·基础研究·

*EZH1/2*抑制剂UNC1999对肝癌细胞的影响

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摘　要：【目的】探讨*EZH1/2*抑制剂UNC1999对肝癌细胞SMMC-7721的影响及其作用机制。【方法】实验设2个组：DMSO（对照组）、UNC1999组，分别加入不同浓度的DMSO、UNC1999并作用不同时间，用CCK-8法检测细胞OD值，筛选药物较佳的作用浓度及时间；EdU细胞增殖实验及克隆形成实验分别检测细胞增殖和克隆形成能力；划痕实验检测细胞迁移能力；Transwell侵袭、迁移实验检测细胞侵袭及迁移的能力；Annexin V-FITC/PI双染法检测细胞凋亡；流式细胞术检测的细胞周期。RNA-seq检测UNC1999对细胞转录组的影响；qRT-PCR法检测药物作用相关基因（*EZH1*、*EZH2*）以及转录水平显著差异基因（*NECTIN4*）的相对表达量；Western Blot法检测EZH1、EZH2、H3K27me3表达情况。【结果】与对照组相比，UNC1999组的肝癌细胞增殖、迁移、侵袭能力均降低（$P < 0.05$）；细胞周期发生G0/1阻滞（$P < 0.05$）；细胞凋亡数量无明显改变（$P > 0.05$）。UNC1999可促进包括多个基因转录水平的改变，首位显著上调的基因为*NECTIN4*（ENSG00000143217）。在基因的转录表达水平上，UNC1999组的*EZH1*、*EZH2*无明显降低（$P > 0.05$）；而在蛋白表达水平上，两者表达降低（$P < 0.05$），同时H3K27me3也表达下降（$P < 0.05$）。【结论】UNC1999能通过在蛋白水平上抑制表观遗传子*EZH1*、*EZH2*的表达及其催化组蛋白甲基化的功能，发挥抑制肝癌的作用；表观遗传子*EZH1*、*EZH2*与肝癌之间具有密切关系，是肝癌治疗具有潜力的靶标；UNC1999处理后*NECTIN4*表达显著升高，该基因异构体与抗癌药物治疗反应性相关，提示表观遗传抑制剂联合抗癌药物可能发挥更佳的治疗作用，这为临床肝癌药物治疗提供了新的思路。

关键词：*EZH1/2*；UNC1999；转录组学；*NECTIN4*；肝癌
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Effect and Mechanism of *EZH1/2* Inhibitor UNC1999 on Hepatocellular Carcinoma Cell: a Preliminary Study

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Abstract:【Objective】The aim of this study was to detect the effect and mechanism of *EZH1/2* inhibitor UNC1999 on hepatocellular carcinoma cell line SMMC-7721.【Methods】Two groups including DMSO group (control group) and UNC1999 group were treated with different concentration of DMSO and UNC1999 for different time, respectively, then OD values were detected by using CCK-8 kit to screen the appropriate action concentration and time of UNC1999. Cell proliferation rate was detected with EdU (5-ethynyl-2-deoxyuridine) Cell Proliferation Kit. The clone formation ability of

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cell was investigated by clone formation assay. Wound healing assay and transwell assay were used to detect the ability of migration and invasion. Annexin V-FITC/PI double staining assay was performed to detect cell apoptosis. Flow cytometry was used to detect cell cycle. RNA-seq was performed to detect the cell transcriptomics. qRT-PCR was conducted to investigate the related genes, including *EZH1*, *EZH2* and *NECTIN4*. Western blot was conducted to detect the expression of EZH1, EZH2 and H3K27me3.【Results】Compared with the control group, the UNC1999 group showed lower cell proliferation, inhibited ability of migration and invasion ($P < 0.05$). In UNC1999 group, G0/1 block occurred in the cell cycle ($P<0.05$), while cell apoptosis had no significant change ($P > 0.05$).【Conclusion】UNC1999 could inhibit HCC by suppressing the expression of *EZH1* and *EZH2* both in protein level, as well as their function of catalyzing histone methylation. *EZH1* and *EZH2* play important roles in HCC, which may be potential targets for HCC treatment. UNC1999 could significantly promote the expression of *NECTIN4* isoform which has been reported to be associated with the response to anti-cancer drug, suggesting that the combination of *EZH1/2* inhibitor and anti-cancer drug may exert greater effect of inhibiting HCC. This can provide a new idea for clinical drug treatment of liver cancer.

Key words: *EZH1/2*; UNC1999; transcriptomics; *NECTIN4*; HCC

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原发性肝癌是常见的、高死亡率的消化道恶性肿瘤，其病理类型绝大部分为肝细胞肝癌(hepatocellular carcinoma, HCC)[1]。在全球癌症病例中，HCC发病率居第6位，死亡率居第4位[2]。中国是肝病大国，HCC在我国的死亡率更是高居第2位[3]。HCC难以早期发现、极易发生转移是治疗难关。目前肝癌的治疗方法包括手术切除、局部消融、肝动脉化疗栓塞等外科治疗及免疫治疗，外科治疗由于癌细胞可早期经血循环转移而容易复发；免疫治疗虽取得了一定进展，但仍然存在某些不良事件[4-6]。所以，直接靶向肿瘤的药物治疗是一个值得研究的突破口。Zeste增强子同源物1(enhancer Zeste of homolog 1, EZH1)和Zeste增强子同源物2(enhancer Zeste of homolog 2, EZH2)，均是多梳蛋白抑制复合物2(polycomb repressive complex 2, PRC2)中具有催化功能的亚基，能够通过催化组蛋白H3的第27位赖氨酸(H3K27)的甲基化，进而调控相关基因，介导转录沉默[7]。PRC2已被报道与多种肿瘤密切相关[8-10]，EZH1/2作为PCR2重要的组成成分，也参与了多种肿瘤的发生发展[11-13]，其中包括肝癌[14-15]。EZH1/2在不同疾病中具有重要作用[16-19]，因此已有多种特异性抑制EZH1/2活性的抑制剂被开发出来，用于多种疾病发生发展机制的辅助研究。UNC1999是一个EZH1/2特异性双重抑制剂[19]，对EZH1与EZH2的抑制效率相差不大，已被报道能抑制多种肿瘤[20-21]。那么，EZH1/2抑制剂UNC1999是否能发挥抗肝癌作用？以及其中的作用机制如何？因此，我们想通过探讨该新的药物对肝癌的影响及作用机制，为肝癌的药物治疗方案提供新的参考。

