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二甲双胍增强胰腺癌放疗敏感性的体外及体内实验研究 *

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摘要 目的：探讨二甲双胍对胰腺癌细胞和胰腺癌裸鼠移植瘤放疗敏感性的影响。**方法：**体外培养人胰腺癌 BxPC-3 和 AsPC-1 细胞，分为二甲双胍处理组和未处理组，处理组给予 10 mmol/L 二甲双胍作用 48 小时后分别给予 0、1、2、4、6、8Gy 射线对两种细胞进行照射，运用克隆形成实验，Giemsa 染色后计算克隆形成率及 SF2，并拟合细胞存活曲线。应用裸鼠皮下注射胰腺癌细胞，建立两种细胞的裸鼠移植瘤模型，裸鼠肿瘤体积达到 100 mm^3 时，作为 0 天，并开始分组，每种移植瘤使用 24 只裸鼠随机分为 4 组：生理盐水对照组、单纯二甲双胍治疗组、单纯照射组、二甲双胍 + 照射组，二甲双胍处理组每天给予 250 mg/kg(50 μL/ 每只)腹腔注射，对照组仅给予等量生理盐水注射(50 μL / 每只)。每 4 天用游标卡尺测量移植瘤的长径和宽径，并绘制生长曲线。当对照组体积达到 200 mm^3 时，照射组和联合组，给予一次性照射 6Gy 射线，当对照组体积达到 1000 mm^3 时，将裸鼠进行麻醉，剥出皮下移植瘤，进行称量和保存，并计算抑瘤率。**结果：**两细胞系经过二甲双胍处理后，经 2、4、6、8Gy 照射后存活分数明显低于未处理组(P<0.01)，随着照射剂量的增大，克隆形成数量明显减少，两个细胞系经二甲双胍处理后进行照射，其 SF2、D0, N 值均较未处理组明显减少(P<0.01)，表明经二甲双胍处理后，BxPC-3 细胞和 AsPC-1 细胞的放射敏感性增强，增敏比分别为 1.2368 和 1.1179。二甲双胍和放射联合处理组的体积、瘤重均明显小于对照组和单独处理组，BxPC-3 和 AsPC-1 移植瘤的抑瘤率分别为 62.14%、61.53%，均明显高于各自单独处理组(P<0.01 或 P<0.05)。**结论：**二甲双胍能够增强胰腺癌的放疗敏感性，在临床上具有潜在的应用价值。

关键词：胰腺癌；二甲双胍；放射敏感性；移植瘤

中图分类号：R-33；R735.9 **文献标识码：**A **文章编号：**1673-6273(2017)13-2418-04

Metformin Sensitizes Pancreatic Cells to Radiation in Vitro and in Vivo*

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ABSTRACT Objective: To examine the potential of metformin (MET) to enhance pancreatic cancer (PC) responses to ionising radiation (IR). **Methods:** Pancreatic cancer cell line BxPC-3 and AsPC-1 were cultured, then they were divided into 2 groups: the control group and metformin treated group, the metformin treated group were given 10mM metformin 48h before irradiated with X-ray irradiation at 0, 1, 2, 4, 6, 8Gy. The clone formation assay were used and Giemsa stained colony formation rate and SF2 were calculated and the fitting cell survival curve were made at the same time. In vivo experiment, the cell suspension of BxPC-3 and AsPC-1 were injected subcutaneously into the right anterior armpit of nude mice, to establish a xenograft model. In each cell line, treatment was initiated when the tu- mor volume reached 100 mm^3, and the 1st day of treatment in both cases was designated as day 0, the 24 injected mice were randomly di- vided into four groups: NS-treated control, MET-treated alone, ionising radiation (IR) treated alone, MET-treated combination IR (n=6, per group), For injection, metformin was dissolved in sterile saline and was intraperitoneally injected once per day at 250 mg/kg(50 μL/mouse). The control group received vehicle only (50 μL saline). Tumor volume (V) was measured by external caliper every 4 days and the growing curve were made. When the volume were reached 200 mm^3, the IR group and the MET-treated combination IR group were given 6Gy X-ray irradiation for once, Mice were sacrificed at the 28^{th} day and the weights of xenografts were measured and the inhabitation rate were calculated. **Results:** The survival fraction were significantly decreased in the MET treated cell group than the untreated group in the both cell lines after irradiated with X-ray of 2, 4, 6, 8 Gy. The colony formation were decreased with the increasing of the X-ray. The SF2, D0 and N were decreased in MET treated group compared with the control group (P<0.01), which indicated that the radiosensitivity after the treatment of the MET, and the ER of the BxPC-3 and AsPC-1 cells were 1.2368 and 1.1179, respectively. In

