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# Inducible overexpression of *Bak* sensitizes HCC-9204 cells to apoptosis induced by doxorubicin

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**KEY WORDS** doxorubicin; vinorelbine; *Bak* genes; apoptosis; hepatocellular carcinoma

## ABSTRACT

**AIM**: To investigate the role of overexpression of *Bak* in apoptotic pathways and drug susceptibility using doxorubicin and vinorelbine in human HCC-9204 cells. **METHODS**: An inducible system, MT-II regulatory system which allowed controlled expression of protein upon addition of $ZnSO_4$ (100 μmol/L) as an external inducer was used. Stable transfection of pMD-*Bak* gene was performed on HCC-9204 cells. Apoptotic cells were measured by morphological criteria, as well as by TUNEL assay and flow cytometry. The ability of *Bak* to decrease clonogenic cell survival was studied by colony-forming assays, while decrease in cell viability was assessed by MTT assay. **RESULTS**: Cells overexpressing *Bak* showed extensive cell death with nucleus fragmentation detected by TUNEL assay. FACS analyses showed that *Bak* could induce significant $G_1$ accumulation and apoptosis in 19.29 % cells 24 h after induction. *Bak* significantly decreased the clonogenic survival following exposure to adriamycin, but not vinorelbine. Furthermore, the time-course of cell viability rates following exposure of HCC-9204/*Bak* cells to adriamycin and vinorelbine was in agreement with the above findings. *Bak* selectively sensitized HCC-9204 cells to death induced by adriamycin while resisted to vinorelbine. **CONCLUSION**: *Bak* may prolong cell cycle in $G_1$ phase, leading to apoptosis and decrease clonogenic survival of HCC-9204 cells in a drug-specific manner.

## INTRODUCTION

*Bak*, another member of *Bcl*-2 pro-apoptotic subfamily with 211 amino acids ($M_r$ 23 400), can promote cell death and counteract the protection from apoptosis provided by *Bcl*-2. *Bak* exhibits marked structural similarity to *Bcl*-2 and other family members, particularly in BH1 and BH2 domains[1]. Overall, *Bak* is identical to *Bcl*-2, *Bcl*-xL, and Bax by 25 %, 22 %, and 19 %, respectively. Moreover, enforced expression of *Bak* induces rapid and extensive apoptosis of serum-deprived fibroblasts, which raises the possibility that *Bak* is directly involved in activating the cell death machinery[2]. However, *Bak* has seldom been reported in regulating the sensitivity of cancer cells to these chemotherapeutic agents despite its similar structure and functions to Bax.

The main objectives of this study were to determine whether transfection of HCC-9204 cells with *Bak* gene may alter the spontaneous rate of apoptosis and the role of *Bak* in response of HCC-9204 cells to death induced by a series of chemotherapeutic agents.

## MATERIALS AND METHODS

**Cells and culture medium** HCC-9204 cell line derived from a human hepatocellular carcinoma and established in our laboratory[3] was cultured in Eagle's medium containing phenol red, with 5 % fetal bovine serum (FBS) (HyClone Laboratories, USA) in a humidified atmosphere of 95 % air, 5 % $CO_2$ at 37 ℃. All media were supplemented with *L*-glutamine 2 mmol/L, benzylpenicillin 100 mg/L, and streptomycin 100 kU/L. Culture medium and supplements were obtained from Gibco BRL. Of the HCC-9204 cell line, p53 was mutated. Medium was changed every 3 d. Cells were removed from culture flasks for passage by washing once with Hanks' balanced salt solution, followed by a 5-min incubation with edetic acid 0.5 mmol/L and 0.05 %

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trypsin at pH 7.4, RT.

**Plasmid construction and DNA transfection** Plasmids were constructed using standard molecular biology techniques. Human *Bak* cDNA (pBluescript SK-*Bak*) was a gift from Dr Thomas Chittenden (Apoptosis Technology, USA)[4]. The cDNA were subcloned into the MT-II inducible vector (pMD-neo) which was constructed by Dr ZHANG Jie (Institute of Radiation Medicine, China) and were placed under the control of MT-II promoter and the SV40 polyadenylation signal. To construct pMD-*Bak*, pBluescript SK-*Bak* was digested with *Bam*H I and *Eco*R I to yield a 1.2 kb fragment. The resulting fragment was then ligated to *Bam*H I and *Eco*R I digested pMD-neo (Fig 1). Clonfectin (Clontech, USA) was used to transfect *Bak* inducible expression vectors into HCC-9204 cells. According to the instructions, clonfectin transfection reagent was an effective liposome transfection reagent for many mammalian cell types with high transfection efficiencies and required only 1 – 4 h incubation for optimal results, especially in serum-containing media. For stable transfection, cells were selected in the above medium containing 0.5 g/L G418 (Life Technologies, Inc, USA) and incubated for 4 wk. Stable cell line of HCC-9204 cell transfected with *Bak*/pMD-neo vector was treated by continuous exposure to $ZnSO_4$ 100 μmol/L.

