Identification and functional analysis of anti-autophagic HCMV miRNAs

SUMMARY OF THESIS

SUBMITTED TO

BABASAHEB BHIMRAO AMBEDKAR UNIVERSITY
LUCKNOW

FOR THE DEGREE OF

Doctor Of Philosophy

IN

BIOTECHNOLOGY

Submitted By

RAJ KUMAR KHALKO

(Enrollment No: 1399/16)

Under the Co-Supervision of

Dr. G SUNIL BABU
Associate Professor

Under the Supervision of

Prof. SANGEETA SAXENA
Professor

DEPARTMENT OF BIOTECHNOLOGY
SCHOOL FOR BIOSCIENCES AND BIOTECHNOLOGY
BABASAHEB BHIMRAO AMBEDKAR UNIVERSITRY
(A CENTRAL UNIVERSITY) (NAAC A++ ACCREDITED)
VIDYA VIHAR, RAEBARELI ROAD, LUCKNOW-226025
UTTAR PRADESH, INDIA

2024

SUMMARY

Human Cytomegalovirus (HCMV), is a ubiquitous human herpesvirus (HHV-5) of the *β-herpesvirinae* subfamily that establishes lifelong latent infections in humans with varying seroprevalence rates ranging from 40% to 100% globally. Though the infections in immunocompetent individuals are asymptomatic, it cause significant clinical challenges in immunocompromised individuals such as HIV/AIDS patients, transplant recipients, people undergoing immunosuppressive therapy and immune naïve individuals. Reactivation in these groups of populations can lead to life-threatening complications. HCMV contains double-stranded DNA as its genome and is ≈235 kb. It is an enveloped virus, showing a proteinaceous icosahedral capsid with a triangulation number (T) of 16. An additional tegument layer surrounds the capsid. Its transmission primarily occurs through contact with bodily fluids, manifesting a spectrum of diseases, including infectious mononucleosis and pneumonia. It causes sensorineural hearing loss and motor deficits in congenital infection in neonates. The virus's ability to establish lifelong infections is partly due to its ability to diversify or subvert innate and/or adaptive immune evasion strategies. This virus dedicated a significant portion of its genome for the above purpose and exhibited various evasive mechanisms, thereby increasing its survival in its human host.

HCMV exhibits a multifaceted subtle mechanism to subvert host immune surveillance, and these strategies are orchestrated by its proteins, RNAs and microRNAs (miRNAs). The existence of HCMV miRNAs was first reported by Pfeffer et al. (2005) and Grey et al. (2005) simultaneously, and now it is known that it can encode 26 mature miRNAs from 15 precursors (miRbase, version 22.1). Several groups discovered the functions of these miRNAs and reported that they exert a regulatory influence on the host's gene expression machinery, fostering an environment conducive to viral survival within the human body. The first identified HCMV miRNA, hcmv-miR-UL112-1 (Grey et al. 2007), was reported to regulate viral replication through translational inhibition, subvert the immune response by targeting and downregulating human protein MICB (Nachmani et al., 2010). Wang et al. (2013) demonstrated that miR-UL148D downregulates IEX-1, promoting anti-apoptosis. We combined with bioinformatics and *in vitro* studies to demonstrate the antiapoptotic and anti-autophagic function of the HCMV miRNAs, hcmv-miR-UL70-3p and miR-UL148D.

This present investigation was intended to identify and functionally characterise HCMV miRNAs with anti-autophagic activity. We adopted both the *in slico* and *in vitro* approaches in predicting and conforming the anti-autophagic functions of HCMV miRNAs. The human autophagy genes included in this study were taken from the Human Autophagy Database (HADb), and their corresponding 3'UTRs from the UTR database (http://utrdb.ba.itb.cnr.it) and the HCMV miRNAs were sourced from miRbase (http://miRbase.org). By using the miRNA target prediction algorithms such as RNAhybrid and RNA22, we probed the potential binding sites for HCMV miRNAs in the 3'UTR regions of autophagy gene mRNAs. The results show that hcmv-miR-UL70-3p has potential binding sites in the 3'UTR regions of autophagy-related genes such as ATG9B, ATG9A, ATG16L2, SQSTM1, EIF2AK2, STK11, MAP2K7, and MAPK1, and the hcmv-miR-US22-3p has potential binding sites in the 3'UTR of the ATG4B gene.