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the experiment of nude mice, the tumor inhibition were obvious in the IRMET-treated combination IR group than the MET alone, IR alone and control group, and the inhibition rate were 62.14% and 61.53% respectively for the BxPC-3 and AsPC-1 xenograft mode(P<0.01 or P<0.05). **Conclusion:** Clinically achievable MET doses inhibit pancreatic cancer cell and tumour growth and sensitise them to IR. Our results suggest that MET can be a clinically useful adjunct toradiotherapy in pancreatic cancer.

Key words: Pancreatic cancer; Metformin; Radiosensitive; Xenograft

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前言

胰腺癌是极具侵袭潜能的恶性肿瘤,早期可发生淋巴转移和远处转移，即使早期行根治性切除其 5 年生存率仅 24%,不能行根治性切除的胰腺癌 5 年生存率低于 2%。胰腺癌患者中位生存时间 <6 个月。在西方国家,肿瘤引起的死亡中,胰腺癌继肺癌、大肠癌、乳腺癌之后居第四位[1-3]。胰腺癌预后较差的主要原因之一在于对于对放疗、化疗的不敏感性,如何提高胰腺癌对放化疗的敏感性是临床亟待解决的问题。

二甲双胍为一种胰岛素增敏剂被广泛用于 II 型糖尿病的一线治疗,近年来发现二甲双胍可明显降低乳腺癌、前列腺癌、胰腺癌、肝细胞癌等的发生率,并可抑制肿瘤的增殖、侵袭、转移和促进肿瘤细胞凋亡[3-7]。近期研究显示二甲双胍能够增强肿瘤细胞对放疗的敏感性[8],本研究从胰腺癌 BxPC-3 细胞核 AsPC-1 细胞的体外细胞实验体外细胞学和体内的胰腺癌移植瘤裸鼠的体内实验,探讨二甲双胍对胰腺癌放疗敏感性的影响。

1 材料与方法

1.1 材料

人胰腺癌细胞株 BxPC-3、AsPC-1 由新疆军区总医院动物实验科保存。二甲双胍购自 Sigma 公司；胎牛血清和高糖 DMEM 培养基购自 Gibco 公司;二甲基亚砜(DMSO)和四甲基偶氮唑盐(MTT)购自 Amersco;SPF 级 BALB/c 裸鼠 24 只购自新疆医科大学实验动物中心,4-5 周龄,体质量 15-20 g,饲养于新疆军区总医院动物实验科 SPF 屏障系统。

1.2 方法

1.2.1 细胞系和细胞培养 BxPC-3、AsPC-1 在含有 5% CO_2 37℃ 95%湿度培养箱中培养。培养液为含 10%胎牛血清和双抗(100 U/mL 链霉素和 100 U/mL 青霉素)的 DMEM 高糖培养基。当细胞融合 90%时,用 0.25%胰酶消化传代,取对数生长期的细胞进行实验。

1.2.2 细胞用二甲双胍药液配置 二甲双胍用高糖 DMEM 稀释为 10 mmol/L 浓度,针式过滤后,4℃保存。

1.2.3 细胞学照射条件 用 ELEKTA Synergy 直线加速器产生 6 MV 的 X 照射细胞，照射距离 100 cm，照射野 16× 18 cm^2,剂量 600 cGy/min。通过多通道电离室适当调整照射剂量,并对培养皿进行衰减矫正,保证细胞接收均匀准确的照射。

