论著·“乳腺癌精准靶向与转化”重点号

通过慢病毒样颗粒载体递送九香虫来源细胞色素c基因诱导线粒体凋亡抑制HER2阳性乳腺癌

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[摘要] **目的** HER2阳性乳腺癌约占全部乳腺癌的20%,其持续激活的HER2/PI3K/AKT信号通路可通过上调Bcl-2/Bcl-xL并抑制内源性细胞色素c(cytochrome c, Cyt c)释放,从而逃避线粒体凋亡。九香虫(*Aspongopus chinensis Dallas*)来源Cyt c具有潜在线粒体促凋亡活性。本研究构建递送九香虫来源Cyt c的慢病毒样颗粒(lentivirus-like particles,VLPs),并系统评价其对HER2阳性乳腺癌的抗肿瘤作用及相关机制。**方法** 从九香虫组织中扩增Cyt c完整编码序列,并克隆至含mCherry报告基因的慢病毒载体pWSLV中,重组质粒与包装质粒共转染HEK293T细胞,构建mCherry-Cyt c-VLPs(滴度≥1×10^8 TU/mL),采用P24蛋白进行Western blot实验及测序验证包装是否成功。采用改良组织块法分离HER2阳性乳腺癌患者原代细胞,通过MUC1、Ki67、HER2、ER及PR免疫荧光进行表型鉴定。体外实验设PBS组、mCherry-VLP组和mCherry-Cyt c-VLP组(MOI=100)。①RT-qPCR和Western blot检测Cyt c递送效率;②MTT法(24 h和48 h)和Transwell实验(24 h)分别检测细胞活力与迁移能力;③流式细胞术分析细胞周期,并以Western blot检测CDK2和Cyclin A表达;④Annexin V-FITC/PI双染检测细胞凋亡,并检测Bcl-2、Bcl-xL、Bax、胞质Cyt c、cleaved Caspase-9及cleaved Caspase-3蛋白表达。建立HER2阳性乳腺癌患者来源异种移植(patient-derived xenograft model,PDX)模型(n=3),肿瘤内注射给药(50 μg/kg,20 μL/次,每2天1次,共20 d),记录肿瘤体积及体质量变化,并通过HE染色、Western blot、血常规及肝肾功能指标评价体内抗肿瘤作用与初步安全性。**结果** 成功构建mCherry-Cyt c-VLPs,P24蛋白表达阳性,提示病毒样颗粒包装完整。分离的原代乳腺癌细胞呈典型上皮样铺路石状形态,MUC1、Ki67、HER2、ER及PR均呈阳性,确认为HER2阳性管腔B型乳腺癌原代细胞。体外实验结果:①基因递送验证:与PBS组及mCherry-VLP组相比,mCherry-Cyt c-VLP组Cyt c mRNA(P<0.000 1)和蛋白水平(P<0.000 1)均显著升高,证实递送成功。②抗增殖与抗迁移:mCherry-Cyt c-VLP组细胞活力(P<0.05)和迁移细胞数(P<0.000 1)显著降低,mCherry-VLP组无显著影响(P>0.05)。③细胞周期阻滞:mCherry-Cyt c-VLP组流式检测显示G1期细胞比例显著增加、S期比例显著降低(P<0.05),Western blot显示CDK2和Cyclin A蛋白水平显著下调(P<0.000 1)。④凋亡诱导:mCherry-Cyt c-VLP组凋亡率约20%

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(P<0.000 1),Bcl-2和Bcl-xL显著下调(P<0.000 1),Bax、细胞质Cyt c、cleaved Caspase-9及cleaved Caspase-3均显著上调(P<0.000 1),提示外源Cyt c表达激活了胞质caspase级联反应,线粒体凋亡通路被激活。体内PDX实验:mCherry-Cyt c-VLP组肿瘤体积和质量均显著低于PBS组(P<0.01),瘤组织HE染色示细胞密度降低、结构紊乱,瘤组织线粒体凋亡相关蛋白变化规律与体外一致,各组小鼠体质量及血液学、肝肾功能指标无显著差异(P>0.05)。**结论** mCherry-Cyt c-VLPs递送的九香虫来源Cyt c在HER2阳性乳腺癌细胞内持续表达,直接激活Caspase-9/3级联反应,导致肿瘤细胞增殖受抑和凋亡,体内外初步研究未见明显系统毒性。

[**关键词**] 乳腺肿瘤;细胞色素c;凋亡;基因载体;九香虫
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Aspongopus chinensis-derived cytochrome c delivered *via* lentivirus-like particles induces mitochondrial apoptosis to inhibit HER2-positive breast cancer

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[**Abstract**] **Objective** Human epidermal growth factor receptor 2 (HER2)-positive breast cancer accounts for approximately 20% of all breast cancer cases. Persistent activation of the HER2/PI3K/AKT signaling pathway enables tumor cells to evade mitochondrial apoptosis through upregulation of Bcl-2/Bcl-xL and suppression of endogenous cytochrome c (Cyt c) release. Cyt c derived from *Aspongopus chinensis* (*A. chinensis*) Dallas exhibits potential pro-apoptotic activity targeting mitochondria. This study aims to construct lentivirus-like particles (VLPs) delivering the *A. chinensis*-derived Cyt c and systematically evaluate their anti-tumor effects and underlying mechanisms in HER2-positive breast cancer. **Methods** The full-length coding sequence of Cyt c was amplified from *A. chinensis* tissues and cloned into the lentiviral vector pWSLV containing mCherry reporter. Recombinant plasmids were co-transfected with packaging plasmids into HEK293T cells to construct mCherry-Cyt c-VLPs (titer $\geqslant 1\times10^8$ TU/mL). P24 protein was detected with Western blotting and sequencing to confirm successful packaging. Primary cells isolated from HER2-positive breast cancer tissues using a modified tissue block culture method were phenotypically characterized by immunofluorescence staining for MUC1, Ki67, HER2, estrogen receptor (ER), and progesterone receptor (PR). *In vitro* experiments included a PBS group, an mCherry-VLP group, and an mCherry-Cyt c-VLP group (MOI=100). ① RT-qPCR and Western blotting were used to evaluate Cyt c delivery efficiency; ② cell viability and migration were assessed by MTT assay (24 h and 48 h) and Transwell assay (24 h), respectively; ③ cell cycle distribution was analyzed by flow cytometry, and CDK2 and Cyclin A expression levels were detected by Western blotting; ④ apoptosis was detected using Annexin V-FITC/PI double staining, and the expression levels of Bcl-2, Bcl-xL, Bax, cytosolic Cyt c, cleaved Caspase-9, and cleaved Caspase-3 were determined by Western blotting. A HER2-positive patient-derived xenograft (PDX) model was further established (n=3 per group). The mice received intratumoral injections (50 μg/kg, 20 μL per injection, once every 2 days for 20 d). Tumor volume and body weight were recorded, and HE staining, Western blotting, blood routine test, and liver

and kidney function tests were applied to evaluate *in vivo* antitumor efficacy and preliminary safety. Statistical analyses were performed using one-way ANOVA and Tukey's post hoc test, with $P<0.05$ considered statistically significant. **Results** mCherry-Cyt c-VLPs were successfully constructed, and positive P24 protein expression confirmed intact VLP viral particle packaging. The isolated primary breast cancer cells exhibited typical epithelial cobblestone morphology and positive expression of MUC1, Ki67, HER2, ER, and PR, confirming them as HER2-positive luminal B primary breast cancer cells. *In vitro* results: ① Gene delivery: compared with the PBS and mCherry-VLP groups, mCherry-Cyt c-VLP treatment significantly increased Cyt c at both mRNA ($P<0.0001$) and protein levels ($P<0.000\ 1$), confirming successful delivery. ②Anti-proliferation and anti-migration: mCherry-Cyt c-VLP treatment significantly reduced cell viability ($P<0.05$) and the number of migrated cells ($P<0.000\ 1$), whereas the empty-particle group showed no significant effect ($P>0.05$). ③ Cell cycle arrest: flow cytometry revealed a significantly increased proportion of cells in G1 phase and a decreased proportion in S phase in the mCherry-Cyt c-VLP group ($P<0.05$), and Western blotting showed significantly downregulated CDK2 and Cyclin A protein levels ($P<0.000\ 1$). ④Apoptosis induction: the mCherry-Cyt c-VLP group exhibited approximately 20% apoptosis rate ($P<0.000\ 1$), with significant downregulation of Bcl-2 and Bcl-xL ($P<0.000\ 1$) and significant upregulation of Bax, cytosolic Cyt c, cleaved Caspase-9 and cleaved Caspase-3 ($P<0.000\ 1$), indicating that exogenous Cyt c expression activated the cytosolic Caspase cascade and thereby the mitochondrial apoptotic pathway. *In vivo* PDX experiments: the tumor volume and weight in the mCherry-Cyt c-VLP group were significantly lower than those in the control groups ($P<0.01$). HE staining of tumor tissues showed decreased cell density and disordered architecture, with mitochondrial apoptosis-related protein changes in tumor tissue consistent with *in vitro* results. No significant differences were found across groups in mouse body weight or in hematological, liver and kidney function indicators ($P>0.05$). **Conclusion** The *A. chinensis*-derived Cyt c delivered by mCherry-Cyt c-VLPs is sustainably expressed in HER2-positive breast cancer cells and directly activates the Caspase-9/3 cascade, leading to suppressed proliferation and apoptosis of tumor cells. No obvious systemic toxicity is observed in either *in vitro* or *in vivo* experiments.

