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Cloning of chicken anemia virus VP3 gene in a mammalian expression vector and analysis of its apoptic activity

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Abstract

The chicken anemia virus VP3 gene was cloned in pTargeT mammalian expression vector and the resultant recombinant plasmid pTargeT.cav-VP3 was characterized by restriction enzyme analysis by Acc III and sequencing. Expression of recombinant clone was confirmed by transfection in Vero cell line by positive immunofluorescence test. The apoptosis inducing potential of CAV-VP3 (apoptin) studied in HeLa cell line confirmed that it induced apoptosis in HeLa cells, which was confirmed by demonstrating the characteristics changes of apoptosis viz. nuclear condensation, fragmentation of the chromatin by acridine orange-ethidium bromide staining, DNA fragmentation by DNA laddering assay and TUNEL assay, plasma membrane alteration by annexin-V-binding assay. Caspase 3 was also detected after 48h of transfection of HeLa cell line by pTargeT.cav-VP3.

Key words: VP3, chicken anemia virus, apoptin, apoptosis, antitumor

Introduction

Chicken anemia virus (CAV), belonging to gyrovirus groups of circoviridae family is the causative agent of chicken infectious anemia (CIA), an immunosuppressive disease primarily of young chickens but also infects the chickens of all age groups. It is one of the smallest avian viruses 23-25 nm in size, icosahedral in shape and non-enveloped, having a 2.3 kb, circular, single stranded DNA genome. Among the viral proteins, VP1 is the major capsid protein (50 kDa) and VP2 is probably a non-structural protein found in the cells in the early stages of the virus replication cycle (Noteborn and Koch, 1995). VP1 and VP2 are the protective proteins that induce neutralizing antibodies (Koch *et al.,* 1995). The third viral

protein, VP3, also named apoptin, is a strong inducer of apoptosis in chicken thymocytes and chicken lymphoblastoid cell lines (Jeurissen *et al.,* 1992).

The VP3 protein is rich in serine-threonine residues and contains 121 amino acids. Because of its death-inducing abilities, the the VP3 gene product was renamed apoptin. Interestingly, the apoptotic activity of apoptin is not restricted to chicken thymocytes. Apoptin also causes apoptosis in various human tumors and transformed cells (Danen-Van Oorschot *et al.,* 1997).The present study was designed to clone VP3 gene of CAV and study its apoptotic activity.

Three putative viral proteins viz. VP1 (45-52 kDa, 449 AA), VP2 (24-30 kDa, 216 AA) and VP3 (13.6-16 kDa, 121 AA) have been well characterized by several workers (Noteborn *et al.,* 1991; Classens *et al.,* 1991; Chandratilleke *et al.,* 1991; Meehan *et al.,* 1992; Buchholz, 1994; Coombes and Crawford, 1994). VP1 in the major structural capsid protein (Todd *et al.,* 1990a, 1994; Koch *et al.,* 1994), though VP2 detection in purified virions revealed it might also to be a structural protein (Buchholz and Bulow, 1994). Both VP1 and VP2 are involved with antigenicity of the virus, and formation of neutralizing antibodies in CAV-infected cells requires the synthesis of both VP1 and VP2 in the same cell (Koch *et al.,* 1994; 1995; Noteborn *et al.,* 1998). VP3 is poorly immunogenic (Cunningham *et al.,* 2001). VP3, also named apoptin, is a strong inducer of apoptosis in chicken thymocytes and chicken lymphoblastoid cell lines (Jeurissen *et al.,* 1992) which is also an important phenomenon during the pathogenesis of CAV (Noteborn *et al.,* 1994; Noteborn and Koch, 1995; Chiu *et al.,* 2001).

Materials and Methods

Gene

The VP3 gene of CAV used in this study was synthesized from Bangalore Genei (India). The nucleotide sequence of VP3 gene of CAV was downloaded from NCBI GenBank. A 372bp VP3 gene was synthesized. The synthetic gene was cloned in pGEM-T easy cloning vector (Promega, USA).

Primers

Primers used in this study were synthesized from Chromous biotech. (India).

Sr. No.	Primer	Primer sequence (5'-3')
1.	VP3 F	ATG AAC GCT CTC CAA GAA G
2.	VP3 R	CTT ACA GTC TTA TAC ACC TT

Host Bacterial strains

Escherichia coli (*E. coli*) $DH_5\alpha$ (Promega,USA) host strain was used for transformation with recombinant plasmids.

Cell culture

HeLa and Vero cell lines were obtained from National Centre for Cell Science (NCCS), Pune. These cell lines were used in the study for apoptotic activity of recombinant plasmid and was maintained in DMEM (Gibco, NY) supplemented with 50 µg/ml gentamicin (Amresco, USA) and 10% fetal calf serum (Hyclone, USA).

Conjugates and Hyper-immune serum

Goat anti-mouse FITC conjugated antibody was obtained from Bangalore Genei, Bangalore, India. Anti VP3-CAV hyperimmune sera were raised in mice using recombinant plasmid. The recombinant plasmid (50 µg/mouse) was administered four times at interval of one week, to 5 mice. The blood was collected from the inner canthus of eye of the mouse and serum was prepared.

