

肝素结合表皮生长因子抑制剂 CRM197 对裸鼠卵巢癌移植瘤的抑制作用 *

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摘要 目的: 研究 CRM197 对裸鼠卵巢癌移植瘤生长的抑制作用探讨其对逆转卵巢癌组织耐药作用及其在卵巢癌治疗中的可行性。方法: 用人卵巢癌亲本细胞 A2780、耐紫杉醇细胞 A2780/Taxol 及耐顺铂细胞 A2780/DDP 分别建立裸鼠卵巢癌移植瘤模型, 观察注射 CRM197 组与阴性对照组裸鼠肿瘤生长情况, 免疫组化法测定各组裸鼠肿瘤组织中 HB-EGF 及 P-gp 表达情况。结果: HB-EGF 及 P-gp 在各肿瘤组织中不同程度的表达, 在注射 CRM197 的裸鼠肿瘤组织中表达明显降低($P < 0.05$), 差异具有统计学意义。结论: CRM197 通过抑制肝素结合表皮生长因子的信号传导通路激活抑制了裸鼠卵巢癌移植瘤的生长, 使裸鼠卵巢癌移植瘤 HB-EGF 及 P-gp 表达明显降低。

关键词 肝素结合表皮生长因子; 卵巢癌耐药; CRM197

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The Depressant Effect of CRM197-A Specific Inhibitor of Heparinbinding Epidermal Growth Factor-for the Xenografts of Ovarian Cancer in Nude Mice*

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ABSTRACT Objective: To investigate the reversal effect of CRM197 on drug-resistant ovarian cancer and its treatment for ovarian cancer by researching the inhibition of CRM197 for the xenografts of ovarian cancer in nude mice. **Methods:** The parental ovarian cancer cell A2780, cells resistant to paclitaxel A2780/Taxol and cisplatin-resistant cells A2780/DDP were used to establish the xenograft model in nude mice, the differentiation for the growth of xenograft tumors in the group which was injected with CRM197 and the negative control group was detected. Immunohistochemistry was used to test the expression of HB-EGF and P-gp in xenograft tumors of each team. **Results:** The expression of HB-EGF and P-gp were in different levels in each group. It was depressed obviously in the xenograft tumors which were injected CRM197. The difference was significant in statistics. **Conclusion:** The inhibition of CRM197 on the activation of the signal transduction pathway of heparin-binding epidermal growth factor inhibited the growth of the xenograft tumors of ovarian cancer and depressed the expression of HB-EGF and P-gp in xenograft tumors.

Key words: Heparinbinding epidermal growth factor; Drug-resistant of ovarian cancer; CRM197

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引言

卵巢癌是病死率高的妇科恶性肿瘤, 化疗是卵巢癌治疗关键手段之一, 肿瘤细胞对抗癌药物的耐药问题是导致化疗失败的常见因素和关键性难题^[1]。细胞信号转导通路在肿瘤细胞生长及化疗耐药形成的过程中具有重要作用, 肝素结合表皮生长因子(heparinbinding epidermal growth factor, HB-EGF)属表皮生长因子(EGF)超家族成员, 是 EGFR 配体之一, HB-EGF 与细胞膜上的 EGFR 结合^[2], 使后者发生磷酸化, 激活下游信号传导通路, 发挥促进肿瘤细胞生长、迁移、入侵及血管形成^[3], 并参与卵巢癌的化疗耐药。应用相应的信号转导通路阻断剂抑制卵巢

肿瘤的生长逆转耐药将成为提高卵巢癌治疗效果的亮点^[4,5,6]。

本文将通过研究 HB-EGF 特异性信号传导通路抑制剂 CRM197 对裸鼠卵巢癌移植瘤的抑制作用, 初步探讨 HB-EGF 信号传导通路与卵巢癌化疗耐药的关系。

1 材料与方法

1.1 材料

人卵巢癌亲本细胞 A2780、耐紫杉醇细胞 A2780/Taxol 及耐顺铂细胞 A2780/DDP 由广州中山三院实验室肖兰博士惠赠。上述三种细胞均为贴壁生长, 采用含 10%胎牛血清的 DMEM 培养液(Dulbecco's Modified Eagle's Medium)培养。

