Original Experimental Research 实验论著

蟾毒灵对裸鼠人肝癌移植瘤的抑制作用及对 Bcl-2 和 Bax 蛋白表达的影响

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目的：探讨蟾毒灵对裸鼠人肝癌原位移植模型的抗肿瘤作用及对 Bcl-2 和 Bax 蛋白表达的影响。
方法：建立裸鼠人肝癌原位移植模型，随机分为蟾毒灵高(1.5 mg/kg)、中(1 mg/kg)、低(0.5 mg/kg)剂量治疗组，阿霉素 8 mg/kg 治疗组和模型组。裸鼠人肝癌原位移植模型第 25 天测量肿瘤体积，取瘤组织做 HE 染色观察肿瘤坏死程度并行电镜观察，DNA 原位末端标记法检测凋亡标记指数，免疫组化法检测肝癌组织中 Bcl-2 和 Bax 蛋白的表达。
结果：蟾毒灵各剂量组肿瘤体积均较模型组明显缩小($P<0.01$)，生存期较阿霉素治疗组及模型组延长($P<0.05$)。电镜观察到蟾毒灵各组肿瘤出现明显的细胞凋亡征象，低、中、高剂量治疗组的凋亡指数分别为 5.87±2.13、8.86±2.96 和 10.60±3.42，明显高于阿霉素治疗组的 3.28±0.98($P<0.05$ 或 $P<0.01$)；蟾毒灵各组瘤组织的 Bax 表达明显增强，而 Bcl-2 表达无明显改变。
结论：蟾毒灵对裸鼠人肝癌原位移植模型有明显的抗肿瘤作用，其作用机制可能与上调 Bax 表达、诱导细胞凋亡有关。

关键词：蟾毒灵；肝癌；细胞凋亡；Bcl-2；Bax；裸小鼠
中图分类号：R735.7；**文献标识码**：A；**文章编号**：1672-1977(2007)02-0155-05

Inhibition action of bufalin on human transplanted hepatocellular tumor and its effects on expressions of Bcl-2 and Bax proteins in nude mice

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Objective: To investigate the anti-tumor effect of bufalin and its regulation on Bcl-2 and Bax proteins in orthotopically transplanted tumor of human hepatocellular carcinoma in nude mice.
Methods: Orthotopically transplanted tumor of human hepatocellular carcinoma was established in nude mice. The mice were randomly divided into five groups: high-dose bufalin-treated group (1.5 mg/kg), medium-dose bufalin-treated group (1 mg/kg), low-dose bufalin-treated group (0.5 mg/kg), adriamycin-treated group (8.0 mg/kg), and normal saline-treated group. After 25 days, mice were sacrificed. The tumor volume was measured, and the pathological changes of tumor tissues were detected by HE staining to observe the tumor necrosis degree. Cell morphological changes were also observed by an electron microscopy. Label index of tumor cell apoptosis was assessed by terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL), and the expressions of Bcl-2 and Bax proteins were determined by immunohistochemical method.
Results: The tumor volume in the bufalin-treated groups was shrunk significantly compared with that in the normal saline-treated group ($P<0.01$). The survival time of the bufalin-treated groups was prolonged compared with that of the adriamycin-treated group and the normal saline-treated group($P<0.05$). Apoptotic characteristics could be seen in tumor tissues of the bufalin-treated groups. The label index of tumor cell apoptosis in the bufalin-treated groups (5.87±2.13, 8.86±2.96 and 10.60±3.42 in low-, medium- and high-dose groups respectively) was higher than that in the adriamycin-treated group (3.28 ±0.98) ($P<0.05$, $P<0.01$). The expression of Bax was up-regulated, while no changes were detected as to Bcl-2 protein in tumors of the bufalin-treated groups.
Conclusion: Bufalin has significant anti-tumor effect on the orthotopically transplanted tumor of human

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hepatocellular carcinoma in nude mice. Its effect might be related to up-regulation of Bax protein and inducement of the tumor cell apoptosis.

