*Full Length Research Paper*

# Co-expression of apoptin (VP3) and antibacterial peptide cecropin B mutant (*ABPS1*) genes induce higher rate of apoptosis in HepG2 and A375 cell lines

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The antibacterial peptide cecropin B mutant (*ABPS1*) gene has a broad range of antibacterial and antiproliferative properties. Apoptin (VP3), a chicken anaemia virus-encoded protein is known to induce apoptosis in human transformed cells. To explore drug combination in human tumor cells, apoptin and ABPS1 eukaryotic expression vector pIRES2-EGFP-apoptin and pIRES2-EGFP-ABPS1 were constructed and their expression effect individually and in combinations were studied in HepG2 and A375 cells. The vector pIRES2-EGFP-ABPS1 and pIRES2-EGFP-apoptin were transfected into tumor cells HepG2 and A375 by the lipofectamine-mediated DNA transfection procedure. At 48 h post transfection, the apoptotic rate obtained by flow cytometry and the morphological changes under light and scanning electron microscope of tumor cells were significant. In contrast, the microvilli on the surface of the control cells were disrupted, decreased and even disappeared. The cell membrane was injured and intracellular substances leaked out. Furthermore, our results indicate that the apoptotic rates of apoptin (27.32% in HepG2 and 9.34% in A375 cells), were higher than ABPS1 (23.79% in HepG2 and 8.33% in A375 cells). Moreover, the co-expression of Apoptin and ABPS1 showed higher apoptotic rates which were 27.66 and 10.33% in HepG2 and A375 cells respectively. However, the apoptotic rates obtained in HepG2 cells treated with apoptin and apoptin and ABPS1 together were closely similar, but, not in A375 cells. Therefore, the results of the present study showed that the combination of Apoptin and ABPS1 has synergistic effect in HepG2 and A375 cell lines.

**Keys words:** Apoptin, ABPS1, apoptosis, co-expression, HepG2, A375.

## INTRODUCTION

Apoptosis is primarily a physiological process necessary to remove individual cells that are no longer needed or that function abnormally (Rohn et al., 2002). It is generally associated with changes in cellular morphology and biochemical features including DNA fragmentation, cytoplasm vacuolation, plasma membrane blebbing, and apoptotic body formation (Kerr et al., 1972; Ravinda et al., 2009). The activation of a family of aspartate-specific cysteine proteases called caspases is responsible for the morphological and nuclear changes associated with apoptosis (Nunez et al., 1998; Thornberry and Lazebnik, 1998; Clarke and Tyler, 2009). Many stimuli can trigger apoptotic cell death (Vinatier et al., 1996). Therefore, the use of genes that can induce apoptosis may be a good way to find potential anti-drugs for cancer therapy.

Chicken anaemia virus (CAV) is a Gyrovirus of the Circoviridae family which is characterized by a negative sense single stranded DNA genome of about 2.3 kb

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(Yuasa et al., 1979; Noteborn et al., 1991; Todd, 2000; Ducatez et al., 2006; Lee et al., 2009). Apoptin or VP3 is one of the three open reading frames (ORFs) present in this ssDNA genome (McNulty, 1991; Rosenberger and Cloud, 1998; Dhama et al., 2002; Natesan et al., 2006a). It encodes a 13 kDa and is known to induce apoptosis in chicken or human tumor cell lines (Noteborn et al., 1994; Murphy, 1999; Noteborn, 2004; Nogueira-Dantas et al., 2006). However, Apoptin is localized in their nucleus, as opposed to the cytoplasm of non-transformed cells (Heilman et al., 2006). Therefore, non-transformed, normal human primary cell types are not killed (Danen-van et al., 1997; Leliveld et al., 2003). These properties make it as a novel and promising candidate anticancer therapeutic.

Recently, drug combination has gained considerable attention because of their beneficial effects in overcoming intrinsic tumor cell resistance to apoptosis. Therefore, finding new combination of treatment strategies for synergistic therapeutic effects is desirable. Antibacterial peptides (ABPs) are small peptides that are produced by many tissues and cell types in a variety of invertebrate, plant and animal species (Brogden, 2005). Cecropin B, which belongs to the cecropin family of ABPs, was originally isolated from immune cecropin haemolymph from the giant silk moth *Hyalophora cecropina* (Hofsten et al., 1985; Xu et al., 2007). Antitumor activities of the ABPs have been reported, but the antitumor mechanisms are still unclear.