1 材料与方法

1.1 材 料

肝癌细胞SMMC-7721来源于中国科学院干细胞库；DMEM培养基、PBS缓冲液、青链霉素、胎牛血清及胰酶均为Gibco产品；EdU细胞增殖检测试剂盒、细胞凋亡试剂盒及细胞周期试剂盒均为Solarbio产品；qRT-PCR引物为英潍捷基产品；cDNA逆转录试剂盒、SYBR Premix ExTaq™ Ⅱ、CCK-8试剂盒及Western blot所用到抗体分别购自Thermo Scientific、Takara、日本同仁及Abcam公司。

1.2 方 法

1.2.1 细胞培养 使用含体积分数100 mL/L胎牛血清及体积分数1%双抗的DMEM高糖培养基培养肝癌细胞SMMC-7721(培养条件为37 ℃、体积分数5% CO_2)。

1.2.2 CCK-8实验 将细胞悬液以2 500个/孔的密度铺于96孔板，每孔100 μL，37 ℃、体积分数5%CO_2培养24 h至细胞贴壁。由于两种药物均溶于DMSO，因此实验设为DMSO组、UNC1999组，分别加入终浓度梯度为0、10、15、20 μmol/L的DMSO溶液、UNC1999药液，并分别作用0、12、24、36 h。后每孔加入CCK-8试剂10 μL，37 ℃孵育0.5 h，后置于450 nm下检测每孔OD值。

1.2.3　EdU 细胞增殖实验　接种细胞于96孔板，每孔 1×10^5 个，每组设置6个复孔，培养24 h至贴壁，分别加入两种药物 15 μmol/L，作用12 h后终止，弃去培养基。每孔加入 50 μmol/L EdU-培养基共 100 μL，培养 2 h 进行标记，后弃去上清。PBS清洗2遍，每次5 min。每孔加入50 μL细胞固定液（含40 g/L多聚甲醛的PBS），室温孵育30 min，弃固定液。每孔加入 50 μL，2 mg/mL 甘氨酸，摇床孵育 5 min，弃甘氨酸溶液。每孔加入 100 μL PBS，脱色摇床清洗 5 min，弃 PBS。每孔加入 100 μL 渗透剂（含体积分数0.5% Triton X-100的PBS）脱色摇床孵育 10 min，PBS 清洗 1 次，5 min。每孔加入 100 μL 1X Apollo 染色反应液，避光、室温、脱色摇床30 min，弃染色反应液。加入每孔加入 100 μL 渗透剂脱色摇床清洗 3 次，每次 5 min，弃渗透剂。每孔加入 100 μL 1X Hoechst 33342 反应液，避光、室温、脱色摇床 30 min，弃染色反应液。每孔 100 μL PBS 清洗 3 次。最后于荧光显微镜下进行观测。

1.2.4　平板克隆形成实验　以每孔500个细胞接种于6孔板中，置于 37 ℃、体积分数 5% CO_2 培养箱中，静置培养24 h至细胞贴壁。分别加入两种药物 15 μmol/L，作用 12 h 后终止。之后每 3 d 更换一次培养基，直至出现肉眼可见的细胞克隆便终止培养。75%乙醇固定 20 min，后用质量分数 0.1%结晶紫染色 30 min，置于缓慢水流下洗去结晶紫，晾干后拍照。

1.2.5　划痕实验　将细胞以 5×10^5 个/孔的密度接种于6孔板中，置于37 ℃体积分数为5% CO_2 培养箱中，静置培养24 h至细胞贴壁。分别加入两种药物 15 μmol/L，作用12 h后终止，弃去培养基，更换为无血清的培养基。使用无菌的 200 μL 枪头的尖端在孔中垂直划3条平行直线，PBS轻轻清洗3次以去除脱落细胞，培养72 h后，于倒置显微镜下拍照。

1.2.6　Transwell 法侵袭、迁移实验　侵袭实验：于 Transwell 小室内铺上 100 μL 基质胶（浓度为 250 μg/L），待胶凝固。用无血清培养基将细胞制成单细胞悬液，并调整密度至 5×10^5/mL。在小室外加入 600 μL 的含体积分数 10%FBS 的 DMEM 培养基，在小室中加入 200 μL 的细胞悬液，置于 37 ℃、体积分数 5%CO_2 培养箱中 12 h。后分别加入两种药物 15 μmol/L，作用 12 h 后终止。取出小室，弃去小室内外的培养液，用棉签轻轻擦去未迁移的细胞，用无钙PBS洗2遍，再用棉签轻轻擦拭一遍。75%乙醇固定 20 min，适当风干。质量分数 0.1%结晶紫染色，室温过夜。在显微镜下选择 5 个具有代表性的视野，进行拍照、计数。迁移实验：不需预先铺胶，其余同侵袭实验。

1.2.7　流式细胞术检测细胞凋亡　以 5×10^5 个细胞/每孔将细胞接种于培养皿中，置于 37 ℃体积分数为 5% CO_2 培养箱中，静置培养 24 h 至细胞贴壁。分别加入两种药物 15 μmol/L，作用 12 h 后终止，弃去培养基，消化细胞得到细胞沉淀。将细胞沉淀重悬在 1 mL binding buffer 中，37 ℃孵育 10 min，1 000 r/min（r=145 mm）离心 5 min，去除上清，按照说明书分别加入染色试剂 Annexin V/PI。室温避光孵育 15 min 后加入 binding buffer，于 1 h 内上机检测。

1.2.8　流式细胞术检测细胞周期　以 5×10^5 个细胞/每皿将细胞接种于培养皿中，置于 37 ℃体积分数 5% CO_2 培养箱中，静置培养 24 h 至细胞贴壁。分别加入两种药物 15 μmol/L，作用 12 h 后终止，弃去培养基，消化细胞得到细胞沉淀。将细胞沉淀样品轻轻弹散，加入适量预冷的体积分数 75%~80%乙醇。样品置于-20 ℃固定过夜。1 000 r/min（r=145 mm）、4 ℃离心 10 min 后，应用预冷 PBS 重悬，洗 1~2 遍。按照说明书加入 0.5 mL 染色剂 PI，室温避光孵育 15~30 min，后上机检测。

1.2.9　RNA-seq　将 DMSO 处理的细胞为对照组，UNC1999 处理的细胞为处理组（作用浓度与作用时间同样分别为 15 μmol/L、12 h），各 3 个生物学重复，每样品约含有 1×10^7 个细胞，提取总 RNA 后使用 Illumina 高通量测序平台进行转录组测序。得到每个样品的序列表达量之后，使用 edgeR 分析每个比较组中的差异基因。用 BH 方法对 P 值进行多重假设检验校正。将 FDR≤0.05 且 |log2FC|≥1 的基因作为候选的差异表达基因。并且对差异基因进行 GO 注释分类和 KEGG 富集分析，探测差异基因的生物学意义。