Keywords: bufalin; liver neoplasms; apoptosis; Bcl-2; Bax; nude mice

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蟾毒灵(bufalin)是从中药蟾酥中提取的一种毒性配基之一,分子式为 $C_{24}H_{32}O_4$,相对分子质量为386.5。研究表明,蟾毒灵具有诱导肿瘤细胞分化和凋亡的作用,主要涉及到白血病、前列腺癌、胃癌、肝癌等人类肿瘤,且仅限于体外研究[1~4]。本文旨在探讨蟾毒灵对人肝癌裸鼠原位移植模型的体内抗肿瘤效应及对 Bcl-2 和 Bax 蛋白表达的影响。

1 材料和方法

1.1 实验材料

1.1.1 *实验动物和瘤株* BALB/C-nu/nu 裸鼠,由复旦大学实验动物中心提供,SCKK(沪)2003-0003,4~5 周龄,体质量 18~20 g,雄性,在无特殊病原菌条件下分笼饲养。人肝癌细胞株 BEL-7402,由中国科学院上海细胞生物学研究所提供,由第二军医大学长海医院中医科实验室传代冻存。

1.1.2 *药物和试剂* 蟾毒灵,美国 Sigma 公司产品;阿霉素(adriamycin, ADM),深圳万乐药业有限公司产品;DNA 原位末端标记试剂盒,Boehringer Mannhein 公司产品;鼠抗人 Bcl-2 和 Bax 蛋白单克隆抗体,Santa Cruz 公司产品。

1.2 实验方法

1.2.1 *模型分组及处理* 参照文献制作裸鼠人肝癌原位移植模型[5,6],随机分为蟾毒灵 1.5、1.0 和 0.5 mg/kg 治疗组,ADM 治疗组和模型组,每组 15 只。各组均于造模后第 15 天用药,蟾毒灵治疗组分别腹腔注射,第 15~24 天,1 次/d;模型组注射等体积的生理盐水(normal saline, NS),方法同蟾毒灵组;ADM 按 8 mg/kg 腹腔注射,仅在造模第15 天给药 1 次。停药次日每组处死荷瘤鼠 10 只,用游标卡尺分别测量瘤体的长径(a)和短径(b),依据公式 $V=ab^2/2$ 计算肿瘤体积;抑瘤率(%)=[1-($V_{治疗组}/V_{模型组}$)]×100。每组剩余小鼠(5 只)观察生存期,计算生命延长率。生命延长率(%)=[(药物组平均存活天数-NS 组平均存活天数)/NS 组平均存活天数]×100。

1.2.2 *组织学及电镜观察* 部分瘤组织用 10%福尔马林固定,石蜡切片,HE 染色作常规病理检查,观察肿瘤组织坏死程度[7]。剩余瘤组织以 3%戊二醛固定 1~3 h,缓冲液冲洗后,用 1%四氧化锇固定 1~2 h,梯度丙酮脱水和 Epon 812 包埋,醋酸双氧铀与柠檬铅双染色、切片,电子染色,透射电镜下观察拍照。

1.2.3 *DNA 原位末端标记检测* 采用 TdT 介导的带生物素 dUTP 缺口末端标记技术(terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling, TUNEL)检测细胞凋亡指数[7]。以 PBS 代替 TUNEL 反应液作为阴性对照,以细胞核有明显棕黄色为阳性细胞。每张切片观察 10 个高倍视野,阳性记数采用双盲法,计数时避开肿瘤坏死区,每 100 个细胞中的阳性细胞数为标记指数(label index, LI)。

1.2.4 *Bcl-2 和 Bax 蛋白的免疫组化检测* 采用过氧化酶标记的链霉卵白素(streptavidin peroxidase, SP)法,DBA 显色,按照试剂说明书的流程进行检测。在光镜下观察,以胞浆内棕黄色细颗粒为 Bcl-2 和 Bax 阳性染色,细胞计数方法同 TUNEL 检测。

1.3 *统计学方法* 以 SPSS 11.0 软件进行统计学分析,数据用 $\bar{x}\pm s$ 表示,进行单因素方差分析。

2 结 果

2.1 *荷瘤裸鼠体质量及肿瘤变化* 治疗前各组肿瘤大小差异无统计学意义($P>0.05$),提示各组瘤体大小具有可比性。与模型组相比,裸鼠人肝癌原位移植模型经蟾毒灵和 ADM 治疗后瘤体均明显缩小($P<0.01$),蟾毒灵高剂量治疗组的抑瘤率高于 ADM 治疗组,差异有统计学意义($P<0.05$);蟾毒灵治疗前后各组裸鼠的体质量无明显改变($P>0.05$)。见表 1。