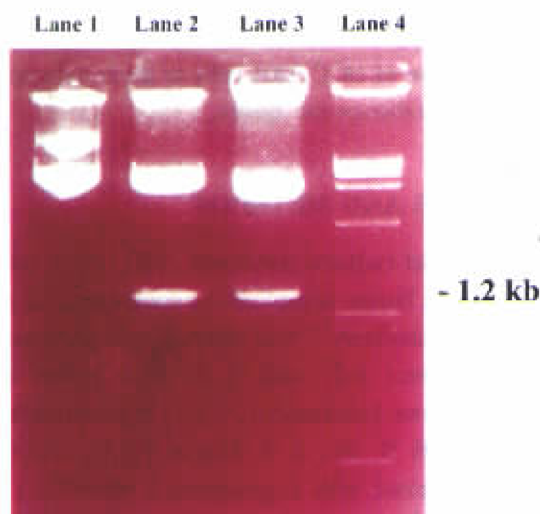


**Fig 1.** **Identification of pMD-*Bak* by restriction enzymes. Lane 1: pMD-*Bak* plasmid; Lane 2: pMD-*Bak*/bamH I + *Eco*R I; Lane 3: pBluescript SK-*Bak*/*Bam*H I + *Eco*R I; Lane 4: DL12000 marker (Takara, Japan).**

**Western blot analysis of transgene expression** Monolayers were rinsed with PBS and lysed with SDS-PAGE loading buffer (Tris 50 mmol/L, HCl pH 6.8, dithiothreitol 100 mmol/L, SDS 2 g/L). Mock and pMD-neo transfected cells served as a control. Samples were analyzed by SDS-PAGE and transferred into Hybond-C super membranes (Amersham, UK). The membranes were blocked with skimmed milk 50 mL/L, Tween-20 1 g/L and then probed overnight with primary antibody, washed in PBS, Tween-20 2 g/L, and then incubated with the appropriate horseradish-peroxidase (HRP) conjugated secondary antibody. After washing, the membranes were developed by DAB detection reagents (Dako Co. USA). To detect *Bak*, rabbit polyclonal antibody (Santa Cruz Biotechnology, USA) followed by HRP-conjugated anti-rabbit IgG (Fc) were used. The level of β-actin was used as a control for equal loading of protein.

**Immunohistochemistry analysis** HCC-9204 cells were harvested at different time of induction with $ZnSO_4$. Cell preparations were fixed with 700 mL/L (vol/vol) ethanol for 2 h, washed in PBS, and incubated with *Bak* antibody diluted at 1:100 in PBS containing 10 mL/L (vol/vol) bovine serum albumin (BSA, Sigma, USA). Immunostaining was performed using LSAB1 kit (Dako, Peroxidase, USA), according to the instructions of the manufacturer.

**Cell cycle analysis** HCC-9204 cells including adherent cells (using trypsin/edetic acid) and nonadherent cells were harvested. Cells were washed in PBS and resuspended and incubated in ethanol 700 mL/L at 4 ℃ for at least 12 h to permeabilize the plasma membrane. Cells were centrifuged at 1200 × *g*, resuspended in RNase 100 mg/L and propidium iodide 10 mg/L, and incubated at 25 ℃ for 15 min in the dark. Single color fluorescent flow cytometry was performed on a FACScalibur flow cytometer (Becton Dickinson, USA). Histograms were analyzed with Multiplus Software Ⅱ. For each sample, 10 000 events were collected.

**Apoptosis analyzed by TUNEL assay** Cells were harvested for TUNEL staining. The proportion of cells showing DNA fragmentation was measured by incorporation of fluorescein isothiocyanate (FITC)-12-dUTP into DNA by using terminal deoxynucleotidyltransferase (TdT)[5]. A kit from Boehringer Mannheim (*In Situ* Cell Death Detection Kit, FITC) was used. Briefly, after a 30-min (RT) incubation with 30 g/L BSA, 200 g/L

normal bovine serum in PBS, pH 7.4, slides were covered with the TUNEL mix [calf thymus TdT, FITC-12-dUTP and cobalt chloride in 1 (reaction buffer) for 1 – 2 h at 37 ℃]. The morphologic features were visualized by fluorescence microscopy. Routine HE staining was also conducted. Negative control was performed by omitting TdT. As a positive control paraffin-embedded sections of HCC were used. Quantitative analysis of apoptosis was represented by Apoptotic Index (AI) as described previously[5]. AI referred to percentage from at least 1000 counted apoptotic and non-apoptotic cells. The following criteria were considered to represent apoptosis[6]: 1) marked condensation of chromatin and cytoplasm (apoptotic cells); 2) cytoplasmic fragments with or without condensed chromatin (apoptotic bodies); 3) intra- and extracellular chromatin fragments (micronuclei).

