[HSM02] Combinatorial gene therapy for the treatment of cancer

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Introduction

VP3 protein is 121 amino acids long, 13.6 kDa, and contains many proline, serine and theonine residues. It has a positively charged C-terminus (Noteborn *et al*., 1991). Danen-Van Oorschot (1997) reported that VP3 contains both a putative nuclear export as well as a putative nuclear localization signal. Moreover, VP3 was found to co-localize with the chromatin in morphologically non-apoptotic cells, and with the condensed DNA in apoptotic ones.

VP3 was proven to induce apoptosis in transformed and human malignant cell lines (Noteborn *et al*., 1994; Danen-van Oorschot *et al*., 1997). More excitingly, apoptosis induced by VP3 gene is p53-independent (Zhuang *et al*., 1995) and cannot be blocked by Bcl-2 (Zhuang *et al*., 1995a,b,c). The protein Bcl-2 is known as apoptosis inhibitor. However, VP3 was shown to induce apoptosis in human malignant blood cells expressing high levels of endogenous Bcl-2 due to a chromosomal translocation. In fact, Bcl-2 enhances VP3-induced apoptosis, and apoptosis process occurs much faster than in transformed blood cells with a normal level of Bcl-2 (Zhuang *et al*., 1995). In contrast, co-expression of VP3 and Bcl-2 in normal non-transformed keratinocytes did not result in cells undergoing apoptosis. Apparently, Bcl-2 does not cause VP3 in normal human cells to induce apoptosis. These results clearly show again that VP3-induced apoptosis benefits a tumorigenic background (Danen-van Oorschot et al., 1997, 1999).

VP3 protein fails to induce programmed cell death in normal lymphoid, dermal, epidermal, endothelial, and smooth muscle cells. In normal cells, VP3 protein was found predominantly in the cytoplasm. Also, VP3 protein has no toxic effect for long-term expression (Danen-Van Oorschot *et al*., 1997).

Granulocyte-monocyte colony stimulating factor (GM-CSF) induces proliferation of granulocyte and monocyte precursor cells. It increases the cytokine production of macrophages and antibody-dependent cellular cytotoxicity (ADCC). In addition, GM-CSF augments the expression of adhesion molecules, respiratory burst and the phagocytic capacity (Ulich *et al*., 1990; Metcalf *et al*., 1987).

Interleukin-12 (IL-12) has shown promising therapeutic effects against a wide variety of murine tumors resulting in tumor regression and reduction of metastases (Hendrazk and Brunda, 1996).

In contrast to its general stimulatory effects on lymphocytes, IL-4 inhibits the tumorigenicity of a variety of tumor cell lines, including those of lymphoid origin (Puri and Siegel, 1993).

Previous studies have identified abundant expression of receptors for IL-4 and IL-13 on several human tumor cell lines (Murata *et al*., 1996; Murata *et al*., 1997a; Murata *et al*., 1997b; Murata *et al*., 1998; Obiri *et al*., 1996). For instance, many human solid tumor cell lines derived from glioblastoma, renal cell carcinoma, AIDS-associated Kaposi's Sacorma, and ovarian carcinoma express high- to intermediate-affinity IL-13R on the cell surface (Debinski *et al*., 1995; Husain *et al*., 1997; Husain and Puri, 2000; Joshi *et al*., 2000; Maini *et al*., 1997). Expression of IL-13R on these cells suggested that receptor-targeted cytotoxins could be developed as an anti-tumor treatment.

Materials and Methods

Cloning, purification and expression of VP3 construct in p-TP plasmid vector

VP3 gene was sourced from recombinant pcDNA3.1/Zeo+ plasmid vector from previous researcher. A set of primers was designed to amplify full length of VP3 gene (367 bp) from recombinant pcDNA3.1 /Zeo+ via polymerase chain reaction (PCR). Forward primer used was **CAA ATG AAC GCT CTC**

CAA GAA, and the Reverse primer used was **TCA GTC TTA GCC TTT TTG C**.