After that, we validated our *in silico* predictions in HEK293T cells using rapamycin as an autophagy-inducing agent. The autophagy induction and inhibitions were analysed by detecting the autophagy markers such as LC3 puncta through fluorescence microscopy, autophagosomes through transmission electron microscopy (TEM) and LC3A/B (autophagy flux) by flow cytometry. Our experimental framework categorised into various groups, including positive and negative controls along with the test groups, i.e., cells transfected with hcmv-miR-UL70-3p followed by the treatment of rapamycin and other test group heaving the cells transfected with hcmv-miR-UL70-3p mimics along with the equimolar concentration of its sequence-specific inhibitor followed by the treatment of rapamycin for autophagy induction. The ectopic expression of hcmv-miR-UL70-3p significantly downregulated the rapamycin-induced autophagy in HEK293 T cells. This effect was further confirmed with the sequence-specific inhibitor of hcmv-miR-UL70-3p. The autophagy flux measured through flow cytometry (LC3-A/B measurements), LC3puncta through confocal fluorescent microscopy and the number of autophagosomes through Transmission electron microscopy followed the same trends, confirming the anti-autophagic activity of hcmv-miR-UL70-3p.

The above experiments substantiated the anti-autophagic activity of hcmv-miR-UL70-3p; however, it was not clear what the autophagy target genes are for this HCMV miRNA. In order to interrogate the above question, we performed qRT-PCR analysis for the *in silico* predicted autophagy gene mRNAs expression in the presence and absence of hcmv-miR-UL70-3p upon rapamycin treatment. Though all the mentioned autophagy gene mRNAs show downregulation

upon hcmv-miR-UL70-3p treatment, the downregulation of ATG9A levels was more pronounced compared to the others. Further, the rapamycin-induced higher expression of ATG9A mRNA levels in HEK293T cells. So, we continued our study with ATG9A and the binding site predicted in the 3'UTR of ATG9A by hcmv-miR-UL70-3p was confirmed through dual-luciferase reporter assays using the 3'UTR of ATG9A vector constructs having binding sites and deleted binding sites (wild type- pEZX-MT06-3'UTR-ATG9A^{WT} and the deleted type-pEZX-MT06-3'UTR-ATG9A^{DEL}). We further investigated whether the hcmv-miR-UL70-3p binding to the 3'UTR of ATG9A leads to the translational repression of the protein through western blotting.

These results confirmed that rapamycin-induced autophagy was inhibited by hcmv-miR-UL70-3p by targeting and repressing ATG9A. These findings are in accordance with our in silico predictions, which indicated that hcmv-miR-UL70-3p has a potential binding site in the 3'UTR of ATG9A. Many studies have reported that autophagy impairment is mediated by various human miRNAs targeting ATG9A. For example, hsa-miR-34a impairs autophagy flux by repressing ATG9A (Pang et al., 2017), and miR-96-5p regulates autophagy by targeting ATG9A (Eroglu et al., 2020). We further investigated sequence homology among the miRNAs targeting ATG9A. TargetScan shows 23 different human miRNAs with target sites in the 3'UTR of ATG9A. The sequence homology search between these 23 conserved human miRNAs and the HCMV miRNA (miR-UL70-3p) revealed a relatively low degree of similarity, showing the complexity in the targeted silencing mechanism of miRNAs, whether human or viral origin. Additionally, functional enrichment analysis of ATG9A indicated its predominant involvement in autophagy-related processes.

In summing up, we have demonstrated the anti-autophagic activity of hcmv-miR-UL70-3p in HEK293T cells and ATG9A as its functional target.

