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华蟾素调控AKT介导的上皮间质转化抑制肝细胞癌肺转移裸鼠模型的作用机制

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摘要: 目的 研究华蟾素通过调控肝细胞癌(HCC)上皮间质转化(EMT)抑制HCC转移的作用和机制。**方法** 将36只6周龄雄性BALB/c裸鼠尾静脉注射MHCC97H细胞建立肝癌肺转移瘤模型,随机分为华蟾素高、低剂量组和对照组。建模当日起分别腹腔注射华蟾素120 μL/kg、60 μL/kg或生理盐水,每周2次。8周后取肺组织行HE染色检测肝癌肺转移率。MHCC97H细胞用华蟾素高、低剂量(2.5 μL/mL、5 μL/mL)干预,通过划痕实验、RT-PCR以及Western Blot检测细胞迁移能力和EMT相关分子的表达。用$CoCl_2$孵育模拟低氧环境诱导MHCC97H细胞,同时加入高、低剂量华蟾素干预,通过划痕实验和Western Blot检测华蟾素对低氧诱导的细胞迁移能力和EMT的影响。使用转录组学分析华蟾素对MHCC97H细胞的效应机制。用Western Blot检验华蟾素干预对MHCC97H细胞的蛋白激酶B(AKT)、磷酸化AKT(P-AKT)表达水平的影响。计量资料多组间比较采用单因素方差分析,进一步两两比较采用LSD-t检验,两组间比较采用成组t检验。**结果** 华蟾素干预组裸鼠较对照组肝癌肺转移率下降。与对照组相比,华蟾素干预使MHCC97H细胞划痕愈合率减小、上皮型分子表达上调(t=2.860,P<0.05),并使EMT转录因子和基质型分子下调(t值分别为3.545、2.022、2.852、2.341,P值均<0.05)。低氧诱导上调MHCC97H细胞划痕愈合率和基质型分子、EMT转录因子表达水平(P值均<0.05),华蟾素干预逆转EMT变化并抑制划痕愈合(P值均<0.05)。肝癌细胞转录组学分析显示,华蟾素组与对照组存在显著的基因差异,华蟾素主要影响了肿瘤、代谢、免疫和信号传导相关基因表达,其中AKT信号转导通路中的差异基因数量最多。进一步检测发现华蟾素干预可下调HCC细胞AKT、P-AKT和P-AKT/AKT的水平(t值分别为2.434、3.401、2.258,P值均<0.05)。**结论** 华蟾素可抑制肝癌转移,尤其对于低氧环境诱导的肝癌转移具有显著抑制作用,调控AKT信号转导通路介导的HCC细胞EMT可能是其部分作用机制。

关键词: 癌, 肝细胞; 肿瘤转移; 华蟾素; 上皮-间质转化

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Mechanism of action of cinobufotalin in inhibiting lung metastasis of hepatocellular carcinoma by regulating AKT-mediated epithelial-mesenchymal transition in a nude mouse model

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Abstract: Objective To investigate the effect and mechanism of cinobufotalin in inhibiting hepatocellular carcinoma (HCC) metastasis by regulating epithelial-mesenchymal transition (EMT). **Methods** A total of 36 male BALB/c nude mice, aged 6 weeks,