1.2.4 克隆形成实验测胰腺癌细胞放疗敏感作用 取对数生长的胰腺癌 BxPC-3 细胞和 AsPC-1 细胞,用 PBS 洗 2 遍后,消化制成细胞悬液。细胞计数后将细胞稀释,按照 10^2-10^5 密度接种到 60 mm 直径平皿,每组设 3 个副孔,细胞贴壁后对照组只换液,BxPC-3 细胞和 AsPC-1 细胞分别分为 2 组:单纯照射组、照射 + 二甲双胍组,根据文献[9]实验组加入 10 mmol/L 二甲双胍作用 48 小时后分别给予 0 Gy、1 Gy、2 Gy、4 Gy、6 Gy、8 Gy 对两种细胞进行照射,照射后对细胞进行换液处理后放回培养箱继续培养至肉眼可见克隆形成终止培养,约 2-3 周,甲醇固定后细胞行 Giemsa 染色，镜下可见大于 50 个细胞为一个集落，计算集落形成率 (PE)=0 Gy 剂量下集落数 / 细胞接种数)100%,各个剂量下细胞存活率(SF)= 某一剂量照射组细胞形成克隆数 / (该细胞种植数× PE，应用单击多靶模型 SF=1-(1-exp-D/D0)N 模型对数据进行处理,并应用 sigmaplot 计算各组放射敏感性参数 D0, N, SF2 值,重复 3 次,并且拟合细胞存活曲线。D0 代表平均致死量。N 值为外推值,反映细胞对放射引起损伤的修复能力。SF2 代表 2Gy 时的存活分数,是代表放射敏感性的重要指标,SF2 越大放射抗拒性越强。并计算放射增敏比(SER)= 对照组 D0 值 / 实验组 D0 值。

1.2.5 胰腺癌移植瘤模型建立及实验分组 取对数生长的胰腺癌 BxPC-3 细胞和 AsPC-1 细胞,调整细胞浓度 1× 10^7/ml,将 0.2 mL 细胞悬液(2× 10^6 细胞)注射到裸鼠右侧腋下,当移植瘤平均体积增到 100 mm^3 时，每种移植瘤使用 24 只裸鼠随机分为 4 组:生理盐水对照组(6 只)、单纯二甲双胍治疗组(6 只)、单纯照射组(6 只)、二甲双胍 + 照射组,二甲双胍溶于无菌生理盐水中,每天给予 250 mg/kg(50 μL / 每只)腹腔注射,对照组仅给予等量生理盐水注射(50 μL/ 每只)。每 4 天用游标卡尺测量移植瘤的长径和宽径,体积 V=0.5236× (长径× 宽径 2),并绘制生长曲线当对照组体积达到 200 mm^3 时，照射组和联合组应用 ELEKTA Synergy 直线加速器产生 6MV 的 X 照射，给予一次性照射 6Gy，剂量率为 300 cGy/min,SSD100 cm,BxPC-3 细胞和 AsPC-1 细胞分别在分组后 8 天和 4 天给予照射，当对照组体积达到 1000 mm^3 时,将裸鼠进行麻醉,剥出皮下移植瘤,进行称量和保存。计算抑瘤率 =(对照组瘤质量 - 处理组瘤质量)/对照组瘤质量。

1.3 统计学分析

使用 SPSS23.0 统计软件处理数据，计量资料以均数± 标准差($\bar{x}$± s)表示,采用单因素方差分析和 t 检验分析组间和组内差异,Sigmaplot12.5 拟合细胞存活曲线并绘制统计图，率的检验用卡方检验,以 P<0.05 为差异有统计学意义。

2 结果

2.1 胰腺癌 BxPC-3 和 AsPC-1 细胞克隆形成实验

胰腺癌细胞经过不同剂量照射后,培养 14-18 天可形成肉眼可见的细胞集落,应用单击多靶模型 SF=1-(1-exp-D/D0)N 模型对数据进行处理,并应用 sigmaplot12.5 拟合 BxPC-3 细胞和 AsPC-1 细胞存活曲线(图 1,图 2),计算各组放射敏感性参数

