Investigation(s) on the HCMV miRNA's role on cellular apoptosis

SUMMARY OF THE THESIS

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SUMMARY

Human Cytomegalovirus (HCMV) is a ubiquitous human pathogen, that belongs to *β-herpesvirus* subfamily, which causes lifelong latent infection in the human host. It is an enveloped virus containing linear double-stranded DNA (~235Kb) as a genome with an estimated global seroprevalence of 83 percent in the general population. Upon primary infection, it establishes lifelong latency after initial infection. Though the HCMV infections are asymptomatic in healthy individuals, they cause significant morbidity and mortality in immune naïve and immunosuppressive individuals such as HIV patients, organ transplant recipients, and autoimmune, cancer patients. Its infection causes infectious mononucleosis, glandular fever, intestinal pneumonia, gastroenteritis, retinitis etc. The long latent infections of HCMV in humans suggest that the virus exists despite the broad antiviral immune arsenal against the virus in the human body. This is due to the several evasive strategies of the virus, as it was thought that it is co-evolved with its human host.

The HCMV evades the antiviral responses of humans through its proteins, accumulating evidence shows even the HCMV encoded miRNAs also took part in this regulation. The existence of HCMV miRNAs came from the pioneer studies of Grey et al. in the year 2005, and later on, other miRNAs were reported. As per the miRbase release 22.1, October 2018, the HCMV was reported to encode 26 mature miRNAs from 15 precursor miRNAs. These miRNAs regulate viral as well as cellular gene expressions and help the virus survival in the human body. The 1st report on the regulation of human genes by the HCMV miRNA came from the studies of Stern-Ginossar et al 2007, where the hcmv-miR-UL-112 targets the immune system gene MICA (MHC class-I polypeptide-related sequence A), in 2009 the same miRNA targets another gene MICB by the same group. In 2013, Wang et al., demonstrated the antiapoptotic activity for the hcmv-miR-UL148D, and in the year 2014 our lab also predicted the antiapoptotic activity to the HCMV miRNAs, hcmv-miR-UL70-3p and miR- UL148D.

So, we initiated this study with the intent to identify the antiapoptotic HCMV miRNAs and their respective apoptotic target genes. The study is broadly divided into two parts, 1st part comprises in *silico* studies where by using various bioinformatic tools we predict the potential antiapoptotic HCMV miRNAs and the potential apoptotic target genes. In the 2nd part by using *in vitro* studies, we evaluate ours *in silico* predictions. To determine the functions of the HCMV miRNAs on the basis of orthologous nature, we examined the sequence similarity between the HCMV miRNAs and human miRNAs using an online alignment tool “T-Coffee” (global). The alignment studies show though the alignment scores between the human and HCMV miRNAs

are “100”, their sequence similarity is not more than “13” and to not continuous. This result suggests that no HCMV miRNA shares the sequence similarity with the human miRNAs, and we were unable to speculate the HCMV miRNA functions on an orthologous basis.

Then we searched the literature related to the HCMV miRNA functions and found that they may target cellular pathways including apoptosis. So, we focussed on the identification of HCMV miRNAs that can target apoptotic genes. So, we retrieved the apoptotic genes from the web (through “death database” and “Thanatos”) and their respective 3'UTRs from the UTRdatabase and the HCMV miRNAs from the miRbase. Then by using miRNA target prediction algorithms such as RNA hybrid and RNA 22, we searched for the potential binding sites for the HCMV miRNAs in the 3'UTR regions of apoptotic gene mRNAs. The results show the hcmv-miR-UL70-3p has potential binding sites in the 3'UTR regions of apoptotic genes, MOAP1, BAK1, TNFRS10C and Caspase 9. The hcmv-miR-UL148D has potential binding sites in the 3'UTR of ERN1 genes.

