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Upregulation of Noxa and p53 upregulated modulator of apoptosis (PUMA) in response to Zika virus infection

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Abstract

Zika virus (ZIKV) is a single stranded RNA virus that belongs to the genus *Flavivirus*, family *Flaviviridae*. The symptoms associated with ZIKV are normally a mild fever, headache, rash and conjunctivitis, but after 2015, ZIKV became a public health concern worldwide because of the occurrence of microcephaly in newborns from mothers infected with Zika virus during pregnancy. Virus replication inside host cells causes accumulation of disordered proteins, and exploitation of ER membranes can cause ER stress that leads to anti-viral defense mechanisms and cell death. Noxa and p53 upregulated modulator of apoptosis (PUMA) belong to Bcl-2 homology domain (BH3) family and induction of both Noxa and PUMA is mediated through p53 in prolonged ER stress conditions. As a recently emerging virus, little is known about strain specific manifestations of this virus and whether ZIKV associated with cases of microcephaly are functionally distinct from normal circulating virus remains unclear. Therefore, this study aimed to analyze expression of Noxa and PUMA in A549 cells after infection with two different Thai strains of Zika virus, namely SV0010/15 isolated from a Thai case of Zika fever, and MU1-2017 isolated from the brain tissue of a fetus aborted for reasons of abnormal development including microcephaly. Results showed significant increase in expression of Noxa and PUMA in A549 cells infected by both viruses, compared with mock infected cells. This shows that ZIKV infection in A549 cell induces p53 mediated ER stress that may lead to apoptosis.

Keywords**:** *Apoptosis, ER-stress, expression of Noxa and PUMA, NOXA, PUMA, Zika virus*

1. Introduction

Zika virus (ZIKV) is a recently emerged, arthropod borne *Flavivirus* belonging to the family *Flaviviridae* that is transmitted by the bite of an infected female *Aedes* (*Ae*.) species of mosquito (*Ae. aegyptii* or *Ae. albopictus*). The genome of ZIKV consists of a single strand, positive sense RNA molecule of 10.8 kilobases and consists of a single open reading frame that encodes 3 structural proteins and 7 nonstructural proteins (Fleming, Ding, Alenko, & Burrows, 2016).

ZIKV was first isolated from Zika forest in Uganda in 1947 from a Rhesus monkey and was subsequently isolated from mosquitoes from the same forest in 1948 (reviewed in Wikan & Smith, 2016). When a person gets infected with ZIKV, the symptoms usually seen are headache, rash and fever. Severe complications include Guillain-Barré syndrome in adults and microcephaly in infants born to women who were infected when pregnant.

When a virus enters inside the host cells, it hijacks the host cell machinery for viral transcription and translation in order to replicate. During the virus replication cycle, the accumulation of misfolded or unfolded proteins or exploitation of the ER membrane can cause ER stress that leads to anti-viral defense mechanisms and cell death. Accumulation of unfolded proteins inside the endoplasmic reticulum mediates the unfolded protein response (UPR) signaling pathway which involve three major molecules PERK, IRE1 and ATF6 to restore ER-homeostasis (Jheng, Ho, & Horng, 2014). However, failure in restoring ER homeostasis leads to induction of apoptosis by expression of CHOP (C/EBP homologous protein) through its interaction with anti-apoptotic B – cell lymphoma 2 protein (Bcl-2) which activates intrinsic apoptosis pathway. There are two major apoptosis pathways, extrinsic or death receptor pathways which is induced by interaction of ligand with death receptors present on the cell surface and intrinsic or mitochondrial pathway which is mediated by internal stimuli such as growth factors, cytokines, toxins, hormones, free radicals and viral infection.

Noxa (also known as Phorbol-12-myristate-13-acetate-induced protein) and p53 upregulated modulator of apoptosis (PUMA) both belong to the Bcl-2 homology domain (BH3) family of proteins, and induction of both Noxa and PUMA is mediated through p53 under prolonged ER stress conditions. Overexpression of Noxa and PUMA activate pro-apoptotic BAK/BAX resulting in release of cytochrome C from mitochondria into the cytosol. The released cytochrome C bind to Apaf-1 which recruits pro-caspase 9 which form apoptosome, that activates caspase 9 and mediates the downstream effector caspase activity (Li, Lee, & Lee, 2006).