It is thought that the peptide acts by causing transmembrane pore-forming mechanisms such as toroidal pore; carpet and Barrel stave (Christensen et al., 1988; Jaynes et al., 1989; Arrowood et al., 1991; Brogden, 2005; Bishop and Finlay, 2006). Here, we used a cecropin B mutant (ABPS1) reported by Yu et al. (2010).

The aim of the present research was to study the apoptosis-inducing activity level of apoptin and *ABPS1*, the two-gene combination in human hepatocellular carcinoma HepG2 and human melanoma A375 cell lines.

## MATERIALS AND METHODS

### Cell culture

The human hepatoma HepG2 and human melanoma A375 cell lines were provided by Beijing Cancer Hospital (China). HepG2 and A375 cells were cultured with Dulbecco's modified eagle medium (DMEM) (Invitrogen Life Technologies, US) and incubated with 5% $CO_2$ at 37°C. The medium were supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin, and 100 mg/ml streptomycin.

### ABPS1 and VP3 insert DNA amplification

The primers were designed by using Primer Premier 5.0 software and provided from Sangon Biotechnology (China). The restriction enzyme sites, EcoR1 and BamH1 (New England BioLabs, Massachusetts, US) were added in the forward and reverse primers

respectively. PCR amplification of ABPS1 from our laboratory and apoptin from Changchun Military Medical Institute (China) were carried out by using the primers set *abps1*-F (5'-atggttaaccgctggcgcctgttccg-3') and *abps1*-R (5'-ctagttaacgcccacggcgcgagcg-3'), *vp3*-F (5'-atgaacgctctccaagaagatactcca-3') and *vp3*-R (5'-tcacagtcttatacgccttttttgcg-3'). The expected sizes of the amplification products were 126 and 372 bp respectively. The reaction mixture was prepared in PCR buffer containing 15 mM $MgCl_2$, 200 mM each of Tris-HCl (pH 8.4) and KCl, 100 mM $(NH_4)_2SO_4$, dNTP (2.5 mM) and 10 µM each primer. Taq polymerase (2.5 unit; TianGen Biotech, China) and 1 µg DNA of apoptin and ABPS1 were added to a total reaction volume of 50 µl. The reaction was carried out in an automated thermal cycler (Bio-RAD) with an initial denaturation for 3 min followed by 30 cycles of denaturation at 94°C for 30 s, specific annealing temperatures for 30 s and extension at 72°C for 1 min and the final extension was carried out at 72°C for 5 min. The PCR-amplified products were purified from the gel by using EasyPure Quick gel-extraction kit (TransGen Biotech, China) and were used for cloning in the pIRES2-EGFP (Takara Biomedical/Clontech, Japan).

### Cloning of the apoptin (VP3) and ABPS1

The vector pIRES2-EGFP and the PCR restriction digested ABPS1 and VP3 were set up by preparing three samples consisting of 14 µl of sterile ddH$_2$O, 2 µl of 10X buffer T4, 1 µl of EcoR1, 1 µl of BamH1, and 2 µl of vector; 6 µl of sterile ddH$_2$O, 2 µl of 10X buffer T4, 1 µl of EcoR1, 1 µl of BamH1, and 10 µl of insert apoptin and ABPS1. The samples were then placed in a 37°C water bath for 2 to 3 h. The digested insert and vector DNA from each sample was then isolated utilizing a 1 to 2% agarose gel electrophoresis. The gel was assembled using 1X Tris base, acetic acid, and EDTA (TAE) and ethidium bromide. The plasmid and the genes of insert Apoptin and ABPS1 were purified in accordance with the TransGen purification kit. The insert DNA were then inserted into the pIRES2-EGFP via ligation reaction. The 1:3 reaction consisted of combining 1 µl of pIRES2-EGFP, 3 µl of insert (apoptin or ABPS1), 1 µl buffer T4, 4 µl ddH$_2$O, and 1 µl T4 ligase (New England BioLabs, Massachusetts, US) (Figure 1). These samples were then incubated for 1 h at 16°C. Post incubation of the two samples were transformed into a volume of 100 µl of *Escherichia coli*. Samples were incubated on ice for 30 min. Samples were then heat in a 42°C water bath for 2 min. The samples were then transferred back to the ice for 5 min. A volume of 800 µl of Luria-Bertani (LB) media was added to each sample and incubated at 37°C for 1 h. A volume of 200 µl of each sample was plated onto the appropriate kanamycin plates. The plates were then incubated at 37°C overnight. The next day the colonies were inoculated in a volume of 10 ml of LB. A volume of 10 µl of kanamycin was added to the media. Samples were incubated for 24 h at 37°C in a shaking incubator 220 rpm. The plasmids were purified in accordance with the EasyPure plasmid mini-prep kit (TransGen Biotech, China). The purified plasmids were used for *in vitro* expression studies.