D0、N、SF2、ER 值(表 1)。结果显示:两细胞系经过二甲双胍处理后，经 2、4、6、8 Gy 照射后存活分数明显低于未处理组(P<0.01),随着照射剂量的增大,克隆形成数量明显减少,两个细胞系经二甲双胍处理后进行照射,其 SF2、D0、N 值均较未处理组明显减少(P<0.01),表明经二甲双胍处理后 BxPC-3 细胞和 AsPC-1 细胞的放射敏感性增强,增敏比分别为 1.2368 和 1.1179。

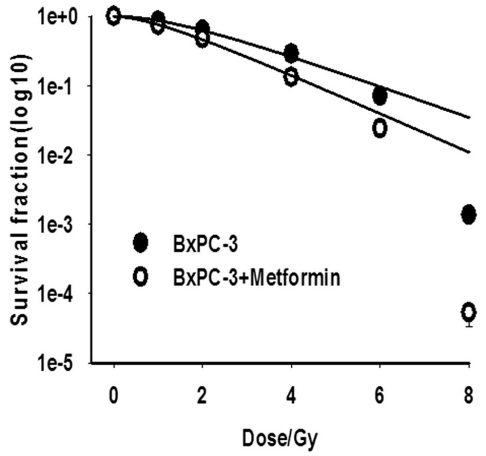

图 1 BxPC-3 拟合细胞存活曲线图

Fig.1 The fitting cell survival curve of BxPC-3

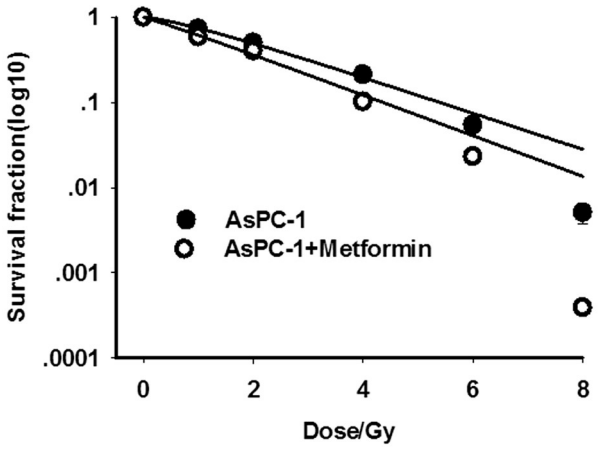

图 2 AsPC-1 拟合细胞存活曲线

Fig.2 The fitting cell survival curve of AsPC-1

表 1 两种细胞放疗敏感性指标

Table 1 The parameters of radiosensitive of two cell lines

Parameters	BxPC-3	BxPC-3+Metformin	AsPC-1	AsPC-1+Metformin
SF2	0.6268	0.4602[a]	0.4932	0.3624[a]
D0	1.9203	1.5504[a]	2.0314	1.8172[a]
N	2.2643	1.9153[a]	1.4528	1.1126[a]
ER	--	1.2368	--	1.1179

Note: Compared with control group [a]P<0.01.

2.2 二甲双胍单独或联合放疗对胰腺癌抑制瘤生长的影响

当肿瘤细胞裸鼠皮下注射约 8-10 天，移植瘤平均体积增到 100 mm^3 时进行分组,计为 0 天,当各组体积达到 200 mm^3 时给予一次性照射 6 Gy。BxPC-3 细胞和 AsPC-1 细胞分别在分组后 8 天和 4 天给予照射，每 4 天用游标卡尺测量瘤体大小,计算出体积,并绘制肿瘤的生长曲线(图 3、图 4)。在分组后 28 天,当对照组体积超过 1000 mm^3 时,将裸鼠进行麻醉,剥出皮下移植瘤,称量瘤体肿瘤,计算抑瘤率(表 2)。结果显示:在分组后 16 天,单独二甲双胍组、单独照射组和二者联合组的移植瘤体积均较对照组减小,其中二甲双胍和放射联合处理组的移植瘤体积、瘤重均明显小于对照组和单独处理组,BxPC-3 和 AsPC-1 移植瘤的抑瘤率分别为 62.14%、61.53%，均明显高于各自单独处理组(P<0.01 或 P<0.05)。