1.2.10　实时荧光定量 PCR 法　使用 Trizol 提取细胞总 RNA，后按照说明书进行实时荧光定量 PCR 法，上机程序为：95 ℃ 5 s，60 ℃ 30 s，共做 40 个 PCR 循环。引物序列如下示（5′-3′）：GAPDH（GGGAAACTGTGGCGTGAT）、EZH2（CCCTGACCTCTGTCTTACTTGTGGA）、EZH1（GCTTCCTTCACCCTTTTCATGCCACCC）、NECTIN4（GCATCTACG-

TCTGCCATGTCAG)。

1.2.11 Western Blot 使用蛋白抽提试剂盒提取细胞蛋白,并用BCA法测浓度。于聚丙烯酰胺凝胶中电泳,PVDF膜电转,封闭完毕孵育一抗4 ℃过夜:β-actin(1∶1 000)、EZH1(1∶1 000)、EZH2(1∶500)、H3K27me3(1∶500);PBST洗膜后孵育二抗1 h,再洗膜。ECl化学发光液显色后于化学发光成像分析系统仪器中曝光。

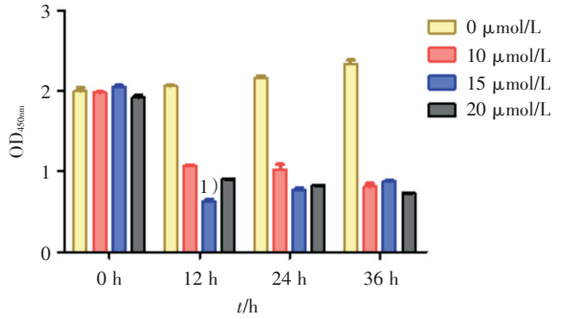
1.3 统计学分析

实验数据均使用Graphpad Prism 5.0软件进行统计。两组间比较,数据呈正态分布且方差齐的数据采用t检验;呈正态分布但方差不齐的数据采用校正t检验。多组间比较数据呈正态分布且方差齐,采用完全随机设计的方差分析,实验组与对照组比较采用Dunnett′t检验,检验为双侧检验。其中$P<0.05$为差异具有统计学意义。

2 结 果

2.1 UNC1999抑制肝癌细胞的活力

将终浓度为0、10、15、20 μmol/L浓度的DMSO、UNC1999分别作用0、12、24、36 h至肝癌细胞SMMC-7721,CCK-8法检测细胞OD_{450nm}值,筛选UNC1999的较佳作用浓度及时间。综合考虑后,选择UNC1999较佳作用浓度及时间分别为15 μmol/L、12 h($F=125.3$,$P=0.0000$),将该结果作为后续的所有实验的处理条件。该处理条件与DMSO组相比,OD_{450nm}值差异具有统计学意义($t=5.132$,$P=0.0068$;图1)。



UNC1999 inhibited the cell viability of SMMC-7721. CCK-8 kit was used to detect the cell viability with OD_{450nm} in different concentration for different action time. The result showed that UNC1999 could inhibit cell viability and the optimal action concentration and time was 15 μmol/L and 12 h, respectively. $n=3$. 1)$P<0.01$.

图1 UNC1999抑制SMMC-7721的活力

Fig.1 The decreased cell viability of SMMC-7721 after UNC1999 treatment

2.2 UNC1999抑制SMMC-7721的增殖和克隆形成能力

进行EdU和平板克隆形成实验,进一步检测UNC1999对细胞增殖和克隆形成能力的影响。通过比较DMSO组(图2A)与UNC1999组(图2B)阳性细胞的比例,发现UNC1999组的细胞增殖比例降低,差异具有统计学意义($t=12.730$,$P=0.0002$;图2C)。将DMSO组(图2D)与UNC1999组(图2E)的细胞克隆数量进行比较后,结果为UNC1999组的细胞克隆数量减少($t=3.138$,$P=0.0349$;图2F)。

2.3 UNC1999抑制肝癌细胞的迁移能力

为探究UNC1999是否对SMMC-7721的迁移能力产生影响,进行划痕实验,分别观察在划痕0 h(图3A,B)以及72 h(图3C,D)后细胞的迁移情况。发现相对于DMSO(图3C),UNC1999组(图3D)中SMMC-7721的划痕距离增大,差异具有统计学意义($t=4.341$,$P=0.0049$;图3E)。

2.4 UNC1999抑制肝癌细胞的侵袭及迁移能力

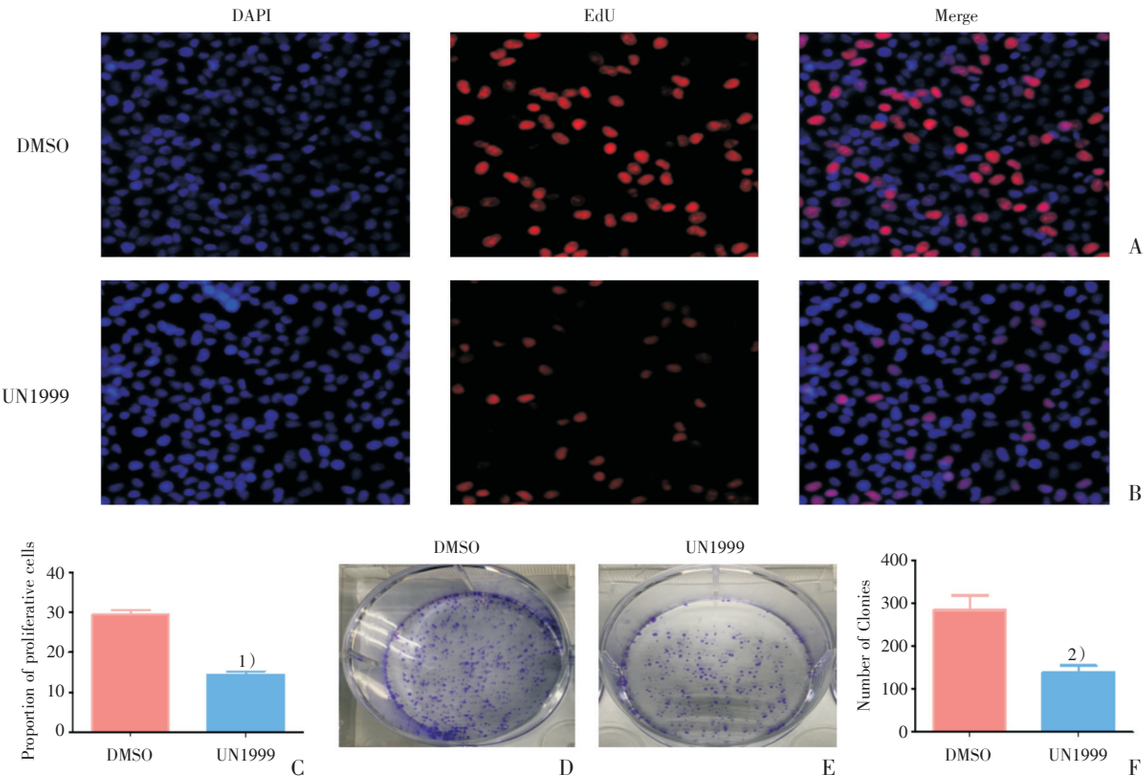
继续对SMMC-7721的迁移、侵袭能力进行Transwell实验的验证。通过对比两组间细胞的侵袭及迁移数量,发现UNC1999(图4B)比DMSO组(图4A)的侵袭数量减少,差异具有统计学意义($t=3.882$,$P=0.0178$,图4E);同时UNC1999组(图4D)比DMSO组(图4C)迁移数量减少,差异具有统计学意义($t=10.630$,$P=0.0004$;图4F)。