were given injection of MHCC97H cells via the caudal vein to establish a model of HCC lung metastasis, and then the mice were randomly divided into high-and low-dose cinobufotalin groups and control group. Since the day of modeling, the mice in the high-and low-dose cinobufotalin groups were given intraperitoneal injection of cinobufotalin at a dose of 120 μL/kg and 60 μL/kg, respectively, and those in the control group were given intraperitoneal injection of normal saline, twice a week. After 8 weeks, HE staining was performed for lung tissue to measure the lung metastasis rate of HCC. MHCC97H cells were treated with high-dose (2.5 μL/mL) or low-dose (5 μL/mL) cinobufotalin for 24 hours, and wound healing assay, RT-PCR, and Western blot were used to measure cell migration ability and the expression of EMT-related molecules. MHCC97H cells were induced in a simulated hypoxic environment with $CoCl_2$ incubation, with high- and low-dose cinobufotalin added for intervention, and wound healing assay and Western blot were used to investigate the effect of cinobufotalin on cell migration ability and EMT induced by hypoxia. Transcriptome analysis was used to investigate the effect mechanism of cinobufotalin on MHCC97H cells, and Western blot was used to observe the effect of cinobufotalin on the expression levels of protein kinase B (AKT) and phosphorylated AKT (P-AKT) in MHCC97H cells. A one-way analysis of variance was used for comparison of continuous data between multiple groups, and the least significant difference *t*-test was used for further comparison between two groups; the independent-samples *t* test was used for comparison of categorical data between two groups. **Results** Compared with the control group, the cinobufotalin group had a significant reduction in the lung metastasis rate of HCC. Compared with the control group, cinobufotalin intervention reduced the wound healing rate of MHCC97H cells, upregulated the expression of epithelial-type molecules (t=2.860, P<0.05), and downregulated the expression of EMT transcription factors (EMT-TFs) and mesenchymal molecules (t=3.545, 2.022, 2.852, and 2.341, all P<0.05). Hypoxia induction upregulated the wound healing rate of MHCC97H cells and the expression levels of mesenchymal molecules and EMT-TFs (P<0.05), and cinobufotalin intervention reversed EMT change and inhibited wound healing (P<0.05). The transcriptome analysis of MHCC97H cells showed significant gene differences between the cinobufotalin group and the control group, and cinobufotalin mainly affected the expression of genes associated with tumor, metabolism, immunity, and signal transduction, with the largest number of differentially expressed genes in the AKT signal transduction pathway. Further measurement showed that cinobufotalin intervention downregulated the expression levels of AKT, P-AKT, and P-AKT/AKT in MHCC97H cells (t=2.434, 3.401, and 2.258, all P<0.05). **Conclusion** Cinobufotalin can inhibit the metastasis of HCC, especially hypoxia-induced HCC metastasis, and regulation of EMT mediated by the AKT signal transduction pathway in HCC cells might be one of its mechanisms of action.

Key words: Carcinoma, Hepatocellular; Neoplasm Metastasis; Hua Chan Su; Epithelial-Mesenchymal Transition

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肝癌是全球第六常见癌症和第三癌症相关死亡原因，在我国占癌症死因的第二位，肝细胞癌（HCC）是其中最常见的类型[1-2]。HCC进展迅速，且具有高度侵袭转移能力，易发生肝内外转移，能通过手术切除患者仅占10%～15%，并且术后转移复发率高[3-4]，导致大多数HCC患者预后较差。近年来研究[5-6]显示，肿瘤细胞上皮间质转化（epithelial-to-mesenchymal transition，EMT）是其侵袭转移能力增强的重要机制，成为现代抗肿瘤转移治疗研究的关键靶标。有多种因素可诱导HCC细胞发生EMT，如炎性微环境、肝纤维化、代谢应激等，其中低氧是包括HCC在内的实体瘤EMT的常见诱因[7]。

中医药在我国HCC治疗中效果确切，单独或联合其他治疗具有抑制进展、减少复发转移、延长生存期等作用。中医学认为肝癌主要病机为本虚标实、正虚邪实，正虚以脾虚为突出，邪实以邪气内蕴、热毒血瘀等为突出，运用清热解毒、疏肝理气、活血散结等治法。蟾蜍在《新修本草》记载："主邪气，破症坚血，痈肿，阴疮，服之不患热病"。华蟾素自中华大蟾蜍提取，是我国自行研制的二类新药和国家中药保护品种，具有清热解毒、化瘀溃坚、利水消肿、止痛等功效，广泛应用于包含HCC在内的中晚期肿瘤的治疗。华蟾素联合肝动脉插管灌注化疗栓塞疗效优于常规药物，并且能够减轻肝癌患者肝脏炎症，改善生存质量[8-9]。实验研究[10-11]发现华蟾素可以抑制HCC的生长和转移，但其相关机制研究目前仍然缺乏。因此，本研究拟基于EMT调控，通过体内外实验研究华蟾素抑制HCC转移的作用和相关机制。

1 实验方法

1.1 动物实验

1.1.1 实验动物和药物 5周龄雄性BALB/c裸鼠36只，

购自上海灵畅生物科技有限公司[许可证号为SCXK(沪)2018-0003；合格证编号为20180003031851]，饲养于龙华医院SPF级动物房裸鼠室，饲养室内温度为(24±2)℃，湿度(55±10)%，光照时间12 h（8:00—20:00）。华蟾素注射液为安徽华润金蟾药业股份有限公司产品(国药准字Z34020273，批号：22G110-1)。根据华蟾素的人常规剂量按照人鼠体表面积换算为低剂量，高剂量为低剂量的2倍。鉴于既往文献[11]更低剂量无明显效应，因此本研究未使用更低剂量。ALT和肌酐检测试剂盒购自南京建成生物工程有限公司(货号：C009-2-1，C011-2-1)。