VP3 PCR product was gel purified and cloned into p-TP expression vector. This recombinant plasmid vector was then being transformed into *E.coli* for multiplication. Later, recombinant plasmids were extracted using alkaline lysis method. The correct orientation of VP3 insert was analyzed via modified PCR technique (aided by T7 forward primer **TAA TAC GAC TCA CTA TAG GG** and GFP reverse primer **GGG TAA GCT TTC CGT ATG TAG C** and confirmed via DNA sequencing. Primer Combination 1 (for detection of correct orientation) comprised VP3 forward primer and GFP reverse Primer, while Combination 2 comprised T7 forward primer and VP3 reverse primer.

Transfection of recombinant p-TP was carried out in a covered 96-well plate and 2-well chamber slide cultured with 80% confluent CT26 cells. Lipofectamine was used as transfection agent *in vitro*.

After the transfection, the cells were monitored at the interval of 24-, 48-, and 72 hours under the light microscope to observe for the signs of cell death, i.e. rounding up of CT26 cells for the treatment group.

Once the sign of cell death is observed, the expression of VP3-GFP fusion protein was screened under immunofluorescence microscope. Also, transfected CT26 cell line was assayed for immunoperoxidase staining at once the sign of death was observed.

The DeadEnd® Colorimetric TUNEL system was used for the detection of apoptosis in CT26 cells transfected with recombinant p-TP plasmid vector. Transfected CT26 cell line was assayed for TUNEL system once the sign of death was observed.

Cloning and purification of Granulocyte-Monocyte Stimulating Factor (GMCSF) and Interleukin-12 (IL-12) construct in in plasmid vector

The plasmid vector used is a 4689bp circular plasmid vector, with 2 multi-cloning sites. High level expression of exogenous DNA cloned into plasmid vector can be achieved by the presence of two glucose regulated protein (GRP) promoter. The human GRP94 and hamster GRP78 promoters were induced in stress conditions prevailing inside tumours, such as glucose deprivation and hypoxia (Little *et al*., 1994).

GMCSF gene and IL-12 gene was sourced from Invivogen, USA. Two sets of primers were designed to PCR-amplify full length of GMCSF gene (584 bp) and IL-12 gene (1651 bp), with restriction sites generated, from the source templates respectively. Forward primer used for GMCSF was **GCA GCA AGA GAC CGG CGA AGG AGG GCC ACC ATG TAC,** and the Reverse primer used was **CGA ACA AAC AGC GCT GGC CTG GGC TTC CTC ATT TTT GGC.** Forward primer used for IL-12 was **GCA GCA AGA ACC ATG GGT CAA TCA CGC TAC CTC CTC,** and the reverse primer used was **CGA ACA AAC GCT AGC ATC CGT TGC ATC CTA GGA TCG**.

GMCSF and IL-12 PCR product was gel purified and cloned into plasmid expression vector, one after another. This recombinant plasmid vector was then being transformed into *E.coli* for multiplication. Later, recombinant plasmids were extracted using alkaline lysis method. The inserts were analyzed and confirmed via DNA sequencing.

Multiplication and purification of plasmid vector IL4/IL13

This recombinant plasmid vector associates the Th2 cytokine IL-4 (Herrick *et al*., 2000) with a IL-13 (Morel and Oriss, 1998), known for its positive influence on the Th2 polarization.

Under strictly sterile condition, lyophilised *E.coli* disk containing recombinant plasmid IL4/IL13 (Invivogen, USA) was multiplied in LB broth, with zeocin 25ug/ml as selective antibiotic.

Mice and tumor induction

BALB/C (6- to 8-week-old female) mice were purchased from Institute for Medical Research, Malaysia. CT26 colon carcinoma cell line used for tumour induction was obtained from ATCC (Manassas, VA). These cells were cultured in RPMI medium supplemented with 10% bovine calf serum. CT26 cells (1×10^6) were injected subcutaneously into flank of mice. Tumour volumes were calculated according to the following formula: $\frac{1}{2} \times \text{length} \times (\text{width})^2$. Statistical analysis was performed by student T-test.

Intratumor delivery of VP3 gene and/or cytokine genes

100ug of recombinant plasmid (containing VP3 or cytokine genes or both) was diluted with normal saline and was delivered intratumorally into each tumor-bearing mouse via a 1ml hypodermic syringe. As for DNA vaccine, the therapeutic gene was delivered at various sites of the tumor. The size of experimental group was 6 mice for the control and treatment group, respectively. For the control group of mice, 100 ul of normal saline was administered instead of therapeutic gene.