[**Key words**] breast neoplasms; cytochromes c; apoptosis; genetic vectors; *Aspongopus chinensis*

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HER2阳性乳腺癌约占所有乳腺癌病例的20%[1],以HER2基因过度表达为核心驱动特征,临床上呈现出侵袭性强、疾病进展迅速及对传统化疗易产生耐药性等特点[2]。尽管靶向HER2的单克隆抗体及抗体-药物偶联物(antibody-drug conjugate,ADC)的临床应用显著改善了患者预后,但原发性或继发性耐药问题依然突出[3]。其根本原因在于:HER2持续激活的PI3K/Akt信号通路可磷酸化促凋亡蛋白Bad、上调并稳定Bcl-2/Bcl-xL[4],使线粒体外膜通透性(mitochondrial outer membrane permeabilization,MOMP)的启动阈值显著升高[5],从而抑制内源性细胞色素c(cytochrome c,Cyt c)的释放,赋予肿瘤细胞对线粒体凋亡通路的特殊抵抗能力。因此,直接从Bcl-2家族"下游"水平旁路激活线粒体凋亡通路,被认为是克服HER2相关耐药的潜在关键策略[4]。

传统中药因其丰富的活性成分和独特的多靶点机制,是创新抗肿瘤候选药物的重要源泉[6-7]。九香虫(*Aspongopus chinensis* Dallas)是中医临床长期应用的传统药材,《本草纲目》记载其具有"治乳岩、消痈散结"之功效,现代药理研究已证实其血淋巴提取物对

乳腺癌细胞具有显著的抗增殖、抗转移及促凋亡活性[8-9];从其体内鉴定的ach-miR-276a-3p可通过调控CDK2-Rb-E2F1信号通路,有效抑制乳腺癌细胞增殖[10-11]。在九香虫的多种活性成分中,Cyt c作为线粒体凋亡通路的核心起始因子具有突出的研究价值。Cyt c在生物进化上高度保守,其铁卟啉(血红素)结合域跨物种保留,可有效参与哺乳动物细胞中Apaf-1/Caspase-9凋亡体的组装,直接激活Caspase级联反应[12-13]。值得注意的是,昆虫来源Cyt c与人Cyt c虽具有功能同源性,但其约10%~15%的序列差异,理论上赋予其规避内源性凋亡抑制蛋白识别的潜在优势[14]。

然而,无论Cyt c来源于何种生物,将其直接作为治疗性蛋白进行给药均面临难以回避的根本性瓶颈。作为大分子蛋白(相对分子质量约12×10^3),Cyt c难以自主穿透细胞膜,在体内极易被蛋白酶降解,且从九香虫组织中的提取纯化难度较高,导致其直接给药的生物利用度极低[14]。本课题组前期以HIV Gag蛋白为基础构建了慢病毒样颗粒(lentivirus-like particles,VLPs)递送平台,成功将活性Caspase-8蛋白递送至乳腺癌细胞内并诱导凋亡[15-19],验证了VLPs平台在递送促凋亡蛋白/基因方面的可行性与有效性。在此基础上,本研究构建mCherry-Cyt c-VLPs,以VLPs递送九香虫Cyt c编码基因,使其在HER2阳性乳腺癌细胞内表达,并从细胞增殖、迁移、细胞周期、凋亡级联和PDX模型抗肿瘤效应等方面进行系统评价。

1 材料与方法

1.1 材料

1.1.1 细胞系与临床样本 HER2阳性人乳腺癌原代细胞系分离自贵州中医药大学第一附属医院甲乳外科2024年9月至2025年12月期间收治的乳腺癌患者肿瘤组织,所有临床组织样本的获取均获患者书面知情同意,研究方案经贵州中医药大学第一附属医院伦理委员会批准(KS2024002)。

1.1.2 动物实验 9只雌性BALB/c裸鼠(4~5周龄,SPF级)购自湖南斯莱克景达实验动物有限公司,在恒温(25±1)℃、恒湿(70%)、12 h光暗循环SPF级动物房中饲养,自由摄食和饮水。动物实验经贵州中医药大学动物伦理委员会批准(20250703013)。实验期间每日观察精神状态、活动、摄食、局部肿瘤破溃、体质量下降和异常死亡等情况;如出现肿瘤溃烂、持续消瘦、活动明显减少或研究者判定存在不可逆痛苦时,提前实施人道终点处置。

1.1.3 主要试剂与仪器 Cyt c、Bcl-2、Bax、Bcl-xL、Caspase-3、Caspase-9、CDK2、Cyclin A及GAPDH抗体均购自Abcam公司;DMEM高糖培养基、胎牛血清(FBS)、胰蛋白酶均购自Gibco公司;TRIzol试剂、反转录及qPCR预混Mix(SYBR Green Premix Pro Taq HS qPCR Kit,Q711-02)购自南京诺唯赞公司;Annexin V-FITC/PI凋亡检测试剂盒(A211-01)和细胞周期检测试剂盒(C1052)均购自上海碧云天公司。主要仪器包括德国耶拿qTOWER 3G实时荧光定量PCR仪、Olympus倒置荧光显微镜(IX73)、流式细胞仪(BD FACSCanto II)及LEICA组织脱水/包埋/切片系统。

1.2 方法

1.2.1 九香虫Cyt c基因克隆与mCherry-Cyt c-VLPs的构建 九香虫于2024年9—10月采自贵州黔东南剑河县,经纯水清洗2遍,滤纸吸干,-80 ℃冻存,从虫体组织中提取总RNA(TRIzol法),经RT-PCR扩增Cyt c基因完整编码序列(引物含*Not* Ⅰ、*Bam*H Ⅰ酶切位点),将PCR产物克隆至携带mCherry荧光报告基因的慢病毒表达载体pWSLV,构建重组质粒pWSLV-mCherry-Cyt c,经*Not* Ⅰ/*Bam*H Ⅰ双酶切及双向测序验证插入序列正确。将鉴定正确的重组质粒与包装质粒(psPAX2和pMD2. G)按三质粒系统共转染HEK293T细胞,收集病毒上清经超速离心(4 ℃,25 000 r/min,90 min)浓缩,P24 ELISA法测定滴度(约1×10^8 TU/mL);同期构建mCherry-VLPs作为空载颗粒对照。

1.2.2 原代乳腺癌细胞培养与表型鉴定 采用改良组织块法从HER2阳性乳腺癌组织中分离原代细胞[20],以含10% FBS的DMEM高糖培养基于37 ℃、5% CO_2条件下培养。差速消化纯化后,取生长状态良好的第3~5代细胞用于后续实验。以免疫荧光染色检测MUC1、Ki67、HER2、ER和PR的表达,鉴定细胞分子分型。

1.2.3 体外细胞分组与慢病毒感染处理 取第3~5代原代细胞以5×10^4/孔接种于6孔板,待细胞融合度达60%~70%时进行感染实验。培养基中加入终浓度8 μg/mL Polybrene(聚凝胺)以增强感染效率。实验分为:空载颗粒组(mCherry-VLPs,MOI=100)、实验组(mCherry-Cyt c-VLPs,MOI=100)和PBS组(加入等体积磷酸盐缓冲液)。感染8~12 h后更换新鲜完全培养基,继续培养24 h。

1.2.4 RT-qPCR检测Cyt c mRNA TRIzol法提取各组细胞总RNA,PrimeScript RT试剂盒反转录合成cDNA。以SYBR Green法在德国耶拿qTOWER 3G系统上进行定量PCR扩增(95 ℃预变性30 s;95 ℃变性

10 s、60 ℃退火延伸30 s,共40个循环)。以GAPDH为内参基因,采用$2^{-\Delta\Delta Ct}$法计算九香虫Cyt c mRNA相对表达量。

1.2.5 Western blot检测 收集细胞或研磨均质后的肿瘤组织,以RIPA裂解液(含蛋白酶抑制剂及磷酸酶抑制剂混合物)提取总蛋白,BCA定量,等量蛋白(30 μg/泳道)经SDS-PAGE分离并转PVDF膜,5%脱脂奶粉封闭1 h,一抗(1∶1 000)4 ℃孵育过夜,HRP二抗(1∶5 000)室温孵育1 h,ECL显影,Image J灰度定量,GAPDH为内参。