Experimental animals

Swiss albino mice, 4 weeks old were procured from Laboratory Animal Resource (LAR), IVRI, Izatnagar and kept in divisional animal experimental shed for raising hyperimune sera.

Subcloning of VP3 gene

Dephosphorylation of pTARGET vector

Dephosphorylation of pTARGET-T vector was done to prevent self ligation of the vector by using Calf intestinal alkaline phosphatase enzyme (CIAP).

Ligation reaction

The gel purified linear plasmid vector (approximately 25 to 50 ng) was ligated with insert in 1:10 ratio using 1U of T_4 DNA ligase.

Colony PCR

The presence of gene insert in right orientation in the recombinant plasmids was confirmed by PCR using T7 primer as forward primer and gene specific reverse primer. The PCR reaction mixture (50 µl) contained 100 ng of recombinant plasmid, 50 pmol each of gene specific reverse primer and T7 promoter specific primer and 3 units of Taq DNA polymerase in 1 x PCR buffer. The reaction mix contained autoclaved distilled water

32µl, Plasmid DNA 5µl, forward primer 2µl, reverse primer 2µl, dNTPs 2µl, buffer (10X) 5µl, Taq DNA polymerase 2µl total volume 50µl.

The VP3 gene was amplified following initial denaturation at 94.C for 5 minutes and 30 cycles of denaturation at 94 for 30 seconds, annealing at 50 for 50 seconds, and amplification at 72 for 50 seconds and a final extension at 72 for 7 minute. After amplification, an aliquot of 10 µl was subjected to agarose gel electrophoresis along with 100bp DNA molecular weight marker through 1.5% agarose gel at 60 volts for 1 hrs for the analysis of PCR product.

Restriction endonuclease analysis for recombinant plasmid

The presence of gene insert in the recombinant plasmids was confirmed by restriction endonuclease analysis using Acc III restriction enzyme. The plasmid DNA (1 µg) was incubated 4 hrs with appropriate 1X restriction endonuclease buffer, acetylated BSA(2µg) and 5 U restriction endonuclease in a water bath at 65.C. The digested DNA was mixed with 1X loading dye and run on 1.5%agarose (CSRL, Mumbai), using Tris acetate EDTA (TAE) running buffer system containing ethidium bromide (0.5 µg/ml) and visualized under UV-transilluminator and photographed.

Sequencing

The recombinant plasmids were further confirmed for gene insert in right orientation by squencing with T7 sequencing primer using primer walking method. The sequence thus obtained was submitted to the GenBank / EMBL database.

***In- vitro* expression analysis**

Expression of pTargeTCAV-VP3 plasmid was studied in Vero cells by indirect fluorescent antibody test (IFAT).

Transfection of Vero cells

The cell monolayer as subcultured and the cells were seeded in 96 well plates for IFAT analysis. The transfection of Vero cells was done using Polyfect transfection reagent (Qiagen, Germany). 0.4 µg DNA dissolved in nuclease free water was diluted with medium containing no serum and antibiotics (OptiMEM, Gibco) to a total volume of 30 µl. Added 2 µl polyfect reagent with optiMEM to a total volume of 20 µl per well. The diluted polyfect reagent was added to the DNA solution. Mixing was done by pipetting up and down. The sample was incubated for 5-10 min at room temperature to allow transfection-complex formation. The growth medium was removed from the well of 90 wells plate. The cells were washed once

with PBS. 50 µl of the solution containing transfection complexes was added directly into one well of a 96-well plate. 50 µl of growth medium was added to the well. The cells were incubated with the transfection complexes for 3 hr at 37^{O}C and 5% CO_2.The medium containing the transfection complexes was removed from the cells by gentle aspiration and the cells were washed once with 100 µl PBS. Fresh growth medium was added. Cells were incubated at 37^{O}C and 5% CO_2. After 24 or 48 hrs of incubated, the cells assayed for expression of the transfected gene.

Indirect fluorescent antibody technique (IFAT)

After 24 h of transfection, Vero cells grown in 96 well plates were fixed with 4% paraformaldehyde. A mock-transfected Vero cells were also fixed as control. To the fixed monolayer, 1:50 mouse diluted mouse anti-VP3-CAV hyperimmune serum was added and incubated at room temperature for 1 h. After the incubation, the tubes were washed with PBS and again incubated with goat antimouse FITC conjugated secondary antibody and incubated further for 1 hour at room temperature. Thereafter, cell monolayer were washed with PBS, mounted in 50% glycerol in PBS and examined under fluorescent microscope (Nikon, Japan) and photographed.

Trypan blue exclusion test

Viable cells are impermeable to trypan blue (Kaltenbach *et al.,* 1958). The HeLa cell monolayer as subcultured and the cells were seeded in 25 cm^2 flask. The Promega Wizard^{R} plus SV Minipreps kit purified plasmid DNA was transfected to 60 to 80% confluent HeLa cells using Polyfect Transfection Reagent (Qiagen, Germany) according to manufacturer's instructions.