### *In vitro* expression studies

HepG2 and A375 cells showing 50% monolayer at 24 h in a six-well culture plate were used for expression study of the pIRES2-EGFP-apoptin, pIRES2-EGFP-ABPS1 plasmids according to the methods reported by Natesan et al. (2006b). The monolayer cells were transfected using Lipofectamine reagent (Invitrogen). 2 µl of plasmid for the individual transfection and 1:1 volume of plasmid for the co-transfection were mixed with OptiMEM medium (Invitrogen Life Technologies, USA) in a total volume of 100 µl, in a 1.5 ml sterile microcentrifuge tube (tube 1) and were allowed to stand at
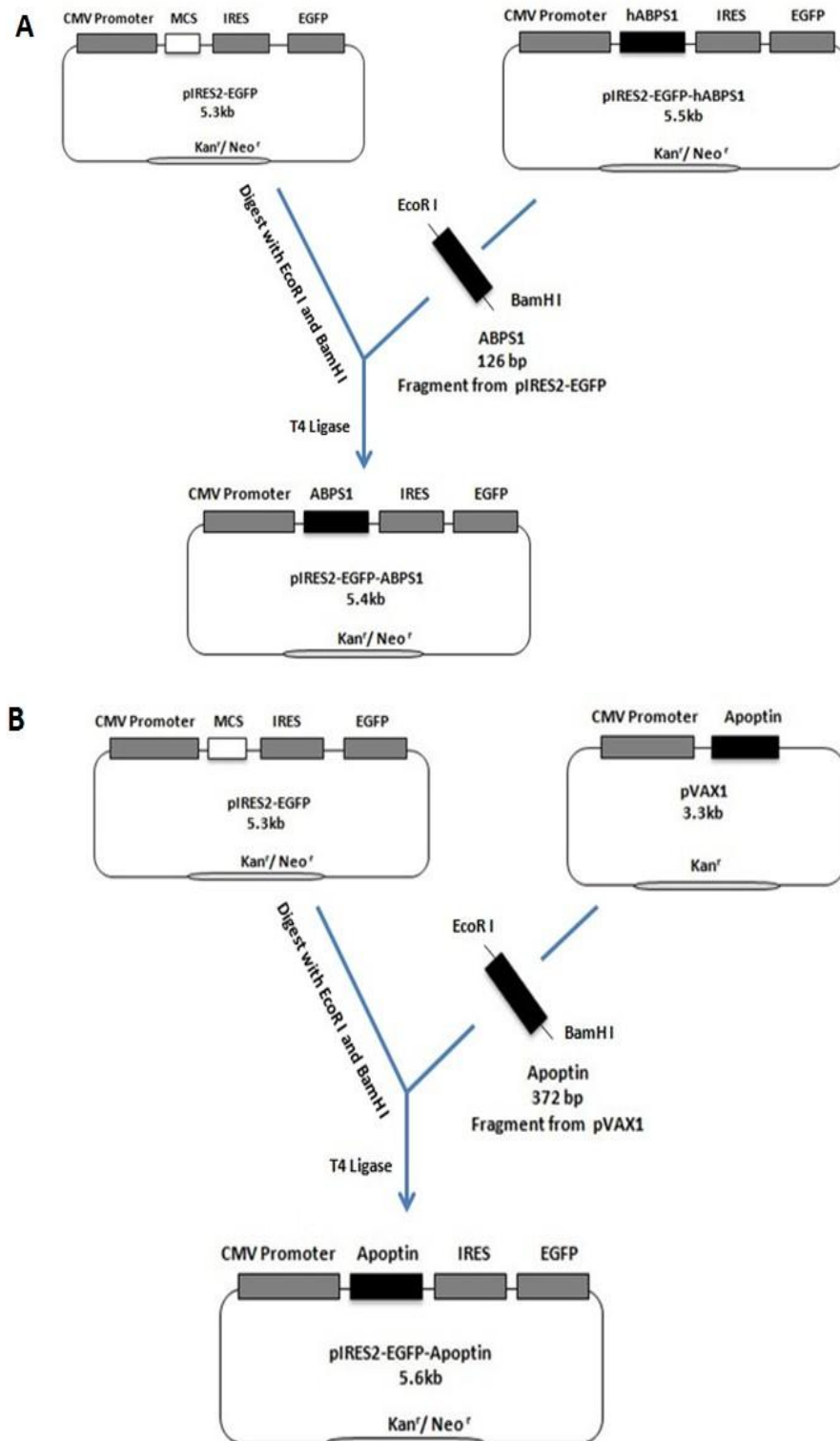
**Figure 1.** Schematic of plasmids construction; A) pIRES2-EGFP-ABPS1, B) pIRES2-EGFP-apoptin.