1.2.6 MTT细胞活力检测 感染24 h和48 h后,向各孔培养基中加入MTT溶液(终浓度0.5 mg/mL),37 ℃继续孵育4 h。小心弃去上清,加入DMSO(150 μL/孔)溶解甲臜晶体,置于酶标仪于490 nm波长处测定吸光度值*OD*(490),以各组*OD*(490)值相对于PBS对照组的比值计算细胞存活率。

1.2.7 Transwell迁移实验 将感染后的细胞消化重悬于无血清DMEM培养基(浓度2×10^5 cells/mL),取200 μL无血清细胞悬液加入Transwell上室(康宁公司,孔径8.0 μm,货号3422),下室加入600 μL含20% FBS的完全培养基作为趋化剂。37 ℃培养24 h后,以4%多聚甲醛固定15 min,0.1%结晶紫染色20 min,PBS轻柔洗涤,棉签擦去上室未穿膜细胞,显微镜下随机选取5个视野计数穿膜细胞数。

1.2.8 流式细胞术检测细胞周期与凋亡 细胞周期检测:收集各组细胞,以预冷70%乙醇于4 ℃固定过夜,PBS洗涤后以PI/RNase A染色液(碧云天,C1052)避光孵育30 min,流式细胞仪检测,FlowJo/ModFit软件分析G1、S、G2/M各时相细胞比例。细胞凋亡检测:参照Annexin V-FITC/PI凋亡检测试剂盒(诺唯赞,A211-01)说明书操作,收集感染48 h后的细胞,依次加入Annexin V-FITC和PI,室温避光孵育15 min,流式细胞仪检测,区分活细胞、早期凋亡、晚期凋亡及坏死细胞群体。

1.2.9 PDX模型建立与体内给药 取新鲜冻存的HER2阳性乳腺癌PDX组织块,于冰上复苏后皮下接种于雌性BALB/c裸鼠(4~5周龄)右侧腋窝皮下。待移植瘤体积生长至约5 mm×4 mm×5 mm(约100 mm^3)时,将小鼠按随机数字表法分为(n=3):PBS组、mCherry-VLP组和mCherry-Cyt c-VLP组,每2天瘤内注射给药1次(剂量50 μg/kg,注射体积20 μL/次,每次以微量注射器缓慢注入瘤体),共给药20 d。每2天以游标卡尺测量肿瘤最大长径(a)和最大短径(b),以公式$V=1/2\times a\times b^2$计算肿瘤体积,同步记录小鼠体质量。实验终点(第20天给药后24 h)颈椎脱臼处死小鼠,剥离肿瘤组织称重,部分组织经4%多聚甲醛固定、石蜡包埋后用于组织学分析,其余组织液氮速冻后保存于-80 ℃用于蛋白提取。

1.2.10 HE组织学检测 石蜡包埋的肿瘤组织切片(厚度4 μm)经二甲苯脱蜡、梯度乙醇(100%、95%、90%、80%和70%乙醇)逐级复水后进行HE染色。具体步骤如下:切片经苏木精染液染色后,经流水冲洗、分化及返蓝处理;随后采用伊红染液进行复染。染色完成后,切片经梯度乙醇脱水、二甲苯透明,并使用中性树胶封片。采用光学显微镜观察各组肿瘤组织的病理形态变化,并采集代表性图像进行分析。

1.2.11 系统安全性评价 为评价mCherry-Cyt c-VLPs体内应用的安全性,于动物实验终点(末次给药后24 h)对各组小鼠进行系统安全性检测。小鼠经异氟烷吸入麻醉后,通过眼眶静脉丛采集全血,部分血液加入含乙二胺四乙酸(EDTA)的抗凝管中,用于血常规指标检测;剩余血液静置凝固后,于4 ℃条件下离心分离血清,用于血清生化指标检测。以全自动血液细胞分析仪检测血常规指标(HB、RBC、WBC、PLT);以全自动生化分析仪检测肝功能指标(AST、ALP、TP)及肾功能指标(BUN、CREA、Cys-C)。

1.3 统计学分析

所有定量数据以$\bar{x}\pm s$表示。采用GraphPad Prism 9.0软件进行统计分析,多组间均数比较采用单因素方差分析(one-way ANOVA)并以Tukey事后检验进行两两比较。$P<0.05$为差异有统计学意义。

2 结果

2.1 mCherry-Cyt c-VLPs的构建与鉴定

从九香虫组织提取的总RNA质量良好(18S/28S条带清晰),以此为模板经RT-PCR扩增获得预期大小的Cyt c特异性条带;经双酶切(Not Ⅰ/BamH Ⅰ)及双向测序鉴定,插入片段与参考序列完全一致,阅读框与mCherry及病毒包装元件正确衔接,重组质粒pWSLV-mCherry-Cyt c构建正确。将重组质粒与包装质粒共转染HEK293T细胞后,Western blot在mCherry-Cyt c-VLPs浓缩上清中检测到明显P24衣壳蛋白条带,PBS组未见表达,提示VLPs包装成功(图1A)。病毒滴度约为1×10^8 TU/mL,可满足后续实验需求。同期构建的mCherry-VLPs用作空载颗粒对照。

2.2 HER2阳性乳腺癌原代细胞的培养与鉴定

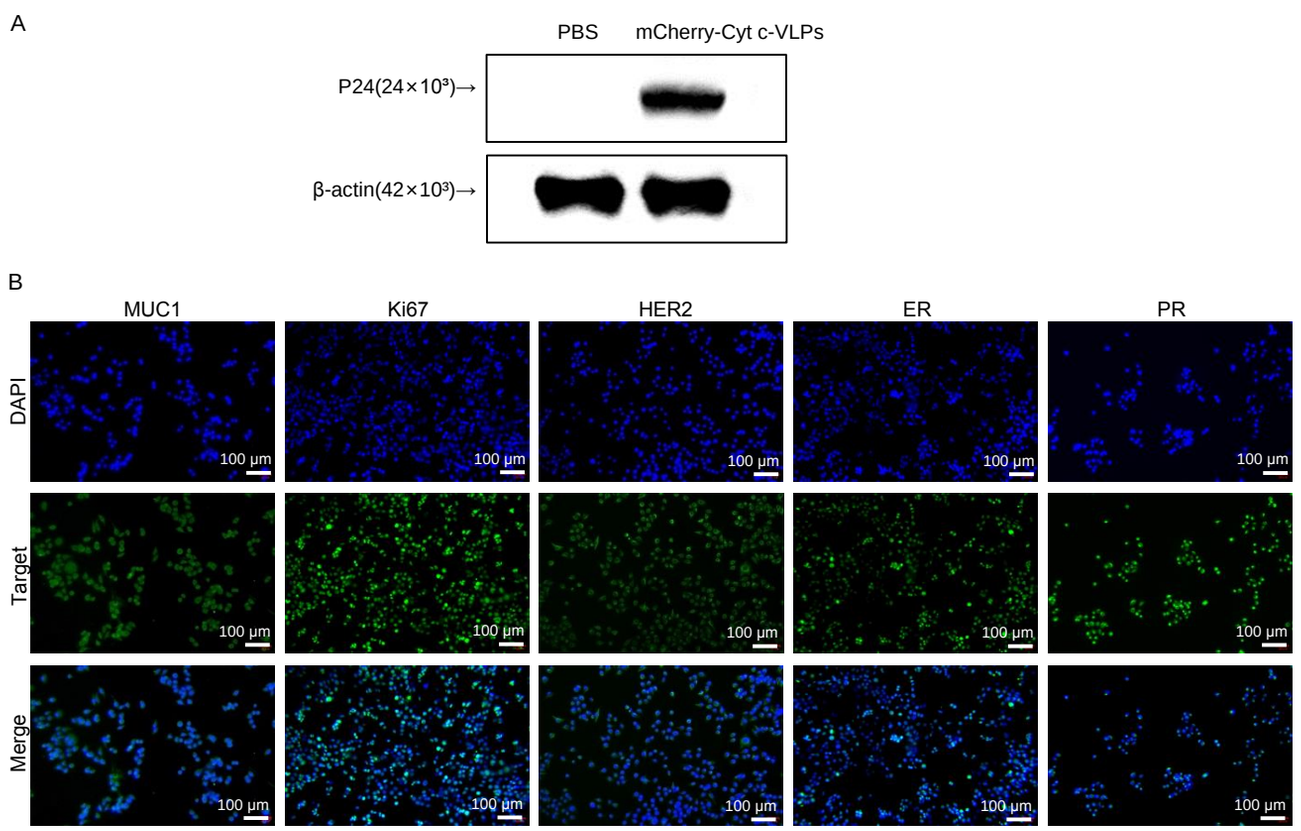
采用改良组织块法分离的原代乳腺癌细胞,差速消化纯化培养后呈典型上皮样铺路石状排列,形态均