2.2 *平均生存期及生命延长率* 蟾毒灵治疗后各组裸鼠的平均生存期均有所延长,蟾毒灵高、中、低剂量治疗组生命延长率分别为 35.9%、29.9%和 17.1%,蟾毒灵各组与模型组生存时间相比,差异均有统计学意义($P<0.05$),以蟾毒灵高剂量治疗组最为明显,而 ADM 治疗组裸鼠生存期未见延长。见图 1。

2.3 *肝肿瘤坏死程度* 治疗后,模型组瘤组织呈轻度坏死,蟾毒灵高、中剂量治疗组和 ADM 治疗组癌灶内有大量纤维结缔组织增生,肝癌组织以中重度

坏死为主。但坏死灶主要集中在肿瘤中央部位,肝癌细胞在癌灶边缘生长仍较为活跃。见图 2。

2.4 肝癌细胞的超微结构 模型组癌细胞表面不规则,核大呈圆形,染色质增多,游离核糖体较多,胞质内可见较丰富的线粒体及内质网。蟾毒灵各治疗组常可见细胞核固缩的肝癌细胞,胞浆浓缩,染色质浓集于核膜内侧呈团块或新月状,胞浆起泡和凋亡小体,并见凋亡小体脱离细胞体,没入间质或被其他细胞吞饮。

2.5 瘤组织凋亡标记指数及 Bcl-2 和 Bax 蛋白的表达 蟾毒灵治疗组肝癌细胞的凋亡指数分别明显高于模型组,ADM 治疗组凋亡指数未见明显增加。蟾毒灵治疗组 Bcl-2 蛋白阳性表达率均有所降低,表达强度较弱,但与模型组比较,差异无统计学意义;瘤组织均见 Bax 蛋白高表达,Bax 蛋白表达标记指数随着蟾毒灵剂量的增加而提高。见表 2、图 3。

表 1 裸鼠人肝癌原位移植模型蟾毒灵治疗前后体质量及瘤体变化

Table 1 Changes of mouse body weight and tumor volume of human hepatocellular orthotopically transplanted tumor induced by bufalin

($\bar{x} \pm s$)

Group	n	Body weight (g)		Tumor volume (mm^3)		Tumor inhibition rate
		Pre-treatment	Post-treatment	Pre-treatment	Post-treatment	
High-dose bufalin-treated	10	20.94±1.00	21.39±1.62*	43.50±5.09	35.21±12.51△△	79.3%*
Medium-dose bufalin-treated	10	20.03±1.16	21.48±1.10*	42.92±4.10	49.83±11.46△△	70.7%
Low-dose bufalin-treated	10	21.06±0.94	21.57±1.14*	43.00±2.97	83.99±24.63△△	50.7%
ADM-treated	10	21.00±1.00	18.90±0.77	42.93±4.23	55.17±16.13△△	67.6%
NS-treated	10	20.95±1.07	20.40±1.23	43.37±4.82	170.39±25.29	

* $P<0.05$, *vs* ADM-treated group; △△ $P<0.01$, *vs* NS-treated group.

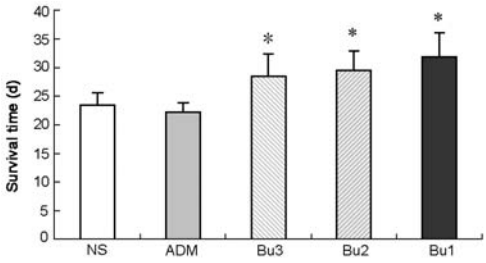

图 1 各组平均生存时间

Figure 1 Mean survival time of five groups

Bu1: High-dose bufalin-treated group; Bu2: Medium-dose bufalin-treated group; Bu3: Low-dose bufalin-treated group; * $P<0.05$, *vs* NS-treated group; $n=5$.