Ethidium bromide-Acridine orange staining

Ethidium bromide-acridine orange staining was done after 48h of transfection to observe the nuclear condensation, fragmentation of nuclei and formation of apoptotic bodies. HeLa cells showing 60% monolayer were transfected.10 µl each of ethidium bromide and acridine orange from stock (10mg/ml) was diluted in 1ml PBS separately. The cells were washed with PBS once. 10 µl each of diluted ethidium bromide and acridine orange was added to each well of 96 wells plate and incubated at room temperature for 5 min. The cells were again washed with PBS and fixed with 4% PFA. The cells were again washed with PBS.50% glycerol in PBS was added and the cells were observed in UV microscope (Nikon, Japan).

DNA fragmentation assay by agarose gel electrophoresis

HeLa cells showing 60% monolayer were transfected as described earlier in the section.After 48h of transfection, the monolayer was trypsinized and collected in a 1.5 ml

tube. The cells were centrifuged at 3000 rpm for 5 min. After centrifugation, the media was removed and the cells were resuspended in 200 µl PBS and the genomic DNA was isolated using QIAmp DNA mini Kit (Qiagen, Germany) using kit protocol. The genomic DNA collected by above procedure was subjected to agarose gel electrophoresis (2%).

TUNEL assay

It was done using APO-Brdu TUNEL assay kit (Invitrogen, USA) following manufacturer's instruction. The principle of the test is based on the fact that the DNA breaks expose a large number of 3'-hydroxyl ends. These hydroxyl groups can then serve as starting points for terminal deoxynucleotidyl transferase (TdT), which adds deoxynucleotides in a template-independent fashion. Addition of the deoxythymidine analog 5 bromo-2'-deoxyuridine 5'-triphosphate (Brd UTP) to the TdT reaction serves to label the break sites. Once incorporated into the DNA, BrdU can be detected by anti-BrdU antibody using standard immunohistochemical techniques. This method of labeling DNA breaks is referred to as Terminal Deoxynucleotide transferase dUTP Nick End Labeling or TUNEL.

Annexin V binding assay

This test was mainly done to detect plasma membrane alteration which occurs during apoptosis. In normal live cells, phosphatidylserine (PS) is located on the cytoplasmic surface of the cell membrane. However, in apoptotic cells, PS is translocated from the inner to the outer leaflet of the plasma membrane, thus exposing PS to the external cellular environment. The human anticoagulant, armexin V, is a 35-36 kD Ca^{2+}-dependent phospholipid-binding protein that has a high affinity for PS. Labeled Annexin V can identify apoptotic cells by binding to PS exposed on the outer leaflet. This test was performed using Vybrant^{R} Apoptosis Assay Kit # 2 (Invitrogen,USA) following manufacturer's instruction. The kit contains recombinant annexin V conjugated to Alexa Fluor^{R} 488 dye. The kit also contains a ready-to-use solution of the red-fluorescent propidium iodide (PI) nucleic acid binding dye. PI is impermeant to live cells and apoptotic cells, but stains dead cells with red fluorescence, binding tightly to the nucleic acids in the cell. After staining a cell population with Alexa Fluor^{R} 488 annexin V and PI in the provided binding buffer, apoptotic cells show green fluorescence, dead cells show red and green fluorescence and live cells show little or no fluorescence.

Caspase 3 detection assay

Activation of caspases plays an important role in apoptosis. Caspase 3 was detected using CaspGLOW^{TM} Fluorescein Active Caspase-3 Staining Kit (Biovision, USA) following manufacturer's instruction. The assay utilizes the caspase-3 inhibitor, DEVD-FMK, conjugated to FITC (FITC-DEVD-FMK) as a marker. FITC-DEVD-FMK is cell permeable, nontoxic, and irreversibly binds to activated caspase3 in apoptotic cells. The FITC label allows for direct detection of activated caspases in apoptotic cells by fluorescence microscopy.

Results and Discussion

The CAV VP3 gene was successfully synthesized and cloned in pGEM-T vector and insert was released from pGEM-T vector by digesting with *Eco*RI enzyme (Fig.4) and then gel purified. It was then ligated to pTARGET mammalian expression vector. The recombinant pTARGET.CAV-VP3 was transformed in *E. coli* DH5 alpha competent cells and the recombinant pTARGET.CAV-VP3 plasmids were isolated. The rec-plasmid digested with *Eco*RI released the whole CAV-VP3 insert (Fig.5). One Acc III site is present in the vector and one site in region of CAV-VP3 gene. When the gene is in correct orientation, two fragments were released viz. 4677 bp and 762 bp (Fig.7). The restriction sites were predicted in pTARGET CAV-VP3 plasmid using DNASTAR software. Colony PCR with T7 promoter primer was done to identify gene in correct orientation (Fig.6). The sequence obtained from sequencing by T7 primer was submitted to GenBank and accession number assigned to sequences is AM904730.

Immunofluorescence analysis of pTARGET CAV-VP3 plasmid transfected Vero cells revealed intracytoplasmic fluorescence in transfected cells (Fig.8a) while, there was no fluorescence in mock transfected Vero cells (Fig.8c) and vector control (Fig.8b) which confirmed the expression of CAV-VP3 gene.