一,折光性良好。免疫荧光检测显示,培养细胞MUC1(CA153)及增殖标志物Ki67均呈高表达,ER和PR在细胞核内呈阳性,HER2在细胞膜上呈强阳性(图1B);该表达谱($ER^+/PR^+/HER2^+$)符合管腔B型HER2过表达乳腺癌细胞特征,提示所培养原代细胞保留了乳腺癌的典型分子特性。

2.3　mCherry-Cyt c-VLPs抑制HER2阳性乳腺癌细胞生长

2.3.1　mCherry-Cyt c-VLPs高效递送Cyt c并抑制细胞活力与迁移　以及RT-qPCR检测各组细胞中Cyt c mRNA水平,以mCherry-Cyt c-VLPs的基因递送效率。结果显示:与PBS组及mCherry-VLP组相比,mCherry-Cyt c-VLP组Cyt c mRNA表达显著升高($P<0.000\ 1$),提示VLPs可有效介导目的基因转录(图2A)。进一步以Western blot检测总蛋白中Cyt c蛋白水平,结果同样显示mCherry-Cyt c-VLP组Cyt c蛋白较两对照组显著增加($P<0.000\ 1$,图2B、C),从mRNA和蛋白双层面确认了外源Cyt c的有效递送与表达。MTT检测结果显示,与PBS组相比,mCherry-Cyt c-VLP组细胞活力显著降低($P<0.05$),而mCherry-VLP组未见明显影响($P>0.05$,图2D、E)。Transwell实验结果显示,mCherry-Cyt c-VLP组穿膜细胞数量显著减少($P<0.000\ 1$),提示细胞迁移能力受到显著抑制;mCherry VLP组无显著变化($P>0.05$,图2F、G)。上述结果表明,mCherry-Cyt c-VLPs可有效递送并表达Cyt c,进而抑制HER2阳性乳腺癌细胞的增殖活性和迁移能力。

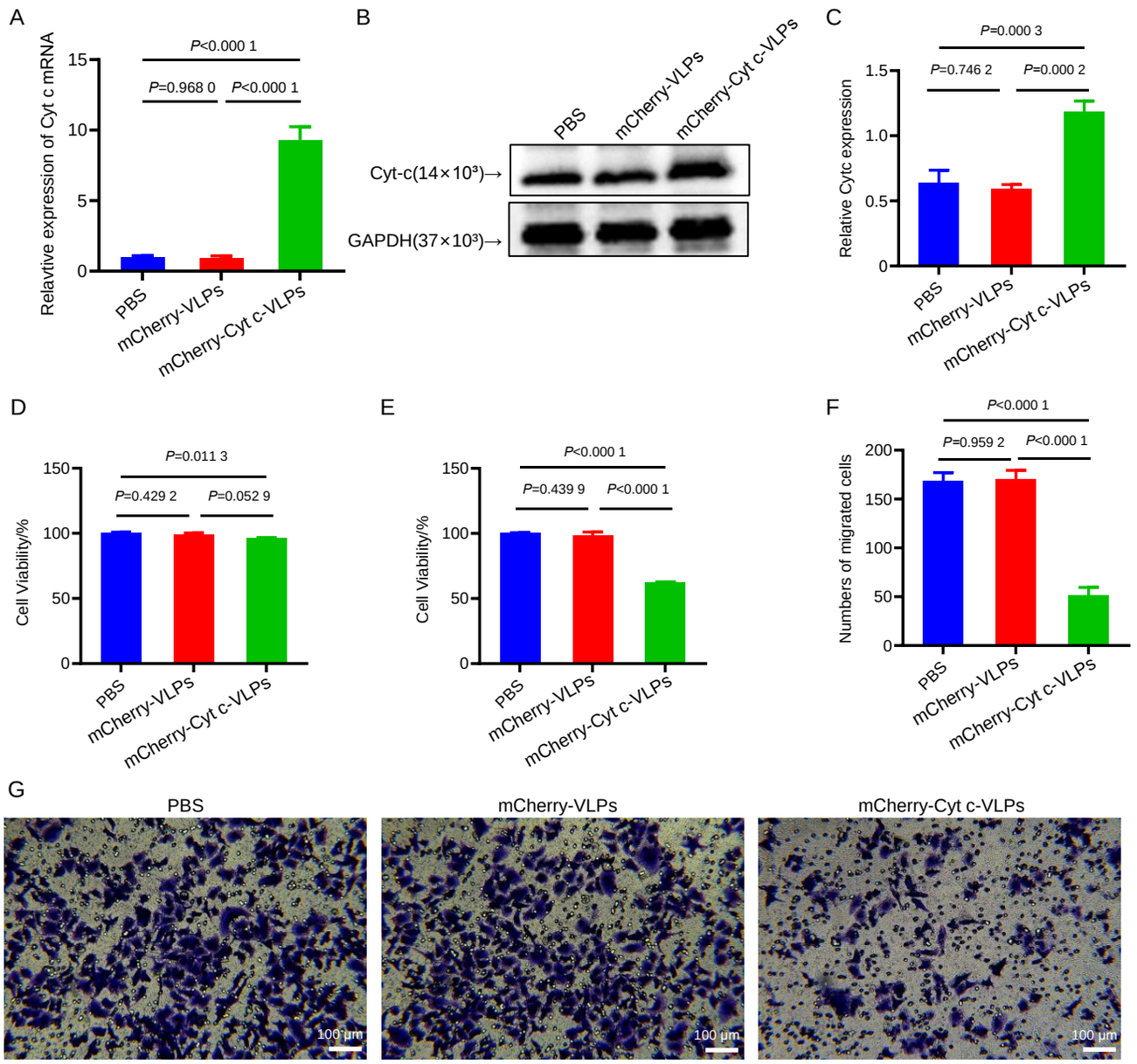
2.3.2　mCherry-Cyt c-VLPs处理后细胞出现G1期阻滞并促进细胞凋亡　为探究mCherry-Cyt c-VLPs抑制细胞增殖的机制,流式细胞术分析细胞周期分布显示,mCherry-Cyt c-VLP较mCherry VLP组G1期细胞比例显著增加($P<0.000\ 1$),S期比例明显降低($P<0.05$,图3A~D),提示细胞在G1/S转换阶段发生阻滞。Western blot进一步显示,mCherry-Cyt c-VLP组CDK2和Cyclin A蛋白表达水平显著下调($P<0.000\ 1$,图3E~G)。



A: Representative Western blotting images of P24 in the PBS control and mCherry-Cyt c-VLP preparations; no P24 band is detected in the PBS control; B: Representative immunofluorescence images of MUC1, Ki67, HER2, ER and PR in primary breast cancer cells; positive staining confirmed the luminal B (HER2-overexpressing) subtype

图1　Western blot鉴定mCherry-Cyt c-VLPs包装及免疫荧光鉴定原代乳腺癌细胞的Luminal B(HER2过表达)分子亚型

Figure 1　Western blotting validation of mCherry-Cyt c-VLP packaging and immunofluorescence identification of the luminal B (HER2-overexpressing) subtype in primary breast cancer cells



A: Relative Cyt c mRNA expression in HER2-positive primary breast cancer cells after treatment with PBS, mCherry-VLPs or mCherry-Cyt c-VLPs, as determined by RT-qPCR(n=3, $\bar{x}\pm s$); B: Representative Western blot images of Cyt c and GAPDH after the indicated treatments; C: Relative Cyt c protein expression normalized to GAPDH(n=3, $\bar{x}\pm s$); D and E: Cell viability (%) at 24 h(D) and 48 h(E) after treatment, as determined by MTT assay(n=3, $\bar{x}\pm s$); F: Number of migrated cells per field quantified by Transwell assay(n=3, $\bar{x}\pm s$); G: Representative images of migrated cells in the Transwell assay. Scale bar indicates 100 μm