2.5 UNC1999对肝癌细胞凋亡无显著影响

为探讨UNC1999对SMMC-7721细胞凋亡情况的影响,采用流式细胞术进行检测。检测的结果包括流式细胞术结果图(图5A,B)、结果统计图(图5C,D)。相比DMSO(图5A),UNC1999的早期凋亡比例($t=1.707$,$P=0.1186$)及晚期凋亡比例($t=1.663$,$P=0.1308$)均无显著改变,差异无统计学差异(图5C-D)。

2.6 UNC1999影响肝癌细胞的细胞周期

肿瘤细胞的异质性之一体现在其细胞周期的变异,为再进一步探究UNC1999是否通过影响肿瘤细胞的细胞周期来发挥作用,我们进行流式细胞术检测细胞周期的情况。结果发现:与对照组相比(图6A),UNC1999组(图6B)出现G0/1期比例增加($t=5.186$,$P=0.0066$,图6C)、S期比例降



UNC1999 inhibited the activity of cell clone formation of SMMC-7721. The cells were fluorescently stained with EdU (red) while the nucleus was stained with Hoechst 33342 (blue). The EdU-positive cells in DMSO and UNC1999 group were shown A and B, respectively. Scale bar = 100 μm. The proportion of EdU-positive cells between two different groups were analyzed in C, 1) $P<0.001$. Cell clone formation assay was conducted to detect the cell clone formation ability of SMMC-7721 after treated with DMSO (D) and UNC1999 (E). The number of clonies between two different groups were analyzed in F, 2) $P<0.05$. $n = 3$.

