In vitro cloning of canine parvovirus NS1 gene and reporter gene GFP in eukaryotic expression vector pVIVO2-mcs and characterization of the double gene construct in mammalian cells


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The use of chemotherapy and/or radiotherapy for treatment of cancer is limited due to genotoxic side effects on healthy cells, involvement of anti-apoptotic signal transduction pathways that prevent cell death, and requirement of functional p53 for induction of apoptosis in cancerous cells. Efforts are being made worldwide to develop new anticancer therapies as an alternative to chemotherapy. And viral gene therapy is one of the most potent therapeutics that is being ventured worldwide. Canine parvovirus-2 (CPV-2) is one of those viruses that have an inherent oncolytic property. The non-structural protein-1 (NS1 protein) of CPV-2 plays a major role in parvoviral cytotoxicity and pathogenicity in permissive cells. The oncolytic potential of CPV2-NS1 has been established in vitro. Prior to taking up the in vivo studies, the present study was undertaken to clone Canine Parvovirus NS1 gene and reporter gene GFP in eukaryotic expression vector pVIVO2-mcs, and to characterize the double construct in mammalian cells. The genes were successfully cloned in pVIVO2-mcs and characterized for their expression as demonstrated by fluorescence microscopy and immunofluorescence staining. This characterized double gene construct will further be used to evaluate the oncolytic potential of CPV-2 NS1 in experimentally induced in vivo tumour model.

Keywords: Cancer, CPV-2, NS1, viral gene oncotherapy

Introduction

Cancer can be defined as a disease in which a group of abnormal cells grow uncontrollably by disregarding the normal rules of cell division. It is the leading cause of death worldwide and remains a challenge to mankind due to its fatality and incurability. The variability within the same kind of tumour is the main reason for variation in response to anticancer treatment. The current use of chemotherapy and/or radiotherapy for treatment of cancer is limited due to genotoxic side effects on healthy cells and involvement of anti-apoptotic signal transduction pathways that prevent cell death. Most of human cancers contain mutated p53 gene and might not undergo apoptosis after the use of chemotherapeutic agents. Such cancers have a very rare chance of responding to chemotherapy. Moreover, over expression of antiapoptotic proteins like Bcl2, Bcl-XL and caspase inhibitors negatively affect the chemotherapeutic treatment of large number of cancers. Resistance to cancer therapy appears to be mediated by resistance to apoptosis due to the simultaneous activation of NF-κB. Therefore, new approaches to cancer therapy that inhibit nuclear translocation of NF-κB may prove to be highly effective in treatment of cancer. Efforts are being made worldwide to develop new anticancer therapies, which are based on induction of apoptosis without the requirement of functional p53 genes and those that are capable to suppress the over expression of anti-apoptotic genes.

Over the last decade, better understanding of the tumour biology and advancement made in molecular biology and genetic engineering have raised the hope for development of viral gene therapy as a potent therapeutics for cancer. Viral gene therapy facilitates immune clearance as well as effectively induces apoptosis in transformed cells by the viral gene expressed proteins. Many viral proteins like VP3 (apoA) from chicken infectious anemia (CIA) and adenovirus protein E4orf4 specifically kill tumour cells, while sparing normal cells unharmed. Canine
parvovirus-2 (CPV-2) is one of those viruses that have an inherent oncolytic property\textsuperscript{10}, which can be harnessed for the treatment of various types of cancers\textsuperscript{11-15}. Further, many workers reported non-structural (NS) protein (NS1) as the major effector of parvoviral cytotoxicity. CPV2-NS1 is a pleiotropic nuclear phospho-protein absolutely essential for viral replication\textsuperscript{16}. Most non-structural proteins show high sequence homology among parvoviruses and are responsible for parvoviral cytotoxicity and pathogenicity in permissive cells, with NS1 playing the main role\textsuperscript{17,18}. Further, it was recently demonstrated that apoptosis induced by CPV-2 NS1 was caspase dependent and p53 independent in HeLa cells in vivo\textsuperscript{19}. To test the efficacy of NS1 gene in experimentally induced in vivo tumour model, it is necessary to clone the gene in eukaryotic expression vector. Plasmid pVIVO2-mcs allows strong and sustained co-expression of two genes of interest due to the presence of two separate transcription units (TUs), each with strong promoter and efficient polyadenylation signal. These features allow cloning and expression of one target gene along with another reporter gene in two different mcs (multiple cloning sites). Expression of reporter gene helps to indirectly monitor the expression of the target gene present in the same vector. Therefore, prior to taking up the in vivo studies, the present study was undertaken to clone CPV-2 NS1 gene and reporter gene GFP (green fluorescent protein) in eukaryotic expression vector pVIVO2-mcs and characterize the double construct in mammalian cells.