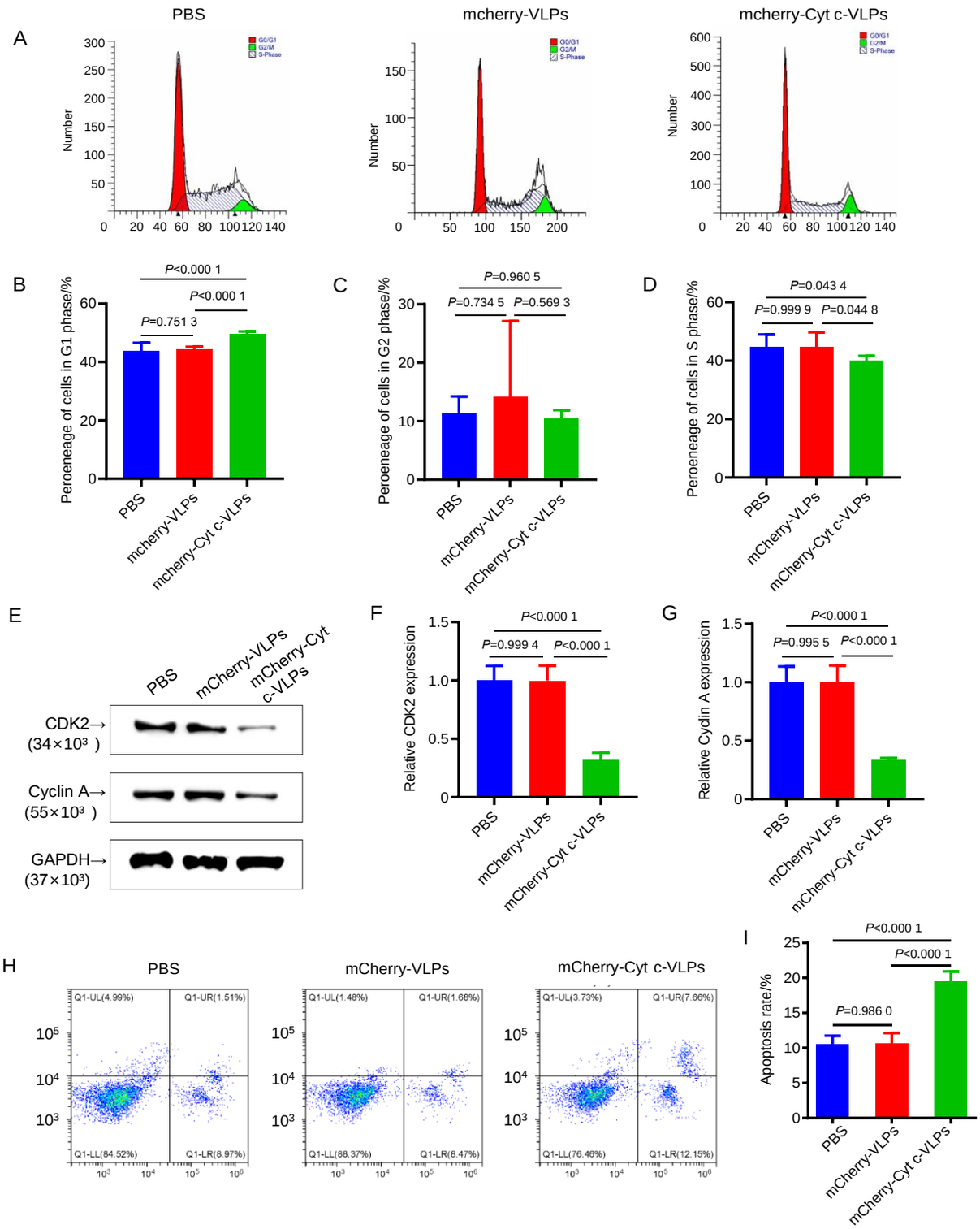
图2 mCherry-Cyt c-VLPs上调HER2阳性原代乳腺癌细胞Cyt c表达并抑制细胞活力与迁移

Figure 2 mCherry-Cyt c-VLPs increase Cyt c expression and inhibit the viability and migration of HER2-positive primary breast cancer cells

CDK2/Cyclin A复合物是驱动细胞越过G1/S检查点的核心执行者,其活性丧失直接导致细胞无法启动DNA复制,与G1期阻滞表型完全吻合。Annexin Ⅴ-FITC/PI双染结果显示,mCherry-Cyt c-VLP组凋亡率约为20%,较PBS组显著升高(P<0.000 1),mCherry-VLP组较PBS组无明显变化(图3H、I)。

2.3.3 mCherry-Cyt c-VLPs激活线粒体凋亡通路

为阐明凋亡机制,检测线粒体凋亡通路相关蛋白的表达变化。Western blot结果显示,与PBS组及mCherry-VLP组相比,mCherry-Cyt c-VLP组抗凋亡蛋



A: Representative flow cytometric histograms of cell-cycle distribution in HER2-positive primary breast cancer cells treated with PBS, mCherry-VLPs or mCherry-Cyt c-VLPs; cell number is plotted against DNA content; B to D: Percentages of cells in the G1 (B), G2 (C) and S (D) phases (n=3, $\bar{x}\pm s$); E: Representative Western blot images of CDK2, Cyclin A and GAPDH; F and G: Relative protein expression levels of CDK2 (F) and Cyclin A (G) normalized to GAPDH(n=3, $\bar{x}\pm s$); H: Representative Annexin V/PI double-staining flow cytometric dot plots of cell apoptosis; I: Total apoptosis rate, calculated as the sum of the percentages of early apoptotic cells (Q1-LR) and late apoptotic cells (Q1-UR)(n=3, $\bar{x}\pm s$)

图3 mCherry-Cyt c-VLPs诱导HER2阳性原代乳腺癌细胞G1期阻滞和凋亡

Figure 3 mCherry-Cyt c-VLPs induce G1-phase arrest and apoptosis in HER2-positive primary breast cancer cells

白 Bcl-2 和 Bcl-xL 显著下调(P<0.000 1),促凋亡蛋白 Bax 上调(P<0.000 1,图 4A~D),Bcl-2/Bax 比值的失衡提示线粒体凋亡通路受到扰动;细胞质中总 Cyt c 蛋白水平显著升高(P<0.000 1,图 4A、E),结合外源 VLPs 递送表达的 Cyt c 及 Bcl-2/Bax 蛋白平衡改变,提示线粒体凋亡通路被激活。细胞质 Cyt c 浓度的升高激活了下游 caspase 级联:cleaved Caspase-9 和 cleaved Caspase-3 水平均显著增加(P<0.000 1,图 4A、F、G)。上述结果表明,mCherry-Cyt c-VLPs 处理后,线粒体凋亡通路相关蛋白发生显著改变,伴随 Caspase-9/3 的激活,提示 Cyt c 表达可直接启动线粒体依赖性凋亡过程。

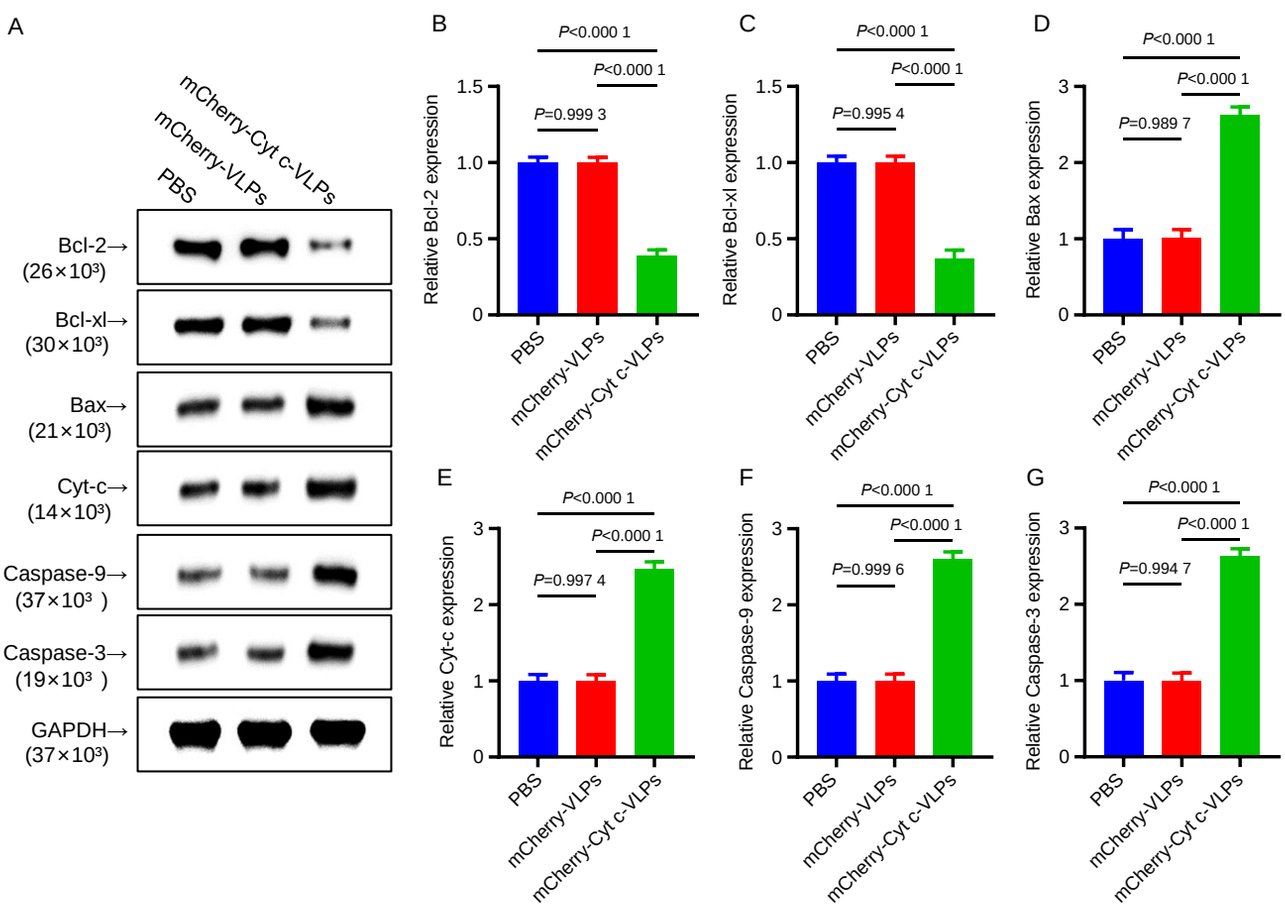
2.4 mCherry-Cyt c-VLPs 在 PDX 模型中抑制肿瘤生长

2.4.1 mCherry-Cyt c-VLPs 显著抑制体内肿瘤生长

为评估体内抗肿瘤效应,建立 HER2 阳性乳腺癌 PDX 模型并进行 20 d 的治疗干预。结果显示,与 PBS 组及 mCherry-VLP 组相比,mCherry-Cyt c-VLP 组显著抑制肿瘤体积增长(图 5A),各组小鼠体质量无明显差异(图 5B),提示无显著系统毒性。实验终点剥离瘤体,大体观察示 mCherry-Cyt c-VLP 组瘤体明显小于 PBS 组和 mCherry-VLP 组,瘤重测定结果与体积变化一致,mCherry-Cyt c-VLP 组肿瘤质量显著降低(图 5C~F,P<0.005)。