room temperature for 10 min. Simultaneously, 6 µl Lipofectamine was mixed with OptiMEM medium in a total volume of 100 µl in tube 2 and kept at room temperature for 10 min. The contents of tubes 1 and 2 were then mixed together and kept at room temperature for 30 min. The monolayer in the six-well plate was washed with sterile phosphate buffered saline (PBS) and 2 ml growth medium

containing 5% FBS was added. Two hundred microlitres of the mixture OptiMEM medium/Lipofectamine/DNA was added drop wise to the well and mixed. The cell monolayer was incubated at 37°C with 5% $CO_2$. After 4 h incubation, the medium over the cells was removed and fresh medium with FBS was added.

## Scanning electron microscope analysis

The transfected and non-transfected cells were cultured in six wells plate containing coverslip. At 48 h post-transfection, cells were fixed in 2.5% glutaraldehyde and 2% paraformaldehyde (PFA) made in 0.1 M sodium phosphate buffer (pH 7.4) for 6 h at 4°C. Post-fixed in phosphate buffered 1% osmium tetroxide for 2 h at 4°C, cells were subsequently washed in PBS destained with Isoamyl acetate. Specimens were then dried using a Hitachi HCP-2 and ion sputtered by Eiko IB-3, and examined using the scanning electron microscope (SEM) (Hitachi S-3400N, Japan).

## Flow cytometry analysis

HepG2 and A375 cells were harvested at 48 h post-transfection by 0.25% trypsin release, centrifuged at 2000 rpm for 3 min and the pellet was collected and 300 μl PBS added. Cells were then stained with 15 μl propidium iodide (PI) for 10 min at room temperature. Samples were processed and screened using FACSCalibur and data were analyzed with CellQuest Pro software (BD Biosciences, US).

## RESULTS

### Lipofection and effect of delivered genes on HepG2 and A375 cells

Figure 2A shows the transfection efficiency using green fluorescence protein (GFP) vector (pIRES2-egfp) on HepG2 and A375 cells the same concentration of lipofectamine with fixed concentration of plasmids, which were selected by GFP and PI staining. According to the higher GFP activity and the flow cytometry rate, 1.2 and 0.8 μg/μl of pIRES2-EGFP-ABPS1 and the pIRES2-EGFP-Apoptin were used, respectively. The *in vitro* expression effect of the plasmids was observed by light microscopy (Figure 2B). The co-expression effect of pIRES2-EGFP-ABPS1 and the pIRES2-EGFP-VP3 in HepG2 and A375 cells showed less viable cell lines compared with ABPS1 and Apoptin individually and with the control. The study reveals that ABPS1 and apoptin in combination had an effect on cell undergoing apoptosis as confirmed by the flow cytometry and scanning electron microscopy assays.

### Scanning electron microscope analysis

To observe the morphological changes on HepG2 and A375 cells after treatment with ABPS1 and apoptin, the scanning electron microscope was used at 48 h post-transfection (Figure 3). The apoptotic cells were identified based on morphological features including the plasma membrane blebbling, pores and microvilli. The pores and membrane blebbling were significant in HepG2 and A375 (Figure 3). The plasma membrane microvilli decreased in cells transfected with apoptin and ABPS1 as compared with the control (cell). The results show a synergistic effect of the two- gene combination.