1.1.2 HCC肺转移裸鼠模型及药物干预 裸鼠适应性饲养1周后，尾静脉注射1×10^6个MHCC97H细胞以建立HCC肺转移模型[12]。随机分为3组：华蟾素高剂量组(n=12)、华蟾素低剂量组(n=10)、对照组(n=12)，建模当日起分别腹腔注射华蟾素高(120 μL/kg)、低剂量(60 μL/kg)或生理盐水，每周2次，持续8周。

1.1.3 肺组织HE染色 8周后麻醉处死裸鼠，取肺组织置于4%中性福尔马林中固定24 h，随后脱水、石蜡包埋，并进行连续切片，厚度为5 μm/片。切片脱蜡后用苏木素染色2 min，伊红染色20 s，自来水冲洗后封片，在显微镜下拍摄。

1.1.4 小鼠血清生化指标检测 依据试剂盒说明检测小鼠血清ALT和肌酐含量。

1.2 细胞实验

1.2.1 实验材料 DMEM培养基、青霉素-链霉素(美国赛默飞世尔科技公司，货号：11965092、15140122)、$CoCl_2$(AR沪试，货号：10007216)、Trizol(美国Life Technologies公司，货号：15596018)、RNA逆转录试剂盒、RNA扩增试剂盒(艾科瑞生物，货号：AG11706、AG11740)、RIPA蛋白裂解液(碧云天生物技术有限公司，货号：P0013B)、微量BCA蛋白定量试剂盒(康为世纪生物科技有限公司，货号：CW0014S)、ECL发光底物(美国Millipore公司，货号WBKLS0500)。抗体来源：vimentin、AKT、P-AKT、N-cadherin、Snail、EPCAM均购自武汉proteintech公司，货号分别为10366-1-AP、10176-2-AP、66444-1-Ig、22018-1-AP、13099-1-AP、21050-1-AP；Slug、β-actin、HRP Goat Anti-Rabbit IgG (H+L)购于武汉ABclonal公司，货号分别为A1057、AC026、AS014。

1.2.2 细胞培养 人肝癌细胞MHCC97H购于复旦大学附属中山医院肝癌研究所，培养于含10%胎牛血清、100 U/mL青霉素和100 μg/mL链霉素的高糖DMEM培养基，置于37 ℃、95%空气湿度和5%CO_2条件下培养。用含有150 μmol/L $CoCl_2$培养基诱导细胞，模拟低氧环境。

1.2.3 划痕实验 将5×10^5个MHCC97H细胞种植于6孔板中，待铺满后用10 μL tip头进行划痕。加入高(5 μL/mL)、低剂量(2.5 μL/mL)华蟾素干预肝癌细胞。低氧实验中加入$CoCl_2$同时孵育，模拟低氧环境。24 h后观察划痕愈合情况，于显微镜下拍照并用Image J分析各组细胞迁移情况。

1.2.4 RT-PCR检测 提取细胞总RNA，运用Evo M-MLVRT Master Mix逆转录试剂盒将RNA逆转录为cDNA。引物由上海闪晶分子生物科技有限公司合成，序列如下：hActin F：TGACGTGGACATCCGCAAAG，R：CTGGAAGGTGGACAGCGAGG；hE-cadherin F：AATTGCTCACATTTCCCAACTC，R：GATTTGATCTGAACCAGGTTTTTAG；hVimentin F：AAATGGCTCGTCACCTTCG，R：CAGATTAGTTTCCCTCAGGTTCA。应用SYBR Green Pro Taq HSqPCR试剂盒在Stepone Plus仪器(美国Applied Biosystems公司)上进行PCR扩增，以β-actin为内参，应用$2^{-\triangle\triangle Ct}$方法计算基因mRNA表达水平。

1.2.5 Western Blot检测 用RIPA裂解液超声下裂解细胞提取蛋白，用BCA法检测浓度。蛋白加入上样缓冲液，经95 ℃变性后用10%的SDS-PAGE电泳分离条带。条带转至PVDF膜，封闭后加入抗β-actin(1∶50 000稀释)、vimentin、N-cadherin(1∶5 000稀释)、AKT、Snail、EpCAM、Slug(1∶1 000稀释)或P-AKT(1∶2 000稀释)抗体于4 ℃孵育过夜。然后膜加入二抗(1∶5 000稀释)，室温孵育1 h，于ECL发光液反应后用Tanon 4600生物发光成像仪(上海天能科技有限公司)进行条带采集分析。

