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敲低 PFKP 联合肉碱棕榈酰基转移酶抑制剂 etomoxir 协同对肾透明细胞癌细胞 Caki-1 的抗肿瘤作用 *

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摘要 目的:研究敲低 P 型磷酸果糖激酶(phosphofructokinase,PFKP)联合肉碱棕榈酰基转移酶抑制剂 etomoxir 对肾透明细胞癌 Caki-1 细胞的影响,并进一步探究其作用机制。**方法:**利用 Western blot 验证对照及 PFKP shRNA 敲低肾透明细胞癌细胞中 PFKP 的敲低效率,分别检测对照组(shCtrl)、PFKP 敲低组、etomoxir 组(shCtrl+etomoxir)、PFKP 敲低联合 etomoxir 组的增殖曲线。使用 Annexin-V/PI 染色并用流式细胞检测对照组、PFKP 敲低组、etomoxir 组、PFKP 敲低联合 etomoxir 组的细胞死亡,研究 PFKP 敲低联合 etomoxir 对细胞存活的影响。分别检测对照组、PFKP 敲低组、etomoxir 组、PFKP 敲低联合 etomoxir 组的 ATP 水平与脂肪酸变化。**结果:**Western blot 结果验证了 PFKP 的敲低效率。流式细胞检测显示,对照组、PFKP 敲低组、etomoxir 组、PFKP 敲低联合 etomoxir 组的平均细胞死亡率分别为 1.1、1.9、13.9、31.3%。PFKP 敲低联合 etomoxir 组 Caki-1 细胞的死亡率显著高于单纯 PFKP 敲低与 etomoxir 组 ($P<0.05$)。PFKP 敲低联合 etomoxir 组 Caki-1 细胞的 ATP 水平显著低于单纯 PFKP 敲低与 etomoxir 组($P<0.05$)。Etomoxir 的加入抑制了 PFKP 敲低引起的游离脂肪酸下降($P<0.05$)。**结论:**PFKP 敲低联合 etomoxir 对 Caki-1 细胞呈现协同的细胞毒抗肿瘤作用。

关键词:PFKP;肾透明细胞癌;细胞死亡;肿瘤代谢

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The Anti-tumor Effect of PFKP Knockdown Combined with Carnitine Palmitoyltransferase Inhibitor Etomoxir on Renal Clear Cell Carcinoma Cell Line Caki-1*

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ABSTRACT Objective: To investigate the effect of P-type phosphofructokinase (PFKP) knockdown combined with carnitine palmitoyltransferase inhibitor etomoxir on the renal clear cell carcinoma Caki-1 cells, and further explore its mechanism of action. **Methods:** The knockdown efficiency of PFKP in renal clear cell carcinoma cells was confirmed by Western blot. The proliferation curves of control group (shCtrl), PFKP knockdown group, etomoxir group (shCtrl + etomoxir) and PFKP knockdown combined with etomoxir group were measured. The effects on cell survival in all groups were detected by flow cytometry using Annevin-V/PI staining. The ATP levels and free fatty acid levels in the control group, PFKP knockdown group, etomoxir group, PFKP knockdown combined with etomoxir group were detected. **Results:** Western blot analysis confirmed the knockdown efficiency of PFKP. Flow cytometry showed that the mean cell mortality rate of control group, PFKP knockdown group, etomoxir group, PFKP knockdown combined with etomoxir group were 1.1, 1.9, 13.9, 31.3% Compared with the control cells, the cell mortality of PFKP knockdown combined with etomoxir group Caki-1 cells was significantly higher than that of PFKP knockdown and etomoxir groups($P<0.05$). The ATP levels in PFKP knockdown combined with etomoxir group were significantly lower than those of PFKP knockdown and etomoxir groups ($P<0.05$). The addition of etomoxir inhibited the decrease in free fatty acids caused by PFKP knockdown ($P<0.05$). **Conclusions:** PFKP knockdown combined with etomoxir showed synergistic cytotoxic and anti-tumor effects on Caki-1 cells.