**Electron microscopy analysis** Cells were pelleted and fixed with 30 mL/L glutaraldehyde in PBS (pH 7.4). Electron microscopy analysis was performed as described previously[7].

**Drugs and strategy of treatment** Vinorelbine (Navelbine, NVB), provided by Pierre Fabre Oncologie, was diluted to 5 mg/L with Eagle's medium. After expression of *Bak* was induced in HCC-9204 cells through addition of zinc in the culture medium for 24 h, HCC-9204/vehicle or HCC-9204/*Bak* cells were treated with NVB 5 mg/L for at least 24 h or 48 h. Adriamycin (Doxorubicin HCl, ADR), provided by Pharmacia & Upjohn, was diluted to 10 mg/L or 20 mg/L with Eagle's medium. The two groups were exposed to ADR at 10 mg/L and 20 mg/L for at least 4 h and were then withdrawn from the culture medium and were kept for 24 h or 48 i. The following investigations were theo conducted.

**Colony forming assay** Culture dishes were fixed and stained with 0.1 % crystal violet at the end of the experiment. Photographs of representative colonies were taken on 7 d after plating. Average colony size and colony cell numbers were determined by examining of several random colonies daily. Cells were tested in triplicate, and similar results were observed in another experiment.

**Cell viability assay** Cell growth was assessed by the 3-(4-,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. First $1 \times 10^4$ cells were seeded to a 96-well plate and left overnight to adhere. Then 100 μL drug solutions were added, and cells were treated for different time periods. After treatment, 50 μL of MTT solution (2 g/L in PBS) was added to each well and incubated at 37 ℃ for another 4 h. The plates were then centrifuged at 200 × *g* for 10 min and supernatant was discarded. Dimethyl sulfoxide 50 μL was added to each well. Plates were then shaken until crystals were dissolved. Reduced MTT was measured spectrophotometrically in a dual beam microtiter plate tracer at 490 nm.

**Statistical analysis** Data were analyzed by the *t* test, and values of $P < 0.05$ were considered statistically significant.

## RESULTS

**Inducible overexpression of *Bak* in HCC-9204 cells at the protein level** To demonstrate the role of *Bak* in the apoptosis response of HCC-9204 cells, a gene transfer technique was used to modulate the expression of *Bak* and analyzed cell death in the presence and absence of additional death stimuli. To obtain tightly regulated *Bak* expression, MT-Ⅱ-regulatable expression system was also utilized. pMD-*Bak* was constructed and transfected into HCC-9204 cells. Stable cell line of HCC-9204 transfected with pMD-*Bak* was obtained by continuous exposure to G418 (0.5 mg/L). $ZnSO_4$ (at a final concentration of 100 μmol/L) was added to the medium after stable transfection.

Immunohistochemistry showed the inducible overexpression of *Bak* by $ZnSO_4$ in HCC-9204 cells (Fig 2). *Bak* positive signal was frequently found on the nuclei membrane or perinuclear region of HCC-9204 cells. Some condensed and rounded cells existed and demonstrated intense staining which widely distributed in the nuclei and cytoplasm of HCC-9204 cells. The *Bak* ectopic expression (translocated into the nuclei) in HCC-9204 cells was specific towards cells with marked condensation of chromatin and cytoplasm (apoptotic cells). Western blotting analysis demonstrated *Bak* expression at 0, 4, 24, 48, and 72 h after addition of $ZnSO_4$. Mock and pMD-neo vector transfected cells, as control, contained no *Bak* levels in presence of 100 μmol/L $ZnSO_4$. However, 4 h after addition of $ZnSO_4$, *Bak* expression increased, with further increasing till it reached a steady level by 24 h as determined by Western blotting analysis (Fig 3).