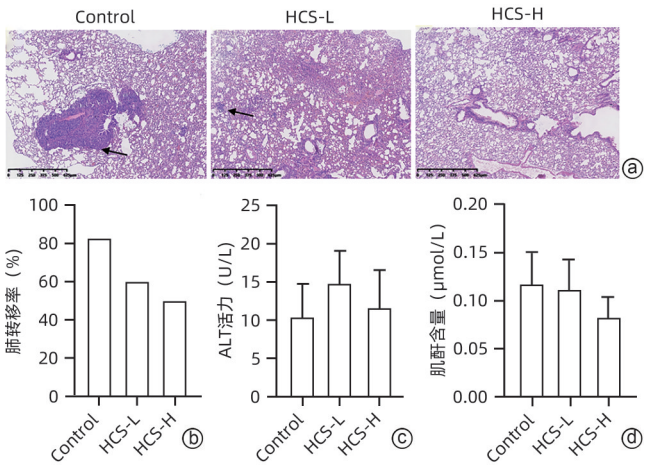
1.3 基因转录组学分析 华蟾素(5 μL/mL)干预24 h的MHCC97H细胞及对照细胞，加入Trizol轻柔吹打后冻存，委托上海伯豪生物技术有限公司进行mRNA测序分析。RNA抽提质检合格后构建测序文库，使用Illumina NovaSeq6000平台，用PE150(Pair-end 150 bp)模式进行测序。

1.4 统计学方法 用SPSS 24.0和Graphpad Prism 9.0软件进行数据统计分析和作图，计量资料采用$\bar{x}\pm s$表示，多组间比较采用单因素方差分析，进一步两两比较采用LSD-t检验；两组间比较采用成组t检验。P<0.05为差异有统计学意义。

2 结果

2.1 华蟾素抑制HCC转移 裸鼠尾静脉注射MHCC97H细胞并干预8周后，图1a、b显示肺组织连续切片HE染色结果。对照组裸鼠多数出现肺转移灶，其肺转移率为83%(12只裸鼠中10只出现转移)，华蟾素低剂量组肺转移率

60%（10只中有6只转移），高剂量组肺转移率50%（12只中有6只转移），表明华蟾素对裸鼠HCC肺转移具有干预作用。图1c、d显示，和对照组相比，用药组血清ALT活力和肌酐含量的差异均无统计学意义（P值均>0.05），表明华蟾素对裸鼠无肝肾毒性。



注：a，肺组织HE染色（×4）；b：肺转移率；c：血清ALT活力（U/L）；d：血清肌酐含量（μmol/L）。Control：对照组；HCS-L：低剂量华蟾素组；HCS-H：高剂量华蟾素组。箭头标示HCC转移灶。

图1　华蟾素对裸鼠肝癌肺转移的影响

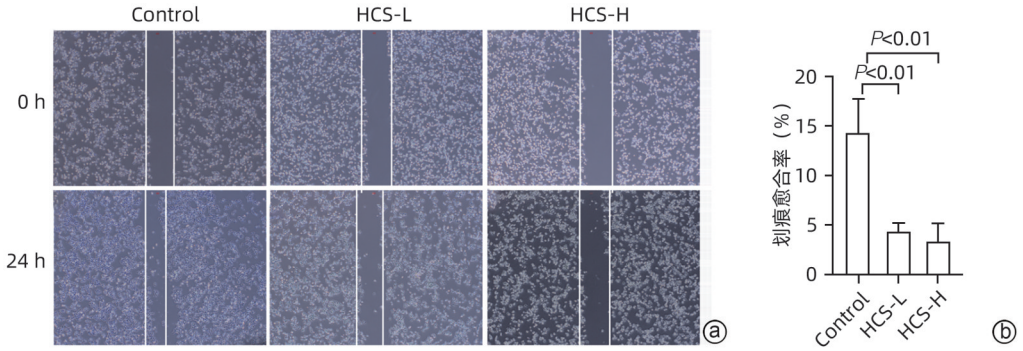
Figure 1　The effect of Cinobufotalin on lung metastasis of HCC in nude mice

MHCC97H细胞划痕实验（图2）显示，划痕24 h后，对照组细胞间隙明显缩小；与对照组相比，华蟾素干预组划痕愈合程度均减少（P值均<0.01）。提示华蟾素可抑制HCC细胞的迁移能力。

2.2　华蟾素调节HCC细胞EMT相关分子的表达水平　为探索华蟾素影响肝癌转移的机制，检测了EMT相关分子的表达水平。RT-PCR结果显示，与对照组相比，华蟾素高剂量干预的MHCC97H细胞E-钙黏蛋白（E-cadherin）的mRNA表达升高（P<0.05）、波形蛋白（vimentin）mRNA水平下降（P<0.05），华蟾素低剂量干预仅有降低趋势（图3a）。因此，后续实验主要选用华蟾素高剂量。Western Blot结果如图3b、c所示，与对照组相比，华蟾素处理的HCC细胞上皮型标志物上皮细胞黏附分子（epithelial cell adhesion molecule，EpCAM）的表达水平升高（t=2.860，P<0.01），而EMT转录因子Snail、Slug表达水平以及基质型标志物vimentin和N-钙黏蛋白（N-cadherin）表达水平降低（t值分别为3.545、2.022、2.852、2.341，P值均<0.05）。

