

VEGF 及 EGFR 抑制药联合放疗抑制肿瘤血管生成及 DSB 修复

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【摘要】 **目的** 靶向 VEGF 及 EGFR 具有抑制肿瘤血管生成作用,与放疗联合的效果尚待研究。本研究建立 FaDu 鳞状细胞癌裸鼠荷瘤模型,了解 VEGF 及 EGFR 抑制药凡德他尼(Zactima™、ZD6474)联合放疗抗肿瘤效果,从机制上探讨对肿瘤微血管生成,肿瘤细胞 DNA 双链断裂(double-strand break, DSB)修复及细胞增殖影响。**方法** 建立裸鼠鳞状细胞癌荷瘤模型,随机均分成 4 组:对照组、放疗组(RT)、凡德他尼组、联合治疗组(凡德他尼+RT)观察肿瘤细胞大体增殖情况,免疫荧光法检测肿瘤组织 CD34、 γ -H₂AX 表达、细胞增殖相关抗原 Ki67,计数微血管密度(microvessel density, MVD),细胞内 γ -H₂AX 荧光焦点平均值以及肿瘤细胞增殖率。**结果** VEGF 及 EGFR 抑制药同步联合放疗组比单药治疗及单纯放疗明显延迟肿瘤细胞增殖时间,同时 CD34、 γ -H₂AX、Ki67 免疫荧光染色显示明显减少微血管密度,增加肿瘤细胞内 DNA 双链断裂程度,并降低肿瘤细胞增殖率($P < 0.05$)。**结论** VEGF 及 EGFR 抑制药与放疗同步,从机制上抑制肿瘤新生血管形成,加重肿瘤细胞 DNA 损伤,抑制肿瘤细胞增殖,从而增加放疗疗效。

【关键词】 血管内皮生长因子;表皮生长因子;放疗;血管生成;DNA 双链断裂修复

【中图分类号】 R732;K9

【文献标志码】 A

【文章编号】 1672-3384(2012)-02-0014-05

Antitumor effect of VEGFR and EGFR inhibitor combined with radiotherapy on angiogenesis and DNA repair

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【Abstract】 **Purpose** To evaluate the antitumor effect of a combination of vascular endothelial growth factor receptor (VEGFR) and epidermal growth factor receptor (EGFR) tyrosine kinase activity inhibitor - ZD6474 and radiotherapy in a human tumor xenograft model (FaDu, squamous cell carcinoma) and to investigate whether the effects of the treatments are related to changes in tumor microvessel density, double-strand break (DSB) repair and proliferation. **Methods** Tumor-bearing nude mice were randomly assigned to either ZD6474 alone, or radiotherapy (RT) alone; or ZD6474 + RT, or control. The antitumor effect of the different treatments was evaluated by the tumor growth delay. The tumor vascularisation was evaluated by CD34 positive vessel segments (tumor vascular density) of immunofluorescence analysis and the DNA repair of tumor cell. Proliferative capacity of the tumor tissue were analyzed by the quantification of γ -H₂AX and Ki67 For the different treatment-groups. **Results** The tumor growth delay induced by the combined treatment (ZD6474 + RT) was greater than that induced by ZD6474 or radiotherapy alone. The application of ZD6474 had significant effects on the tumor vasculature and DNA repair, which was due to reducing neoangiogenesis and increasing the expression of γ -H₂AX. Moreover, the proliferative capacity of the tumor tissue was significantly decreased by ZD6474 ($P < 0.05$). **Conclusion** When irradiation is combined with VEGFR and EGFR blockade, significant enhancement of antiangiogenic, DNA injury and antitumor effects were observed. These data provide support for clinical trials of biologically targeted and conventional therapies for the treatment of cancer.