The cell viability was determined using Trypan blue exclusion test after 48h of transfection of HeLa cells by pTargeT.cav-VP3. The percentage of viable cells was 70%.The apoptosis inducing potential of CAV-VP3 (apoptin) when evaluated in HeLa cells by ethidium bromide-acridine orange staining, clear apoptotic changes of nuclear condensation, fragmentation of nuclei were observed in VP3 transfected cells (Fig.9a) while no such type of changes were found in control (Fig.9b,c).When DNA laddering assay was done, nucleosomal

laddering was detected on agarose gel electrophoresis (Fig.10c) while control showed no such laddering pattern (Fig.10a,b). For detecting apoptotic activity using TUNEL assay which revealed almost 27% cells showed apoptosis (Fig.11b). In vector control and in mock transected cells, cells showed some apoptotic activity (Fig.11c,a). In annexin-V-binding assay, apoptotic cells showed bright green fluorescence (Fig.12a) whereas in control live cells showed faint green fluorescence and necrotic cells showed red fluorescence (Fig.12b,c). In caspase detection assay, caspase-3 was detected. Caspase-3 positive cells showed green fluorescence (Fig.13a). Whereas control cells no such changes were found (Fig.13 b,c).All these five assays revealed that CAV-VP3 showed good apoptotic activity in cultured cells.

Intracytoplasmic expression was found in these cells which confirmed the *in-vitro* expression of VP3 gene. The apoptotic activity of VP3 gene was studied in neoplastic HeLa cell line. The results of present study indicated that the apoptosis in HeLa cells was due to the action of VP3 protein or apoptin, which was confirmed by *in-vitro* studies. Induction of apoptosis in HeLa cells was observed by employing ethidium bromide/acridine orange staining, DNA laddering assay, DNA fragmentation detection by TUNEL assay, caspase-3 detection assay and annexin-V-binding assay. In ethidium bromide/acridine orange staining, we found characteristic cytomorphological changes of nuclear condensation, fragmentation of chromatin. Similar characteristics morphological changes of apoptosis have been reported by using same techniques (Lam and Vasconcelos, 1994; Vasconcelos and Lam, 1994). In DNA laddering assay, nucleosomal laddering was detected in lane on agarose gel electrophoresis. The nucleosomal laddering is a hallmark of apoptosis. These findings were found to be similar as reported by other (Jeurissen *et al.,* 1992). DNA fragmentation was also confirmed by TUNEL assay. By using TUNEL assay, we were able to determine the percentage of apoptosis in HeLa cells. Almost 20% cells were found to be apoptotic as compared to control and it is consistent with results of other workers (Lee *et al.,* 2007).

Externalization of phosphatidlyserine (PS) is a hallmark of the changes in the cell surface during apoptosis (Koopman *et al.,* 1994; Martin *et al.,* 1995; Emoto *et al.,* 1997). It is an early event of apoptosis (Enderson *et al.,* 1995). In our study also this plasma membrane alteration was detected by Annexin-V-binding assay. Apoptotic cells showed green fluorescence while necrotic cells showed both green and red fluorescence. CAV-VP3 is a very small protein of 13 kDa in size, which is produced abundantly during the virus replication in the host cells and it is poorly antigenic in nature (Cunnigham *et al.,* 2001). The apoptosis inducing property of chicken anemia virus was demonstrated by elecron

microscopy and biochemical methods in cortical thymocytes after *in vivo* infection and in lymphoblastoid cell line after *in vitro* infection (Jeurissen *et al.,* 1992). Later, Noteborn *et al.* (1994) reported that chicken anemia virus VP3 protein alone induced apoptosis in chicken mononuclear cells and lymphoblastoid cell line. Following these reports, the research work was focused to exploit its apoptotic potential against the neoplastic cells.

The activation of the caspase cascade plays a central role in execution of apoptosis by cleaving a large number of proteins (Cryns *et al.,* 1998; Green *et al.,* 1998; Nunez *et al.,* 1998). Danen-van Oorschst *et al.* (2000) showed that apoptin requires activation of downstream caspases (Caspare 3, 6 and 7) for rapid induction of apoptosis. Among the downstream caspases, caspase-3 plays a central role in the execution phase of apoptosis. Active caspase-3 was also detected in apoptotic HeLa cells.Caspase-3 positive cells showed green fluorescence.

Pietersen *et al.* (2004) reported that adenovrial vector delivery of VP3 gene completely eradicated cholagiocarcinoma cells, a biliary tract cancer cells. Xu et *al.* (2006) showed the killing effects of VP3 gene of CAV in nasopharyngeal carcinoma cell line CNE-2 cells. They used PCDNA 31(-) CMV as plasmid expression vector. They used MTT assay to determine the killing effects of VP3 gene on nasopharyngeal carcinoma cell-line CNE-2. They found that the growth of CNE-2 cells that expressed VP3 gene was inhibited, while the growth of CNE-2 cells that did not express VP3 gene was not inhibited. They concluded that VP3 gene can kill nasopharyngeal carcinoma cell CNE-2. Mitrus *et al.* (2005) used synthetic VP3 gene for induction of apoptosis in COS-7, Renca and B16 (F10) cell lines. They used electroporation for delivery of VP3 gene into these cell lines. Li *et al.* (2006) constructed a recombinant fowlpox virus expressing the apoptin protein (vFV-apoptin) and compared the tumor-killing activity of the recombinant virus with that of wild type fowlpox virus in human hepatoma cell line HepG2. They found that although cells were somewhat resistant to the basal cytotoxic effect of wild type fowlpox virus, infection with vFV-apoptin caused a pronounced, additional cytotoxic effect. Natesan *et al.* (2006) showed that antineoplastic effect of chicken anemia virus VP3 protein (apoptin) in Rous-sarcoma virus induced tumour in chicken. They used pVAX expression vector for delivery of VP3 gene. They investigated *in vitro* antineoplastic effect of VP3 protein (apoptin) in Rous sarcoma virus (RSV)-transformed chicken embryo fibroblast (CEF) cells and in RSV-induced tumors of specific