2.3　华蟾素对低氧诱导的HCC细胞迁移及EMT的作用　肝癌作为一种实体瘤，瘤组织中往往存在血供不足造成的低氧微环境，这与EMT发生密切相关[7]，因此本研究进行了低氧诱导下细胞迁移能力及EMT相关检测。划痕实验显示，低氧诱导的MHCC97H细胞划痕愈合率增加（P<0.05），华蟾素干预使其下调（P值均<0.05）（图4a、b）。同时，低氧诱导使肝癌细胞EMT相关基质型分子N-cadherin、vimentin和EMT转录因子Snail表达上调（P值均<0.05）；华蟾素可逆转这些表达变化（P值均<0.05）（图4c、d）。

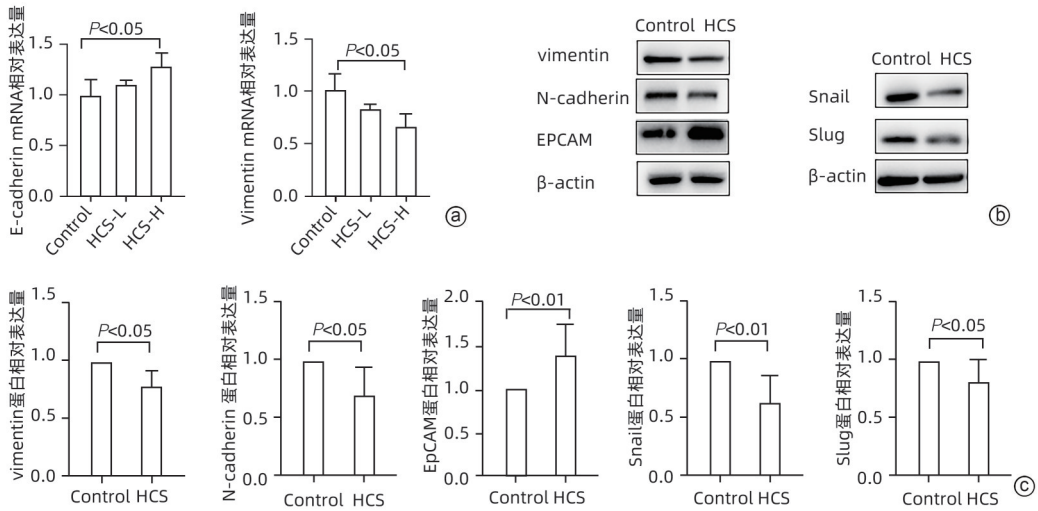
2.4　华蟾素干预的HCC细胞转录组学分析　mRNA sequence数据聚类分析热图显示华蟾素组和对照组MHCC97H细胞间存在显著的基因表达差异（图5a）。火山图显示华蟾素干预后共有3 112个基因表达差异，其中2 346个基因上调、766个基因下调（图5b）。KEGG富集分析显示，华蟾素主要影响了肝癌细胞与肿瘤、细胞生长运动、代谢、免疫和信号传导等相关基因的表达（图5c）。在信号传导通路中，磷脂酰肌醇3-激酶（phosphatidylinositol-3-kinase，PI3K）/蛋白激酶B（protein kinase B，AKT）信号转导相关差异基因数量最多，有75个基因在华蟾素干预后表达发生改变，包括磷脂酰肌醇3-激酶调节亚基1（1PIK3R1）、磷酸酶PTEN、磷酸酶PHLPP2等表达上调，纤维连接蛋白1、血管内皮生长因子α、血小板源性生长因子β等表达下调。