**Materials and Methods**

**Plasmids and Bacterial Strain**

*Escherichia coli* DH5α strain was used as host cell for transformation of recombinant plasmids. The vector pVIVO2-mcs (InvivoGen, USA) was used for cloning and expression studies.

**Cell Line and Culture Medium**

MDCK cells were obtained from the National Centre for Cell Sciences (NCCS), Pune. The cells were adapted to grow in DMEM (high glucose) supplemented with 10% FBS, penicillin 100 U/mL, streptomycin 100 µg/mL, 10 µL fungi zone and 10 mM HEPES buffer (Duchefa Biocheme, Netherlands) at 37°C under 5% CO2 in 25 cm² flasks (Nunc).

**Cloning and Characterization of GFP Gene in mcs2 of pVIVO2-mcs Mammalian Expression Vector**

The primers (forward: 5′GCCGAA TTCATG GTGAGC AA GGGCGAGGA3′ and reverse: 5′GCCGCT AGCCTA CACATT GATCCT AGCAGA AGC3′) for GFP gene were designed using Lasergene software (DNASTAR, Inc., USA) and their specificity was checked by BLAST (http://ncbi.nlm.nih.gov/BLAST/). The restriction sites for EcoRI and *NheI* were added in forward and reverse primers of GFP gene, respectively to facilitate cloning in multiple cloning site-2 of pVIVO2-mcs. pd1EGFP-N1 vector (Clontech) was used as template to amplify the GFP gene by the above mentioned primers. The amplification reaction was carried out in a 50 µL reaction volume containing 1× PCR buffer with 2.0 mM Mg\textsuperscript{2+}, 0.4 µM of each forward and reverse primer, 200 mM of dNTPs mix, 2.5 U of *Taq* DNA polymerase with *Pfu* DNA polymerase (15:1) and 2 µg pd1EGFP-N1 plasmid. After an initial denaturation at 95°C for 5 min, 35 cycles of amplification was performed as follows: denaturation at 95°C for 60 sec, annealing at 72°C for 60 sec and extension at 72°C for 60 sec. A final extension at 72°C for 10 min ended the reaction. The PCR amplicon was resolved in 1% agarose gel and visualized on ultraviolet transilluminator.

The PCR product of GFP was recovered from low melting point agarose, purified (QIAEX II gel-extraction kit, Qiagen) and cloned at EcoRI and *NheI* site of pVIVO2.mcs and called pVIVO.gfp. The desired recombinant plasmid pVIVO.gfp was confirmed by colony PCR and restriction enzyme digestion with EcoRI/NheI.

**In Vitro Expression Analysis of Recombinant pVIVO.gfp Clones in MDCK Cells**

To check the functional activity of the pVIVO.gfp recombinant clone, in vitro expression study was carried out in MDCK cells. For this, transfection grade pVIVO.gfp and pVIVO2.mcs were prepared using EndoFree Plasmid Maxi kit (Qiagen) as per the manufacturer’s protocol. Purified clones were used to transfect 70-80% confluent MDCK cells in a six well plate using Lipofectamine\textsuperscript{TM} 2000 (Invitrogen, USA) transfection reagent. For this, 4 µg plasmid DNA was mixed with 10 µL Lipofectamine in 500 µL of optiMEM (Invitrogen, USA). The mixture was incubated for 30 min at room temperature for complex formation. Meanwhile, cells were washed once with 1 mL of warm PBS and 1.5 mL of fresh cell growth medium without antibiotics (DMEM with 10% FBS). After incubation, DNA-lipofectamine complexes were added over the cells and plate was incubated at 37°C for 4 h. After 4 h, medium was replaced with fresh growth medium and incubated at
37°C under 5% CO₂ for 18 h. Cells were examined for expression of green fluorescence under Fluorescence Microscope (Nikon, USA) after excitation with blue light. The mock transfected MDCK cells were used as control.

Cloning and Characterization of CPV-2 NS1 in mcs1 of pVIVO2-mcs Mammalian Expression Vector

The primers for CPV2-NS1 gene (forward: 5’GCATCA TGAATG TCTGCG AACCAG TATACT GAGA3’ and reverse: 5’GTTCTT AGGT TA ATCCAA GTCGTC TCGAAA ATC3’) were designed as described before. The restriction sites for BspHI and AvrII were added in forward and reverse primers, respectively to facilitate cloning in mcs1 of pVIVO2-mcs. pcDNA.CPV.2.NS1 construct²⁰ was used as template to amplify the NS1 gene by the above primers. The PCR amplification was carried out as described above with annealing at 68°C for 45 sec and extension at 72°C for 2 min. The PCR product was cloned at BspHI-AvrII site of pVIVO2.mcs plasmid and was named as pVIVO2.ns1. Presence of CPV2-NS1 insert in recombinant plasmid was confirmed by colony PCR and restriction enzyme digestion.