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1.2 方法

1.2.1 细胞培养 人卵巢癌亲本细胞 A2780、泰素耐药细胞 A2780/Taxol 及顺铂耐药 A2780/DDP 分别于含有 10%胎牛血清的低糖型 DMEM 培养液中, 37℃ 5%CO₂ 饱和湿度下培养, 0.25%胰蛋白酶消化传代。A2780/Taxol 细胞耐药指数为 8.0, A2780/DDP 细胞耐药指数为 5.0。

1.2.2 细胞处理 当上述三种细胞生长至 90%融合时, 弃培养液, 用 PBS 洗涤细胞 2 次, 清除死亡脱落的细胞, 0.25%胰蛋白酶消化细胞, PBS 重悬细胞, 1000rpm、室温下离心 3min, 弃上清, 用 PBS 充分洗涤细胞 2 次, 以去除可能诱发裸鼠体内非特异性免疫反应的血清成分, 离心后用 PBS 液重悬成浓度为 1×10⁷/mL 的单细胞悬液。

1.2.3 裸鼠皮下移植瘤模型的建立及用药方法 BALB/C nu/nu 雌性裸鼠 30 只, 3~4 周龄, 体重 15~18g, 购自北京维通利华公司, 按照《实验动物管理条例》规范化饲养管理 1 周, 待裸鼠状态稳定后随机分成 A、B、C 三组, 每组 10 只。取配置好的上述三种细胞的单细胞悬液分别接种于三组裸鼠右前肢腋窝皮下, A 组接种亲本细胞, B 组接种 A2780/Taxol 细胞, C 组接种 A2780/DDP 细胞, 每只接种 0.2mL。成瘤后每 3 天用游标卡尺测量 1 次皮下肿瘤长径 (a) 及短径 (b) 的变化。根据公式 $V(\text{mm}^3) = ab^2 / 2$ 计算肿瘤体积, 裸鼠移植瘤平均体积为 100mm³ 时开始给药。HB-EGF 特异性信号传导通路抑制剂 CRM197 (美国 segma 公司), 三组裸鼠每组随机选取 5 只给药, 给药组分别记为 A⁺、B⁺、C⁺, 按 1mg/kg 腹腔内注射^[7], 其余腹腔注射 PBS 液作为阴性对照, 每周 1 次, 共 4 次, 给药结束 1 周后处死裸鼠。

1.2.4 免疫组化检测瘤体内 HB-EGF 及 P-gp HB-EGF 鼠抗人单克隆抗体 (Abcam 公司), P 糖蛋白鼠抗人单克隆抗体 (武汉博士德公司), 免疫组化采用链酶素抗生物素过氧化物酶连接法 (S-P 法), S-P 试剂盒购于北京中山公司。免疫组化主要反应步骤按试剂盒说明书操作。PBS 代替一抗作为阴性对照。

1.3 免疫组化结果判断

HB-EGF、P-gp 着色部位均在胞膜或胞浆。每张片计数 100 个肿瘤细胞, 阳性细胞数 <5% 为 -, 1%~25% 为 +, 26%~50% 为 ++, >50% 为 +++。

1.4 统计学处理

用 SPSS17.0 软件对结果进行分析, 计量资料的比较采用 t 检验, 计数资料的比较采用 X² 检验。

2 结果

2.1 各实验组小鼠及肿瘤生长情况

各组中阴性对照组肿瘤大体所见界限不清, 与周围组织粘连, 不易剥离, 血管粗大, 质地软, 生长较用药组快, HE 染色见肿瘤细胞生长活跃, 极少见坏死及凋亡, 各组中用药组肿瘤大体所见界限清楚, 与周围组织无粘连, 易剥离, 质地硬, 生长速度较慢, 肉眼未见血管形成, HE 染色见肿瘤边缘细胞退变, 易见坏死及凋亡细胞。亲本细胞组肿瘤生长速度稍快。用药过程中小鼠体重平稳增长, 未发生药物毒性反应, 实验前后各用药组与对照组小鼠体重如表 1 所示, 组内比较 P>0.05, 差异无统计学意义。用药组肿瘤生长受到抑制, 各组内部肿瘤质量比较 P<0.05, 差异有统计学意义。