针对肿瘤靶向治疗主要包括两大类,一是靶向与肿瘤密切相关肿瘤血管,即血管内皮生长因子(vascular endothelial growth factor, VEGF)系统,影响肿瘤血管生成,从而诱导细胞凋亡;二是靶向肿瘤增殖活性,抑制肿瘤内表皮生长因子受体(epidermal growth factor receptor, EGFR)及其信号系统表达。近期研究发现,EGFR 信号系统参与细胞 DNA 双链断裂(double-strand break, DSB)修复过程,抑制 EGFR 及其信号系统,干扰 DSB 正常修复过程,从而抑制细胞增殖^[1]。多项研究发现,放疗不论联合 VEGF 或 EGFR 抑制药均具有放射增敏作用^[2],凡德他尼(Zactima™, ZD6474)^[3-4]是一种新型兼具靶向 VEGFR 及 EGFR 双通道药物,其与放疗联合效果及机制尚不明确。本研究建立 FaDu 鳞状细胞癌裸鼠荷瘤模型,研究 VEGFR 及 EGFR 抑制药——凡德他尼联合放疗抗肿瘤效果,并从机制上了解对肿瘤微血管生成、细胞 DSB 修复、细胞增殖的影响。

1 材料与方法

1.1 材料

1.1.1 主要试剂 兔抗人 Ki67 单克隆抗体,鼠抗人 CD34 单克隆抗体,鼠抗人 γ -H₂AX 单克隆抗体。

1.1.2 实验动物 nu/nu 裸鼠,雌性,共 40 只,8~2 周龄,体重 20~25g(德国萨尔大学放射肿瘤实验室)。

1.1.3 药物和癌细胞株 凡德他尼(ZD6474); FaDu 鳞状细胞癌细胞株(德国萨尔大学放射肿瘤实验室)。

1.2 实验动物分组及处理

1.2.1 裸鼠鳞状细胞癌荷瘤模型的建立 FaDu 鳞状细胞癌细胞悬浮于含 10% 胎牛血清(FBS)的 RPMI-1640 培养基,37℃、5% CO₂、饱和湿度下培养传代维持单层细胞生长,收集培养肿瘤细胞与 RPMI-1640 以 1:1 比例制成瘤细胞悬液,细胞密度约为 2×10^7 mL⁻¹,用于动物实验。取 40 只裸鼠,植入前 48h 给予所有裸鼠单次 4Gy 全身放疗,抑制排斥反应,以 1mL 注射器吸取 0.1mL 相应肿瘤细胞注入小鼠右后腿皮下,观察 5~7d,直到肿瘤直径达 1cm。

1.2.2 实验动物分组及治疗 将 40 只裸鼠随机分为 4 组:对照组;ZD6474 组($50\text{mg} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$ 经灌胃给药);放疗组(RT, $2\text{Gy} \cdot \text{d}^{-1}$, d1-5);联合组(凡德他尼 + RT):采用常规分割放疗剂量 $2\text{Gy} \cdot \text{d}^{-1}$, d1-5;ZD6474 以 $50\text{mg} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$ 灌胃给药,均于每日放疗前 2h 给予)。治疗期间定期观察裸鼠的生活状态及裸鼠体重变化。

1.3 观测指标及方法

1.3.1 肿瘤体积测定 从治疗开始,隔日以卡尺测量获得肿瘤最长径(L)及最短径(W),依据公式^[4]: 肿瘤体积 = $(L \times W^2)/2\text{mm}^3$,记录肿瘤生长时间、体积,到肿瘤 2.5cm^3 时停止,比较所需时间差别。

1.3.2 免疫荧光染色检测肿瘤组织 CD34、 γ -H₂AX、Ki67 表达 依据上述分组,完成 5d 连续治疗后间隔 24h,于每组中随机选择 4 只裸鼠,完整分离肿瘤组织,用 10% 中性甲醛固定,石蜡包埋,连续切片,切片厚度 $4\mu\text{m}$,通过荧光标记的 CD34、 γ -H₂AX、Ki67 抗体特异性标记,PI 进行核染色,免疫荧光染色和激光共聚焦显微镜观察组织内 CD34、 γ -H₂AX、Ki67 表达。

1.3.3 MVD 测定 CD34 是骨髓祖细胞抗原,同时表达在内皮细胞,近年来用于监测、诊断肿瘤新生血管^[5]。通过荧光标记显现 CD34 阳性内皮细胞,于 $100 \times$ 荧光显微镜下观察,在肿瘤区域选择 4 个血管密集区,随后 $600 \times$ 镜下计数上述 4 个不同区域血管数,取平均值,为该切片 MVD 值^[6]。

1.3.4 γ -H₂AX 免疫荧光在肿瘤组织表达分析 当细胞内存在 DSB,磷酸化的 H₂AX 迅速形成并聚集在 DSB 处,通过免疫荧光染色形成可以分辨的焦点(foci),在不同干预治疗下肿瘤组织中,分别计数 80~100 个肿瘤细胞内 γ -H₂AX 荧光焦点,取平均值以量化分析 γ -H₂AX 表达^[1]。