Results

Cloning, purification of VP3 construct in p-TP plamid vector

VP3 gene was successfully amplified from recombinant pcDNA3.1/Zeo+ plasmid using designed primers (FIGURE 1). The size of insert was as expected, i.e. approximately 370bp.

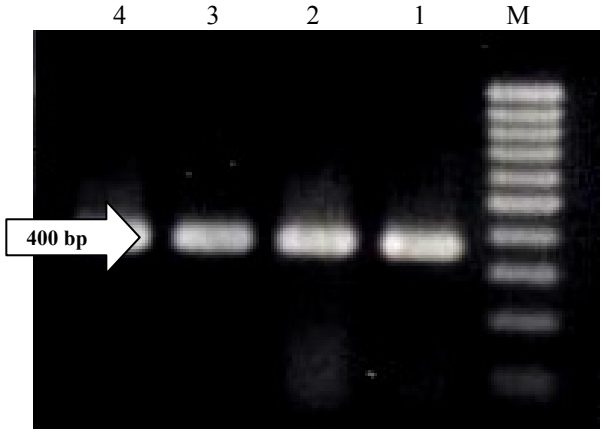


FIGURE 1 PCR amplification of VP3 gene from recombinant pcDNA3.1/zeo+. The amplified products were sized by ethidium bromide agarose gel electrophoresis. Lane 1 to 4 represented 4 samples from different clones. (M=100bp marker, L1-L4= PCR product)

Modified PCR amplification technique successfully identified 7 clones with the correct insert orientation out of 12 clones, by using designed primer combination. The correct clone shown to have the insert size of approximately 550bp for primer combination 1 and 450bp for primer combination 2, and 370bp for VP3 gene primers (FIGURE 2).

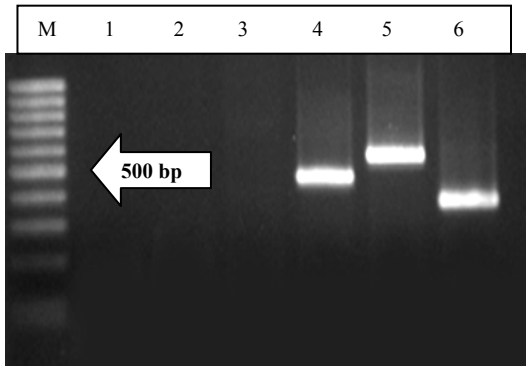


FIGURE 2 PCR amplification of VP3 gene from recombinant p-TP vector. The amplified products were sized by ethidium bromide agarose gel electrophoresis. PCR analysis of shown one of the correct clones of VP3 gene using primer combination 1 (Lane5), 2 (Lane4), and control using VP3 primer set (Lane 6)

The sequencing result, as analysed by Bioedit software shown that VP3 gene was intact and without mutation.

Cells transfected with recombinant p-TP-VP3 plasmid vector showed the sign of cell death 48 hours post-transfection. FIGURE 3 showed the control CT26 whereas FIGURE 4 showed the transfected CT26 48 hours post-transfection. The most obvious sign of death was slight rounding-up of CT26 cells in the treatment group.

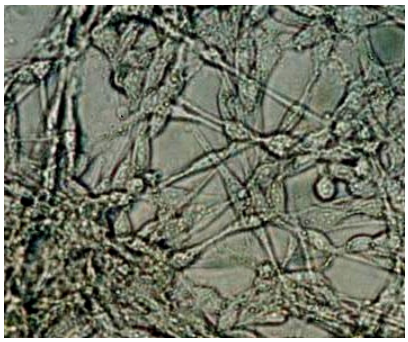


FIGURE 3 Non-transfected Control CT26 at the magnification of 100X (48 hours)

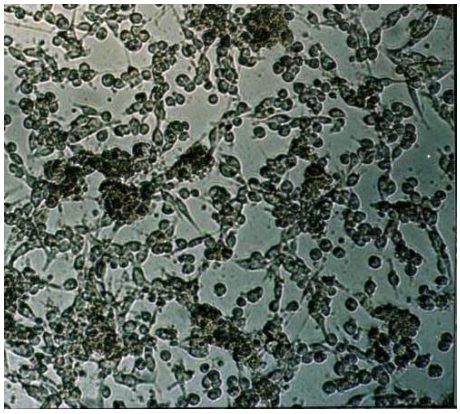


FIGURE 4 VP3 transfected CT26 (48 hours post-transfection) at the magnification of 100X.