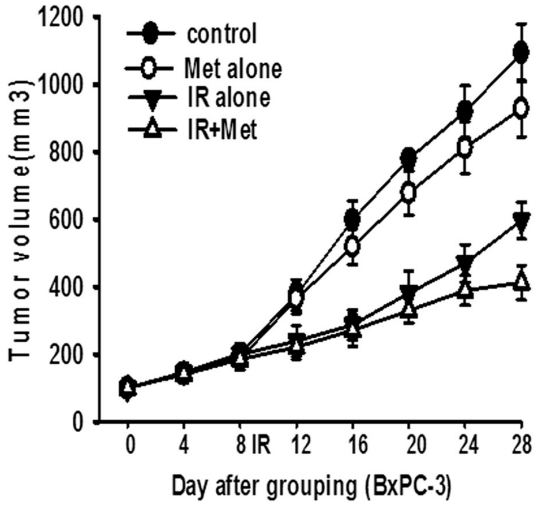

图 3 BxPC-3 移植瘤生长曲线

Fig.3 The growth curve of BxPC-3 xenograft

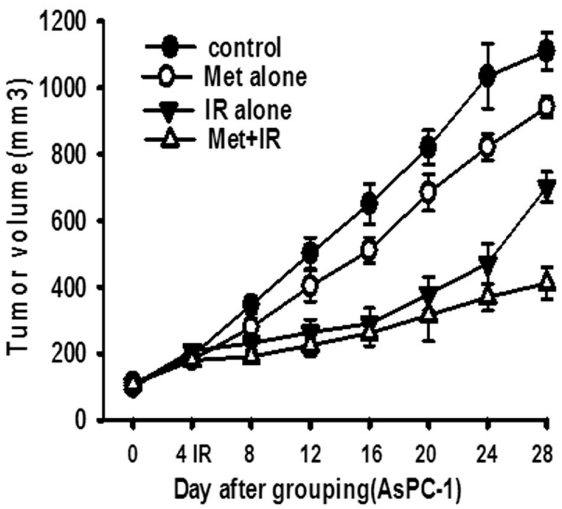

图 4 AsPC-1 移植瘤生长曲线

Fig.4 The growth curve of AsPC-1 xenograft

表 2 不同处理组的移植瘤体积、重量及抑瘤率

Table 2 The tumor volume, weight and the inhabition rate of the different groups

Groups	Volume		Weight		Inhabition rate (%)	
	BxPC-3	AsPC-1	BxPC-3	AsPC-1	BxPC-3	AsPC-1
Control	1093.41± 86.00[a]	1109.67± 56.35[a]	1.03± 0.09[a]	1.04± 0.07[a]	-	-
Met	928.47± 83.19[a,c]	941.39± 30.54 [a,c]	0.88± 0.08[a,c]	0. 91± 0.09[a,d]	14.56[a]	12.5[a]
IR	596.49± 54.88[a,c]	701.45± 45.23[a,c]	0.54± 0.03[a,c]	0.68± 0.03[a,c]	47.57[b]	34.62[a]
Met+IR	412.47± 51.13c	411.56± 47.36 c	0.39± 0.05c	0.40± 0.04 c	62.14	61.53

Note: Compared with combination group: [a]P<0.01, [b]P<0.05;

Compared with control group: [c]P<0.01, [d]P<0.05;