1.3.5 细胞增殖测定 增殖抗原 Ki67 能监测除 G₀ 期细胞增殖状态。通过免疫荧光染色显现 Ki67 阳性肿瘤细胞,计数 500~1000 个肿瘤细胞 Ki67 阳性肿瘤细胞数,计算 Ki67 指数(Ki67 Labeling index, Ki67LI)^[7]。

1.4 统计学分析

采用 SPSS 13.0 软件进行分析,定量检测结果采用均数 ± 标准差 ($\bar{x} \pm s$) 表示,采用单因素方差分析进行多组间均数比较,两组间比较采用组间 q 检验,以 $P \leq 0.05$ 为差异具统计学意义。

2 结果

2.1 肿瘤增殖大体观察评价

于肿瘤细胞植入后 1 周,所有动物均存活,可于裸鼠后腿部触及肿瘤结节,平均直径在 1cm,依据研究分组治疗。结果显示,对照组经 28d 达到所需体积,而凡德他尼组、RT 组及联合组分别需 40、45 及 49d,联合治疗组明显延长肿瘤增殖所需时间。结果见图 1。

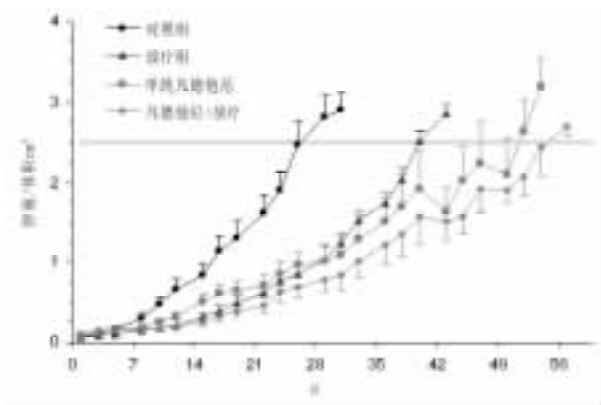


图 1 不同分组的肿瘤体积随时间的变化

2.2 肿瘤组织微血管评价

对照组每视野下微血管密度 (MVD) 为 30.9 ± 1.4 , RT 及凡德他尼组均减少 MVD 值,而联合组相较于前二组进一步减少组织新生血管形成。见表 1。

2.3 γ -H₂AX 免疫荧光在肿瘤组织的表达

在不同干预治疗下肿瘤组织中,分别计数 80 ~ 100 个肿瘤细胞内 γ -H₂AX 荧光焦点,取平均值以量化分析 γ -H₂AX 表达。结果显示,联合治疗组较 RT 及凡德他尼组显示存在较多荧光标记 γ -H₂AX,增加细胞内 DNA 双链断裂程度。见表 1。

2.4 肿瘤细胞增殖评价

对照组中肿瘤细胞 Ki67 呈现高表达, Ki67

指数高,细胞增殖活跃; RT 及凡德他尼组 Ki67 阳性表达减少,细胞增殖受抑制,联合治疗组则 Ki67 表达相较于前两组明显下降,细胞增殖活性明显受抑制。见表 1。

表 1 各实验组肿瘤增殖时间、MVD、 γ -H₂AX、Ki67LI 表达 ($\bar{x} \pm s$)

组别	肿瘤增殖时间/d	MVD	γ -H ₂ AX foci/ tumor cell	Ki67LI
对照组	27.5 ± 1.2	30.9 ± 1.4	0.56 ± 0.03	50% ± 1%
RT	40.0 ± 0.7**	21.6 ± 1.7**	1.23 ± 0.12*	21% ± 2%**
ZD6474	45.2 ± 3.0	17.5 ± 2.3△	0.59 ± 0.07△△	15% ± 1%△
ZD6474 + RT	48.9 ± 2.8	12.5 ± 0.6	1.52 ± 0.16	9% ± 1%

各组间差别在肿瘤增殖时间、MVD、 γ -H₂AX、Ki67LI 差别明显 $P < 0.01$; 联合组 (凡德他尼 + RT) 与 RT 比较, * $P < 0.05$, ** $P < 0.01$; 联合组 (凡德他尼 + RT) 与凡德他尼组比较, △ $P < 0.05$, △△ $P < 0.01$ 。