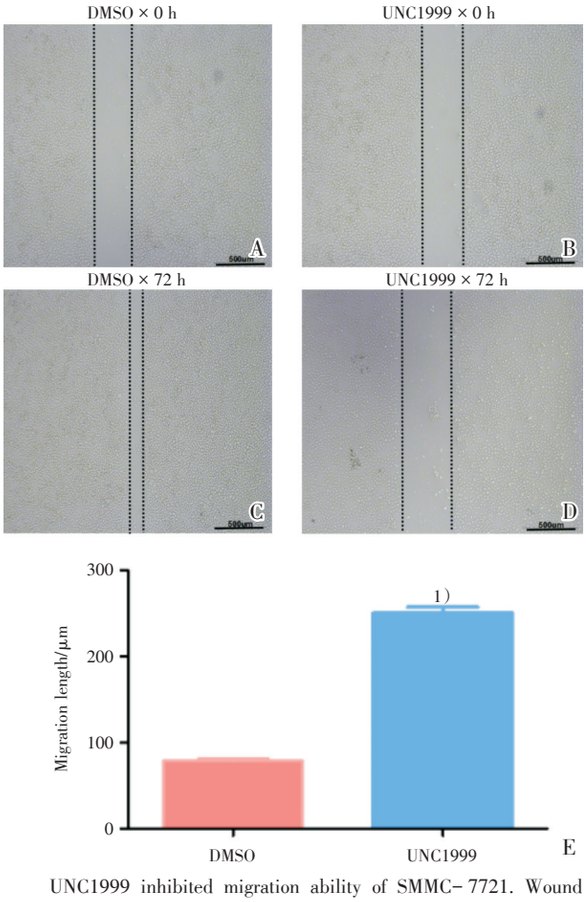
图2　UNC1999 抑制 SMMC-7721 的增殖和克隆形成能力

Fig.2　UNC1999 inhibited cell growth and colony formation of SMMC-7721

低($t = 6.766$, $P = 0.0025$；图6D)，差异具有统计学意义。

2.7　UNC1999 处理 SMMC-7721 后细胞转录组变化情况检测与分析

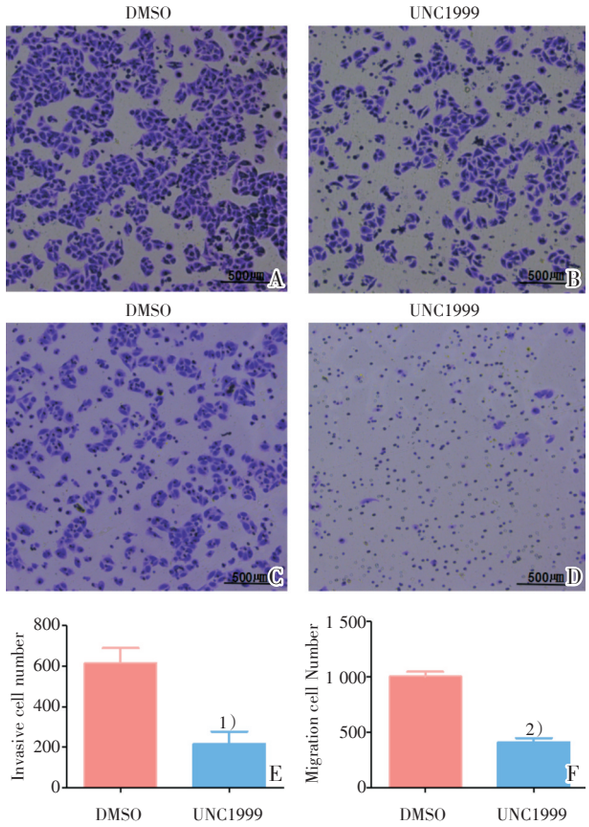
通过一系列的细胞实验，我们发现 UNC1999 能够抑制肝癌的多种生物学功能，为进一步研究 UNC1999 发挥功能的机制，进行了 RNA-seq 检测分析。将样品所测得的序列与所选的参考序列进行比对，统计序列与每一个参考序列的比对结果，如表1所示。并对两组之间基因的转录情况进行统计比较，以期探究 UNC1999 处理后对 SMMC-7721 基因表达的影响，结果发现某些基因出现显著性改变(表2、3)。同时对 UNC1999 作用的两个基因 *EZH1* 及 *EZH2* 基因进行统计(表4)，检测药物在转录水平上对两个基因的影响情况。进而，通过对两组细胞的转录组学结果进行基因差异分析及富集分析，图7A 为差异表达基因的火山图，每个点代表一个基因，图中红色的点代表统计检验显出差异的基因；图7B 为上下调差异基因分别富集在生物过程(BP)、细胞组分(CC)和分子功能(MF)的情况；图7C 分析统计了差异基因富集的通路所在的级别——A 级(代谢)；图7D 为差异基因 *KEGG* 富集的点图，点的大小表示此通路中差异表达基因个数多少，而点的颜色对应于不同的 *P* 值范围。分析统计结果发现：与 DMSO 组相比，UNC1999 处理后 SMMC-7721 存在某些的基因转录差异；差异的基因富集在细胞代谢、细胞粘附结合、细胞免疫应激等多个功能种类上(图7B~D)。[备注：FPKM(fragments Per Kilobase per Million mapped reads)为每百万序列中来自某一基因每千碱基长度的片段。FDR 是对 *P* 值做多重校正后的值。]



UNC1999 inhibited migration ability of SMMC-7721. Wound healing assay was performed to investigate the migration ability of SMMC-7721 by measuring the migration length. The migration length of SMMC-7721 treated with DMSO was measured after 0 h (A) and 72 h wound healing assay (C). The migration length of SMMC-7721 treated with UNC1999 was measured after 0 h (B) and 72 h wound healing assay (D). The migration distance between two groups were analyzed in E, 1) $P < 0.01$. (Each histogram showed the migration length at one high magnification, scale bar = 500 μm, $n = 4$).

图3 UNC1999抑制SMMC-7721的迁移能力

Fig.3 The decreased migration ability of SMMC-7721 after UNC1999 treatment



UNC1999 inhibited the invasive and migrated ability of SMMC-7721. Transwell assay was conducted to investigate SMMC-7721 invasion after treated with DMSO (A) and UNC1999 (B). The invasive cell number of SMMC-7721 between two different groups were analyzed in E, 1) $P<0.05$. Transwell assay was conducted to investigate the migrated ability of SMMC-7721 after treated with DMSO (C) and UNC1999 (D). The migrated cell number of SMMC-7721 between two different groups were analyzed in F, 2) $P < 0.001$. (Each histogram showed the invasive cell number at one high magnification, scale bar = 500 μm, $n = 5$).

图4 UNC1999抑制SMMC-7721的侵袭及迁移能力

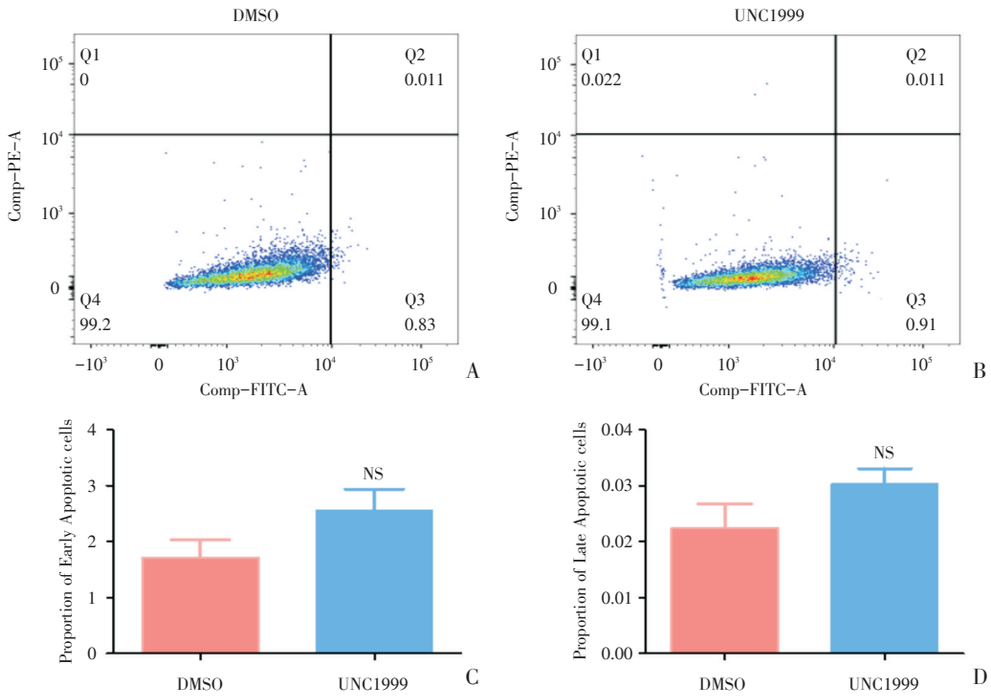
Fig.4 The inhibited invasive and migrated ability of SMMC-7721 after UNC1999 treatment

2.8 UNC1999促进肝癌细胞NECTIN4的表达

在分析了两组细胞转录学结果之后,发现UNC1999在转录水平并未影响*EZH1*及*EZH2*的表达水平,于是使用qRT-PCR进行验证;同时发现UNC1999处理后,SMMC-7721首位显著上调的基因为*NECTIN4*(ENSG00000143217),该基因与肿瘤细胞的药物敏感性有关,这引起了我们的注意,遂同样采用qRT-PCR进行验证。结果发现:与DMSO组对比,UNC1999组的*EZH1*($t = 2.279$, $P = 0.0849$;图8A)与*EZH2*(图8B)基因相对表达水平无明显改变($t = 1.608$, $P = 0.1832$, $P > 0.05$),与转录组学结果一致;*NECTIN4*($t = 8.239$, $P = 0.0012$;图8C)基因相对表达水平上调明显,差异具有统计学意义($P < 0.05$)。