pathogen free (SPF) chicks *in vivo.* Lee *et al.* (2007) showed VP3 gene of the CAV induced apoptosis in malignant canine mammary tumors but not in non-neoplastic canine mammary gland epithelial cells. The present study also clearly showed the apoptotic activity in neoplastic HeLa cell line. Apoptin can induce apoptosis in cell lines derived from a variety of human tumors, e.g. hepatoma, lymphoma, leukemia, cholangiocarcinoma, melanoma, breast and lung tumor and colon carcinoma (Noteborn *et al.,* 1991). In contrary, apoptin does not induce apoptosis in "normal" non-transformed human diploid cells, such as fibroblasts, keratinocytes, smooth muscle cells, T cells or endothelial cells. The possible cause for this phenomenon is that, in tumor cells, apoptin is located in the nucleus, whereas in normal cells it is present in the cytoplasm. Long-term expression of apoptin in normal human fibroblasts revealed that it has no toxic or transforming activity in these cells (Danen-van Oorschot *et al.,* 1997). These differences in the localization pattern are suggested to be the main mechanism by which normal cells show resistance to apoptin-mediated cell killing. Although the mechanism of action of apoptin is presently unknown, it seems to function by the induction of programmed cell death (PCD) after translocation from the cytoplasm to the nucleus and arresting the cell cycle at G_2/M, possibly by interfering with the cyclosome (Teodoro *et al.,* 2004). In 1972, Kerr *et al.* described a distinct morphology of dying cells and called it apoptosis. The term was coined based on the fact that the release of apoptotic bodies by dying cells resembled the picture of falling leaves from deciduous trees, called in Greek "apoptosis" (Kerr *et al.,* 1972). Apoptosis is a physiological form of cell death characterized by nuclear chromatin condensation, cytoplasmic shrinking and membrane blebbing (Lauber *et al.,* 2004; Schmitz *et al.,* 2000). This form of programmed cell death is predominantly induced by cancer therapy (Kawanishi and Hiraku, 2004; Wesselborg and Lauber, 2005). The two best-studied cell death signalling pathways include the extrinsic/cell death receptor pathway and the intrinsic/mitochondrial-initiated pathway (Greil *et al.,* 2003; Los *et al.,* 1999). The death receptor family [the tumor necrosis factor receptor (TNF-R) superfamily] includes CD95 (Fas/APO-1), TNF R1, DR3, DR4 (TRAIL-R1) and DRS (TRAIL-R2) receptors. Upon ligation, these receptors recruit the FADD-adapter protein and the apical caspase-8 (or caspase-10), resulting in the formation of the death-inducing signalling complex (DISC). Following this, caspase-8 is activated and released from the DISC, and further acts on downstream effector molecules (Los *et al.,* 1999; Sartorius *et al.,* 2001). The mitochondrial

death pathway is activated in the presence of diverse apoptotic stimuli, such as anticancer drugs, UV and gamma-irradiation (Hill *et al.,* 2003). The tumor suppressor gene p53 and the orphan steroid receptor Nur77 have been proposed as being the main molecules capable of transmitting the apoptotic signal from the nucleus to the mitochondria (Erster and Moll, 2004; Jeong *et al.,* 2003; Lane and Hupp, 2003; Li *et al.,* 2000). The initial common event during activation of the intrinsic/mitochondrial death pathway is the release of cytochrome C. Cytochrome C triggers the formation of a multimeric Apaf-1/cytochrome C/dATP/procaspase-9 protein complex called the apoptosome, and leads to the activation of caspase-9. Caspase-9 then cleaves and activates downstream caspases, including caspase-3, caspase-6 and caspase-7. This pathway is regulated at several levels. The mitochondrial apoptotic pathway is negatively modulated by the anti-apoptotic Bcl-2 family members, which block the release of cytochrome C from the mitochondria. Furthermore, caspase activation and the activity of already active caspases can be inhibited by 'the inhibitor of apoptosis proteins' (IAPs). In turn, IAPs can be inactivated by Smac/DIABLO or OMI / Htr A2, two regulatory proteins that are released from mitochondria.