注：a：划痕愈合图（×4）；b：3组划痕愈合率比较。Control：对照组；HCS-L：低剂量华蟾素组；HCS-H：高剂量华蟾素组。

图2　华蟾素对肝癌细胞迁移能力的影响

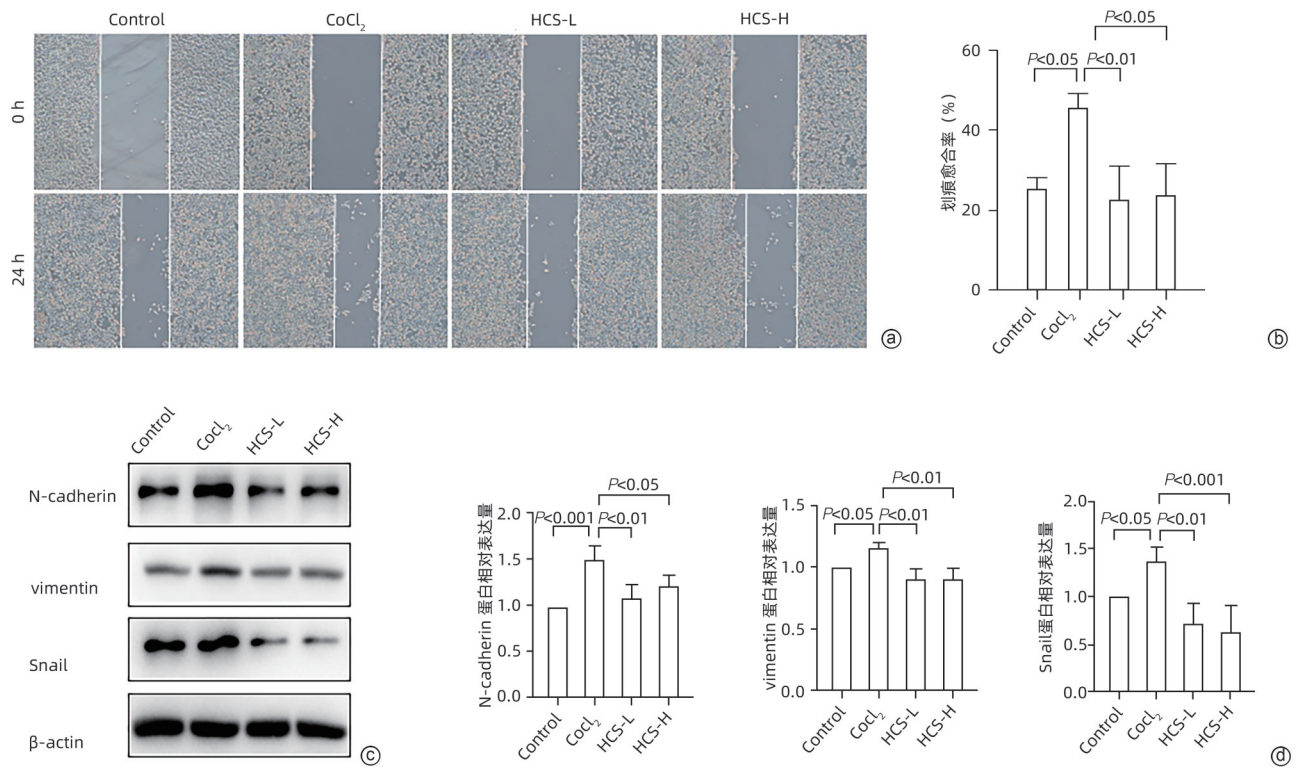
Figure 2　The effect of Cinobufotalin on the migration ability of HCC cells



注：a，EMT相关分子mRNA表达水平；b，EMT相关分子蛋白表达水平；c，蛋白表达分析。Control：对照组；HCS-L：低剂量华蟾素组；HCS-H：高剂量华蟾素组；HCS：华蟾素组。

图3　华蟾素对肝癌细胞EMT相关分子表达水平的影响

Figure 3　The effect of Cinobufotalin on the expression of EMT-related molecules in HCC cells