### Detection of apoptosis by flow cytometry

To investigate the possible synergistic effect of apoptin and ABPS1, and the induction of apoptotic cell death, PI (FL2-H) staining (detected by flow cytometry) was used as criteria to distinguish between the apoptotic cells (Figure 4). A375 and HepG2 cells were evaluated by flow cytometry 48 h post treatment with VP3 and ABPS1 each alone and in combination. All data were analyzed by specific software, CellQuest Pro software (BD Biosciences, US). The rate of apoptosis was obtained after 48 h post treatment. Control cells rates treated with PI were not significant. As shown in Figure 3, apoptotic rates were 23.79 and 8.33% in HepG2 and A375 cells, respectively after treatment with ABPS1. Apoptotic rates were 27.32 and 9.34% in HepG2 and A375 cells respectively after treatment with apoptin, and 27.66 and 10.33% in HepG2 and A375 cells respectively after ABPS1-apoptin treatment.

## DISCUSSION

This study was undertaken to characterize the effect of apoptin and ABPS1 individually and in combination in inducing apoptosis *in vitro* in HepG2 and A375 cells. We cloned the apoptin and ABPS1 in the pIRES2-EGFP expression vector and the recombinant plasmids pIRES2-EGFP-Apoptin and pIRES2-EGFP-ABPS1 were used. Thereafter, we attempted to determine whether the co-expression of ABPS1 and apoptin induce a synergistic apoptotic effect in HepG2 and A375 cells. For this purpose, the co-expression effect of ABPS1 and apoptin was