Cycle-3 GFP was expressed on the 48 hours post-transfection. The fluorescent protein was found in cytoplasm as well as the nucleus of the transfected cells, under the fluorescence microscopy examination.

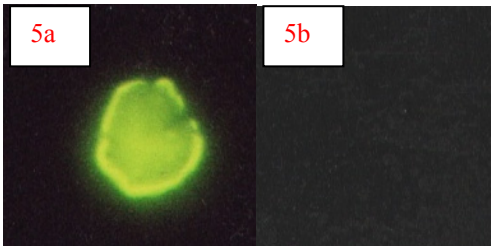
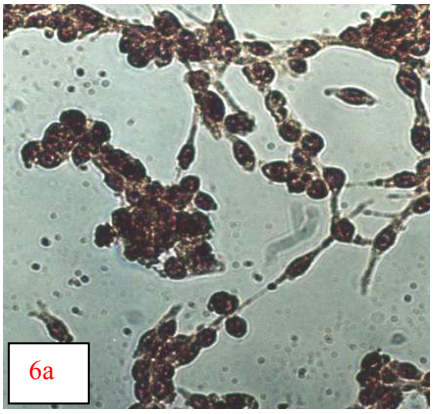


FIGURE 5 a) VP3-transfected CT26 (48 hours post-transfection) at the magnification of 400X. b) Non VP3-transfected CT26 (48 hours post-transfection) at the magnification of 400X**.**

Immunoperoxidase test showed that VP3 was expressed in transfected CT26 cells (FIGURE 6). The expression was mostly located in the nucleus of CT26 cells.



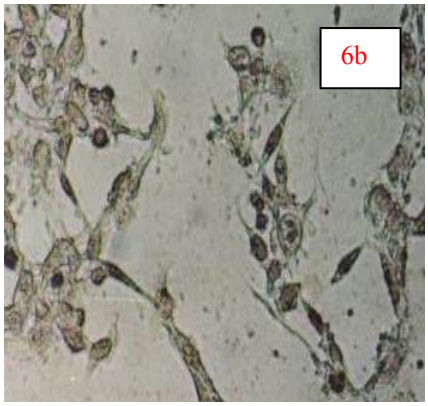
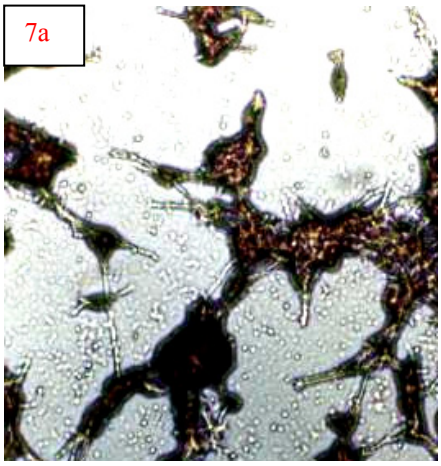


FIGURE 6 a) Immunoperoxidase Staining for VP3-transfected CT26 (48 hours post-transfection) at the magnification of 100X. b) Non-transfected cells.

DeadEnd TUNEL Test showed that VP3 caused apoptosis in transfected CT26 cells (FIGURE 7). The apoptosis was mostly found in the nucleus of CT26 cells.



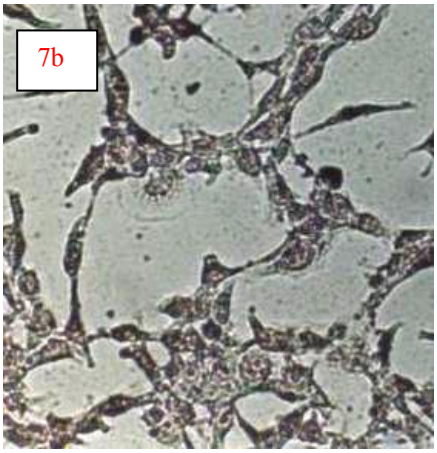


FIGURE 7 DeadEnd Tunnel Assay for a) VP3-transfected CT26 (48 hours post-transfection) at the magnification of 100X. Apoptosis detected. b) Non-transfected cells.

