP沉默后，细胞内Rh-123含量明显增高。因为Rh-123同为ABCB1和ABCC2的运送底物，所以细胞对Rh-123的吸收可以间接地反映细胞对药物的吸收。我们又通过Western blot和QRT-PCR检测细胞中ABCB1和ABCC2的表达，证实他们的表达受YAP的调节。YAP作为转录共活化蛋白，可与转录因子TEADs结合，介导*RUNX*2、*ITGB*2和*ErbB*4等下游基因的表达[14]。在肿瘤细胞中，*RUNX*2过表达促进细胞增殖，促进表皮样到间质样转变，临床研究发现，*RUNX*2过表达与患者预后不良密切相关[15-16]，而沉默或抑制*RUXN*2活性可抑制肿瘤生长[17]。有研究显示，在肿瘤细胞中YAP可上调ITGB2表达，促进肿瘤细胞生长[18]。ErbB4则常与其同家族的成员相互作用，在肿瘤的生长、恶化和耐药性中发挥重要作用[19-21]。本研究显示，PC9/Adr高表达此类蛋白，而沉默YAP后它们的表达均下降，暗示它们可能在PC9/Adr的耐药中发挥作用。

在肿瘤的发生、发展中发挥重要作用的AKT信号通路也与Hippo/YAP信号通路关系密切[22]。在结肠癌细胞中发现，二基甲烷以AKT依赖的方式调节YAP活性，抑制肿瘤细胞生长[23]。本研究显示，沉默YAP后，AKT信号通路的活性受抑制，说明后者在YAP介导的多柔比星耐药中也发挥作用。

综上所述，YAP在PC9/Adr的多柔比星耐药中发挥作用，沉默YAP可逆转其耐药性，其机制可能与通过调节耐药相关基因的表达、促进细胞凋亡有关。

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