Key words: PFKP; Renal clear cell carcinoma; Cell death; Cancer metabolism

Chinese Library Classification(CLC): R33; R737.11 Document code:A

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前言

代谢异常是肾脏透明细胞癌的一项基本特征[1,2]。肿瘤代谢

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可能是肾透明细胞癌新的靶点[1,2]。我们前期研究发现在肾透明细胞癌临床样品中糖酵解的关键酶,磷酸果糖激酶 P 型(phosphofructokinase,PFKP),是肾透明细胞癌中主要的磷酸果糖激酶 1(PFK1)亚型并且在肿瘤中显著高表达[3]。有意思的是,在肾脏透明细胞癌细胞中敲低 PFKP 阻断糖酵解后,在阻断初期细胞凋亡增加,而利用 shRNA 稳定 PFKP 敲低 2 周后,细胞逐步适应了这种变化;细胞死亡率与对照细胞无显著差异[3]。在代谢方面,细胞内 ATP 水平、AMPK 通路状态均没有明显改变,细胞的氧耗明显增加,细胞中的游离脂肪酸显著下降,提示在阻断糖酵解后,癌细胞可以切换至氧化磷酸化模式,并且可能转而利用脂肪酸氧化供能[3]。因此,我们猜测只有同时抑制有氧糖酵解及脂肪酸氧化磷酸化才能真正杀死肾透明细胞癌。我们发现敲低 PFKP 联合肉碱棕榈酰基转移酶抑制剂 etomoxir[4]能够显著诱导肾透明细胞癌细胞 ATP 水平下降及细胞死亡率增加。本课题利用已建立的敲低 PFKP 的稳转肾透明细胞癌株,通过流式细胞术等技术,探索敲低 PFKP 联合肉碱棕榈酰基转移酶抑制剂 etomoxir 对肾透明细胞癌的抗肿瘤作用及机制。

1 材料与方法

1.1 实验设计

PFKP 敲低效果验证部分利用已构建的 Caki-1 对照(shCtrl)与 PFKP(shPFKP)稳定敲低细胞株,进行 Western blot 验证 shPFKP 的敲低效果。为探索敲低 PFKP 联合肉碱棕榈酰基转移酶抑制剂 etomoxir 对肾透明细胞癌的作用,设置了 shCtrl 与 shPFKP 稳转细胞株分别联合 etomoxir 处理, 即 shCtrl、shPFKP、shCtrl+etomoxir、shPFKP+etomoxir 组, 分别进行细胞增殖、Annexin-V/PI 流式、ATP、脂肪酸水平检测。

1.2 细胞系与培养

Caki-1 细胞系及含对照 shRNA 与 PFKP shRNA 的慢病毒稳转 Caki-1 细胞株均为实验室保存。稳转细胞系构建相关信息详见[3]。细胞培养于添加了 10%胎牛血清,2 mmoL/L 谷氨酰胺,100 unit/mL 青霉素,100 mg/mL 链霉素的 RPMI 1640(Invitrogen Life Technologies,USA)培养基中。细胞培养温度 37℃,CO_2 浓度 5%。每 2 至 3 天更换一次培养基。

1.3 Etomoxir 处理

Etomoxir 购自 Selleck 公司。Etomoxir 最终工作浓度为 100 μM, 肾透明细胞癌细胞在含 etomoxir 的培养基中培养 24 小时,进一步进行流式、代谢相关检测。

1.4 Western blot

提前将细胞接种于 6 孔板,待细胞长至 60%-80%时,使用 RIPA 裂解液提取细胞总蛋白,利用 BCA 试剂盒测定浓度。在转膜后,5%脱脂牛奶室温封闭 1 h, 一抗 4 ℃ 过夜, 二抗室温孵育 1 h,然后加曝光底物曝光。一抗抗人 PFKP 抗体购自 Cell Signaling Technology,抗人 β-Actin 抗体购自 Sigma-Aldrich。

1.5 Annexin -V/PI 染色

提前将细胞接种于 6 孔板, 每种细胞各设置 3 个复孔,待细胞长至 60%-80%时,按照步骤收细胞,按产品说明先后使用 Annexin-V、PI 染色,上流式测定。