examined by using light microscopy, flow cytometry and scanning electron microscopy. In the cells, expression of apoptin and ABPS1 could be seen at 48 h post-transfection. Under light microscopy, cells at different stages of apoptosis with an opaque zone in the centre surrounded by healthy cells with normal nuclei were observed. These morphological changes of apoptosis have been typically observed in cells treated with apoptin alone and in combination with ABPS1 (Figures 2b, c, d, g and h). However, apoptin is known to be localized in the nuclear of transformed cells. Similar characteristic have been reported by using the same technique (Vasconcelos and Lam, 1994; Lam and Vasconcelos, 1994; Natesan et al., 2006b). From these observations, we concluded that ABPS1 may not be involved in the morphological changes occurring in the nucleus. Subsequently, the scanning electron microscopy
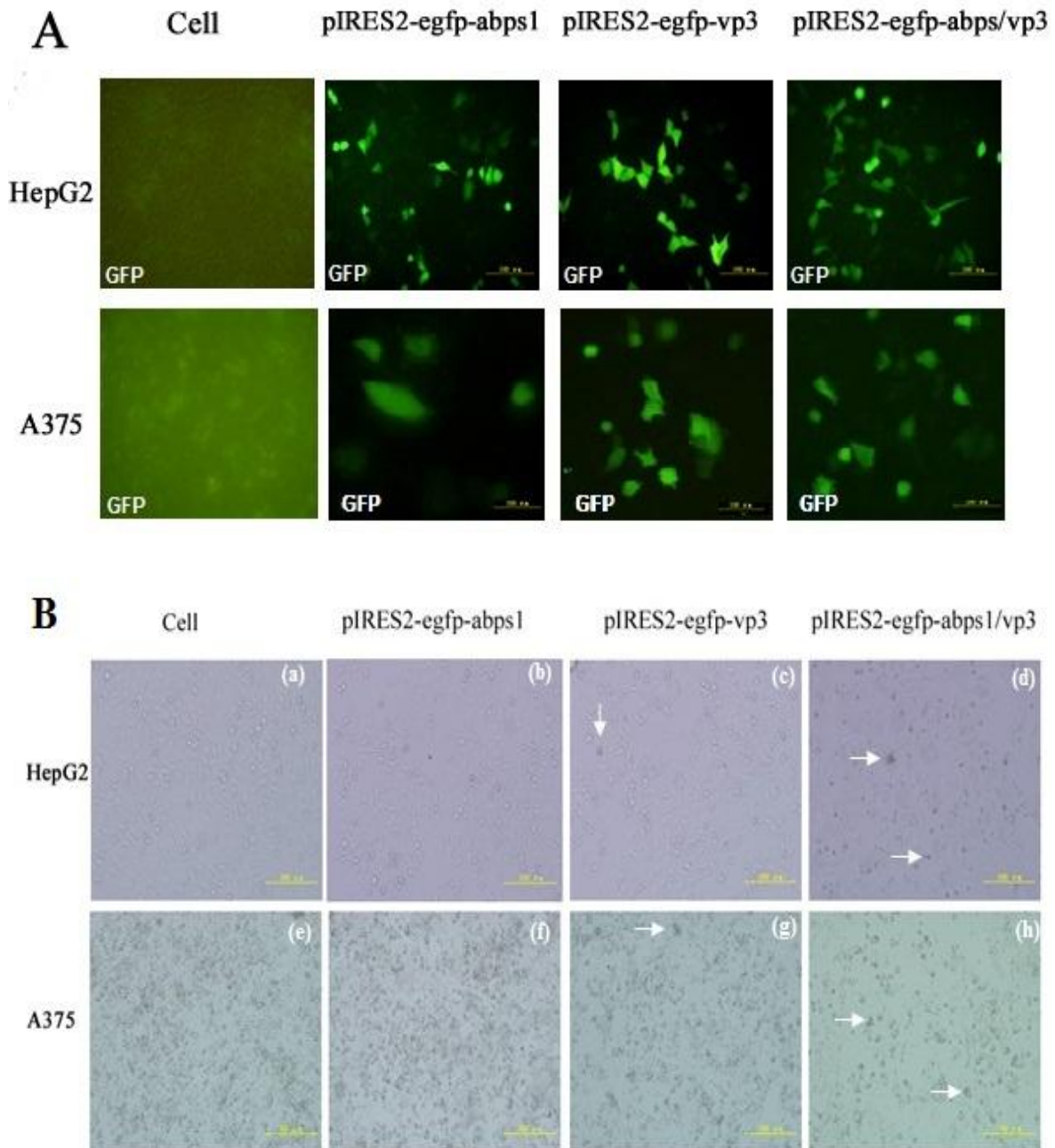
**Figure 2.** Expression of ABPS1 and apoptin individually and in combination in HepG2 and A375, showing the efficiency of the transfection by fluorescence microscopy at 24 h post-transfection with pIRES2-egfp-ABPS1, pIRES2-egfp-VP3, pIRES2-egfp-ABPS1/pIRES2-egfp-VP3, and empty vector or control (cell) (A). The effects of ABPS1 and VP3 individually and in combination were analyzed by light microscope at 48 h post-transfection with pIRES2-egfp-ABPS1, pIRES2-egfp-VP3, pIRES2-egfp-ABPS1/pIRES2-egfp-VP3 and empty vector or control (cell) (B).