**Overexpression of *Bak* stably induced apoptosis in HCC-9204 cells** *Bak* transfected cells were stably established after G418 selection. Western blotting showed that *Bak* was overexpressed after induction of

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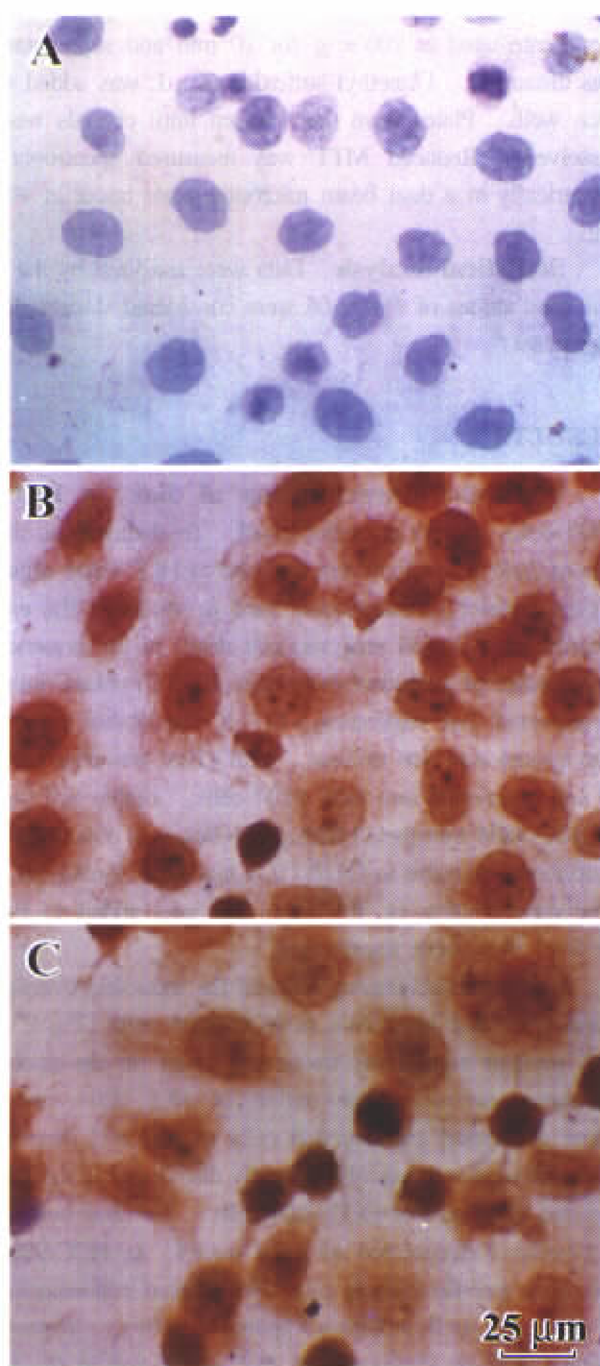


**Fig 2. The inducible overexpression of *Bak* before (A) or 4 h (B), 24 h (C) after addition of $ZnSO_4$ in HCC-9204 cells. (Original magnification × 400).**

$ZnSO_4$ in a time-dependent manner. *Bak* transfected cells displayed morphological characteristic of apoptotic cell death as early as 24 h after induction. Cells became rounded and shrunken, with blebbing of the cytoplasmic membrane asobserved under Nikon Eclipse TE200 inverted microscope (Fig 4). In contrast, mock- and null-transfected cells did not exhibit any of these changes.

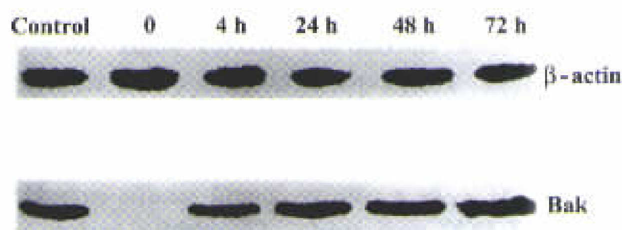


**Fig 3. Induction of Bak expression by $ZnSO_4$ in HCC-9204 cells. Western blotting analysis of Bak expression in (−)$ZnSO_4$(0 h) or (+)$ZnSO_4$ for 4, 24, 48, and 72 h. The level of β-actin was used as a control for equal loading of protein.**

To gain further evidence that the cell death induced by overexpression of *Bak* was apoptotic, nuclei of transfected cells were stained with apoptosis specific detection assay, such as TUNEL.

Compared to the untransfected HCC-9204 cells, *Bak* transfected cells showed obvious morphological changes. Some apoptotic cells appeared rounded with condensed cytoplasm and highly fragmented chromatin. Others showed typical crescent-shaped chromatin margination, nuclear fragmentation and formation of numerous micronuclei (Fig 5). In addition, compared to the untransfected HCC-9204 cells, apoptotic index significantly increased in *Bak* transfected HCC-9204 cells 24 h after addition of $ZnSO_4$ 100 μmol/L (Fig 6). *Bak* was highly enriched in apoptotic cells, greatly facilitating the morphometry in a time-dependent mode. Since the increase in apoptotic cells correlated with an increased ectopic expression of *Bak*, this ectopic expression of *Bak* induced susceptibility of HCC-9204 cells to cell death significantly.