Several strategies have been pursued to develop cancer selective therapies. The most broadly applied approaches explore defects in the regulation of the cell cycle (cell cycle check-points), which are commonly found in cancer cells (Blagosklonny and Darzynkiewicz, 2005; Westwell, 2004). Other strategies for selective cancer therapy involve molecules that act on and kill cancer cells preferentially, leaving normal cells unaffected. Several viruses require (rapidly) dividing cells for the completion of their lifecycle, thus viral proteins have gained attention as potential cancer selective therapeutics (Cassens *et al.,* 2003; Cornelis *et al.,* 2004; Oro and Jans, 2004). Tumor cell specificity is an important prerequisite for successful cancer therapy (Fischer and Schulze-Osthoff, 2005). Apoptin was identified as a protein inducing cell death by apoptosis in chicken mononuclear cells (Jeurissen *et al.,* 1992). *In vitro,* expression of apoptin induces cell death by apoptosis in cells that are susceptible to CAV (Noteborn *et al.,* 1998). Danen Van Oorschot *et al.,* (1997) have shown that apoptin fails to induce cell death by apoptosis in normal diploid cells, dermal cells, epidermal cells, endothelial cells and smooth muscle cells *in vitro*. Normal cells become susceptible to apoptosis by apoptin after they have become transformed.

It has been proven by cloning of the gene coding for VP3 protein in plasmid and transfecting in MDCC-MSB-1 cells, which showed induction of apoptosis and necrosis by propidium iodide staining of cell nuclear and DNA laddering pattern.

Zhuang *et al.* (1995) reported that apoptin also includes cell-death by apoptosis in a variety of human transformed cells, regardless of whether they express wild-type, mutant, or no tumor-suppressor gene product p53. He first screened this protein in three inbreed mice lines of p53 mutant, p53 normal and p53 deficient to study the role of p53 in apoptin mediated apoptosis and concluded that apoptin induced apoptosis independently in all the three mice lines. Danen van Oorschot *et al.* (2000) have analyzed the role of caspases in apoptin-induced apoptosis using a specific antibody, active caspase-3 was detected in cells expressing apoptin and undergoing apoptosis. They concluded that apoptin employed cellular apoptotic factors for induction of apoptosis, although activation of upstream caspases was not required but activation of caspase-3 and possibly other downstream caspases were essential for rapid apoptin induced apoptosis. Teodoro *et al.* (2004) have shown that apoptin associates with the anaphase-promoting complex and induce G2/M arrest of the cell cycle and apoptosis in the absence of p53. Apoptin triggers apoptosis by activating the mitochondrial pathway (intrinsic apoptosis), and acts independently of the death receptor pathway (extrinsic apoptosis). The process is modulated by Bcl-2, is associated with cytochrome C release from mitochondria, activation of caspase-3 and caspase-7 and requires Apaf-1 (Maddika *et al.,* 2005).

Pietersen and Noteborn (2000) have also reported that tumor cells were resistant to apoptosis induced by cytotoxic agents than normal cells. However, the resistance could be a direct consequence of mutation in certain tumor suppressor gene (P^{53}) or of certain proto-oncogens (Bcl-2). Apoptin acted independently of P^{53} was stimulated by Bcl-2, and insensitive to BCR-ABL which meant that apoptin induced apoptosis in cases where current therapeutic agents would fail.

Apoptin is transported into the nucleus but nuclear localization per se is not sufficient for apoptin to become active (Danen-Van Oorschot *et al.,* 2003; Rohn and Noteborn, 2004). Activation of the protein requires phosphorylation, and these steps appear to be much less efficient in normal cells as compared to transformed cells. Rohn *et al.* (2002) have reported the existence of a Kinase that phosphorylates apoptin. This kinase is present in tumor cells but at low levels only in normal cells.Apoptin forms highly stable, multimeric biologically active complexes consisting of 30-40 monomers and higher-order nucleoprotein complexes

with DNA conformation found predominantly in transcriptionally active, replicating and damaged DNA therefore, apoptin may trigger apoptosis by interfering with DNA transcription and synthesis (Leliveld *et al.,* 2003a). Apoptin has been shown to bind several cellular proteins known to be involved in cell-death by apoptosis. Danen-van Oorschot et. al. (2004) have reported that apoptin binds to DEDAF, a protein that binds to the DED domains of pro-apoptotic proteins. Cheng *et al.* (2003) have reported that apoptin binds to Hippi, a protein interactor and apoptosis co-mediator of Huntingtin interacting protein 1. These two proteins appear to co-localize in the cytoplasm of normal cells but not in cancer cells and may play a role in the suppression of apoptosis in normal cells.

Zhang *et al.* (2003) also showed that formation of uniform district, stable multimeric complex inside the cell on cytoplasmic micro-injection. Their further studies revealed that recombinant mannose binding protein-apoptin multimer retain its biological activity similar to the ectopically expressed wild type apoptin, namely the complexes translocated to the nucleus of the tumor cells and induced apoptosis, whereas they remained in the cytoplasm of normal primary cells and extend no apparent toxic effect.

LeLiveld *et al.* (2003b) later reported that apoptin predominantly co-localized with heterochromatin and nucleoli within tumor cells on the basis of immunoelectrochemistry. They have also reported that apoptin co-operatively forms distinct superstructures with DNA *in vitro* and these superstructures do not go beyond 20 multimeric apoptin complexes and approximately 3 kb of DNA. Further, they have showed that a single apoptin multimer to have eight independent nonspecific DNA binding sites which predominantly bind strand ends.