results showed plasma membrane pores on cells transfected with ABPS1 (Figures 2B and F), when compared with control and Apoptin. In previous studies, Brogden (2005) demonstrated that the hydrophobic peptide region of ABPs aligns with the lipid core region of bacterial membrane and that the hydrophilic peptide region forms the interior region of the pore but the mechanism of ABPs in eukaryotic cells is not yet elucidated. However, we found that ABPS1 created membrane pores in HepG2 and A375 tumor cell lines and its combination with apoptin increased the morphological changes of apoptosis (Figures 3D and H). Otherwise,
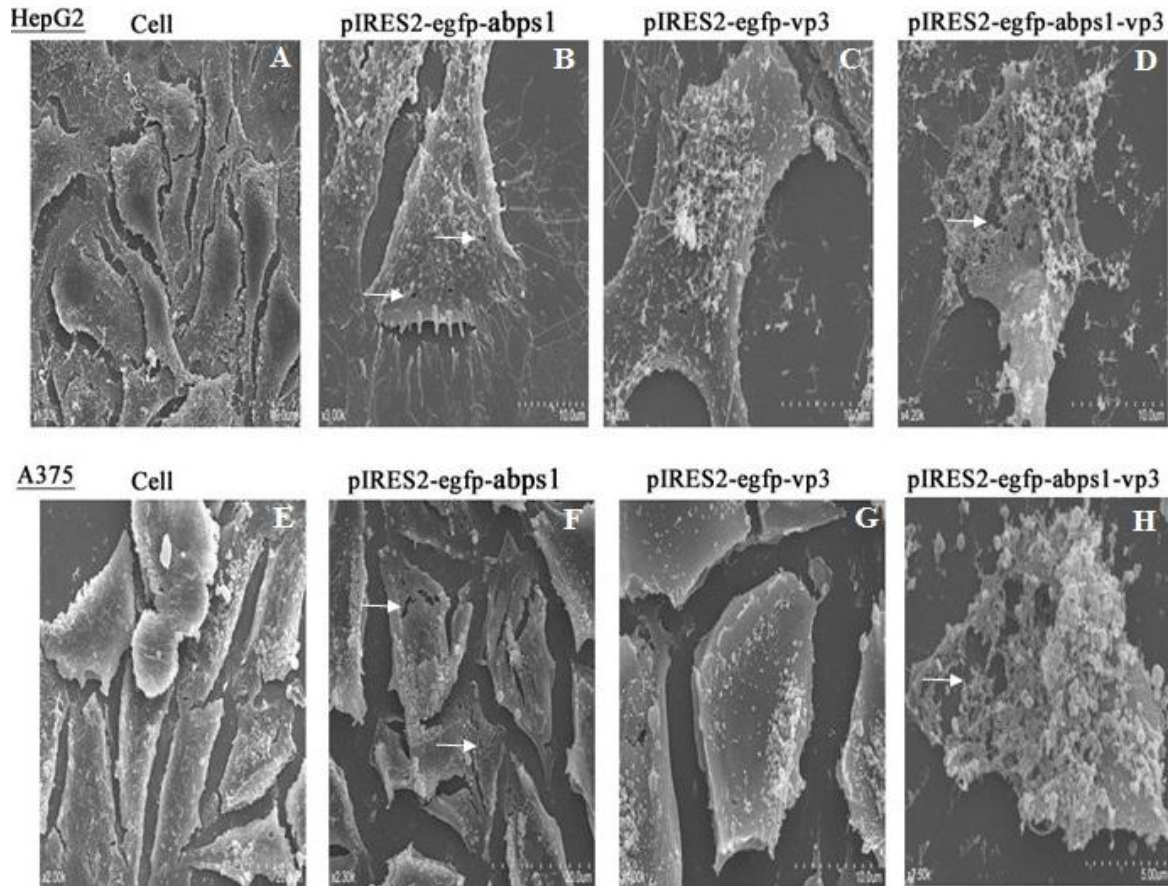
**Figure 3.** Scanning electron microscope analyses of apoptosis in A375 and HepG2 cells. Cells transfected with ABPs1, apoptin and the two-gene combination or the control vector, were harvested, fixed and subsequently washed in PBS as described in materials and methods. After dehydration with ethanol, cells were stained with Isoamyl acetate. The pictures were taken 48 h post-transfection.