3 讨论

肿瘤细胞增殖及转移是一个依赖血管生成过程^[8]。早在 1971 年, Folkman^[9] 研究发现缺乏充足血供, 肿瘤生长体积将 $< 2\text{mm}^3$, 而这正是营养物质通过血管向周围组织弥散最大距离, 如果需要进一步增殖, 则需要新生血管形成, 因此提出抑制肿瘤血管生成在肿瘤发生、发展各个阶段均可有效发挥抗肿瘤作用。研究发现, 肿瘤组织内在微环境异常及乏氧状态能刺激 VEGF 及 EGFR 表达, VEGF 与其受体 VEGFR-2 结合能刺激血管内皮细胞及内皮祖细胞增殖、迁移、抑制凋亡、促进内皮网状结构形成并诱导血管高渗透性^[9]; 同时 VEGF 能诱导 EGFR 表达, 后者则通过其下游 MAPK 信号通路促进多种生长因子如 TGF- α 、TGF- β 、bFGF 等分泌, 均进一步促进 VEGF 表达, 从而诱导肿瘤血管生成^[10-11]。多数研究显示, 靶向 VEGF 信号, 抑制肿瘤血管生成具有抑制肿瘤增殖作用。

凡德他尼是 VEGF 抑制药。体外研究中, 凡德他尼能抑制内皮细胞增殖, 同时与放疗联合时能增强内皮细胞放射敏感性^[12]。在本研究中, 与单药治疗及单纯放疗组比较, 联合治疗组明显延迟肿瘤增殖时间并明显减少微血管密度, 差异性显著。

研究发现多数肿瘤组织呈现 EGFR 过度表达, EGFR 及其信号系统在诱导肿瘤增殖中起重要作用;而且抑制 EGFR 信号系统具有放射增敏作用,但机制尚不明确。部分研究显示,抑制 EGFR 信号系统可能通过影响细胞 DSB 修复过程实现放射增敏^[13]。DSB 是细胞致死性损伤,干扰 DSB 修复已被认为是放射增敏新途径,当细胞内存在 DSB,磷酸化的 γ -H₂AX 迅速形成并聚集在 DSB 处形成荧光下可以分辨的焦点,随着 DNA 损伤消除(如被修复) γ -H₂AX 将随之被去磷酸化而消失^[14]。因此荧光标记的 γ -H₂AX 已被认为是识别细胞内 DSB 有效工具,细胞内残留 γ -H₂AX 焦点量随细胞种类的不同而异,代表细胞损伤修复能力,与细胞放射敏感性有密切的相关性^[15]。在本研究中,与单药治疗及单纯放疗组比较,联合治疗组增加细胞内 γ -H₂AX 荧光焦点数,伴随 γ -H₂AX 表达增加, Ki67 表达下降,组织增殖受抑制,提示作为 EGFR 抑制药凡德他尼在与放疗联合治疗中通过增加细胞 DNA 损伤实现放疗增敏疗效。其机制可能是放疗诱导细胞 DSB 形成,而凡德他尼通过抑制 EGFR 及其信号系统干扰与 DSB 修复相关蛋白功能,从而使放疗后细胞内出现 DNA 损伤——DSB 无法修复而抑制肿瘤细胞增殖。

早在 2006 年, Hoang 等^[16]研究发现凡德他尼于体外能抑制血管内皮细胞增殖及血管网状结构形成;进一步于体内研究显示凡德他尼联合放疗能抑制 A549 细胞在裸鼠体内增殖。但研究着重于肿瘤血管生成抑制,对肿瘤内在增殖影响及与 DSB 修复相关性未涉及。Mahoud 等^[17]体外研究显示抑制 EGFR 信号可干扰 A549 肿瘤细胞辐射后 DSB 修复,增加细胞内 DNA 双链断裂程度,但未进行相应动物体内研究。凡德他尼是 VEGF 及 EGFR 双通道抑制药,我们建立裸鼠肿瘤模型,研究显示 VEGF 及 EGFR 抑制药与放疗联合,通过靶向 VEGF 抑制肿瘤新生血管形成,靶向 EGFR 诱导细胞 DSB 形成抑制肿瘤细胞增殖,从而增加放疗疗效,与上述研究者结论一致。本研究为临床常规治疗及生物靶向治疗提供进一步支持。

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