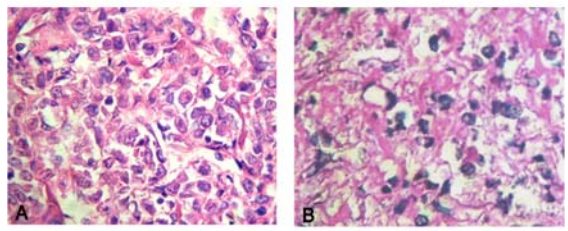

图 2 瘤组织坏死程度的病理形态(HE 染色, ×250)

Figure 2 Pathological characteristics of tumor necrosis (HE staining, ×250)

A: NS-treated group; B: High-dose bufalin-treated group.

表 2 肝癌组织细胞凋亡及 Bcl-2、Bax 表达的标记指数

Table 2 Label index of tumor cell apoptosis and expressions of Bcl-2 and Bax in tumor tissues

($\bar{x} \pm s$)

Group	n	Label index (%)		
		Apoptosis	Bcl-2	Bax
High-dose bufalin-treated	10	10.60±3.42**	8.76±5.18	90.70±16.82**
Medium-dose bufalin-treated	10	8.86±2.96**	8.52±3.57	80.21±21.23**
Low-dose bufalin-treated	10	5.87±2.13*	11.34±6.24	79.54±10.75**
ADM-treated	10	4.26±2.12	10.59±6.92	47.56±15.31*
NS-treated	10	3.28±0.98	9.05±4.97	31.25±12.50

* $P<0.05$, ** $P<0.01$, *vs* NS-treated group.

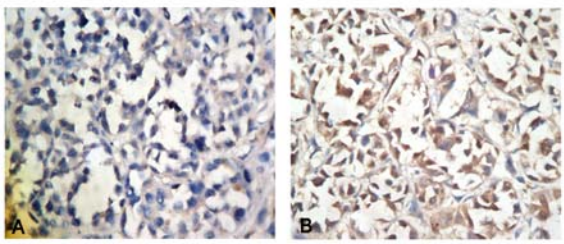

图 3 裸鼠原位肝内移植瘤 Bax 蛋白的表达(SP 法, ×200)

Figure 3 Expression of Bax in orthotopically transplanted tumor of human hepatocellular carcinoma in nude mice (SP method, ×200)

A: Bax expressed weakly in NS-treated group. B: High expression in high-dose bufalin-treated group.

3 讨 论

蟾酥及其相关制剂一直是临床治疗肝癌最有效的中药之一[8]。蟾酥的化学成分复杂,通过对蟾酥的分离和鉴定,主要的化学成分已得到部分确认[9,10]。蟾毒灵是从中药蟾酥中提取的毒性配基之一,属于细胞拓扑异构酶Ⅱ抑制剂。蟾毒灵对血液肿瘤、前列腺癌、胃癌等人类肿瘤的抑制作用已有报道,但未见有对人肝癌细胞的体内研究报道。在对蟾毒灵抗肿瘤的前期研究中发现蟾毒灵在体外对人肝癌 SMMC-7721 和 BEL-7402 细胞株具有较强的杀伤作用,诱导肝癌细胞凋亡及细胞周期 G_2/M 期阻滞,这可能是蟾毒灵抗肝癌的作用机制[4,11],但蟾毒灵在体内抗人肝癌的作用仍不清楚。

蟾毒灵对小鼠的 LD_{50} 为 2.2 mg/kg[12],本实验预实验也发现 2 mg/kg 的一次性给药剂量就可使小鼠发生一过性的痉挛性抽搐,但未引起小鼠中毒性死亡。鉴于此,本实验主要研究给予 1.5 mg/kg 及以下剂量蟾毒灵时动物体内的抗肿瘤作用。人类肿瘤裸鼠原位移植瘤模型是抗癌药物筛选和机制研究较为理想的动物模型,我们成功建立了裸鼠人肝癌原位移植模型,在此基础上观察蟾毒灵对裸鼠原位人肝癌移植模型的抗肿瘤效果,结果表明蟾毒灵按总量 15、10 和 5 mg/kg 分 10 d 连续给药治疗裸鼠原位人肝癌移植性模型在瘤体缩小方面具有与阿霉素 8 mg/kg 相同的疗效,且在带瘤生存期的延长及模型生活质量(体质量的增减)的改善方面,蟾毒灵各治疗组明显优越于阿霉素治疗组。提示蟾毒灵在体内具有良好的抗肝癌作用。