1.6 ATP 检测

ATP 定量试剂盒 (ATP Colorimetric/Fluorometric Assay Kit)购自 biovision 公司;收集 1× 10^6 细胞,利用去蛋白试剂盒(biovision)准备样品,取上清,按照步骤进一步处理,室温反应 30 分钟,测 570 nm 处的吸光值,根据吸光值来反应细胞中的 ATP 水平。

1.7 脂肪酸检测

游离脂肪酸定量试剂盒(Free Fatty Acid Quantification Colorimetric Assay Kit)购自 biovision 公司;收集 1× 10^6 细胞,利用 200 μL 的 1% Triton X-100 的氯仿抽提脂质成分, 放入 50℃烘箱,使氯仿蒸发,按照步骤进一步处理,37℃反应 30 分钟,测 570 nm 处的吸光值,根据吸光值来反应细胞中的游离脂肪酸含量。

1.8 细胞增殖实验

胰酶消化细胞,进行细胞计数,然后以每孔 4000 个细胞重新铺在 6 孔板中。每组设置 3 个复孔,在第二、三、四、五天后计数。

1.9 统计学分析

使用 R 软件处理数据,两组间比较用 t 检验。P<0.05 时认为差异有统计学意义(用 * 在图中标注)。

2 结果

2.1 PFKP 敲低效果验证

培养已构建的 Caki-1 对照(shCtrl)与 PFKP(shPFKP)稳定敲低细胞株,利用 Western blot 验证 shPFKP 的敲低效果。结果显示 PFKP 敲低组的 PFKP 水平显著下降(图 1)。

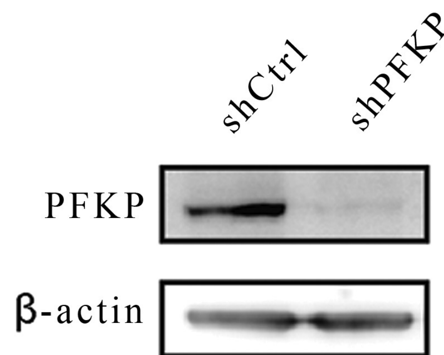

图 1 Western blot 检测稳转细胞中的 PFKP 蛋白敲低水平

注:Caki-1 对照(shCtrl)与 PFKP(shPFKP)稳定敲低细胞株,分别进行 Western blot 验测 PFKP 的表达水平。

Fig.1 Western blot detection of PFKP protein expression level in Caki-1 stable cell lines

Note: Caki-1 control (shCtrl) and PFKP (shPFKP) stably knockdown cell lines were subjected to Western blot. The expression of PFKP was detected by Western blot.

2.2 敲低 PFKP 联合肉碱棕榈酰基转移酶抑制剂 etomoxir 对肾透明细胞癌的作用

Caki-1 shCtrl 与 shPFKP 稳转细胞株分别联合 etomoxir 处理,shPFKP 与 etomoxir 处理均可以抑制 Caki-1 细胞的增殖(图 2)。联合 shPFKP 与 etomoxir 可以显著杀伤 Caki-1 细胞,使细胞数量明显下降至起始细胞量以下, 联合 shPFKP 与 etomoxir 组的细胞数显著少于 shPFKP 组与 shCtrl+etomoxir 组(P分别为 <0.001 与 0.0016)(图 2)。利用 Annexin-V/PI 染色与

流式细胞术，我们发现 shCtrl+etomoxir 组与联合 shPFKP 与 etomoxir 组的细胞死亡以细胞坏死与晚期凋亡为主。shCtrl、shPFKP、shCtrl+etomoxir、shPFKP+etomoxir 组的平均细胞死亡率分别为 1.1、1.9、13.9、31.3%。shPFKP 对 Caki-1 细胞的死亡率无显著改变，etomoxir 可以轻度增加 Caki-1 细胞的死亡率。联合 shPFKP 与 etomoxir 使 Caki-1 细胞的死亡率显著高于 shPFKP 组与 shCtrl+etomoxir 组(P 分别为 0.0001，0.0014)(图3)。