In Vitro Expression Analysis of Recombinant pVIVO.ns1 Clones in MDCK Cells

For checking the expression of CPV2-NS1 genes, MDCK cells were transfected using the protocol described before and expression was analyzed by Immuno-fluorescence (IF) assay. For the IF assay, we followed previously published protocol²¹. Briefly, cells were fixed in 4% PFA (paraformaldehyde), permeabilized with 0.2% Triton× 100, blocked with 2% bovine serum albumin and were incubated with rabbit anti-NS1 specific primary antibody²² (1:500 dilution). The cells were washed thrice with PBS and incubated with TRITC labeled secondary goat anti-rabbit IgG (Sigma, USA, Cat#T6778, 1:400 dilution) and nuclei were stained with DAPI (Lonza, Cat#PA3013), followed by two washing with PBS and observed under Inverted Floreescence Microscope (Nikon, USA).

Cloning and Expression of CPV2-NS1 in pVIVO2.gfp Vector

CPV2-NS1 fragment was released from pVIVO.ns1 construct using BspHI and AvrII restriction enzymes and further cloned in pVIVO.gfp plasmid at the same site. The plasmid was verified by restriction digestion and by transfection of MDCK cells, following procedures described above.

Results and Discussion

Development of Gene Construct

The present study was undertaken with the objective to clone the NS1 gene of CPV-2 and GFP gene in eukaryotic expression vector pVIVO2-mcs. Initially both the genes CPV-2 NS1 and GFP were cloned individually into mcs1 and mcs2 of pVIVO2-mcs. Later, the NS1 gene from pVIVO.ns1 was cloned into mcs1 of pVIVO.gfp.

Cloning and Expression of GFP Gene in mcs2 of pVIVO2-mcs Eukaryotic Expression Vector

The pd1EGFP-N1 was used as template for amplification of GFP gene, which was cloned at mcs2. This amplified product (758 bp) (Fig. 1a) was gel purified, digested with EcoRI and NheI and cloned at mcs2 of vector pVIVO2-mcs. The recombinant clones were screened by colony PCR (Fig. 1b) and further confirmed by restriction digestion with EcoRI and NheI enzymes, which released the desired fragment of 750 bp (calculated from the enzyme cut site from both ends) (Fig. 1c). The recombinant pVIVO.gfp was prepared in bulk, using Endofree maxi columns. The purity of DNA preparation was checked by measuring absorbance at 260/280, which was found to be 1.8 indicating that the DNA preparation contains pure double stranded DNA, free from protein and RNA contamination. The in vitro expression analysis of purified recombinant pVIVO.gfp was performed by transfecting MDCK cells in 6 well plates using 4 µg of recombinant DNA in combination with 10 µL of Lipofectamine™ 2000 (per well) transfecting agent as per manufacturer’s protocol. After 18 h post transfection, the cells were fixed in 4% PFA, stained with nuclear stain DAPI and visualized under Fluorescent Microscope. The expression of cloned gene GFP at protein level was confirmed by the characteristic green fluorescence (Fig. 1d).

Cloning and Expression of NS1 Gene of CPV-2 in mcs1 of pVIVO2-mcs Eukaryotic Expression Vector

The pcDNA.CPV.NS1 (already available in the laboratory) was used as template for amplification of NS1 gene (insert I) of 2007 bp (calculated from the enzyme cut site from both ends) (Fig. 2a). This NS1 gene was cloned in the backbone of mammalian expression vector pVIVO2-mcs at mcs1. The amplified product was gel purified, digested with BspHI and AvrII, and cloned at mcs1 of vector pVIVO2-mcs. The recombinant clones were
confirmed by restriction digestion with *Bsp*HI and *Avr*II enzymes, which released the desired fragment of 2017 bp (Fig. 2b). The recombinant pVIVO.ns1 was prepared in bulk, using Endofree maxi columns. The purity of DNA preparation was checked by measuring absorbance at 260/280, which was found to be 1.8 indicating that the DNA preparation contains pure double stranded DNA, free from protein and RNA contamination. The *in vitro* expression analysis of purified recombinant pVIVO.ns1 was performed by transfecting MDCK cells with 4 µg of recombinant/well in 6 well plates using 10 µL of Lipofectamine™ 2000 transfection reagent. After 24 h post transfection, cells were fixed in 4% PFA and stained with DAPI for detection of nuclei. A. Photomicrograph of MDCK cells under bright field; B. MDCK cells showing nuclei stained with DAPI; C. NS1 protein detected in MDCK cells as red fluorescence; & D. Merged image of fields shown in B and C.
were visualized under Fluorescent Microscope. The expression of NS1 protein was confirmed by the characteristic red fluorescence (Fig. 2c).