Once, we identified the potential HCMV miRNAs and their respective cellular targets, we initiated *in vitro* studies using HEK293T cells to validate our *in silico* predictions. The HCMV miRNA targets predicted were to be the apoptotic genes, so, we induced apoptosis in HEK293T cells with H_2O_2 and staurosporine and evaluated the effect of hcmv-miR-UL70-3p and miR-UL148D. The apoptotic induction and inhibitions were evaluated through characteristic features of the apoptotic cell such as chromatin condensation, nuclear fragmentation by cell nucleus staining by DAPI, and membrane ruffling/blebbing by the scanning electron microscopy, Caspase 3/7 activity by Caspase Glo 3/7 assay and membrane flipping by flow cytometry. The results show on H_2O_2 (0.4mM; 5h exposure) treatment, the apoptotic cell characters appear in the cells indicating the apoptosis induction, while on hcmv-miR-UL70-3p treatment there were significant reductions in these characters. Further, when we block the effect of hcmv-miR-UL70-3p through its inhibitor, the apoptotic characters in the cells reappear, suggesting that the HCMV miRNA inhibits the H2O2-induced apoptosis. We further searched for the functional target for this HCMV miRNA, while doing so, we considered our *in silico* results, which predicts, modulator of apoptosis-1 (MOAP1) mRNA is the functional target for the hcmv-miR-UL70-3p. The ectopic expression of this HCMV miRNA significantly reduced the MOAP1 mRNA. This miRNA binding to the 3'UTR of MOAP1 is validated/confirmed with the dual-luciferase reporter assays using the wild and deleted 3'UTR of MOAP1vector constructs (pEZX-MT06-3'UTR$^{WILD/DEL}$ - MOAP1). The western blotting studies also show a significant reduction of MOAP1 protein in the cells treated with hcmv-

miR-UL70-3p suggesting the translational repression of MOAP1 by the HCMV miRNA. In addition, we compared the inhibitory effects of hcmv-miR-UL70-3p and siRNA of MOAP1 on the expression of MOAP1 mRNA and protein showing both the siRNA and miRNA downregulate mRNA and protein levels of MOAP1, however the siRNA exhibits higher levels of inhibitions. These results demonstrate that hcmv-miR-UL70-3p attenuates the H2O2-induced apoptosis by the translational repression of MOAP1.

The *in silico* studies predict another HCMV miRNAs i.e., hcmv-miR-UL148D targets the ER-stress signaling molecule ERN1. So, to evaluate these results we used the staurosporine which can induce ER-stress as well as ER-stress induced apoptosis, and evaluated the effect of hcmv-miR-UL148D on staurosporine-induced apoptosis through microscopy, Cas 3/7 activities and flow cytometry. The results how the ectopic expression of this HCMV miRNA significantly downregulated the staurosporine-induced apoptosis. The qRT-PCR results show in the presence of this miRNA the ERN1mRNA expressions were downregulated suggesting that it is the functional target. With the help of dual-luciferase assays we confirmed the hcmv-miR-UL148D binding to the 3'UTR of ERN1 mRNA. The ERN1 gene encodes IRE1 protein which exists in 2 isoforms in humans, i.e., IRE1α and IRE1β. So, we evaluated the effect of hcmv-miR-UL148D on the protein level, i..e, IRE1 α protein and found that this protein is significantly downregulated in the presence of hcmv-miR-UL148D. This result suggests that the ectopic expression of hcmv-miR-UL148D attenuates the staurosporine-induced apoptosis in HEK293T cells by translational repression of ERN1mRNA. The ERN1encoded IRE1α plays an important role in deciding the fate of the cell, i.e., survival or death. Upon ER stress the IRE1α activates the pathway of XBP1 splicing leading to the XBP1 protein which is a transcription factor for the transcription of Endoplasmic reticulum-associated protein degradation (ERAD), thereby relives the ER-stress. Upon prolonged UPR, the same molecule switches another pathway where by combining with TRAF2-p38 and phosphorylate JNK leading to the ER-stress induced apoptosis. We analyzed the levels of XBP1 splicing and JNK-phosphorylation in the presence and absence of hcmv-miR-UL148D and found they were reduced when compared to the controls. These results indicate that though the home-miR-UL148D doesn't directly target either XBP1 mRNA or the JNK mRNA, however by reducing the IRE1α levels by repressing the ERN1 mRNA, it could regulate the ER-stress induced apoptosis.

In summing up, through this study we have demonstrated the antiapoptotic activities of HCMV miRNAs, i.e., hcmv-miR-UL70-3p and miR-UL-148D in HEK293T cells, and their functional

targets are identified to be MOAP1 and ERN1. Based on these results we can say that these HCMV miRNAs might regulate the cellular apoptosis during the infection in humans and pave a conducive environment for viral survival in the human body.