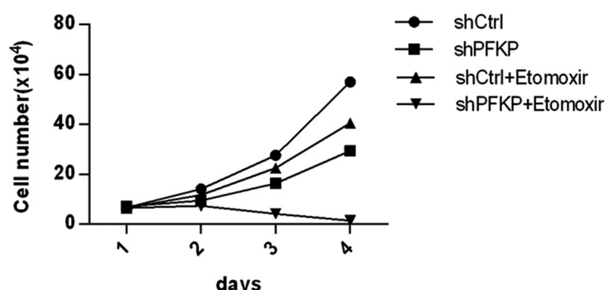

图 2 敲低 PFKP 联合 etomoxir 对细胞增殖的影响

注：shCtrl、shPFKP、shCtrl+etomoxir、shPFKP+etomoxir 组 Caki-1 细胞分别进行细胞增殖实验。

Fig.2 The effect of PFKP knockdown and etomoxir on cell proliferation

Note: The proliferation of Caki-1 cells in shCtrl, shPFKP, shCtrl + etomoxir and shPFKP + etomoxir groups was studied.

2.3 敲低 PFKP 联合 etomoxir 显著下调细胞 ATP 水平

利用 ATP 检测，我们发现单纯使用 shPFKP 与 shCtrl+etomoxir 均不会改变细胞的 ATP 水平(P 分别为 0.65，0.20)，而联合 shPFKP 与 etomoxir 会显著下调细胞 ATP 水平(P<0.05)(图 4)。利用脂肪酸检测，我们发现 etomoxir 可以部分抑制 shPFKP 引起的细胞内脂肪酸的下降，提示 etomoxir 可以抑制 PFKP 敲低引起的脂肪酸氧化利用增加(图 5)。

3 讨论

90%的散发肾透明细胞癌患者存在 VHL(Von Hippel Lindau)基因的突变或高甲基化失活[5]。VHL 的失活可使下游靶基因缺氧诱导因子 HIF 积累并激活糖酵解相关靶基因，如 GLUT1、PGK1、LDHA 与 PDK1 等[5-8]。在肾透明细胞癌中，脂肪酸氧化代谢与其他有氧磷酸化代谢被显著抑制，这与肾透明细胞癌中 HIF 通路的激活是一致的[8-10]。因此，阻断有氧糖酵解被认为是可行的靶向治疗肾透明细胞癌的方法[11]。可是我们在实验中发现，肾透明细胞癌的代谢网络具有一定的可塑性，当我们敲低有氧糖酵解的关键酶磷酸果糖激酶 1 时，肾透明细胞癌转而利用其他代谢方式提供能量。因此，单纯阻断糖酵解仅抑制了细胞增殖，但未真正杀死肿瘤细胞。