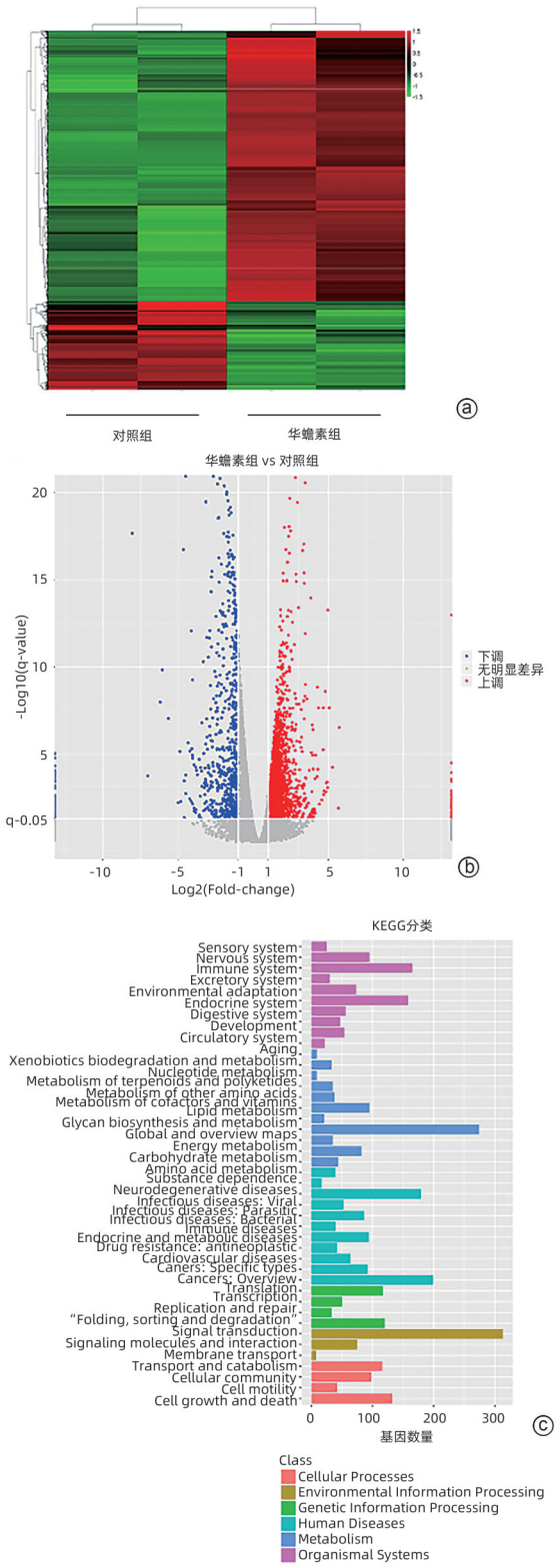


注：a，划痕愈合实验图；b，划痕愈合率；c，EMT相关分子蛋白条带；d，EMT相关分子蛋白分析。Control：对照组；$CoCl_2$：模型组；HCS-L：低剂量华蟾素组；HCS-H：高剂量华蟾素组。

图4　华蟾素对低氧环境诱导的肝癌细胞迁移能力和EMT的影响

Figure 4　The effect of Cinobufotalin on hypoxia-induced cell migration ability and EMT of HCC cells

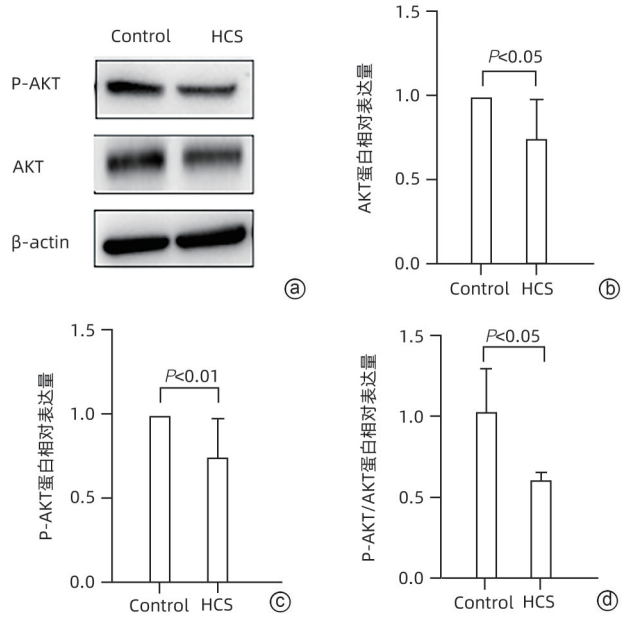
2.5　华蟾素抑制HCC细胞PI3K/AKT信号转导通路　基于转录组学分析，对MHCC97H细胞PI3K/AKT信号转导通路活化水平进行检测。结果显示，和对照相比，华蟾素处理的HCC细胞中AKT、磷酸化AKT（phospho-AKT，P-AKT）及P-AKT/AKT水平均下调（t值分别为2.434、3.401、2.258，P值均<0.05）（图6）。



注：a，对照组和华蟾素组之间的差异基因聚类热图；b，表达量差异火山图；c，核心靶点的KEGG富集分析图。

图5　华蟾素干预的HCC细胞转录组学分析

Figure 5　Seq-RNA analysis of Cinobufotalin -treated HCC cells



注：a. 蛋白条带图；b：蛋白表达分析。Control：对照组；HCS：华蟾素组。

图6　华蟾素对肝癌细胞AKT信号通路的调控作用

Figure 6　The regulatory effect of cinobufotalin on AKT signaling pathway in HCC cells