2.4.2 mCherry-Cyt c-VLPs 改善体内肿瘤组织病理形态

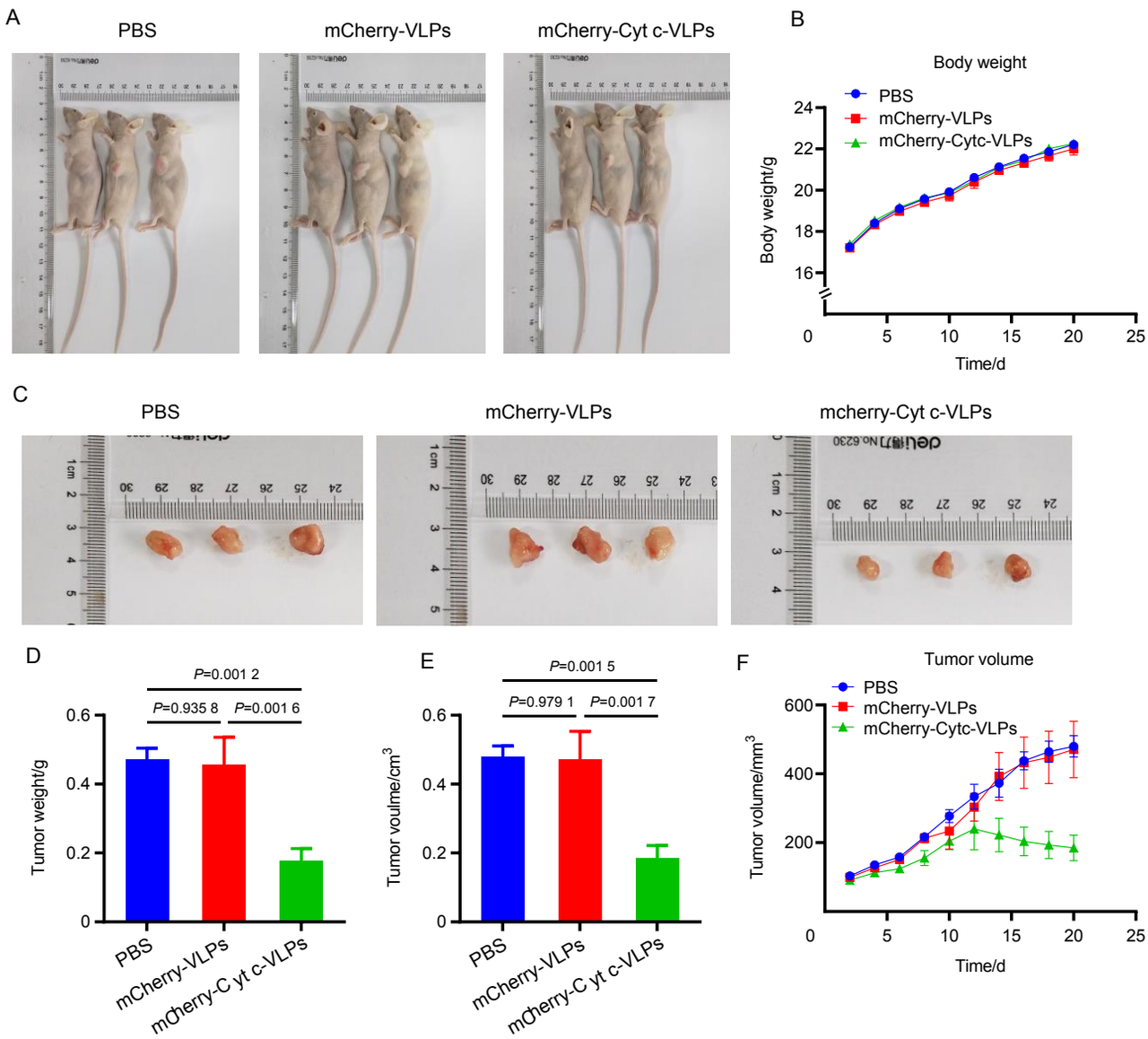
HE 染色显示,PBS 组及 mCherry-VLP 组肿瘤组织细胞排列致密、核分裂象多,窦状毛细血管丰富;mCherry-Cyt c-VLP 组细胞密度明显降低,组织结构紊乱,窦状毛细血管数量减少(图 6A),提示 mCherry-Cyt c-VLP 处理可显著改变体内肿瘤组织的病理形态,造成肿瘤组织退变,与体外抑瘤结果相一致。



A: Representative Western blot images of Bcl-2, Bcl-xL, Bax, cytoplasmic Cyt c, cleaved Caspase-9, cleaved Caspase-3 and GAPDH in HER2-positive primary breast cancer cells treated with PBS, mCherry-VLPs or mCherry-Cyt c-VLPs; B to G: Relative protein expression levels of Bcl-2 (B), Bcl-xL (C), Bax (D), cytoplasmic Cyt c (E), cleaved Caspase-9 (F), and cleaved Caspase-3 (G)(n=3, $\bar{x}\pm s$)

图 4 mCherry-Cyt c-VLPs 激活 HER2 阳性原代乳腺癌细胞线粒体凋亡通路

Figure 4 mCherry-Cyt c-VLPs activate the mitochondrial apoptotic pathway in HER2-positive primary breast cancer cells



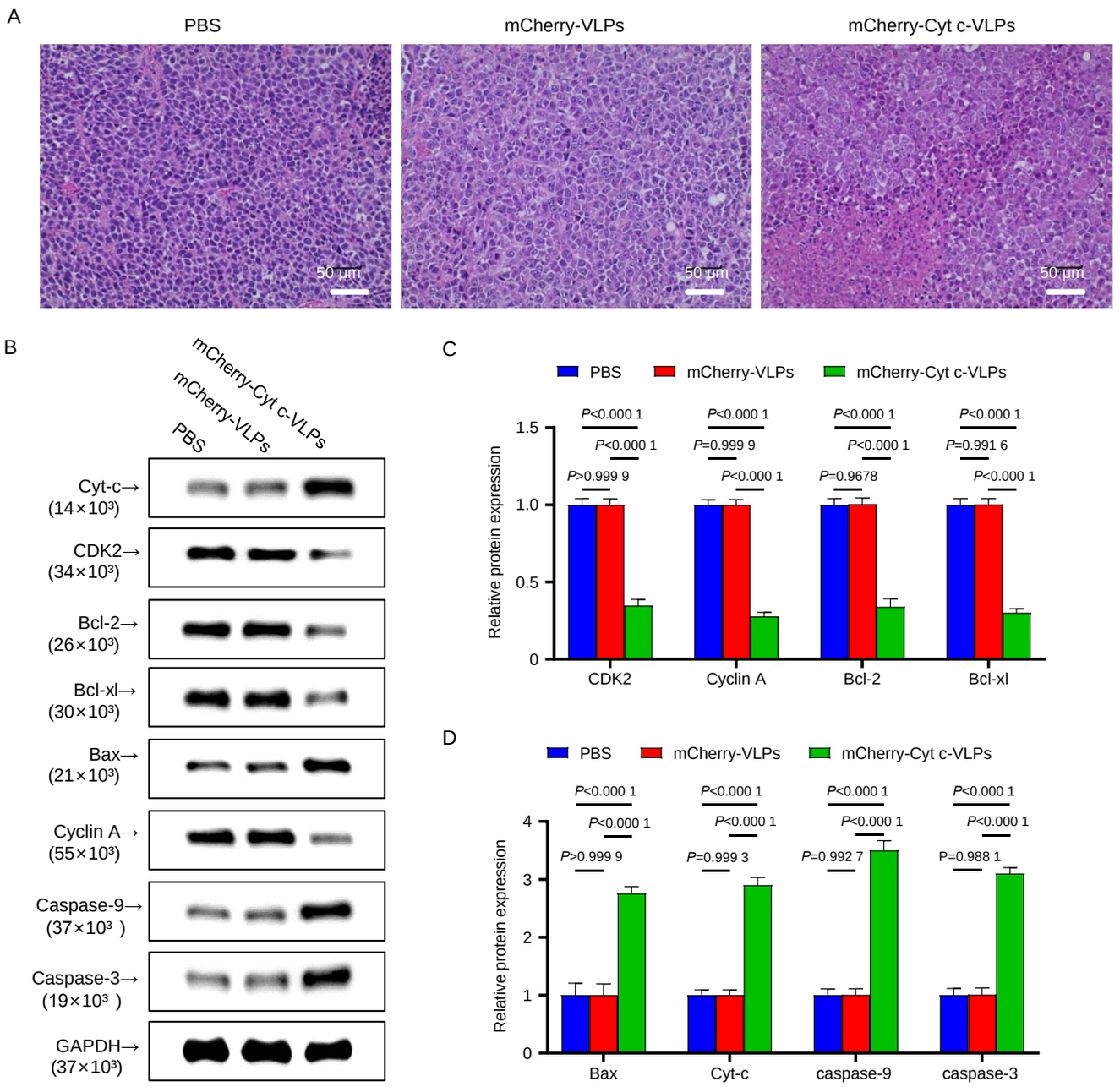
A: Representative gross images of HER2-positive breast cancer-bearing mice treated with PBS, mCherry-VLPs or mCherry-Cyt c-VLPs; B: Changes in body weight of tumor-bearing mice over time during treatment(n=3, $\bar{x}\pm s$); C: Representative gross images of resected tumors from the indicated treatment groups; D and E: Quantitative analyses of resected tumor weight (D) and volume at the experimental endpoint; F: Changes in tumor volumeover time during treatment(n=3, $\bar{x}\pm s$)