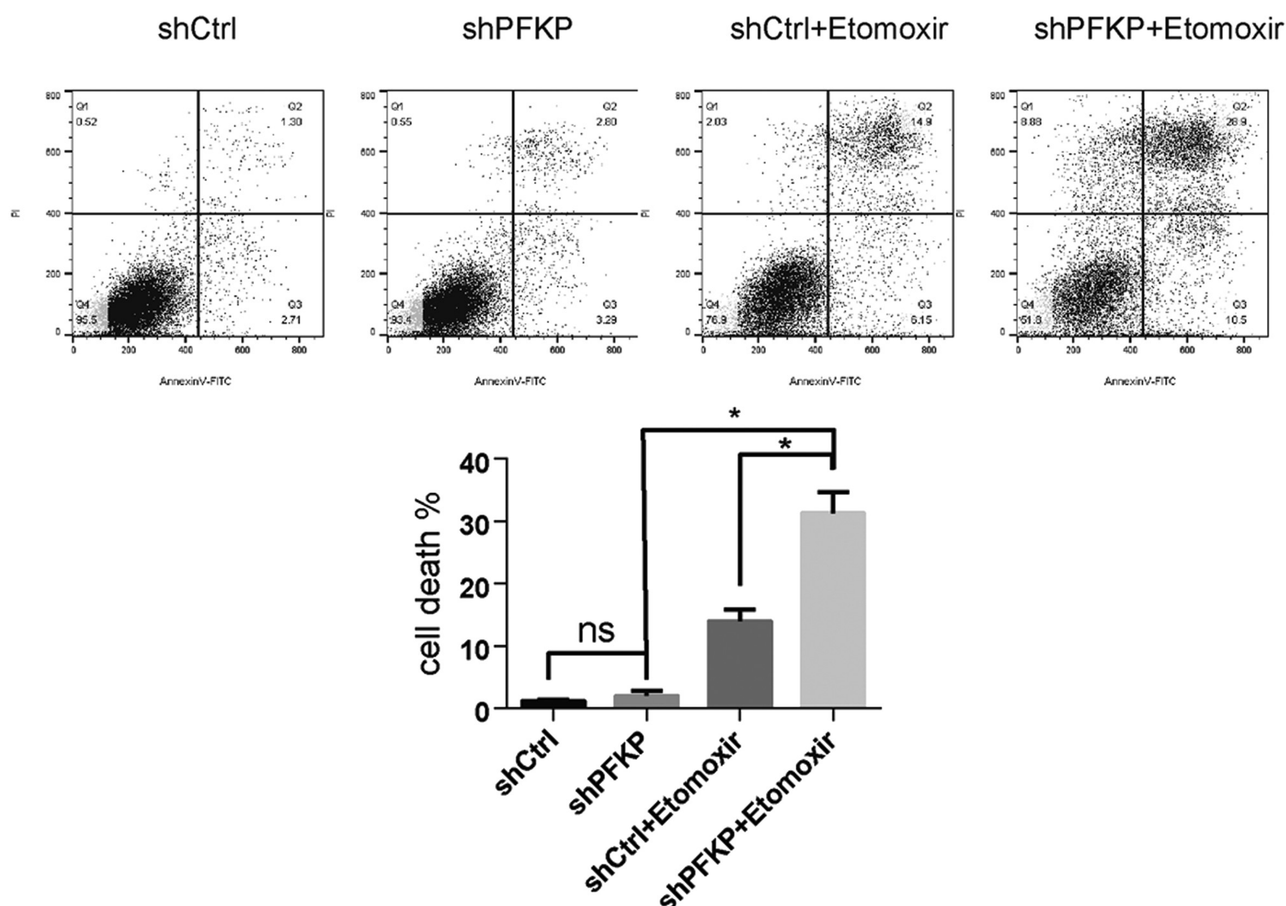

图 3 敲低 PFKP 联合 etomoxir 对细胞生存的影响

注：shCtrl、shPFKP、shCtrl+etomoxir, shPFKP+etomoxir 组 Caki-1 细胞分别进行 Annexin-V/PI 流式检测与定量结果。

Fig. 3 The effect of PFKP knockdown and etomoxir on cell survival

Note: Annexin-V/PI flow cytometry was used to detect in shCtrl, shPFKP, shCtrl+etomoxir, and shPFKP+etomoxir groups respectively. The quantifications of death rate were presented as bar graph.

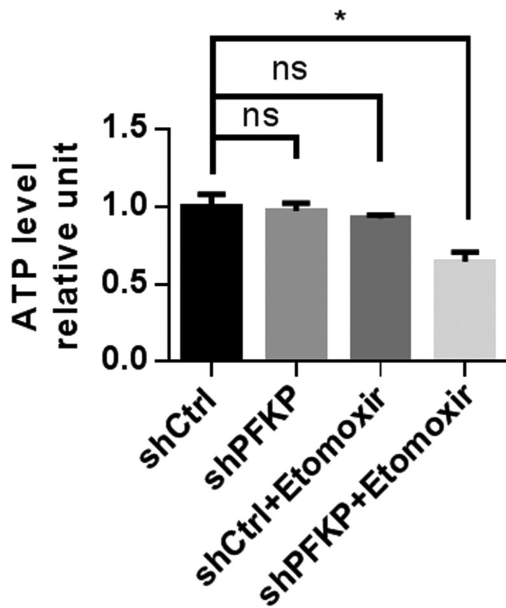

图 4 敲低 PFKP 联合 etomoxir 对细胞内 ATP 水平的影响

注:shCtrl、shPFKP、shCtrl+etomoxir、shPFKP+etomoxir 组 Caki-1 细胞分别进行 ATP 相对水平检测。

Fig. 4 The effect of PFKP knockdown and etomoxir on cellular ATP levels

Note: Levels of ATP were detected in shCtrl, shPFKP, shCtrl+etomoxir and shPFKP+etomoxir groups respectively.