2.9 UNC1999抑制肝癌细胞*EZH1*、*EZH2*、*H3K27me3*的表达

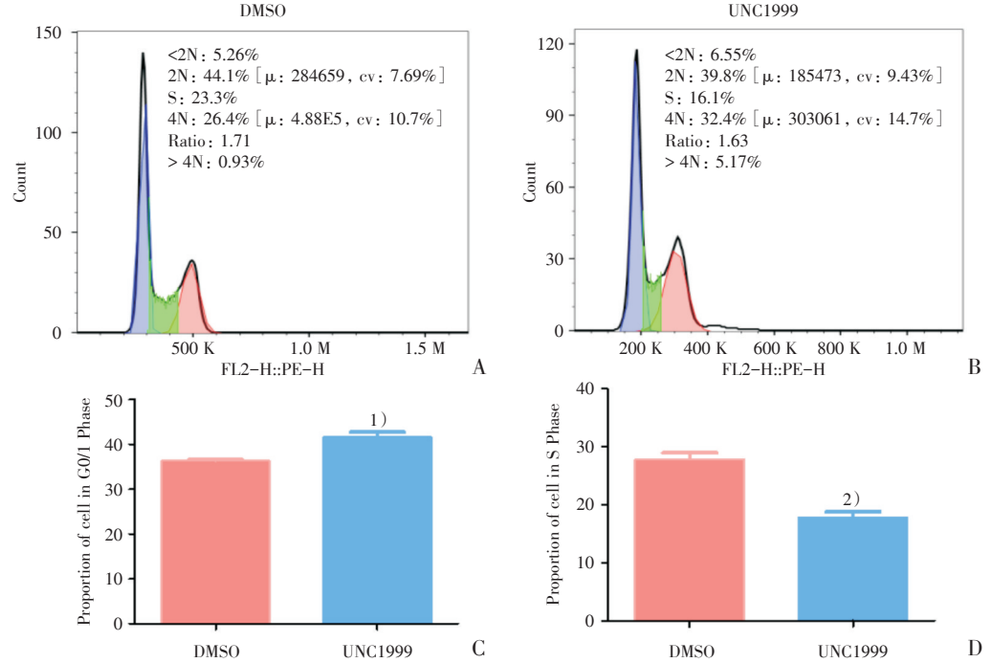
我们在检测*EZH1*及*EZH2*的基因表达水平之后,发现在基因水平上,UNC1999并未明显抑制两种基因的转录水平,遂进一步在蛋白水平上验证。结果发现UNC1999能抑制*EZH1*($t = 4.032$,



UNC1999 could not influence apoptosis of SMMC-7721. Apoptosis state including early apoptosis (Annexin V positive and PI negative) and late apoptosis (Annexin V-PI double positive) were shown in cell percentage by flow cytometry (A-B). The proportion of SMMC-7721 in early and late apoptosis phase between two different groups were analyzed in C and D, respectively ($n = 6$).

图5　UNC1999不影响SMMC-7721细胞凋亡

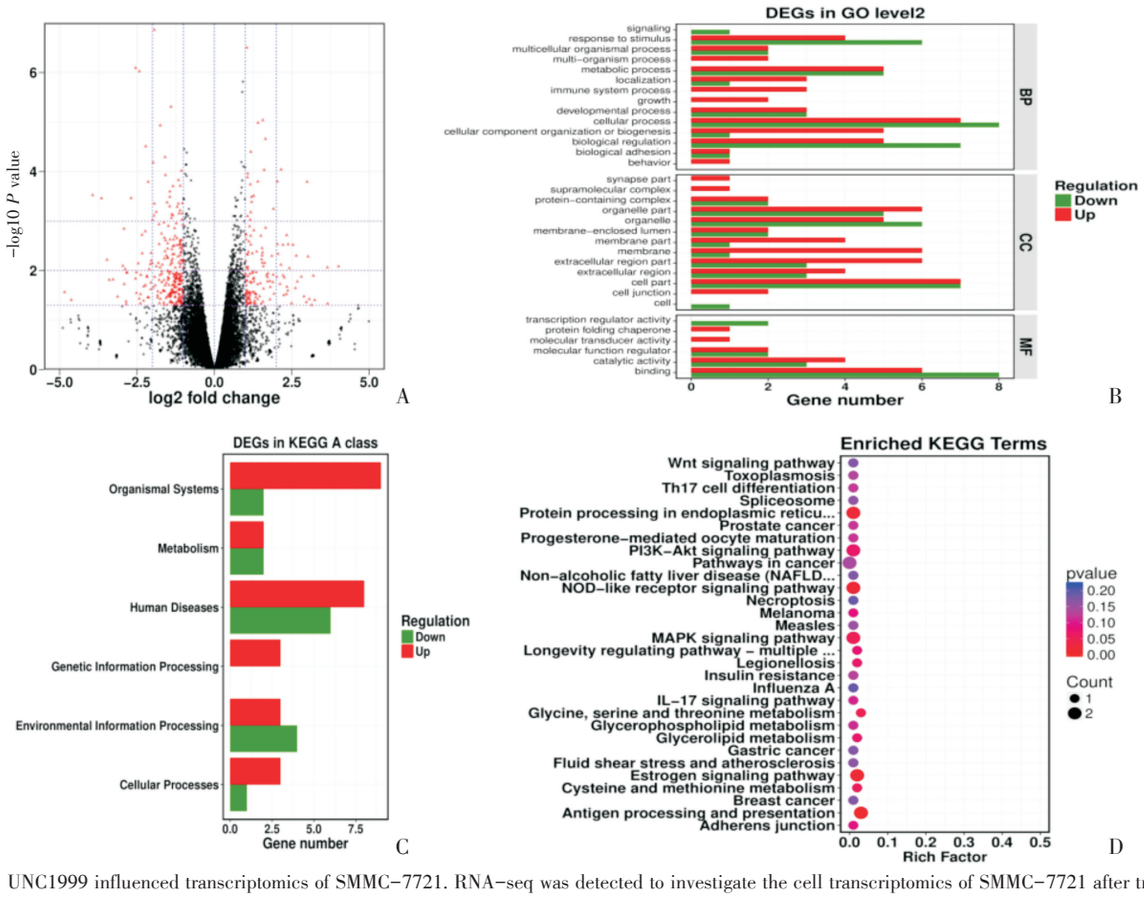
Fig.5　UNC1999 could not influence cell apoptosis of SMMC-7721



UNC1999 influenced cell cycle of SMMC-7721. Flow cytometry was performed to investigate the cell cycle of SMMC-7721 after treated with DMSO (A) and UNC1999 (B). The proportion of SMMC-7721 in G0/1 phase between two different groups were analyzed in C, 1) $P<0.05$. D showed the proportion of cell number in S phase in two groups and the differences between them, 2) $P<0.05$. $n = 3$.