Liu *et al.* (2006) showed involvement of sphingolipids in apoptin-induced cell killing-apoptin activates acid sphingomylinase (ASMase), which generates ceramide ; inturn, ceramide acts as a second messenger and signals apoptotic response (Ogretmen and Hannun, 2004).

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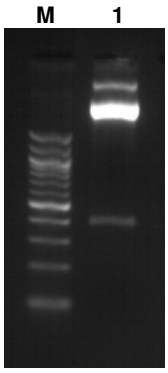

Fig. **VP3 insert released by digestion with EcoRI of pGEMTCAV.VP3**
Lane M: 100 bp ladder, 1: Release of 372 bp VP3 from pGEMTCAV-VP3

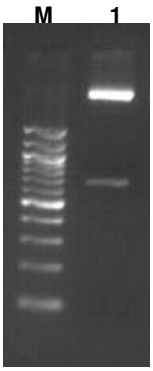

Fig. **Orientation conformation by R.E. analysis (digested with AccIII)**
Lane M: 100 bp ladder; 1: Release of insert of from pTagrgeTCAV-:VP3 confirming the right orientation of the gene

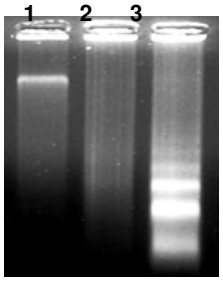

Fig. **DNA laddering assay**
Lane 1: healthy control ,2: Vector control,3 : pTargeTCAV-VP3

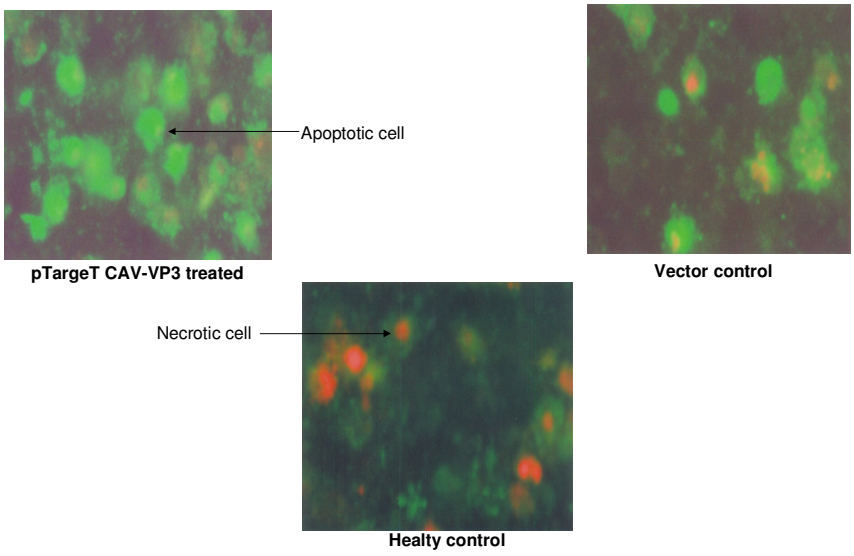

Fig. Annexin-V-binding assay of pTargeTCAV-VP3 in HeLa cells.

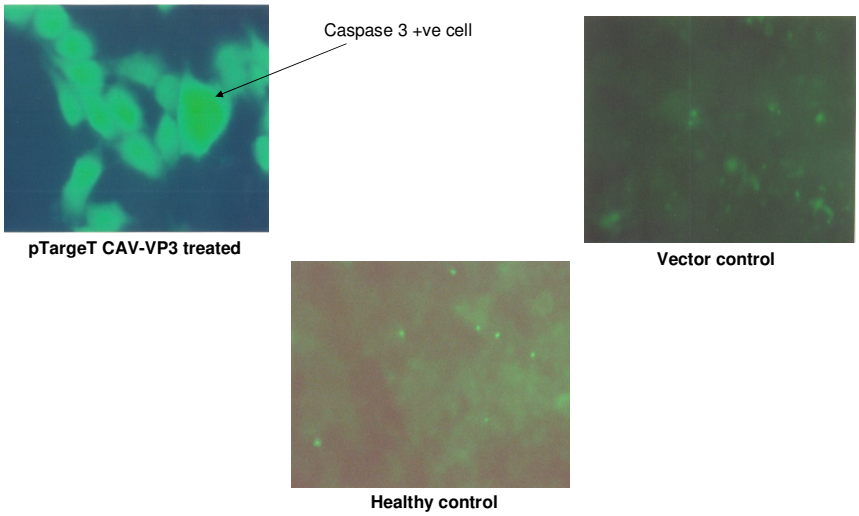

Fig. Caspase 3 detection assay of pTargeT CAV-VP3 in HeLa cells.

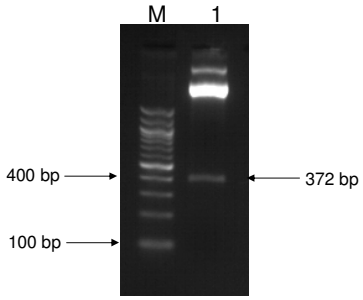

Fig. VP3 insert release by digestion with EcoRI of pGEMTCAV.VP3 Lane M: 100 DNA bp ladder;1: Release of 372 bp VP3 insert.