**Effect of overexpression of *Bak* on cell cycle**

In order to investigate whether *Bak* may affect cell growth or not, FACS analysis was performed on the cell cycle distribution of HCC-9204 cells 0 h, 4 h, and 24 h after addition of $ZnSO_4$. Overexpression of *Bak* showed significantly higher apoptotic peak than normal control as indicated by the "sub-$G_1$" peak, confirming that overexpression of *Bak* sensitized HCC-9204 cells to apoptosis. HCC-9204/*Bak* showed proportionally more cells in $G_1$, fewer cells in S and about the same percentage in $G_2$/M 22.65 % when compared with parental HCC-9204 and HCC-9204/pMD-neo control cells (Tab 1). Furthermore, electronic microscopy also confirmed that overex-

pression of *Bak* induced apoptotic cell death in HCC-9204 cell line as shown in Fig 7.

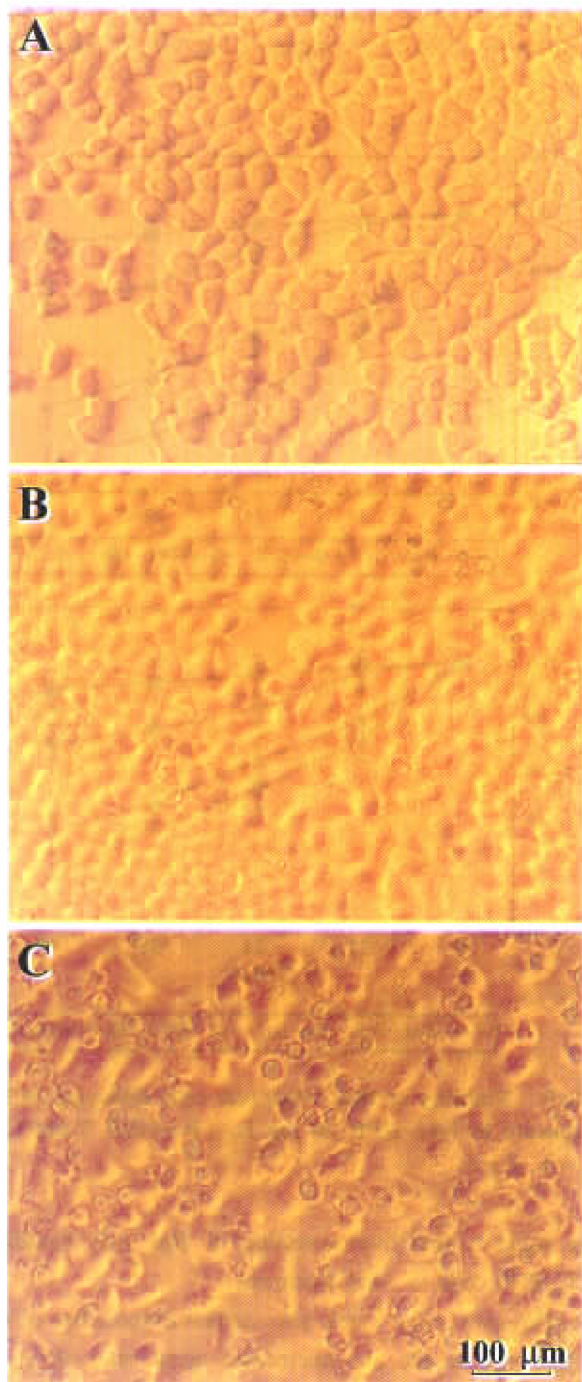


**Fig 4. Morphological changes in HCC-9204/*Bak* cells 0 (A), 24 h (B), and 48 h (C) after addition of $ZnSO_4$ as observed by inverted microscopy. (Original magnification ×100). Cells exhibited typical apoptotic phenotype including cell shrinkage, dramatic convolution, blebbing, and condensed nuclei.**

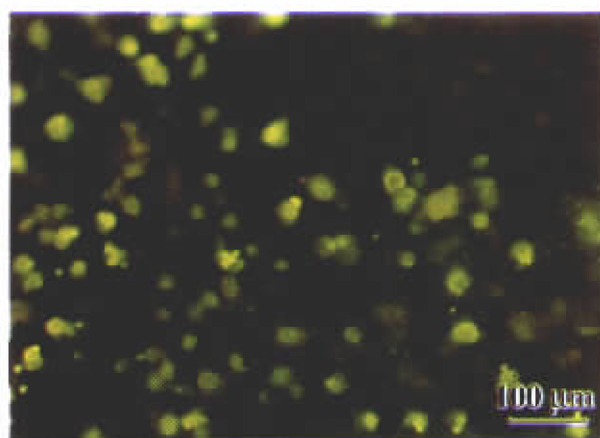


**Fig 5. TUNEL assay demonstrating marked morphological changes in HCC-9204/*Bak* 24 h after addition of zinc. (Original magnification ×100).**