Cloning and Expression of both NS1 and GFP Genes in pVIVO2-mcs Eukaryotic Expression Vector

For the cloning of NS1 into mcs1 of pVIVO.gfp, recombinant pVIVO.ns1 and pVIVO.gfp were digested with BspHI and AvrII. The NS1 insert released from pVIVO.ns1 was cloned into pVIVO.gfp recombinant. The presence of NS1 and GFP genes in recombinant pVIVO2.gfp.ns1 was confirmed by restriction digestion using BspHI and AvrII (for NS1 gene), and NheI and BamHI (for GFP gene), respectively. The release of desired fragments of GFP gene (750 bp) and NS1 gene (2017 bp) was checked by agarose gel electrophoresis, which confirmed the respective inserts in the recombinant clone (Fig. 3). The recombinant pVIVO.gfp.ns1 was prepared in bulk using Endofree maxi columns and the in vitro expression analysis was performed by transfecting MDCK cells with Lipofectamine™ 2000, following manufacturer’s instruction. The expression of NS1 and GFP gene at protein level was confirmed by immunofluorescence staining assay (IFAT) using polyclonal sera raised in rabbit against NS1 protein, followed by TRITC labeled anti-rabbit secondary antibody (Sigma) and by simple fluorescence microscopy after nuclear staining with DAPI, respectively. Results demonstrated both bright red and green fluorescence in pVIVO.gfp.ns1 transfected MDCK cells, only green fluorescence in case of pVIVO.gfp and no fluorescence in mock control cells (Fig. 4).

In the present study, the CPV-NS1 gene was cloned along with the reporter gene in the commercially available vector pVIVO2-mcs, which had two independent transcription units driven by two ubiquitous ferritin promoters. Human ferritin heavy chain promoter drives expression of NS1 from canine parvovirus (CPV2-NS1) and human ferritin light chain promoter regulates GFP expression. As ferritin levels are elevated in individuals suffering from malignant tumours, presumably due to high oxygen demand for excessive proliferation of malignant cells, the gene construct pVIVO2.gfp.ns1 is expected to express NS1 in a wide variety of tumour cells. Though the oncolytic potential of CPV-NS1 is established in vitro, it needs to be evaluated in vivo in tumour models. Further, the reporter gene GFP used in this study would code for the reporter protein that is

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**Fig. 3**—Generation of pVIVO.gfp.ns1 plasmid construct: Confirmation of recombinant pVIVO.gfp.ns1 by RE digestion. Plasmids were isolated from clones and digested with BamHI, NheI, BspHI and AvrII REs. Desired fragments (2017 bp for NS1 and 750 bp for GFP) were released and run in 1% Agarose gel (Lanes 1-4, 2017 bp fragment for NS1 and 750 bp fragment for GFP; & Lane M, 1 Kb DNA ladder(# SM0312)).

**Fig. 4**—(A-H)—Functional assessment of pVIVO.gfp.ns1 plasmid construct: Expression of GFP and NS1 proteins in MDCK cells after transfection. MDCK cells were transfected with mock reagent (A-D) or with pVIVO.gfp.ns1 (E-H). [A. Bright field image of mock transfected cells; B. No red fluorescence was detected in mock transfected cells; C. DAPI stained nuclei in mock transfected cells; D. Merged image of A and C with enlarged image in inset; E. Expression of GFP in MDCK cells transfected with pVIVO.gfp.ns1 plasmid; F. Red fluorescence was detected in pVIVO.gfp.ns1 transfected cells confirming the expression of NS1; G. DAPI stained nuclei in pVIVO.gfp.ns1 transfected cells; & H. Merged image of E, F and G with enlarged image in inset.]
non-toxic to most mammalian cells and does not interfere in cellular metabolism. Presence of GFP would allow to measure the efficiency of delivery, tracking of malignant cells, and also to study their fates in a variety of cells. The gene construct pVIVO2.gfp.nS1 proven for its expression of NS1 and delivery can now be used to assess the oncolytic potential of CPV-2 NS1 gene in in vivo tumour models.

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References