图5 mCherry-Cyt c-VLPs抑制HER2阳性乳腺癌荷瘤小鼠的肿瘤生长

Figure 5 mCherry-Cyt c-VLPs inhibit tumor growth in mice bearing HER2-positive breast cancer

2.4.3 体内瘤组织中细胞周期及线粒体凋亡相关蛋白验证 对PDX瘤组织提取蛋白进行Western blot检测,结果与体外实验高度一致。与PBS组和mCherry-VLPs组相比,mCherry-Cyt c-VLP组CDK2、Cyclin A、Bcl-2、Bcl-xL表达均下降(P<0.000 1,图6B~C),Bax、Cyt c、cleaved Caspase-9和cleaved Caspase-3表达均显著上调(P<0.0001,图6B、D)。上述体内蛋白数据从分子层面进一步证实了mCherry-Cyt c-VLPs通过激活细胞周期阻滞和线粒体凋亡通路发挥体内抗肿瘤作用。

2.4.4 系统安全性评价 实验终点血常规检测显示,mCherry-Cyt c-VLP组HB、RBC、WBC及PLT水平与PBS组相比均无显著差异(图7A~D,P>0.05),提示对造血系统无明显影响。肝肾功能检测结果显示,

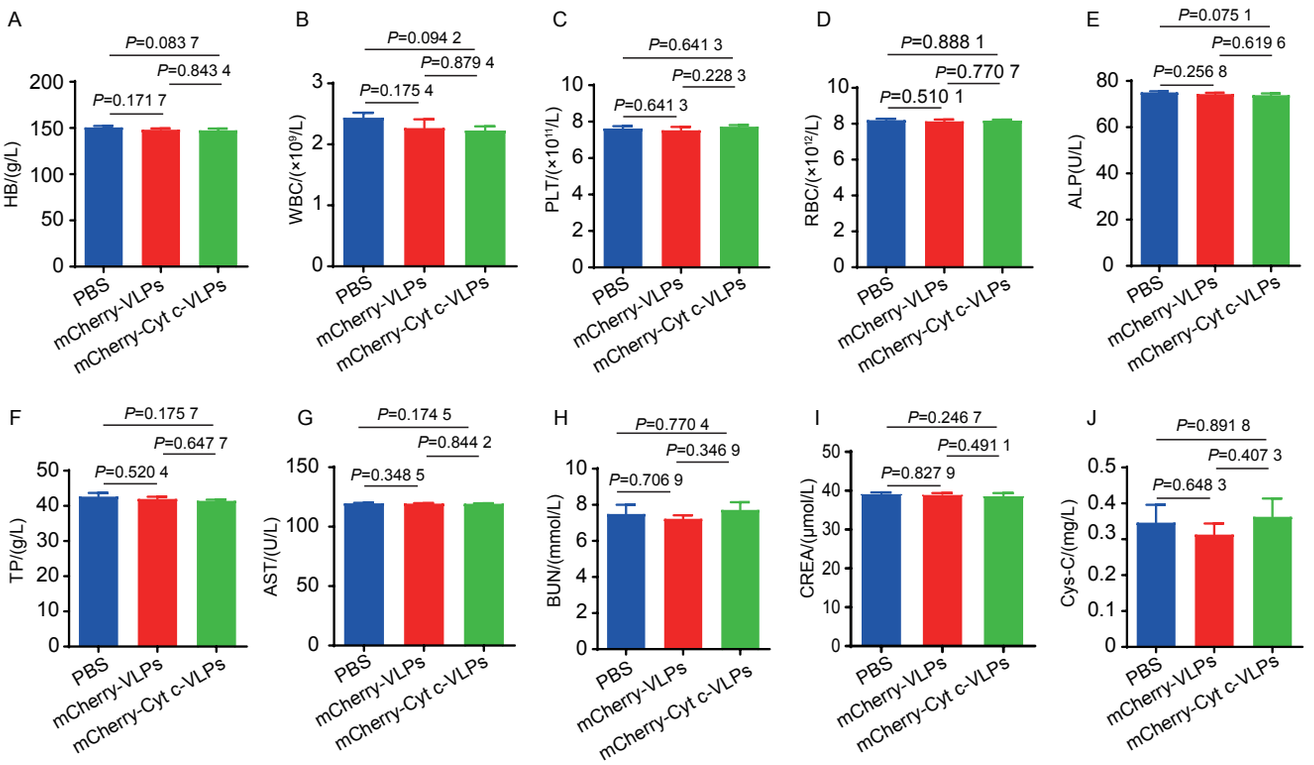


A: Representative HE-stained sections of PDX tumor tissues from mice treated with PBS, mCherry-VLPs or mCherry-Cyt c-VLPs. Scale bar indicates 50 μm; B: Representative Western blotting images of Cyt c, CDK2, Bcl-2, Bcl-xL, Bax, Cyclin A, cleaved Caspase-9, cleaved Caspase-3 and GAPDH in PDX tumor tissues; C: Relative protein expression of CDK2, Cyclin A, Bcl-2 and Bcl-xL normalized to GAPDH (n=3, $\bar{x}\pm s$); D: Relative protein expression of Bax, Cyt c, cleaved Caspase-9 and cleaved Caspase-3 normalized to GAPDH(n=3, $\bar{x}\pm s$)

图6 mCherry-Cyt c-VLPs处理后HER2阳性乳腺癌PDX瘤组织病理形态及细胞周期和线粒体凋亡相关蛋白表达

Figure 6 Histopathological changes and expression of cell cycle- and mitochondrial apoptosis-related proteins in HER2-positive breast cancer PDX tumors after treatment with mCherry-Cyt c-VLPs

ALP、TP、AST及肾功能指标(尿素氮、肌酐、Cys-C)在各组间均无统计学差异(图7E~J,P>0.05)。上述安全性数据表明,mCherry-Cyt c-VLPs在本研究剂量和方案下未引起明显的血液学及肝肾功能异常,具有良好的体内安全性。



A: Hemoglobin (HB) concentration in peripheral blood from mice treated with PBS, mCherry-VLPs or mCherry-Cyt c-VLPs; B to D: Counts of White blood cells (WBC, B), platelets (PLT) and red blood cells (RBCs); E: Serum alkaline phosphatase (ALP) activity; F to J: Serum levels of total protein (TP, F), aspartate aminotransferase (AST, G), blood urea nitrogen (BUN, H), creatinine (CREA, I), and cystatin C (Cys-C, J)

图7 mCherry-Cyt c-VLPs的体内血液学及肝肾功能安全性评价 (n=3, $\bar{x}\pm s$)

Figure 7 *In vivo* evaluation for hematological and hepatorenal safety of mCherry-Cyt c-VLPs (n=3, $\bar{x}\pm s$)