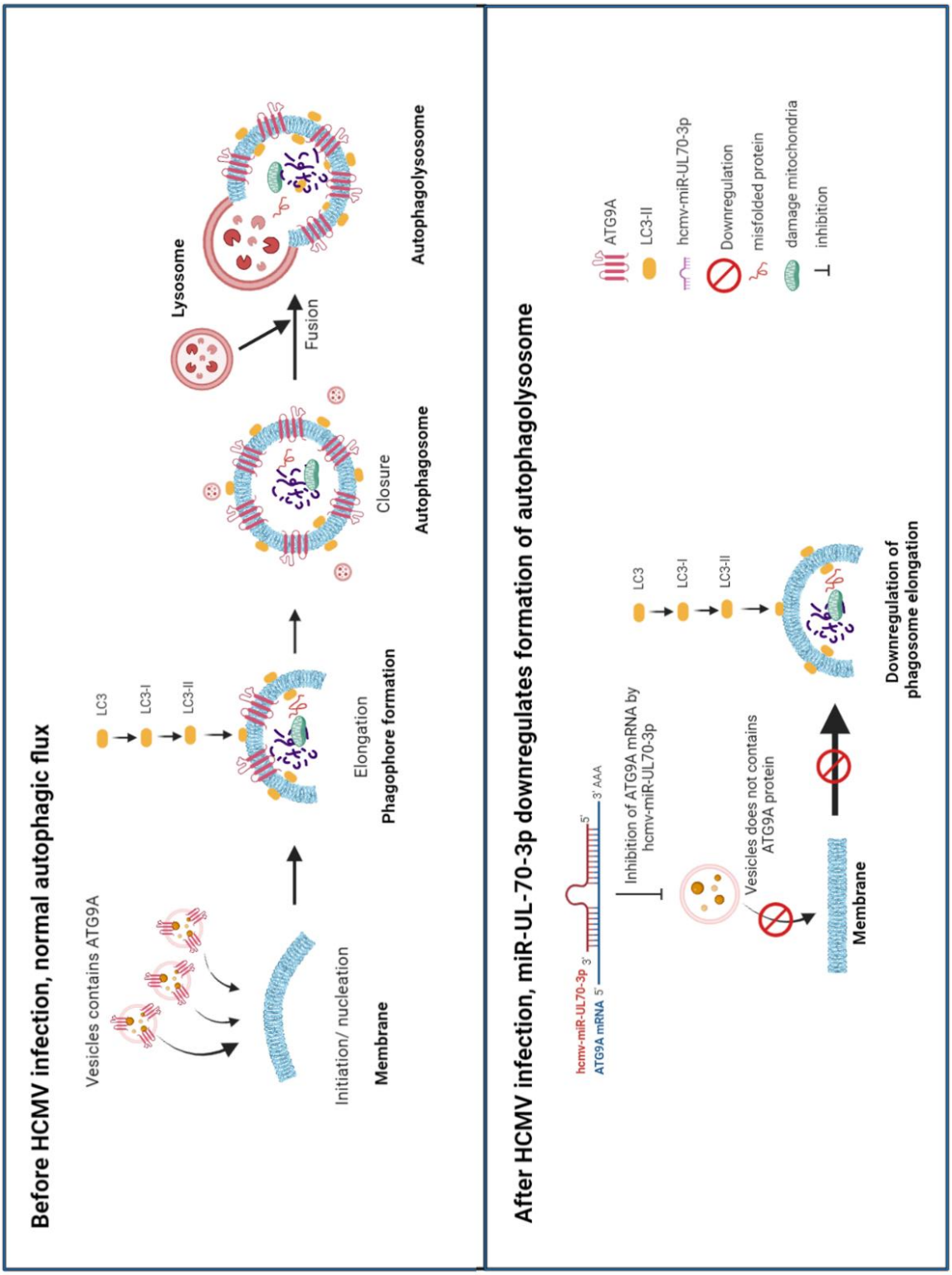

Figure: This graphical abstract represents the role of hcmv-miR-UL70-3p targets the ATG9A during infection and modulate the cellular autophagy for conducive environment for viral survival in the host.