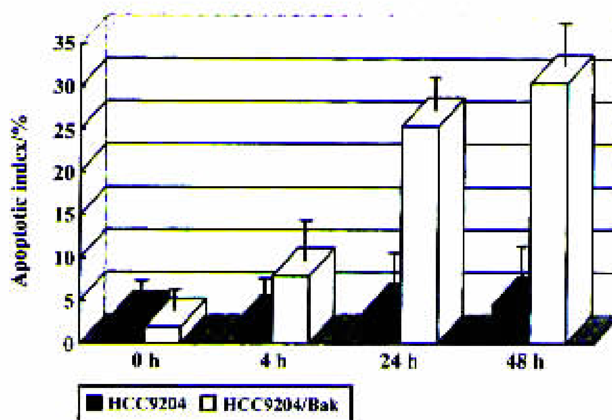


**Fig 6. Induction of apoptosis in HCC-9204/*Bak* cells after addition of zinc. $n=4$, $\bar{x}\pm s$.**

**Tab 1. Effects of *Bak* overexpression on cell cycle in HCC-9204 cells. $^bP<0.05$ indicates significant differentiation by the Fisher's test.**

| Population | $ZnSO_4^-$ | $ZnSO_4^+$ 4 h | $ZnSO_4^+$ 24 h |
|---|---|---|---|
| Pre $G_1$ | 0 % | 9.60 % | 19.29 %$^b$ |
| $G_1$ | 35.21 % | 52.72 % | 76.26 %$^b$ |
| S | 40.32 % | 21.62 % | 1.09 % |
| $G_2$/M | 24.47 % | 25.65 % | 22.65 % |

***Bak* mediated decrease in clonogenic survival** The survival of HCC-9204 cells was tested by clonogenic assay following 24-h exposures to adriamycin and vinorelbine. The data presented in Fig 8 clearly demonstrated that *Bak* overexpression caused decrease in clonogenic survival following exposure to adriamycin, but

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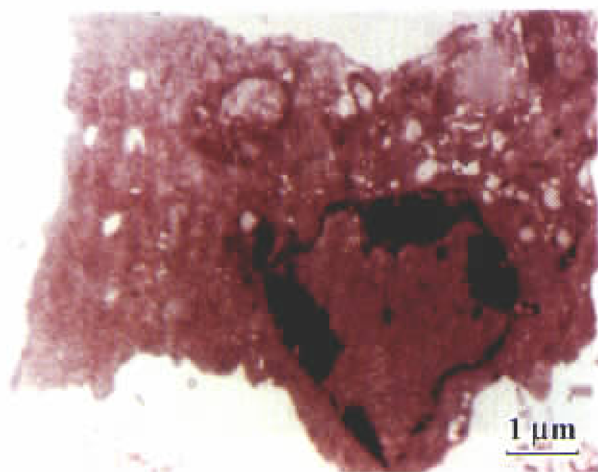


**Fig 7. Ultrastructural changes associated with apoptosis in *Bak* sensitized HCC-9204 cells. × 10 000.**

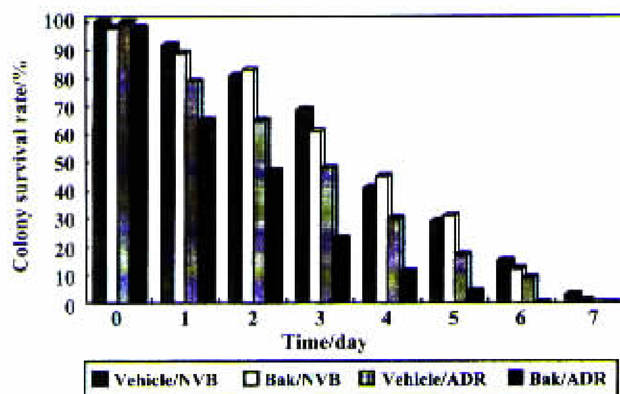


**Fig 8. Increased colony growth inhibition by inducible overexpression of *Bak* before treatment with ADR or NVB.**

not vinorelbine. Clonogenic survival rate was decreased from 50 % (HCC-9204/vehicle) to 23 % (HCC-9204/*Bak*) 3 d after ADR withdrawal ($P<0.01$). However, clonogenic survival rate had no constant significant variance between HCC-9204/vehicle group and HCC-9204/*Bak* group after exposure to NVB ($P>0.01$).

