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Oncolytic effect of chicken anemia virus vp3 protein (apoptin) in *in-vitro* cultured bovine horn cancer cells

HIMANSHU SINGH[1], N S JADON[2], A K TIWARI[3], G K SINGH[4], PRIYANKA PANDEY[5] and SANJAY KUMAR [6]

Govind Ballabh Pant University of Agriculture and Technology, Pantnagar, Uttarakhand 263 145 India

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Therapeutics for cancer, one of the world's most dreaded and incurable diseases, are costly and also not safe and effective for longer period, because of either mutation in P53 gene and BCl2 protein. In animals also it is formidable and animal suffers in silence. Cancer virotherapy (use of viruses for cancer treatment) a new therapeutic approach, was found more efficacious and less toxic than conventional radio-chemotherapy. Viruses can attack cancer cells in multiple, but targeted ways. The self replicating property amenability to genetic manipulation make viruses perfect candidates for new generation smart therapeutics for cancer and seem to have tremendous potential for cancer management in future. The concept of using replicating lytic viruses for cancer treatment dates back to the beginning of 20th century, when it was observed that patients with various malignancies experienced spontaneous tumor regression following rabies vaccination (Dock 1904). Chicken anemia virus released protein namely VP3 or apoptin, smallest protein (13 kDa), having a unique oncolytic induction property which selectively target the cancer cell (Noteborn *et al.* 1994, Noteborn 1999), make them a potential anti-cancer agent (Maddika *et al.* 2006). The development of cancer therapeutics requires intensive screening, and it is a difficult task to screen the drug *in vivo* or in natural condition. So *in-vitro* cultivation of cancer cell may help to screen the drug faster and intensive. Considering the above, the present investigation was done to find out the effect of VP3 gene on bovine horn cancer.

Twenty cases of bovine horn cancer were subjected to this study. All the animals were subjected to surgical intervention for the removal of tumor growth under standard protocol of anesthesia. For the confirmation of malignancy, tumor samples were collected in 10% buffer formalin beside a small piece of tumor collected in a vial containing DMEM supplemented with 2%FBS and 5X antibiotics, viz. streptomycin, penicillin, gentamycin and nystatin for *in vitro* culture. Sections of 6 to 8 microns were stained with H&E stain. Microscopically, degree of differentiation, type of growth pattern of malignant cells, degree of malignancy and cellular characteristics were recorded. Bovine horn cancer cells were cultured *in vitro* as per Lee *et al.* (2003). Bovine horn cancer cells in suspension were sub cultured for 6 passages continuously. After sixth passage bovines horn cancer cells (BHCs) in suspension were transfected by VP3 gene of CAV, cloned in PcDNA 3.1 eukaryotic expression vector. Cells were harvested by trypisinization at 24 and 48 h post transfection and resuspended in 1X annexin-binding buffer to a density of 1×10^6 cells/ml. 5µl of Alexa Fluor 488 annexin V and 1µl of the 100µg/ml PI working solution was added to 100µl of cell suspension and cells were incubated at room temperature for 15 min. Thereafter, 400µl of 1X annexin-binding buffer was added and cells were kept on ice and analysed by flow cytometry using FL1 (530nm) and FL3(575nm) bandpass filters. Oncolytic potential of recombinant PcDNA.ciav.VP3 was established in BHCs by Annexin V binding assay and TUNEL assay.

The biopsy specimens for cell culture and microscopic studies were collected aseptically. Histopathological findings revealed that all the bovine (100%) were squamous cell-carcinoma. The cultured cells of biopsy material from the bovine horn cancer cells were passaged serially and survived for last 6 months. All the cells were grown in suspension culture. One lineage of BHC cells was used to study the oncolytic effect of the virus. The molecular mechanism of apoptosis induced by CAV revealed that initially virus utilizes mitochondria mediated pathway to kill the cells followed by death receptors and endoplasmic reticulum mediated mechanism at later stages (72 h or later). Similar findings were reported by Maddika *et al.* (2005). DNA fragmentation

Present address: [1,6]Ph.D.Scholar (himanhappy @rediffmail.com), [2]Professor and Head (drjadonnns12 @rediffmail.com), [4]Dean, College of Veterinary and Animal Science. [3]Principal Scientist (aktiwari63@gmail.com), [5]SRF (shuklapriyanka5@gmail.com), Division of Animal Biotechnology, IVRI, Izatnagar.

by TUNEL assay confirms that the VP3 gene of CAV cloned in PcDNA 3.1 eukaryotic expression vector was found functionally active and have oncolytic potential, which is also confirmed by the findings of Chiu *et al.* (2001). Annexin V binding study also revealed that VP3 protein obtained from Indian isolate of CAV can induce cell death among tumor cells of bovine origin *in vitro*. In this study, most of the VP3 protein in BHC cells was expressed in the nucleus, where it aggregated in the form of a fine granular pattern. The results obtained in the study are in accordance with the finding of Noteborn *et al.* (1998). They mentioned that CAV-associated VP3 protein is believed to be dependent on its ability to localize in the nuclei of transformed cells, but not in the nuclei of primary or non transformed cells.

SUMMARY

Horn cancer is very common in bovine, and development of cost effective therapeutics is required for saving animals. Considering this, the present study was undertaken, to assess the oncolytic effect of chicken anemia virus VP3 protein (apoptin) *in vitro* in cultured malignant tumors of bovines. Twenty bovine subjected to horn cancer were surgically operated, histopathological examinations were done. Histopathological examination revealed that the subjected tumours were 100% squamus cell carcinoma. Further, the biopsy material was cultivated in laboratory and cell lines were developed. These cell lines were transfected with 3 groups of treatments– mock (no treatment), vector (lipofactimine alone), and vector plus VP3 cloned in Pc DNA. Assessment of oncolysis was done using Annexin V binding assay and TUNEL assay. This *in-vitro* study confirmed that VP 3 gene/apoptin induced oncolysis in cultured malignant bovine tumor cells and regression extent was 13%. Our results revealed that apoptin had oncolytic effect and further improvements in the dose, delivery methods and delivery frequency of apoptin expressing recombinant vector may help to develop apoptin as an oncolytic drug.

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