表 1 各组小鼠体重、抑瘤率及肿瘤质量及 P 值

Table 1 The body weight, inhibition rate and tumor weight of nude mice in each group and P value

	Body weight(g)		Inhibition rate	Tumor weight(g)	P value	
	Begin	end			HB-EGF	P-gp
Group A	16.31± 0.78	20.61± 1.09	-	2.80± 0.27		
Group A ⁺	16.29± 0.77	20.58± 1.03	43%	1.30± 0.15	0.019	0.026
Group B	16.69± 1.02	20.61± 1.08	-	1.76± 0.23		
Group B ⁺	16.60± 1.01	20.63± 1.07	68%	0.56± 0.09	0.026	0.026
Group C	15.99± 1.45	20.59± 1.77	-	1.78± 0.25		
Group C ⁺	16.01± 1.42	20.49± 1.82	52%	0.88± 0.13	0.018	0.030

2.2 各种卵巢组织中 HB-EGF、P-gp 的表达

用药后 A、B、C 三组中 HB-EGF、P-gp 表达明显低于对照

组。对各组肿瘤组织中用药前后 HB-EGF、P-gp 的表达结果进行 Pearson 卡方检验, 结果如表 2 所示, P 值均小于 0.05, 差异有统计学意义, 见图 1 及图 2。

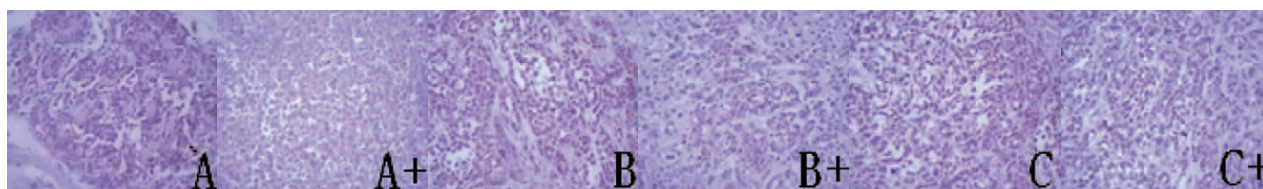


图 1 各组肿瘤组织中 HB-EGF 的表达。标 + 为用药组(400×)

Fig.1 Immunohistochemical stains for the expression of HB-EGF of the tumors (400×)

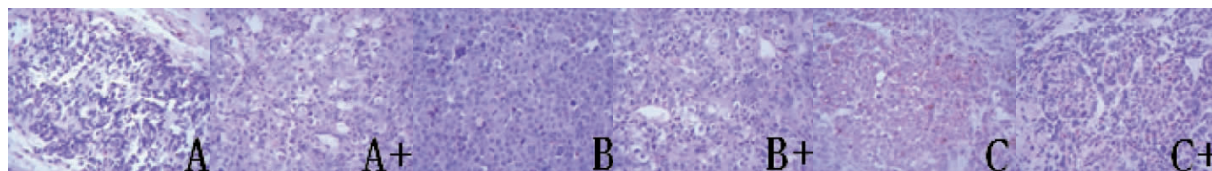


图2 各组肿瘤组织中 P-gp 的表达。标 + 为用药组(400×)

Fig.2 Immunohistochemical stains for the expression of P-gp of the tumors(400×)

