

## Coxsackievirus B3 protease 3C induces cell death in eukaryotic cells

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**Abstract:** Coxsackievirus B3 (CVB3) is the most common agent known to cause viral myocarditis. The viral genome encodes a single polyprotein that is cleaved to produce several proteins by virally encoded proteases. Most of this proteolytic processing is catalyzed by a cysteine protease called 3C. The 3C protease plays major role in viral replication and cellular damage. To understand the mechanism of 3C function in virus infected cells and also development of antiviral agents against the virus, a 3C expressing plasmid was constructed. The cDNA of 3C protease was synthesized using CVB3 infected cells through reverse transcription process and was cloned in pcDNA3.1-. The constructed plasmid was confirmed by sequencing and restriction enzyme analysis. By transfection of the constructed plasmid into HeLa and MCF-7 cells, we showed that 3C protease induced cell death through multiple converging pathways, such as down regulation of cellular factors and decreasing of mRNA transcripts. This affect on HeLa cells as stronger than MCF-7 cells.

**Keywords:** Protease 3C • Cloning • Cell death • Coxsackievirus B3

### Introduction

Coxsackievirus B3 (CVB3) is a positive single-stranded RNA virus in the genus Enterovirus of the family Picornaviridae. It is one of the six serotypes of coxsackievirus B group and its genome contains a single open reading frame flanked by the 5' and 3' untranslated regions (UTRs). The two virally encoded proteases, 2A and 3C, are the key proteins that control the virus replication cycle (12, 18). The structure of catalytic site of 3C protease is similar to the serine proteinase chymotrypsin; however, 3C protease uses a cysteine instead of serine as the nucleophilic amino acid in the catalytic triad (15, 17). Protease 3C mediates cleavage of TATA-box binding protein (TBP), a transcription factor, which in turn prevents RNA polymerase II-mediated cellular transcription, whereas TBP cleavage by 2A was unable to inhibit the function of TBP (7, 24).

Protease 3C can also cleave ribosome-associated poly (A)-binding protein (RABP), removing its C-terminal domain that is important for interactions with other translation factors (13). Like protease 2A, 3C protease cleaves cellular proteins important in host gene expression. Therefore, these enzymes are attractive therapeutic targets for development of antiviral therapeutic agents (14).

Several picornaviruses, including poliovirus and coxsackievirus, are able to kill cells by apoptosis in a variety of situations (4, 5, 23). This process may provide an efficient way to help virus spread to neighboring cells, to protect progeny viruses from host immune defenses, and to avoid an inflammatory response (3, 22). Virus-induced apoptosis could play a key role in the cytotoxicity associated with several human diseases of viral origin (2, 20).

The mentioned past studies on 3C protease function mainly focused on poliovirus (PV). The recent researches, demonstrated that 3C protease of CVB3 induced apoptosis through multiple converging pathways, such as induced cell death through cleavage or down regulation of cellular factors for translation and transcription (6).

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To more evaluate the 3C function and also to make an in vitro system for studying the effects of anti-proteolytic agents on picornaviruses in the present study, we cloned the CVB3 cDNA segment encompassing the 3C coding region and then HeLa and MCF-7 cells were transfected with the constructed plasmid. After transfection, we showed the cells morphological changes by phase contrast microscopy and measured mRNA transcripts by Semi-quantitative RT-PCR.

### Materials and methods

#### Cells and virus

HeLa and MCF-7 cells were cultured in DMEM (Dubelcco Minimal Eagle Medium)(Gibco,UK.) supplemented with 10% fetal bovine serum(FBS) (Gibco), 0.2% sodium bicarbonate (sigma) and 100 IU/ml penicillin. HeLa cell line was used for CVB3 propagation (Nancy strain, American type tissue culture number VR-30).

#### Bacterial strains and plasmids

Escherichia coli DH5 $\alpha$  was used as a host during the cloning experiments and utilized for propagation of the plasmids. The expression vector pcDNA3.1 (Invitrogen, USA), was used for cloning and expression of truncated CVB3 3C protease gene.

#### Viral RNA preparation

HeLa cells at 80% confluency were infected with CVB3 at moi of 10 PFU in a medium containing 1% FBS. Infected cells were collected after 24 h by centrifugation at 300  $\times$ g for 10 min. Total RNA was extracted by the guanidine isothiocyanate based RNX-plus solution (SinnaGen INC., IRI) according to the manufacturer protocol as described elsewhere (11).