**Effect of *Bak* on cell viability** Owing to the ability of *Bak* to decrease clonogenic survival in an agent-specific manner in HCC-9204 cells, experiments were performed to determine the effects of ADR or NVB on cell viability by MTT assay. *Bak*-transfected HCC-9204 cells were compared to nontransfected HCC-9204 cells 48 h after exposure to a single optimum concentration of ADR (20 mg/L) or NVB (5 mg/L). Fig 9A indicates that the effects of *Bak* on cell viability following ADR exposure may be significant as compared to the vehicle-transfected HCC-9204 cells ($P<0.01$). Cell viability rate decreased from 57.2 % to 35.7 % at 24 h when withdrawal of ADR ($P<0.01$). Also at 24 h the decrease in cell viability rate was higher in *Bak*/ADR (59.9 %) group as compared to *Bak* (8.0 %) or ADR treatment (38.4 %) alone (Fig 9). Thus, the above

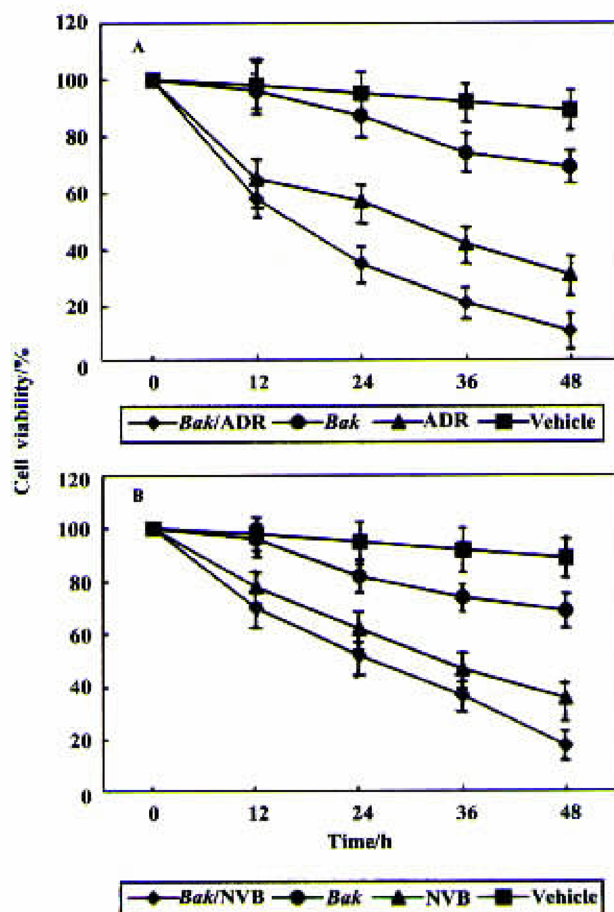


**Fig 9. Viability of HCC-9204/*Bak* cells following 24 h exposure to (A) ADR 20 mg/L, (B) NVB 5 mg/L. $n=3$. $\bar{x}\pm s$.**

data suggests that overexpression of *Bak* may sensitize HCC-9240 cells to ADR treatment.

However, *Bak* levels also seemed to modulate cellular response to the anti-microtuble agent vinorelbine as shown in Fig 10 ($P<0.01$), which was seemingly contradictory to the results of colony formation assay.

## DISCUSSION

Recent advances towards understanding the mode of the diverse nature of anticancer drugs, most of them have indicated that regardless of the diverse nature of anticancer

drugs, most drugs elicit apoptosis in the target cells. Many toxic stimuli have been shown to induce apoptosis, even at doses or concentrations insufficient to cause a general metabolic dysfunction. The ability of tumor cell to detect cellular damage and activate the apoptotic response may determine the ultimate success of cancer chemotherapy. Since apoptosis plays a major role in cell death induced by DNA-damaging agents, Brown has provided a hypothesis that cancer cells when treated with chemotherapeutic drugs die of apoptosis, and the cells resistant to apoptosis are resistant to anticancer therapy[8].

During the past decade, interest of basic scientists and clinicians in the influence of apoptosis on the sensitivity of tumors to anticancer treatment has been evoked. Apoptosis is a defined program of cell death that is markedly influenced both positively and negatively by a variety of genes, many of which are mutated and/or dysfunctionally regulated in human cancers. Among the most important of these are the tumor suppressor gene p53 and members of the *bcl*-2 gene family. Previous studies have suggested that tumors with mutations in p53, high levels of *bcl*-2, or high ratios of *bcl*-2: bax should be resistant to cancer treatment[9]. Introduction of functional p53 into CEM cells enhances their sensitivity to the DNA-damaging agent doxorubicin, but not to the tubulin-active compound vincristine. Thus, mutational p53 inactivation in acute lymphoblastic leukemia might entail relative resistance to DNA-damaging, but not to tubulin-destabilizing, chemotherapy[10].