3 讨论

HB-EGF 是 1988 年由 Besne 等在人的巨噬细胞培养液中发现的一种分子量约 20-22kD 肽类生长因子,其可由巨噬细胞、平滑肌细胞、血管内皮细胞等分泌产生^[8,9],以其膜前体的形式即跨膜形式(proHB-EGF)存在于细胞表面。在某些刺激因素作用下,proHB-EGF 胞外域脱落形成成熟可溶性 HB-EGF (sHB-EGF),sHB-EGF 可以直接结合并激活 EGFR,使其发生自身磷酸化,启动下游的 MAPK 等信号通路,发挥促进细胞增殖、分化等作用^[10]。HB-EGF 广泛分布于多种组织和器官,与某些癌症如肝癌、乳腺癌、卵巢癌的发生有关^[11]。近年研究发现 HB-EGF 可参与肿瘤发生发展的各个环节,并可通过激活 Ras/Raf/MAPK 及 AKT 信号传导通路对抗凋亡,导致肿瘤细胞化疗耐药^[12,13]。

P-gp 是人类 mdrl 基因编码的分子量为 170KD 的跨膜糖蛋白,为一种具有 ATP 酶活性的能量依赖性转运排出泵,在化疗耐药的肿瘤组织中过度表达^[14],能将肿瘤细胞内包括多种抗肿瘤药物的底物泵出细胞外,造成细胞内药物积聚量减少,降低化疗药物对肿瘤细胞的毒性,使细胞发生化疗耐药而导致化疗的失败^[15]。

CRM197 是白喉毒素 52 位的甘氨酸突变为谷氨酸后形成的无毒突变体,是 HB-EGF 的特异性信号传导通路抑制剂,可与 proHB-EGF 及 sHB-EGF 的 EGF 样结构域特异性结合抑制 MAPK 等信号通路的激活,发挥抑制肿瘤生长逆转化疗耐药的作用^[16,17]。本实验结果显示注射 CRM197 后裸鼠移植瘤质量小于对照组,差异具有统计学意义,表明 CRM197 有效的抑制了裸鼠移植瘤的生长。HB-EGF、P-gp 表达实验组明显低于对照组,表明 CRM197 抑制 HB-EGF 的表达的同时也抑制了 P-gp 的表达,抑制肿瘤生长同时逆转卵巢癌耐药,并且其逆转卵巢癌耐药的机制不仅仅是抑制了信号传导通路的激活,还可能通过减少细胞中 P-gp 的数量减少化疗药物的外排,从而使细胞内保持有效的化疗药物浓度,提高化疗的疗效,这与 2009 年 Yagi 等报道的 CRM197 与紫杉醇连用后抗肿瘤作用增强的结果相符合^[18],表明 CRM197 不但可以单独作为一种抗肿瘤药物,在联合化疗中也可发挥显著疗效。2008 年 Yagi 报道 HB-EGF 在胃癌及卵巢癌中参与上皮间质转换(EMT),介导肿瘤的腹膜种植^[19]推测 CRM197 可能在抑制卵巢癌转移中也能发挥重要作用。在本研究中用药组裸鼠体重与未用药裸鼠体重差异无统计学意义,实验过程中无药物毒性反应,国外有学者研究证明 CRM197 生物利用度合理,因治疗而产生的毒性也在可接受范围^[20]因而 CRM197 有望成为一种新型的有效的抗肿瘤药物。

参考文献(References)