#### DNA recombinant technology

All molecular techniques including PCR and cloning were performed according to the standard methods (21). Briefly, after RNA extraction the CVB3 3C cDNA was synthesized by RT-PCR, using random hexamers. The flowing primers were used for amplification of 3C cDNA: 3Cf (5'-CAAGCAAATGCAAGG-3') and 3Cr (5'-CTCTCAATAAATTATATTTCACC-3'). The BamHI and XbaI sites and start and stop codons were included at the 5' ends of forward and reverse primers, respectively. After digestion with BamHI and XbaI, the PCR product was inserted into the BamHI and XbaI sites of the vector pcDNA3.1-under the control of CMV promoter. The resulted construct was verified by colony PCR, restriction

enzyme analysis and DNA sequencing ( SEQLAB, Germany).

#### Cell transfection

HeLa and MCF-7 cells grown in 6-well plates were transfected with the plasmid (pcDNA3C) using Lipofectamin2000 (Invitrogen,USA). 3C-less plasmids-transfected cells were used as negative controls. The transfected cells were examined for morphological changes at 24, 48 and 72 h post transfection.

#### Multiplex RT-PCR amplification and Semi-quantitative analysis

Total RNA was isolated from cells 36h after transfection using RNX-plus solution.  $\beta$ 2 microglobulin mRNA was used as an indicator for total mRNAs isolates.

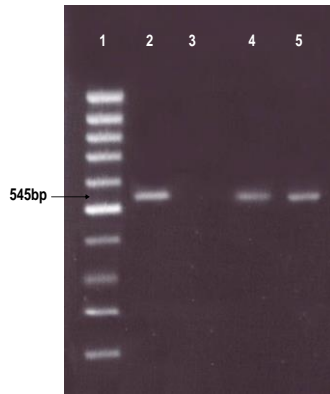
To compare the amount of  $\beta$ 2 microglobulin mRNA with a known concentration of RNA in semi-quantitative RT-PCR test, 1 $\mu$ g of entro virus RNA was added to each sample before RNA extraction. PCR amplification was performed with two sets of specific primers in one tube included the sense enterovirus (5' ctg aat gcg gct aat cc 3'), anti sense enterovirus (5' tgt cac cat aag cag cca 3'), sense b2m (5' tct ggg ttt cat cca tcc 3'), anti sense b2m (5' tac ctg tgg agc aac ctg 3'). PCR products were run on 2% agarose gel and photographed on top of a 280nm UV light box after ethidium bromid staining. The gel images were digitally captured with a CCD camera and analyzed with the National Institutes of Health (NIH) Imager beta version 2 programs.  $\beta$ 2 microglobulin mRNA content of each sample was measured semi quantitatively by band densitometry ratio of  $\beta$ 2 microglobulin to enterovirus bands.

### Results

#### PCR of 3C gene

Coxsackievirus B3 was propagated in HeLa cells and the virus was harvested after 24h while maximum cytopathic effect was observed. The titer of harvested virus was determined to be 10<sup>-5</sup> TCID50/ml when calculated through Spearman-Kärber formula.

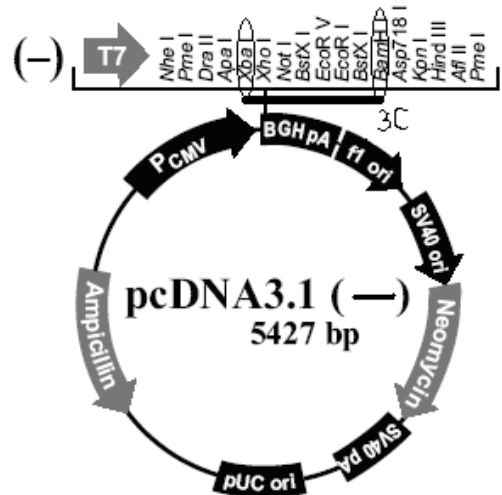
PCR was performed using designed specific primers to obtain the 3C gene and the product of PCR (a 545 bp band) was evaluated through electrophoresis on 0.8% agarose gel (Fig. 1).



**Fig. 1:** Gel electrophoresis of PCR product of 3C amplification.

Cloning of coxsackievirus B3 cDNA encoding 3C protease plasmid construction was done with reference to the CVB3 restriction map (Fig. 2) derived from the nucleotide sequence reported by Lindberg et al (16), and constructed plasmid was checked by colony PCR and restriction enzyme map analysis.