Earlier work showed that p53 might induce apoptosis through direct activation of killer genes, such as *bax*, or down-regulate survival genes, such as *bcl*-2 in some cell types. In addition, $G_1$ arrest and apoptosis appeared to be an alternative of p53-induced outcomes. Because mutation in the p53 gene is one of the most frequent genetic alterations in human hepatocellular carcinoma, it is possible that in malignant liver cells apoptosis caused by DNA damage is p53-independent. Therefore, those cells where p53 gene was mutated and/or dysfunctionally regulated, other gene families would be responsible for the regulation of apoptosis and sensitivity of these tumor cells to chemotherapeutic agents. The *bcl*-2 family proteins appear to regulate the commitment to survive or die on challenge with various apoptotic stimuli by controlling the flux of ions and proteins through intercellular membranes. Pro-apoptotic members of the family include Bax, *Bak* and *bcl*-xS whereas anti-apoptotic members include *bcl*-2, *bcl*-xL, and *bcl*-w. Thus, investigation of the expression and regulation of the *bcl*-2 family proteins may provide insight to the mechanisms of susceptibility of HCC to apoptosis induced by chemotherapeutic agents[11]. Since p53 was mutated in HCC-9204 cells, we presumed that inducible overexpression of *Bak* might be leading to apoptosis. This study observes the sole contribution of *Bak* mediated drug resistance in human hepatocellular carcinoma. Our study has established that cells overexpressing *Bak* might be resistant to vinorelbine during induction of apoptosis. Elevated *Bak* expression induced by exogenous transfection can be considered parallel to induction of cell cycle elongation in $G_1$ phase. Since Navelbine blocks mitosis in phase $G_2$/M and induces cell death at interphase or at the following mitosis, HCC-9204 cells were initially made to undergo cell cycle elongation in $G_1$ phase through inducible overexpression of *Bak* in our experiments, and loss of sensitivity to NVB might be exerting its effect in the mitotic phase. Therefore, we were not able to observe the exact increase in susceptibility of HCC-9204/*Bak* cells to NVB.

In the present investigation, however, the results of MTT assay (short-term assay) contradict with the results from the colony-forming assay. Dead cells can be readily identified and quantitated since they have many distinct morphological features, lose their metabolic functions, and fail to exclude dyes such as propidium iodide and trypan blue. However, cells do not die immediately after treatment as this highly depends upon the cell type and the toxic agent being investigated, and this can lead to an underestimation of the overall level of dead cells. Despite this, many investigators assess cellular sensitivity to genotoxic agents by total population staining MTT or trypan blue exclusion was in short time period (1 – 4 d) after treatment[12].

Colony forming assay tests every cell in the population for its ability to undergo unlimited division hence this assay is widely used for assessing the response to cytotoxic agents. The ability of cells to undergo unlimited proliferation has become the "gold standard" for assessment of cellular sensitivity to cytotoxic treatments[13].

Since the colony forming assay is more reliable than MTT or trypan blue exclusion, we can conclude that *Bak* selectively sensitizes HCC-9204 cells to cell death induced by adriamycin while resists cell death induced by vinorelbine.

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## 可诱导型 *Bak* 基因过度表达对多柔比星诱导 HCC-9204 细胞凋亡的增敏作用

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**关键词**　多柔比星；长春瑞滨；*Bak* 基因；细胞凋亡；肝细胞癌

**目的**：研究 *Bak* 基因过度表达在 HCC-9204 细胞凋亡途径中的作用以及对多柔比星和长春瑞滨的可能的增敏作用． **方法**：采用 MT-II 可调控性表达载体系统，通过外加 $ZnSO_4$(100 μmol/L)诱导 *Bak* 基因表达，并获得稳定转染子． 以形态学标准并结合 TUNEL 或流式细胞仪检测细胞凋亡． 克隆形成实验反映克隆细胞存活率，MTT 法检测细胞活力． **结果**：*Bak* 基因过度表达的细胞出现显著的细胞死亡，TUNEL 证实为一种凋亡性细胞死亡． 流式细胞仪的结果显示 *Bak* 能够显著地诱导细胞在 $G_1$ 期聚积并在阿霉素诱导后 24 h 19.26 %的细胞发生凋亡． *Bak* 基因过度表达只能显著降低阿霉素处理组的克隆存活率，而对长春花碱组没有显著效果． MTT 实验的结果类似，提示 *Bak* 基因能够选择性对阿霉素诱导的细胞死亡具有增敏作用，而对长春花碱没有这种作用． **结论**：*Bak* 基因的过度表达使 HCC-9204 细胞的细胞周期在 $G_1$ 延长，导致细胞凋亡并选择性对化疗药物具有增敏作用．

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