图6　UNC1999影响SMMC-7721的细胞周期

Fig.6　UNC1999 could altered cell cycle of SMMC-7721



UNC1999 influenced transcriptomics of SMMC−7721. RNA−seq was detected to investigate the cell transcriptomics of SMMC−7721 after treated with DMSO and UNC1999. The differentially expressed genes (DEGs) between two different groups were showed by red dots in A, and the up−regulated genes were represented in the right side while the down−regulated genes in the left. B showed the enrichment result of up−regulated and down−regulated GO Terms in biological processes (BP), cellular components (CC) and molecular functions (MF). The class of the pathways of DEGs were analyzed in C. DEGs KEGG enrichment plot was showed in D, and the colors of the points correspond to the different *P*−value range.

图7 UNC1999影响SMMC−7721的转录组学结果

Fig.7 UNC1999 altered transcriptomics of SMMC−7721

表1 UNC1999组与DMSO组的RNA−seq的统计学结果

Table 1 The statistical result of RNA−seq map in DMSO group and UNC1999 group

Sample	Total paired reads	Paired mapped reads	Unpaired mapped reads	Unmapped reads	Total mapped
DMSO	19428372.5	0.939548333	0.014048578	0.046403089	0.953596911
UNC1999	19447736.0	0.951100735	0.012692377	0.036206888	0.963793112

P = 0.0157;图9A)、*EZH2*(t = 7.197, *P* = 0.0006;图9A)的蛋白表达,差异具有统计学意义(图9C,D)。由于*EZH1/2*显著的功能之一是对H3K27进行三甲基化,于是同时检测H3K27me3的蛋白表达情况(t = 3.489, *P* = 0.0251;图9B),发现UNC1999能抑制其表达(图9B),差异具有统计学意义(图9E)。

3 讨 论

在本研究中,我们通过采用*EZH1/2*的特异性抑制剂UNC1999处理肝癌细胞SMMC−7721,检测了用药后SMMC−7721的多个生物特性,并初步探讨了药物对肝癌影响的作用机制。结果显示

表2　UNC1999组中上调的基因列表

Table 2　The up-regulated genes in UNC1999 group

Gene_ID	FPKM_UNC1999	FPKM_DMSO	log2FC	FDR	*P*	Name
ENSG00000143217	1.74	0.29	2.152648148	0.048659509	9.11E-05	*NECTIN4*
ENSG00000139438	1.975	0.66	1.647722677	0.021521034	2.19E-05	*FAM222A*
ENSG00000088854	0.36	0.12	1.606731287	0.048659509	9.27E-05	*C20orf194*
ENSG00000135636	3.065	1.205	1.562729637	0.011105304	9.17E-06	*DYSF*
ENSG00000109971	1642.745	614.3	1.428968541	0.000101237	1.86E-08	*HSPA8*
ENSG00000224411	37.165	14.52	1.424949529	0.000101237	1.93E-08	*HSP90AA2P*
ENSG00000080824	429.605	175.635	1.409644806	0.011604621	1.03E-05	*HSP90AA1*
ENSG00000107984	44.635	20.29	1.331497529	0.000314318	7.98E-08	*DKK1*
ENSG00000143797	4.445	2.05	1.17636482	0.032464612	4.00E-05	*MBOAT2*
ENSG00000142669	102.695	51.495	1.046316055	0.000826652	3.15E-07	*SH3BGRL3*

表3　UNC1999组中下调的基因列表

Table 3　The down-regulated genes in UNC1999 group

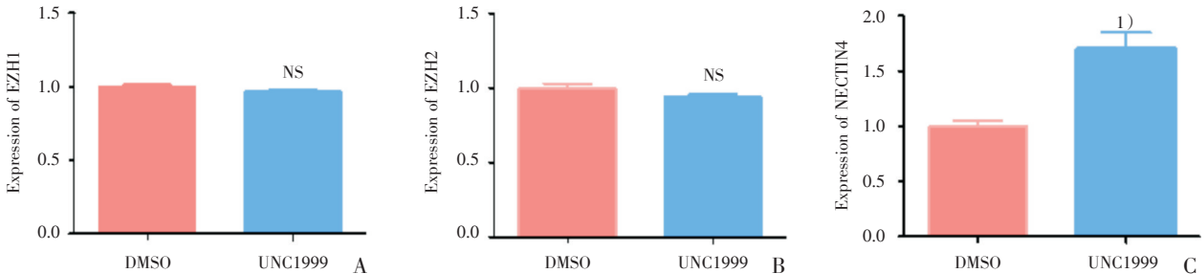
Gene_ID	FPKM_UNC1999	FPKM_DMSO	log2FC	FDR	*P*	Name
ENSG00000244879	2.765	4.43	−1.068884042	0.048872151	9.62E-05	*GABPB1-AS1*
ENSG00000063438	42.175	105.325	−1.403743141	0.006435532	4.90E-06	*AHRR*
ENSG00000189060	103.575	327.685	−1.594535781	0.038163655	5.09E-05	*H1F0*
ENSG00000160323	0.38	1.55	−1.751740686	0.012291274	1.17E-05	*ADAMTS13*
ENSG00000160200	11.155	42.415	−1.852740172	2.90E-07	1.84E-11	*CBS*
ENSG00000009950	2.25	8.58	−1.943027288	0.000431587	1.37E-07	*MLXIPL*
ENSG00000234432	0.34	1.405	−1.977148908	0.043576332	6.58E-05	*AC092171.3*
ENSG00000148225	0.135	0.5	−2.218736644	0.02856755	3.08E-05	*WDR31*
ENSG00000265972	13.14	76.66	−2.425709669	0.00185489	9.42E-07	*TXNIP*
ENSG00000116183	0.2	1.235	−2.544902167	0.001819164	8.09E-07	*PAPPA2*
ENSG00000264058	0.005	0.28	−5.115716573	0.002501605	1.59E-06	*AC073508.2*
ENSG00000105550	0.055	2.695	−5.430543468	0.043576332	6.64E-05	*FGF21*