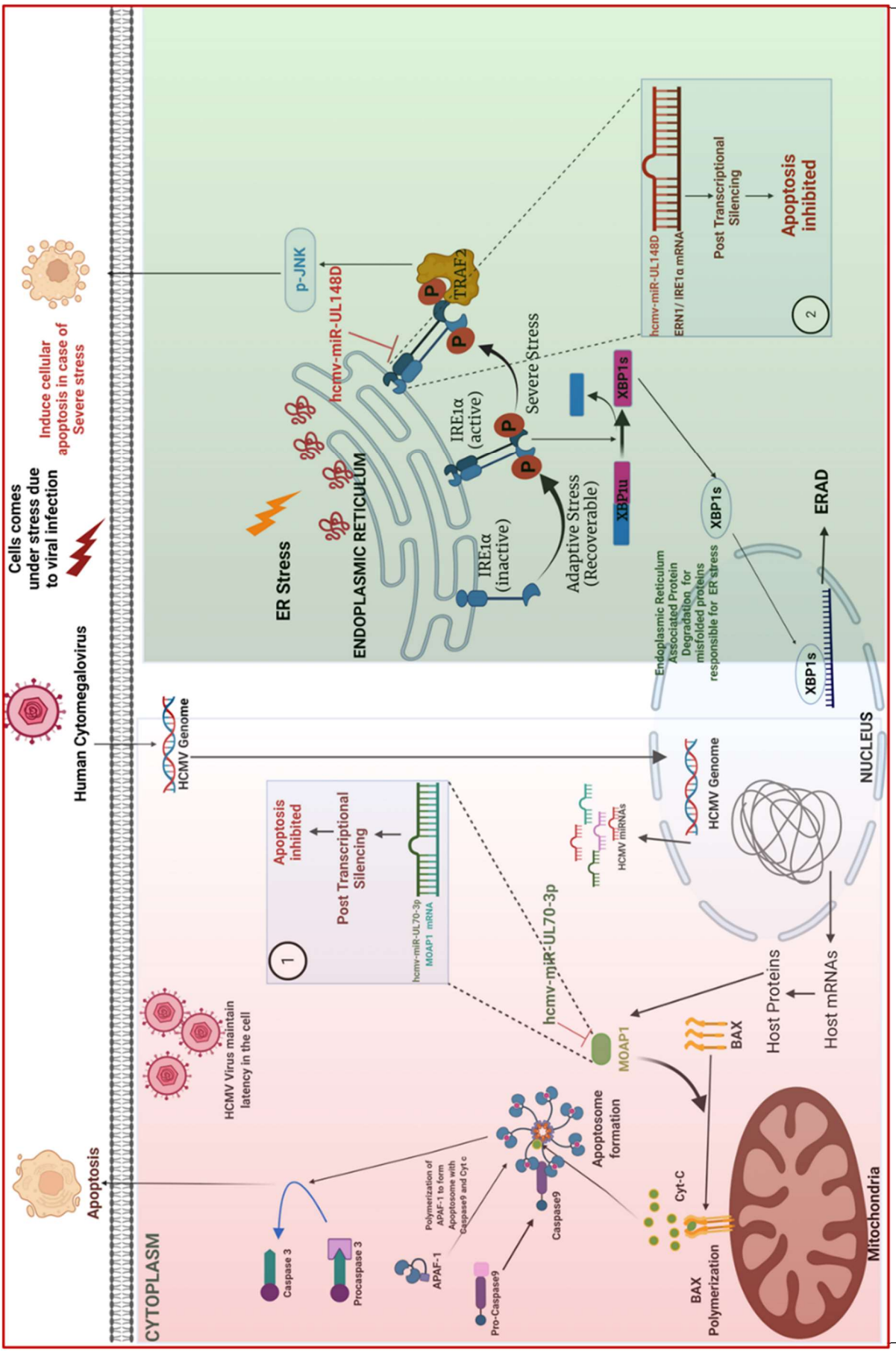

Figure: Role of hcmv-miR-UL70-3p & hcmv-miR-UL148D encoded by Human Cytomegalovirus on cellular apoptosis process in humans.