病理学及 TUNEL 检测结果发现,高、中剂量蟾毒灵不仅能导致肝癌组织中、重度坏死及大量纤维结缔组织增生,而且能原位诱导肝癌细胞凋亡,凋亡标记指数与蟾毒灵给药呈剂量依赖性。细胞凋亡是由于细胞内外环境变化或死亡信号触发以及在基因调控下所引起的细胞主动死亡的过程。Bcl-2 基因家族是细胞凋亡研究中最受重视的基因,目前已发现几个与 Bcl-2 同源的基因,包括 Bcl-xL、Bcl-xs、Bax、Bak、mcl-1、A1、Bag-1、Bfl-1、ced9、BHRF-1 等,构成了 Bcl-2 家族。其中 Bax 基因是 Bcl-2 家族中的一员,其生物学作用是拮抗 Bcl-2,与 Bcl-2 形成二聚体,抑制或促进细胞凋亡。Bcl-2 和 Bax 的比例决定细胞的凋亡状态:Bcl-2 表达水平高于 Bax 时,Bcl-2 和 Bcl-2 形成同源二聚体,细胞凋亡受抑制;Bax 表达水平高于 Bcl-2 时,则形成 Bax-Bax 同源二聚体,细胞凋亡增强[13]。有资料显示,Bcl-2 蛋白在肝癌组织和肝癌细胞系中,一般只呈低水平的表达或不表达,而 Bax 蛋白则呈较高水平的表达[14]。本研究中裸鼠人肝癌原位移植瘤的免疫组化结果显示,Bcl-2 蛋白阳性表达率仅为 9.05%,而 Bax 蛋白阳性表达率为 31.25%,这与文献报道一致。蟾毒灵治疗后均未见瘤组织 Bcl-2 表达率的明显变化,而 Bax 表达却明显提高,瘤组织 Bax 蛋白阳性标记指数与凋亡标记指数有正相关趋势($r=0.876\ 8$),提示上调 Bax 蛋白表达、诱导肝癌细胞凋亡可能是蟾毒灵体内抗肝癌的机制之一。

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Society for Acupuncture Research
Announcement and Call for Papers

The Status and Future of Acupuncture Research: 10 Years Post-NIH Consensus Conference

University of Maryland at Baltimore
November 8-11, 2007
Baltimore, MD, USA

Co-sponsors include the University of Maryland, Harvard Medical School's Osher Institute, Shanghai University of Traditional Chinese Medicine, Journal of Chinese Integrative Medicine, Chinese University of Hong Kong, Kyung He University, Guanxi Medical University, Korea Institute of Oriental Medicine, Meiji University of Oriental Medicine, The World Federation of Acupuncture-Moxibustion Societies, and co-sponsors in Australia, Europe, and the USA.

Abstract submission deadline April 1, 2007

Purpose

This special event will mark the 10th anniversary of the landmark 1997 NIH Consensus Development Conference on Acupuncture.

- Leading researchers in the field from the U.S. and abroad will be invited to give keynote and overview presentations assessing progress in the past decade, and challenges and opportunities for future research.
- Members of the national and international acupuncture research communities will present original presentations in three major areas: clinical, basic science and methodology.
- Key issues will be addressed in panel discussions, break-out sessions, and poster sessions.
- Pre-conference workshops will focus on fundamentals of Oriental medicine (OM) research for OM practitioners, students, and educators new to this field.

Abstracts are solicited for presentations in the areas of clinical research, basic science and research methodology

Abstract submission

Please email your abstracts to helene.langevin@uvm.edu by April 1, 2007. Only email submissions will be accepted. Abstracts submitted after this date may not be considered. Abstracts will be reviewed by the Program Committee and prospective speakers will be informed by August 1, 2007 as to whether their papers have been accepted for oral or poster presentation.

Abstract format information and submission forms can be found on the SAR website www.acupunctureresearch.org.