- [1] Sood AK, Abu-rustum NR, Barakat RR, et al. Fifth international conference on ovarian cancer:challenges and opportunities [J]. Gynecol Oncol, 2005, 97 :916-923
- [2] Raab G, Klagsbrun M. Heparin-binding EGF-like growth factor [J]. Biochem Biophys Acta, 1997, 1333:179-199
- [3] Pat P, Ongusaha, Jennifer C. Kwak, Andrew J. Zwible, et al. Angiogenesis HB-EGF Is a Potent Inducer of Tumor Growth and Angiogenesis [J]. Cancer Research, 2004, 64:5283-5290
- [4] Luciano JC, Robert MG, Harry AD. Upstream Signaling Inhibition Enhances Rapamycin Effect on Growth of Kidney Cancer Cells [J]. Urology, 2007, 69 (3):596-602
- [5] Doherty L, Gigas DC, Kesari S, et al. Pilot study of the combination of EGFR and mTOR inhibitors in recurrent malignant gliomas[J]. Neurology, 2006, 67: 156-158
- [6] Buck E, Eyzaguirre A, Brown E, et al. Rapamycin synergizes with the epidermal growth factor receptor inhibitor erlotinib in non-small-cell lung, pancreatic colon, and breast tumors[J]. Mol Cancer Ther, 2006,5 (11):2676-2684
- [7] Shingo M, Michinari H, Ayano Y, et al. Heparin-Binding EGF-Like Growth Factor Is a Promising Target for Ovarian Cancer Therapy[J]. Cancer Research, 2004, 64:5720-5727
- [8] ThomPosn SA, Higashiyama S, Wood K, et al. Characterization of sequences within the heparin-binding EGF-like growth factor that mediate interaction with the heparin[J]. Biol Chem, 1994, 29:2541-2549
- [9] Miyoshi E, Higashiyama S, Nakagawa T, et al. Membrane-anchored heparin-binding epidermal growth factor-like growth factor acts as a tumor survival factor in a hepatoma cell line [J]. Biol Chem, 1997, 272 (22):14349-14355
- [10] Shigeki H, Hidehiko I, Chie M, et al. Membrane-anchored growth factors, the epidermal growth factor family: Beyond receptor ligands [J]. Cancer Sci, 2008, 99(2):214-220
- [11] Iwamoto R, Mekada EK. Heparin-binding EGF-like growth factor: a juxtacrine growth factor [J]. Cytokine Growth Factor Rev, 2000, 11: 335-344
- [12] Johnson FM, Saigal B, Donato NJ. Induction of heparin-binding EGF-like growth factor and activation of EGF receptor in imatinib mesylate-treated squamous carcinoma cells [J]. Cell Physiol, 2005, 205(2):218-27
- [13] Yotsumoto F, Oki E, Tokunaga E, et al. HB-EGF orchestrates the complex signals involved in triple-negative and trastuzumab-resistant breast cancer[J]. Cancer, 2010, 127(11):2707-2717
- [14] Naomi W, Annemarie L, Susan K, et al. Expression of multidrug resistance markers ABCB1 (MDR-1/P-gp) and ABCC1 (MRP-1) in renal cell carcinoma[J]. BMC Urology, 2009, 9:6-11 (下转第 4263 页)