cells treated with apoptin showed a decrease of membrane surface microvilli (Figure 3C and G). Furthermore, apoptin is well known to induce G2/M arrest and apoptosis of tumor cells (Heilman et al., 2006). Therefore, a stop of cell division involves a loss of activity of microvilli. We found that the plasma membrane changes induced by apoptin were not immediate compared with the ABPS1 and the reason could be its nuclear-localization (Danen-van et al., 2003). Furthermore, the morphological changes of apoptosis observed under light microscopy and scanning electron microscopy in cells transfected with apoptin and ABPS1 individually were observed with the two-gene combination.

The quantitative apoptotic level determined by flow cytometry analyses showed that the two-gene in combination induced higher rate of apoptosis compared with their individual expression and the control (Figure 4). The results also reveal a difference of sensitivity between HepG2 and A375 cells to apoptin and ABPS1. The HepG2 cells were more sensitive than A375 cells.

However, the anti-tumor activity depends on the efficient delivery of apoptin or ABPS1 and also on the importance of the target part in the cell.

In summary, the results from this analysis of co-transfection of apoptin and ABPS1 support the hypothesis that co-expression of apoptin and ABPS1 induces higher levels of apoptosis in HepG2 and A375 cell lines. Further improvements in dose and delivery method of the apoptin and ABPS1 could help to develop new anti-cancer drug.
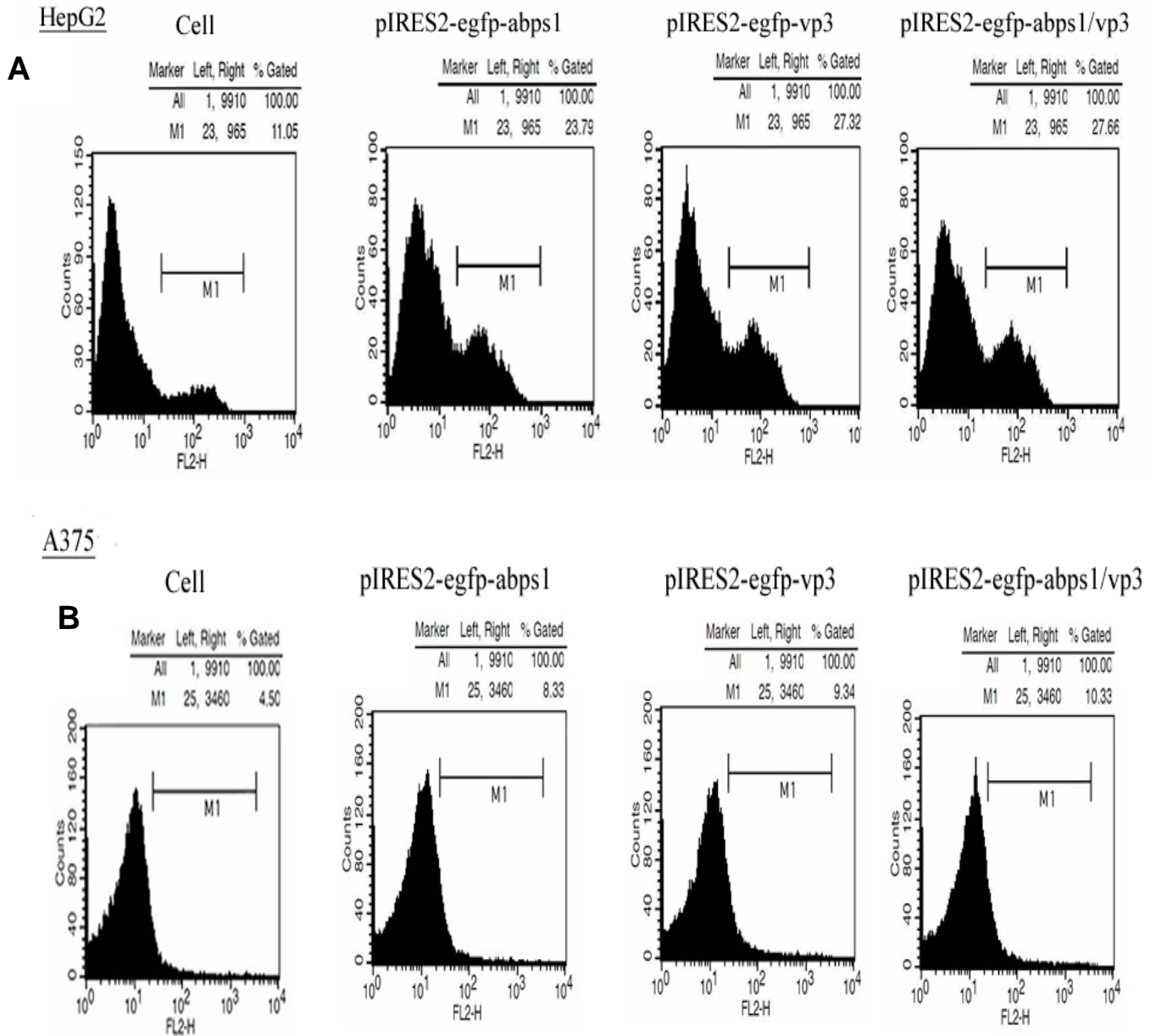
## ACKNOWLEDGEMENTS

**Figure 4.** Flow cytometry analyses of apoptosis in A) HepG2 and B) A375 cells by propidium iodide (FL2-H) labeling. HepG2 and A375 cells untreated (cell) or treated with ABPS1 and VP3 individually or in combination for 48 h, and were stained with propidium iodide. FACS analyses were then performed to quantify the apoptotic rates of cells following each treatment. Cells binding only PI were considered as necrotic.

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