3 讨论

越来越多的研究表明二甲双胍作为抗糖尿病药物在肿瘤的预防和治疗过程中也发挥了重要的作用,尤其近年来实验表明二甲双胍能够增加肿瘤对放射线的敏感性,回顾性分析表明对于需要用二甲双胍控制血糖的糖尿病患者在合并肿瘤时,此类患者在进行放射治疗的效果明显增加[8]。本研究分别应用 10 mM 二甲双胍作用于胰腺癌 BxPC-3 细胞和 AsPC-1 细胞后经照射发现,其 SF2 值分别为 0.6268 和 0.4932,表明 AsPC-1 细胞对放射敏感性较 BxPC-3 细胞高,经二甲双胍处理后,二者的 SF2 值分别为 0.4602 和 0.3624,D0、N 值均较未处理组下降表明二甲双胍均能增加两种细胞的放射敏感性,本研究中两种细胞的增敏比分别为 1.2368 和 1.1179, 表明二甲双胍对于对 BxPC-3 的增敏作用更强。从移植瘤裸鼠的瘤体生长情况看,二甲双胍联合放射组无论是在瘤体的体积、重量还是抑瘤率上均明显低于对照组、单纯二甲双胍组和单纯放射组,表明二甲双胍在体内实验也可明显增加胰腺癌的放射敏感性。

二甲双胍对肿瘤放射敏感性的机制目前正在研究中,目前认为二甲双胍增加放疗敏感性的机制是多方面的,主要有以下几个方面:(1) 调节细胞周期并抑制 DNA 修复和诱导细胞凋亡:Jeong YK 等 [10] 研究认为二甲双胍即使在对放疗不敏感的 HCT116 $p53^{-/-}$ 结肠癌细胞和肿瘤也能通过诱导细胞周期 G2/M 捕获和减少 DNA 修复蛋白的表达来增加放疗敏感性, 因而认为二甲双胍是一种 P53 缺失的结肠肿瘤的放疗增敏剂。Wang Z 等[9]等研究发现二甲双胍可通过消除胰腺癌 MIA PaCa-2 和 PANC-1 细胞周期 G2 期检查点和抑制 DNA 损伤修复来增加细胞的放射敏感性。Li H 等[11]研究发现二甲双胍对鼻咽癌拥有很强的增敏作用,这种增敏效应与通过抑制 HR 和 NHEJ 修复信号通路对 DNA 双链断裂的修复有关, 从而增加辐射诱导的细胞凋亡。(2)影响细胞代谢,改善细胞缺氧:Matthews Q 等[12]发现在二甲双胍作用下的乳腺癌 MCF7 细胞,其辐射诱导下的糖原明显减少,二甲双胍对糖原的抑制与肿瘤细胞的放射敏感性增加密切相关。肿瘤缺氧是肿瘤预后差的因素,部分原因是缺氧引起的放疗不敏感性。Zannella VE 等[13]研究发现二甲双胍能够提高肿瘤的氧供并增加其对放疗的敏感性。Sesen J 等[14]研究发现二甲双胍减少人恶性胶质瘤细胞有丝分裂依赖的 ATP 产物和氧耗,提高乳酸和糖酵解的 ATP 产物。(3)影响信号通路:二甲双胍诱导细胞增殖的减少、细胞周期捕获、自噬、细胞凋亡和死亡与 AMPK、Redd1 通路激活和 mTOR 通路抑制有关[14]。Fasih A 等[15]研究认为应用微摩尔浓度的二甲双胍可增加胰腺癌 MiaPaCa-2 和 Panc1 细胞对放疗的敏感性, 是通过 AMPK 通路, 并影响 DNA 的损害通路。Zhang T 等 [16] 研究发现由 EGFR/PI3K/Akt 下调引起的 DNA-PKcs 磷酸化减少是二甲双胍增加前列腺癌放疗敏感的重要途径。Storozhuk Y 等[17]在对非小细胞肺癌研究发现辐射和二甲双胍能够诱导肿瘤持续的 ATM-AMPK-p53/p21(cip1)通路的激活和 Akt-mTOR-4EBP1 通路抑制有关。(4)调节活性氧簇(ROS):ROS 对细胞的损伤是放射生物学主要作用原理之一。Zhang Y 等[18]研究认为二甲双胍增强乳腺癌细胞放疗敏感性是通体调控 Trx 家族蛋白的表达来改变细胞内 ROS 水平来实现的。

尽管二甲双胍增强肿瘤放射敏感性的作用机制尚未完全阐明,本研究通过体外细胞学实验和体内肿瘤生长的抑制实验证实二甲双胍能够增强胰腺癌的放疗敏感性,在临床上具有潜在的应用价值。

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