DNA sequence of the viral cDNA in pcDNA3.1- was verified by sequencing (SEQLAB, Germany). Morphological changes induced in cells expressing CVB3 3C protease to determine whether CVB3 3C protease expression affects cell viability, HeLa and MCF-7 cells were transfected with the constructed plasmid and cell morphological changes were observed by inverted microscope (Fig. 3). The



**Fig. 2:** Diagrams of the plasmid pcDNA.1- which used for the insertion of desired 3C gene.

appearance of cell shrinkage and loss of cell adherence began after 24h for HeLa cells and 36h for MCF-7 cells. After 96h most cells had died, as evidenced by the detached and rounded cells floating in the culture media. Cell death after post transfection of HeLa cells were more than MCF-7 cells.

HeLa and MCF-7 cells transfected with CVB3 3C protease gene were observed by invert microscope at the indicated time point post transfected. 3C-less plasmid transfected and untransfected cells were used as control.

**Semi-quantitative analysis**

Decreasing of mRNA was measured semi-quantitatively by the band densitometry ratio of

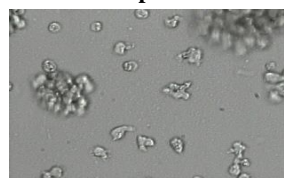
**3C-less plasmid HeLa cells**



**24h pt**



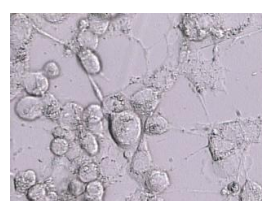
**96h pt**



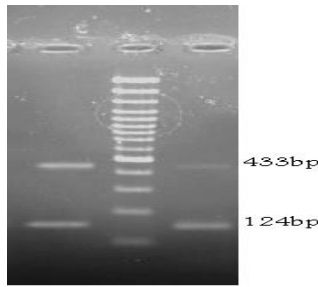
**nontransfected**



**MCF-7 cells**



**Fig. 3:** Expression of CVB3 3C protease induced HeLa and MCF-7 cell death.



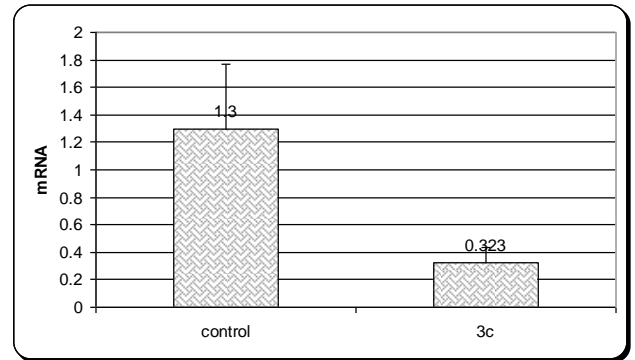
**Fig. 4:** Multiplex PCR was performed with enterovirus and  $\beta 2m$  set primers in one tube. The size of enterovirus and  $\beta 2m$  PCR product are 433 and 124 bp, respectively.

$\beta 2m$  to enterovirus (Fig. 4). Values of  $P \leq 0.05$  were taken as significant (Fig. 5). The decrease of mRNA content for both cells was almost equal.

### Discussion

Coxsackievirus B3 is an important human pathogen that has been associated with severe diseases. Clinically, coxsackievirus infections are known to be associated with acute and chronic viral myocarditis as well as pancreatitis (1, 18). It is well demonstrated that the picornavirus proteases 2A and 3C cleave cellular proteins involving in translation like the translation initiation factor eIF4G (9), and the poly (A) binding protein (10), leading to a host translation shut-off. Two major and distinct modes of eukaryotic cell death are known: apoptosis and necrosis (8). Apoptosis is a common cellular response to a variety of viral infections which promotes virus release from apoptotic host cells results in the spread of virus particles (19). Recent study showed that the 3C protease of CVB3 induces apoptosis through multiple converging pathways (6).

In the present study the 3C gene of CVB3 was used to study the proteolytic effect of 3C which is conserved among all picornaviruses. The cDNA from CVB3 3C protease was prepared and cloned into eukaryotic expression vector, and then the constructed plasmid was transfected into HeLa and MCF-7 cells. Regarding the fast growing nature of these cells, HeLa and MCF-7 were chosen for better monitoring of cell changes. Investigation of the effect of the plasmid on the cell lines showed that cell death begun after 24h post transfection. We showed this effect by cell morphological changes and semi-quantitative RT-PCR. These results suggest that although 3C changes protein content of cells (7, 13), it may affect mRNAs level



**Fig. 5:** The  $\beta 2m$  mRNA level in transfected cells. The results showed that mRNA level in transfected cells (3c) has significantly decreased comparing to non-transfected cells (control).

by probable degradation of proteins involving in mRNA processing.

The results of this study, suggest that 3C protease is thought to be a suitable target for antiviral chemotherapy.

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