- Liu Fang, Xing Xiao-ming, Li Yu-jun, et al. Colorectal Adenocarcinoma Grp78 Expression and Its Significance [J]. Qingdao University Medical College, 2009, 45(3):253-254
- [4] Zheng HC, Nakamura T, Zheng Y, et al. SV40 T antigen disrupted the cell metabolism and the balance between proliferation and apoptosis in lens tumors of transgenic mice[J]. J Cancer Res Clin Oncol, 2009, 135(11):1521-1532
- [5] Lee AS. GRP78 induction in cancer:therapeutic and prognostic implications[J].Cancer Res, 2007, 67(8): 3496-3499
- [6] 吴倩,张红,张和平. 组织芯片技术研究 cd147 和 mmp-2 在胃癌中的表达[J]. 安徽医科大学学报, 2010, 45(1):43-46
- Wu Qian, Zhang Hong, Zhang He-ping. Tissue Microarray Technology Cd147 and Mmp-2 Expression in Gastric Cancer [J]. Anhui Medical University, 2010, 45(1):43-46
- [7] Jemal A, Siegel R, Ward E, et al. Cancer statistics [J]. CA Cancer J Clin, 2008, 58(2):71-96
- [8] Silvestri GA, Alberg AJ, Ravenel J. The changing epidemiology of lung cancer with a focus on screening[J]. BMJ, 2009, 339:451-454
- [9] Li J and Lee AS. Stress induction of GRP78/BiP and its role in cancer [J]. Curr Mol Med, 2006, 6(1):45-54
- [10] Ortiz C, Cardemil L. Heat shock responses in two leguminous plants : a comparative study[J]. Exp Bot, 2001, 52(361):1712-1719
- [11] Yang Y, Turner RS, Gaut JR. The chaperone BiP/GRP78 binds to amyloid precursor protein and decreases Abeta 40 and Abeta 42 secretion [J]. Biol Chem, 1998, 273(40):25552-25555
- [12] 姜莉,张黎川,王琪. GRP78 表达下调可降低肺腺癌细胞对 VP-16 的耐药[J]. 中国肺癌杂志, 2009, 12(11):1159-1163
- Jiang Li, Zhang Li-chuan, Wang Qi. GRP78 Expression Down Can Reduce Lung Adenocarcinoma Cells Resistant To VP-16 [J]. Chinese Journal of Lung Cancer, 2009, 12(11):1159-1163
- [13] Fernandez PM, Tabbara SO, Jacobs LK, et al. Over expression of the glucose regulated stress gene GRP78 in malignant but not benign human breast lesions[J]. Breast Cancer Res Treat, 2000, 59(1):125-126
- [14] Fu Y, Lee AS. Glucose regulated proteins in cancer progression, drug resistance and immunotherapy [J]. Cancer Biol Ther, 2006, 5(7):741-744
- [15] Jianze Li, Amy S. Lee, et al. Stress induction of GRP78/BiP and its role in cancer Current Molecular Medicine, 2006, 6:45-54
- [16] Fernandez PM, Tabbara SO, Jacobs LK, et al. Over expression of the glucose regulated stress gene GRP78 in malignant but not benign human breast lesions[J]. Breast Cancer Res Treat, 2000, 59(1):15-26
- [17] Zheng H, kahashi. Overexpression of GRP 78 and GRP94 are marker smarkers for aggressive behavior and Poor Prognosis in gastric carcinomas[J]. Hum Pathol, 2008, 39(7):1042-1049
- [18] 张莹,孙黎光.GRP78 的研究进展[J]. 国外医学·生理、病理科学与临床分册, 2005, 25(3):251-253
- Zhang Ying, Sun Li-guang. Advances In Research On GRP78[J]. Foreign Medical Physiology, Pathology, Scientific and Clinical Journal, 2005, 25(3):251-253
- [19] Zhuang L, Scolyer R A, LEE C S, et al. Expression of glucose-regulated stress protein GRP78 is related to progression of melanoma [J]. Histopathology, 2009, 54(4):462-470
- [20] 王琪,安利佳,陈誉华,等.内质网分子伴侣 GRP78 在人肺癌组织中的高表达及其临床意义[J]. 中华医学杂志, 2003, 83(2):148-150
- Wang Qi, An Li-jia, Chen Yu-hua, et al. Endoplasmic Reticulum Molecular Chaperone GRP78 in Human Lung Cancer Tissue and Its Clinical Significance [J]. Chinese Medicine Journal, 2003, 83(2):148-150

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- [15] O'Driscoll L, Walsh N, Larkin A, et al. MDR1/P-glycoprotein and MR-P-1 drug efflux pumps in pancreatic carcinoma [J]. Anticancer Res, 2007, 27:2115-2120
- [16] Kageyama T, Ohishi M, Miyamoto S. Diphtheria toxin mutant CRM-197 possesses weak EF2-ADP-ribosyl activity that potentiates its anti-tumorigenic activity[J]. Biochem, 2007, 142(1):95-104
- [17] Fridman JS, Caulder E, Hansbury M, et al. Selective inhibition of ADAM metalloproteases as a novel approach for modulating ErbB pathways in cancer[J]. Clin Cancer Res, 2007, 13(6):1892-1902
- [18] Yagi H, Yotsumoto F, Sonoda K, et al. Synergistic anti-tumor effect of paclitaxel with CRM197, an inhibitor of HB-EGF, in ovarian cancer[J]. Int J Cancer, 2009, 124(6):1429-1439
- [19] Yagi H, Yotsumoto F, Miyamoto S. Heparin-binding epidermal growth factor-like growth factor promotes transcoelomic metastasis in ovarian cancer through epithelial-mesenchymal transition [J]. Mol Cancer Ther, 2008, 7(10):3441-3451
- [20] Buzzi S, Rubboli D, Buzzi G, et al. CRM197 (nontoxic diphtheria toxin): effects on advanced cancer patients[J]. Cancer Immunol Immunother, 2004, 53(11):1041-1048