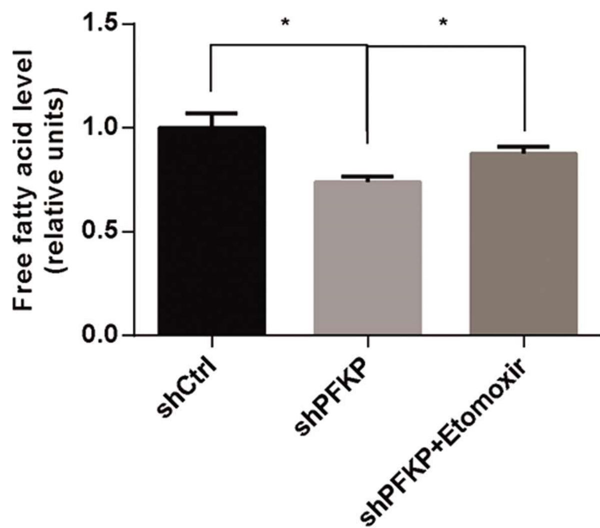

图 5 敲低 PFKP 联合 etomoxir 对细胞内游离脂肪酸水平的影响

注:shCtrl、shPFKP、shPFKP+etomoxir 组 Caki-1 细胞分别进行游离脂肪酸相对水平检测。

Fig. 5 The effect of PFKP knockdown and etomoxir on levels of cellular free fatty acid

Note: Levels of free fatty acid were detected in shCtrl, shPFKP, and shPFKP+etomoxir groups respectively.

肉碱棕榈酰基转移酶是脂肪酸线粒体转运中的关键酶,在肾透明细胞癌转而利用脂肪酸代谢中起重要作用。PFKP 是有氧糖酵解中的限速关键酶。在本研究中,我们通过敲低 PFKP 与抑制肉碱棕榈酰基转移酶同时阻断了有氧糖酵解与脂肪酸氧化,肾透明细胞癌 Caki-1 细胞出现了真正的 ATP 下降。而且有意思的是其死亡方式以细胞坏死与晚期凋亡为主。在本研究中我们发现较长时间的 etomoxir 培育可以取得比较好的杀伤作用,因此,也不完全排除在短时间 etomoxir 处理后,呈现以早起凋亡为主要死亡方式的可能。

人体多数组织同时表达三种不同的亚型:M 型、L 型、P 型[12-14]。在不同组织中,PFK-1 四聚体的组成不同。如在成熟的肌肉细胞中仅表达 M 型,肝脏中有 L 型表达,正常肾脏以 M 型、L 型为主。肾透明细胞癌组织中的 PFKP 占 PFK 总和的 80%以上,且因其在正常组织丰度低,可被其他同工酶代偿。因此,靶向 PFKP 有望实现较高的肿瘤特异性。并且除了肾透明细胞癌,PFKP 已被发现在乳腺癌、脑胶质瘤、口腔鳞状细胞癌中发挥作用[15-18]。

可惜的是,目前尚没有特异阻断 PFKP 的抑制剂。3PO[19]与 PFK15[20]可以通过抑制 PFKFB3 间接影响 2,6- 磷酸二果糖的浓度,并调控 PFK1 的活性。因此,3PO 与 PFK15 无法实现亚型的特异性。而且,3PO 化学性质不稳定,需现用现制备,影响其应用。PFK15 能否与 etomoxir 联合使用,并发挥协同作用有待进一步研究评价。

总之,PFKP 敲低联合 etomoxir 对肾透明细胞癌 Caki-1 细胞呈现协同的细胞毒抗肿瘤作用。

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(上接第 4414 页)

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