Combinatorial Gene Therapy in an Animal Model

Figure 8 showed the tumor mass grown at the flank region 30 days post-inoculation.

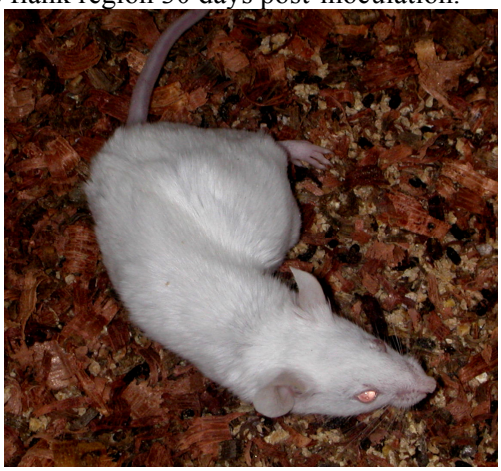


FIGURE 8 Typical tumor growth on the left flank of a Balb/c Mouse (Day-35 Post inoculation).

This project is in collaboration with Majlis Kanser Nasional (MAKNA). Due to confidentiality and the issue of intelectual properties, *in vivo* results will be discussed further during seminar presentation.

Discussions

VP3 gene successfully caused apoptosis in CT26 colon cancer cells *in vitro* and *in vivo*. Since VP3 protein is located in the nucleus of apoptotic cells, this may suggest that optimal VP3 protein expression is related to its locality (Danen-Van Oorschot, 1997).

Literatures showed that, a mutant VP3 protein that lacks a part of the positively charged amino acids remained partially localized in the cytoplasm and had a reduced apoptotic activity. The positively-charged characteristic of VP3 may allow interaction with nucleic acids, consistent with its nuclear localization. The presence of VP3 in the chromatin structure and its high proline content may cause disturbance of the supercoiled organization, which could then result in apoptosis. Another possibility is that VP3 acts as a transcriptional regulator of genes influencing the apoptotic process (Noteborn et al., 1998a,b,c).

GM-CSF induces proliferation of granulocyte and monocyte precursor cells. Destruction of tumor cells in which macrophages are directly involved is thought to occur by two distinct mechanisms, i.e. (1) macrophage-mediated tumor cytotoxicity and (2) ADCC (Ulich *et al*., 1990; Metcalf *et al*., 1987).

IL-12 has shown superior antitumor activity in contrast to other cytokines (Mu *et al*., 1995; Cavallo *et al*., 1997; Rakhmilevich *et al*., 1997; Takashi *et al*., 1995). Moreover, IL-12 has been demonstrated effective in preventing and inhibiting the growth of primary tumors induced by chemical carcinogens (Takashi *et al*., 1995) or spontaneous tumor growth in mice expressing rat HER-2/neu oncogene in the mammary gland (Boggio *et al*., 1998).

All tumors must generate a brisk angiogenic response to support their progressive growth (Folkman, 1995), and it is possible that IL-4 limits tumor growth in part by inhibiting angiogenesis. Two observations in the literature suggest that IL-4 might have such an inhibitory activity. First, tumor cells that secrete IL-4 can induce concomitant tumor resistance (Prehn, 1993). For example, the growth of B16F10 melanoma cells engineered to secrete IL-4 at one site in a mouse can retard the growth of parental cells implanted at a distant site (Ohira *et al*., 1994). In other systems, this ability to hold distant tumors in check has been shown to be due to the production by the first tumor of inhibitors of angiogenesis that accumulate in the circulation (O'reilly *et al*., 1994; O'relly *et al*., 1997; Volpert *et al*., 1998). A possible antiangiogenic role for IL-4 is also supported by the recent finding that vessel density in tumors resulting from the injection of C6 glioma was halved if the tumor cells were secreting IL-4 (Saleh *et al*., 1997).

Based on the above observations, we believed that pVP3 has synergistic effect with pGM-CSF/IL12 gene in cancer cells removal.

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