表4　UNC1999组与DMSO组中EZH1及EZH2的表达统计情况

Table 4　The statistics of EZH1/2 in UNC1999 group and DMSO group

Gene_ID	FPKM_UNC1999	FPKM_DMSO	log2FC	*P*	FDR	Name
ENSG00000108799	7.1	9.34	−0.434821271	0.279017167	0.916852919	*EZH1*
ENSG00000106462	39.325	43.905	−0.107841252	0.742187553	1	*EZH2*

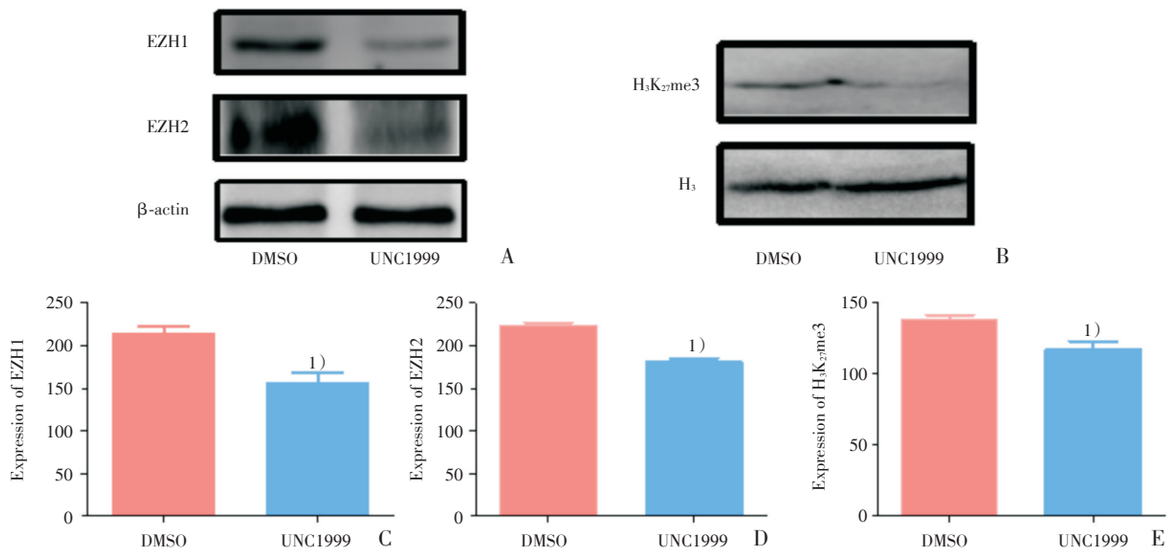
UNC1999能抑制肝癌细胞的增殖、侵袭、迁移能力；并能阻滞肝癌细胞由G0/1期向S期转化，但影响程度较小；而对细胞凋亡无明显影响。提示UNC1999对肝癌的抑制作用不是通过促进凋亡或阻滞细胞周期来实现。为进一步研究其中的作用机制，进行RNA-seq测序发现UNC1999能影响多个基因的表达，并验证发现UNC1999能通过在蛋白水平上抑制*EZH1*、*EZH2*及其催化H3K27me3的功能从而发挥抑制肝癌作用。*EZH1/2*阻断后诱导肝癌受到抑制，这一过程可能同时存在一种或者多种调节，例如：引发了自然杀伤细胞介导的肝癌细胞清除[15]；诱导了多种抑癌microRNA表达上调[22-23]；抑制了肝癌干细胞的自我更新[24]。这些可能存在的机制需要进一步的验证。另外，



UNC1999 promoted *NECTIN4* expression in SMMC-7721. qRT-PCR was performed to investigate the expression of *EZH1* (A), *EZH2* (B) and *NECTIN4* (C) in DMSO group and UNC1999 group, respectively. 1) $P < 0.05$; $n = 3$.

图8 UNC1999促进NECTIN4的表达

Fig.8 UNC1999 promoted NECTIN4 expression in SMMC-7721



UNC1999 inhibited EZH1, EZH2 and H3K27me3 expression of SMMC-7721 in protein level. Western blot detected the expression levels of relative proteins after drug administration. The expression of EZH1, EZH2 (A) and H3K27me3 (B) were detected. The differences of the proteins between two groups were shown in C-E. 1) $P < 0.05$; $n = 3$.

图9 UNC1999抑制SMMC-7721的EZH1/2及H3K27me3蛋白的表达

Fig.9 UNC1999 inhibited EZH1, EZH2 and H3K27me3 expression in protein level

UNC1999处理后*NECTIN4*显著上调,该基因属于免疫球蛋白样跨膜细胞粘附分子蛋白的成员[25],是某些病毒(如麻疹病毒)入侵宿主细胞过程中的细胞受体,已被报道参与抑制AKT-mTOR信号通路从而引起细胞自噬[26]。自噬能够促进和维持肝癌进展[27-28],SMMC-7721转录组结果提示UNC1999作用后,肝癌细胞发生改变的基因所富集的信号通路之一是PI3K-AKT-mTOR(图7D),所以我们推测UNC1999发挥抑制肝癌的机制之一,可能为通过*NECTIN4*抑制AKT-mTOR信号通路进而抑制SMMC-7721自噬,最终抑制肝癌。这一推测同样需要后续的研究。*NECTIN4*的特殊异构体被报道能预测抗肿瘤药物对肿瘤的细胞毒性和靶向治疗的反应性[29]。临床上,肝癌的常用抗癌药物为索拉非尼,但是据报道索拉非尼只对约30%的肝癌病人有效,且会出现继发性耐药[30-31],肝癌的耐药性是其化疗的严重障碍之一。UNC1999处理肝癌细胞之后*NECTIN4*表达水平明显升高,该结果启示我们:将UNC1999与抗肝癌药物联合应用,或许能增加肝癌细胞对抑癌药物的敏感性从而改善耐药问题,甚至两者相互协同发挥更大抗癌作用。这一启发值得深入探讨。

综上,我们发现并研究了特异性的表观遗传子*EZH1/2*抑制剂UNC1999对肝癌细胞的抑制作

用及其机制，揭示表观遗传子 *EZH1/2* 与肝癌细胞密切关系；同时发现与抗癌药物治疗反应性相关的 *NECTIN4* 特殊异构体，在 UNC1999 处理后显著升高，提示表观遗传抑制剂与抗癌药物的联合应用可能成为肝癌的治疗新策略。

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