3　讨论

临床研究[13-14]显示，华蟾素在肝癌、胰腺癌、肺癌、淋巴瘤等中晚期肿瘤中均表现出抑制肿瘤复发转移的作用，可延长患者生存期，改善中晚期肿瘤患者生活质量。另外，Meta分析[15]表明，与单独TACE治疗的HCC患者相比，TACE联合华蟾素注射液辅助治疗能够显著延长患者总生存期，提高患者生活质量。华蟾素联合解毒颗粒可推迟肝癌术后肿瘤复发和转移，提高术后生存率[16]。通过体内实验，本研究也证实华蟾素抑制裸鼠肝癌肺转移的作用，体外实验表明该作用可能由于其抑制HCC细胞的迁移能力。

研究[17]表明EMT与肿瘤细胞侵袭和迁移能力密切相关。EMT是上皮型细胞转换为具有运动能力的间充质型细胞表型并伴随有细胞黏附分子的表达和细胞骨架的改变[18]。在此过程中，受EMT转录因子Snail、Slug、Twist等调控，肿瘤细胞逐渐丢失上皮细胞的部分特征，如E-钙黏蛋白水平下降，导致细胞的黏附力降低，同时获得间质细胞的某些特殊属性，vimentin、N-cadherin等表达升高，使细胞骨架改变形态趋向纺锤形，细胞运动迁移能力增强，从而促进肿瘤细胞的扩散和转移[19-21]。本研究发现华蟾素干预可下调EMT转录因子Snail、Slug及基质型相关分子的表达，上调上皮型分子的表达水平，表明华蟾素可以抑制HCC细胞的EMT变化。

肿瘤生长迅速，对氧及能量物质需求增加，且肿瘤组织内往往血液供应不足，有90%的实体肿瘤存在低氧微环境，其中HCC是低氧最严重的恶性肿瘤之一[22-23]。研究[24]发现低氧能够上调血管扩张剂刺激磷酸化蛋白（vasodilator-stimulated phosphoprotein，VASP）表达，VASP通过激活AKT和ERK信号通路促进EMT和基质金属蛋白的表达，促进HCC侵袭转移。低氧诱导CSN8（COP9 signalosome subunits 8）在结直肠细胞中高表达，通过调控EMT促进结直肠细胞侵袭和转移能力[25]。本研究应用$CoCl_2$模拟低氧环境，发现低氧诱导使HCC细胞迁移能力较对照组增加，华蟾素干预使其明显下调。并且低氧诱导加剧HCC细胞EMT，华蟾素干预使其逆转。

为进一步探讨华蟾素对肝癌的效应机制，进行HCC细胞基因转录组学分析。结果显示，华蟾素影响了肝癌代谢、免疫、细胞生长运动等多方面，差异基因KEGG富集分析发现华蟾素干预对PI3K/AKT信号通路影响显著。PI3K/AKT被认为是抑制肿瘤细胞EMT的重要信号通路[26-27]，激活该通路可调控EMT转录因子促进肿瘤细胞EMT转化[28-29]。其中AKT是该信号传导通路的关键激酶。研究[30]发现，Osteoglycin（OGN）通过抑制PI3K/Akt信号激活，从而抑制乳腺癌细胞EMT，降低乳腺癌细胞的迁移和侵袭能力。PI3K/AKT信号通路也是TGF-β诱导肺癌细胞EMT转化的关键，抑制该通路可抑制EMT进而抑制肺癌转移[31]。此外，药物可通过调控PI3K/AKT信号介导抑制EMT效应。如野黄芩苷可下调PI3K/AKT活化抑制黑色素瘤细胞EMT和血管生成[32]。蟾毒灵、黄酮苷可通过诱导PI3K/AKT信号失活从而抑制肝癌细胞EMT，发挥抗肝癌转移的作用[33-34]。本研究结果显示华蟾素干预HCC细胞可下调AKT的表达和活化水平。另外，PTEN及PHLPP2可下调AKT活化通路中的第二信使而具有负调控作用[35]，本研究中转录组学检测到在华蟾素干预组上调这两种蛋白磷酸酶。这些结果提示该药物可能通过对PI3K/AKT信号调控抑制HCC细胞EMT。

综上，本研究结果表明，华蟾素可抑制肝癌的转移，尤其对于低氧环境诱导的肝癌转移具有显著抑制作用，其机制可能与通过调控AKT信号转导通路介导的HCC细胞EMT有关。本研究有助于阐释华蟾素对肝癌效应机制的认识，为中医药治疗肝癌提供科学依据。中药提取物作用靶点广泛复杂，因此后续本课题组将依据转录组学及体内外实验结果，对华蟾素调控EMT及其他肝癌生长和转移相关的分子机制进一步深入探讨。

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