3 讨论

3.1 九香虫来源Cyt c的研究依据及VLP递送价值

九香虫为传统药用昆虫,现代药理研究显示其血淋巴提取物能够抑制乳腺癌细胞的增殖和转移,其来源的ach-miR-276a-3p可通过靶向APPL2调控CDK2-Rb-E2F1信号通路,从而诱导乳腺癌细胞周期阻滞[8-11]。这些结果表明,九香虫中存在具有抗肿瘤活性的生物大分子,为进一步筛选和评价其候选效应分子提供实验依据。本研究据此选择九香虫来源Cyt c作为候选效应分子,并不将其预设为九香虫抗肿瘤作用的唯一物质基础,而是重点评价其经VLPs递送后能否在HER2阳性乳腺癌模型中产生可重复的生物学效应。Cyt c兼具线粒体电子传递和细胞凋亡调控功能。经典研究证实,进入细胞质的Cyt c可参与Apaf-1寡聚化及凋亡小体形成,进而激活Caspase-9和下游Caspase级联反应[12-13,21-25]。尽管Cyt c在进化上具有较高保守性,不同物种Cyt c与人源Apaf-1的结合及其促凋亡效能仍需通过功能实验加以验证,不能仅依据序列差异推断其作用优势。因此,本研究的选题价值主要建立在Cyt c已知的凋亡生物学功能、九香虫来源活性大分子的研究基础以及本研究获得的体内外功能学证据之上。既往研究已证明,基于慢病毒结构蛋白构建的VLPs可将Gag-CASP8等效应分子递送至乳腺癌细胞,并抑制肿瘤细胞生长[15,17,19]。在此基础上,本研究将递送载荷拓展为九香虫来源Cyt c基因,并通过P24蛋白检测、Cyt c mRNA及蛋白表达分析证实其包装和胞内表达。与直接递送活性蛋白相比,基因递送可在受体细胞内形成一定时间范围的蛋白表达,但其表达动力学、亚细胞定位和有效剂量仍需进一步定量。

3.2 mCherry-Cyt c-VLPs诱导线粒体凋亡的实验依据

本研究结果显示,与PBS组和mCherry-VLPs组相比,mCherry-Cyt c-VLPs组HER2阳性原代乳腺癌细胞中Bcl-2和Bcl-xL蛋白表达降低,Bax、Cyt c、cleaved Caspase-9和cleaved Caspase-3蛋白表达升高,同时细

胞凋亡率增加。上述变化表明,mCherry-Cyt c-VLPs处理与线粒体凋亡通路激活密切相关。经典的内源性凋亡通路中,细胞质Cyt c在ATP或dATP参与下促进Apaf-1组装为凋亡小体,募集并激活ProCaspase-9,继而促使Caspase-3等下游效应性半胱天冬酶活化[21-25]。近期研究进一步显示,将Cyt c直接递送至细胞质可在纳摩尔水平诱导Caspase-3依赖性凋亡[26];单细胞动态监测亦观察到Cyt c信号升高先于Caspase-3活化[27]。本研究中Cyt c、cleaved Caspase-9和cleaved Caspase-3同步升高,与上述Cyt c介导的caspase级联过程一致,支持mCherry-Cyt c-VLPs通过Cyt c相关凋亡通路诱导乳腺癌细胞死亡。但本研究未直接检测Apaf-1寡聚化、凋亡小体形成及caspase酶活性,因而现有结果是对该机制的支持性证据,尚不能视为对完整因果链的直接证明。Bcl-2、Bcl-xL和Bax是调控线粒体外膜通透性的关键蛋白。本研究观察到抗凋亡蛋白Bcl-2和Bcl-xL下调、促凋亡蛋白Bax上调,提示mCherry-Cyt c-VLPs处理同时伴随Bcl-2家族蛋白平衡重塑。PDX瘤组织中cleaved Caspase-9和cleaved Caspase-3的变化方向与体外结果一致,且与肿瘤体积和质量下降以及HE染色所示的组织学改变相互印证,说明该处理在体内亦可产生与凋亡激活相一致的抗肿瘤效应。

3.3 细胞周期阻滞与抗肿瘤效应

除诱导凋亡外,mCherry-Cyt c-VLPs处理还使HER2阳性原代乳腺癌细胞G1期比例升高,并伴随CDK2和Cyclin A蛋白表达降低。CDK2可在不同细胞周期阶段与Cyclin E或Cyclin A形成复合物,其中Cyclin A-CDK2主要参与DNA复制及S期进程[28-29]。因此,本研究中CDK2和Cyclin A下调与G1期细胞积聚共同提示G1/S转换及后续DNA复制过程受到抑制。九香虫来源ach-miR-276a-3p通过APPL2-CDK2-Rb-E2F1通路诱导乳腺癌细胞G0/G1期阻滞的研究结果[11],也从另一类九香虫来源分子的角度提示CDK2相关细胞周期调控可能是其抗乳腺癌效应的重要环节。需要强调的是,本研究并未通过CDK2或Cyclin A的过表达回补、药理学干预及连续时相检测验证其与G1期阻滞之间的直接因果关系,因此不宜将CDK2/Cyclin A下调表述为唯一或已被证实的上游机制。现有证据更适合说明,mCherry-Cyt c-VLPs处理后凋亡增强与细胞周期进程受抑同时发生,二者共同构成其降低细胞活力和迁移能力的细胞学基础。既往Gag-CASP8-VLPs在HER2过表达及三阴性乳腺癌原代细胞中亦可引起凋亡和细胞周期改变[17,19],本研究进一步表明,以九香虫来源Cyt c为载荷的VLPs同样能够在临床来源细胞模型中产生细胞死亡和增殖抑制效应。在PDX模型中,mCherry-Cyt c-VLPs经20 d处理后可降低肿瘤体积和质量,并伴随瘤组织细胞周期及凋亡相关蛋白的相应变化。实验期间小鼠体质量、血常规及肝肾功能指标未见明显异常,提示在本研究给药方式、剂量和观察周期内具有初步的耐受性。上述结果将体外的凋亡和细胞周期变化与体内抑瘤效应相结合。

3.4 本研究的主要贡献、局限性与展望

本研究主要解决了2个问题:①建立了九香虫来源Cyt c基因的VLPs递送体系,并在HER2阳性原代乳腺癌细胞中证实其可实现胞内表达及抗肿瘤效应;②在原代细胞和PDX模型2个层面观察到凋亡增强、细胞周期进程受抑及肿瘤生长减缓,为传统药用昆虫来源大分子与VLPs递送技术的融合提供了概念验证。

本研究仍存在以下局限。①机制因果关系尚未完全建立。外源Cyt c表达、Bcl-2家族蛋白变化、Caspase级联激活及CDK2/Cyclin A下调之间的先后顺序和因果方向尚不明确。活化的Caspase通路可能导致细胞周期调控蛋白的继发性改变,线粒体能量代谢和氧化还原状态变化也可能参与细胞周期调控。后续应通过连续时相检测、Caspase抑制、Apaf-1或Caspase-9干预以及CDK2/Cyclin A回补实验验证关键环节。②内源性与外源性Cyt c的贡献尚未区分。本研究检测的是细胞质总Cyt c,未能确定其升高主要来自VLPs递送后的外源表达,还是同时包含线粒体内源性Cyt c释放。同时,研究未直接检测线粒体膜电位、Bax/Bak寡聚化及线粒体外膜通透化。后续可采用线粒体与细胞质分离、标签或物种特异性检测、活细胞亚细胞定位、JC-1或TMRM染色以及Bax/Bak活化分析,对Cyt c来源和线粒体功能变化进行验证。③递送靶向性和全身给药可行性尚未评价。本研究体外采用直接感染,体内采用瘤内注射,主要反映VLPs到达靶细胞后的药效学作用。现有颗粒未设置HER2特异性识别元件,不能据此推断其经全身给药后的肿瘤选择性。已有研究表明,利用HER2结合Affimer等配体修饰VLPs可提高其对HER2表达细胞的靶向递送效率[30]。后续应开展包膜靶向改造,并系统评价全身给药后的药代动力学、生物分布、肿瘤富集、网状内皮系统清除、免疫原性及脱靶效应。④体内疗效与安全性证据仍属初步。当前PDX实验样本量和观察周期有限,且血常规、肝肾功能及体质量正常不能完全替代系统毒理学评价。后续需扩大样本量和观察时间,增加主要脏

器组织病理学、炎症因子、免疫反应及重复给药毒性检测,并在具有完整免疫系统的模型中评价疗效和安全性。在完成机制验证和主动靶向优化后,可进一步评估mCherry-Cyt c-VLPs与抗HER2治疗或其他细胞周期靶向药物联合应用的可行性。

综上,本研究证实mCherry-Cyt c-VLPs可在HER2阳性原代乳腺癌细胞和PDX模型中产生凋亡增强、细胞周期进程受抑及肿瘤生长减缓等效应,且在当前实验条件下未见明显血液学及肝肾功能异常。该研究为九香虫来源Cyt c的抗肿瘤递送提供了初步证据,但其完整作用机制、全身靶向递送能力及长期安全性仍需进一步验证。

利益冲突 本文所有作者声明不存在利益冲突

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