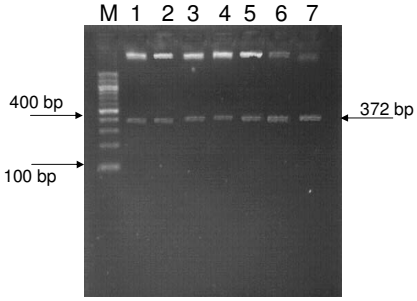

Fig confirmation of Insert bydigestion with EcoRI;Lane M:100 bp DNA ladder;1-7 release of 372 bp fragment.

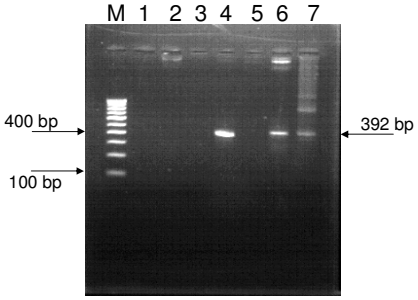

Fig Right Orientation confirmation of clones by PCR using T7 primer and gene specific reverse primer.Lane M: 100 bp DNA ladder;4,6&7: amplified product;1,2,3&5: No amplified product

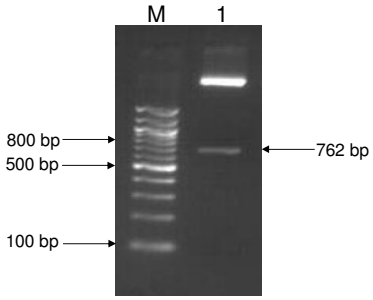

Fig. Orientation conformation by digestion with AccIII;Lane M: 100 bp DNA ladder,1: Release of 762 bp insert

TUNEL ASSAY

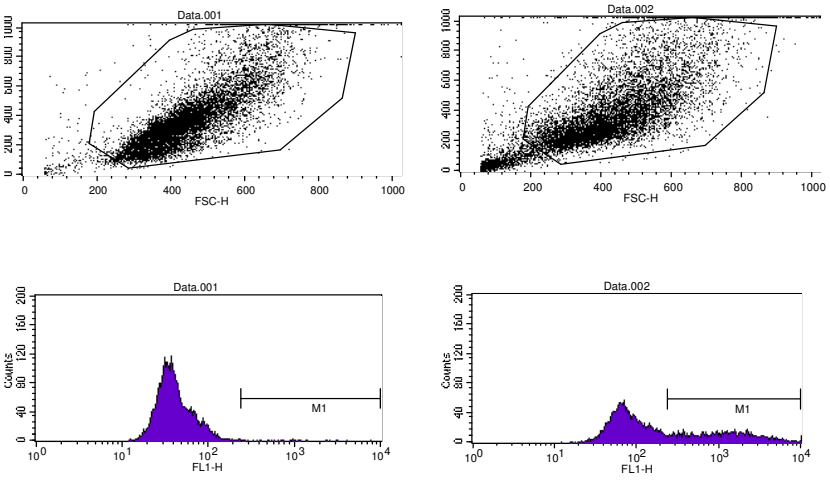

Fig.1 Mock transfected cells
Percentage of apoptotic cell- **0.37**%

Fig.2 pTARGET CAV-VP3 Transfected cells
Percentage of apoptotic cell- **27.18**%

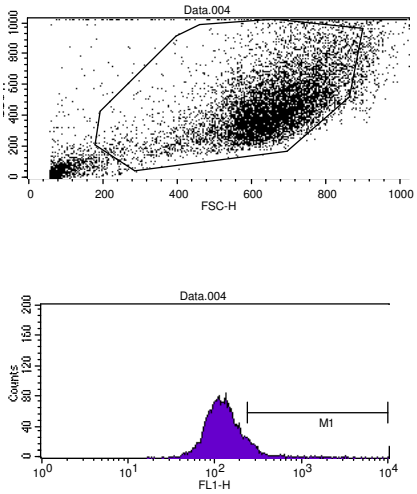

Fig. 3 Vector control
Percentage of apoptotic cell- **6.39%**

EXPRESSION ANALYSIS BY IFAT

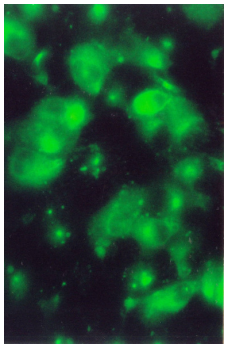

Fig pTARGET CAV-VP3 transfected Vero cells

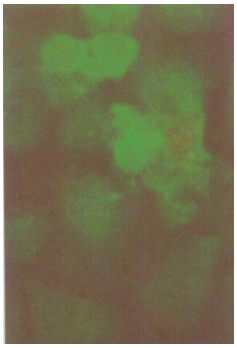

Fig Vector control

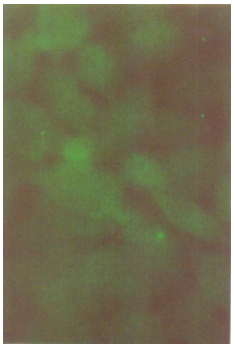

Fig Mock transfected vero cells

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