In the study conducted by Li and colleagues, Noxa and PUMA activation under ER-stress conditions contributed to ER stress induced apoptosis in genetically deficient mouse embryo fibroblasts (Li, Lee, & Lee, 2006). Similarly, ZIKV infection has been shown to induce ER-stress and finally leading to apoptosis in ZIKV infected progenitor cells as well as mouse embryos (Gladwyn-Ng et al., 2018). ZIKV infection leading to apoptosis is also shown in human umbilical vein endothelial cells (HUVECs) (Anfasa et al., 2019). Studies have also shown that ZIKV infection activates the UPR in both *in vivo* and *in vitro* studies (Tan et al., 2018). This shows that there are several studies conducted that have investigated ZIKV infection leading to apoptosis in both *in vitro* as well as *in vivo* model and also in different cell types. However, little is known about the apoptosis process as well as expression of Noxa and PUMA with two different strains of ZIKV, one associated with case of microcephaly and another normally circulating virus. The virus selected for investigation were Zika SV0010/15 (ZIKV-15) isolated from the serum of Thai with self-limiting Zika fever, and Zika MU1-2017 (ZIKV-MU1) isolated from an aborted fetus with ZIKV associated abnormalities (Thai strain) and cell lines used was A549 (Human lung carcinoma cell line) cells. The result shows significant increase in expression of NOXA and PUMA in response to infection for both strains of ZIKV.

2. Objectives

The major objective of this study was to analyze the expression of Noxa and PUMA in A549 cells infected with two different Thai strains of ZIKV.

3. Materials and methods

3.1 Cell and virus culture

A549 (Human lung carcinoma cell line) cells were used in this study. A549 cells were grown and maintained in Dulbecco's modified eagle's medium with 10% heat-inactivated fetal bovine serum (FBS) and 100 units/ml of penicillin/streptomycin (PAA Laboratories GmbH, Pasching, Austria). Cells were incubated at 37°C with 5% CO_2.

Virus: Zika SV0010/15 (ZIKV-15) isolated from the serum of Thai with self-limiting Zika fever, and Zika MU1-2017 (ZIKV-MU1) isolated from an aborted fetus with Zika virus associated abnormalities (Thai strain) was used in this study. Virus was propagated in *Aedes albopictus* C6/36 cells at 28°C until the appearance of cytopathic effect. The virus was harvested and stored at -80°C. Sequencing of virus was done and virus titer was determined by plaque assay on Vero cells.

3.2 Virus infection

A day prior to infection, A549 cells were seeded in 6-well plates. At the time of infection, medium was aspirated and cells were inoculated with both Zika SV0010/15 (ZIKV-15) and Zika MU1-2017 (ZIKV-MU1) diluted in ice cold FBS-free DMEM at appropriate multiplicities of infection for 2 hours at 37 °C with gentle rocking in every 10 minutes. After 2 hours, medium was removed and cells were supplemented with pre warmed DMEM enriched with 10% FBS. Cells were then incubated at 37 °C with 5% CO_2 until required. As a control cells were mock infected and were cultured in parallel to each ZIKV-infected cell. All experiments were performed independently in triplicate.

$$\text{MOI (Multiplicity of infection)} = \frac{\text{Volume of virus (µl) X Virus titer (PFU/ml)}}{\text{Total number of cells}}$$

3.3 Quantitation of virus infection

Cells that were mock-infected or infected with ZIKV-15 and ZIKV- MU1 were trypsinized and harvested on the selected day. Flow cytometric analysis was undertaken after staining with mouse monoclonal antibody HB112 and a FITC-conjugated goat anti-mouse IgG antibody (KPL, Guilford, UK) exactly as previously described elsewhere

(Diteepeng, Khongwichit, Paemanee, Roytrakul, & Smith, 2019). All experiments were performed independently in triplicate.

3.4 Cell morphology

A549 cells were seeded at an appropriate cell density. On the next day cells were either mock treated or inoculated with Zika SV0010/15 (ZIKV-15) and Zika MU1-2017 (ZIKV-MU1) independently, diluted in ice cold FBS-free DMEM at appropriate multiplicities of infection for 2 hours. After 2 hours, medium was removed and cells were supplemented with pre-warmed DMEM enriched with 10% FBS. Cells were then incubated at 37 °C with 5% CO_2 until required. Cell morphology was visualized under an inverted microscope with 100X magnification at the appropriate day. All experiments were performed independently in triplicate.

3.5 Semi-quantitative PCR

Total RNA was extracted from mock infected or ZIKV-15 and ZIKV-MU1infected cells by using TRIzol reagent (Ambion, Life Technologies, Waltham, MA). cDNA was synthesized from 1µg of total RNA extracted by using Oligo (dT) primer and RevertAid™ reverse transcriptase enzyme (Thermo scientific™, MA, USA). Specific primers for Noxa, PUMA and β-actin were used to amplify the synthesized cDNA by using DreamTaq™ DNA polymerase. Expression of Noxa and PUMA was quantitated against β-actin using ImageJ analysis software.

3.6 Statistical analysis

All data are plotted and analyzed using GraphPad Prism program version 5 and data are presented as mean ± SEM. The statistical significance was analyzed by independent t-test using PASW statistics 18. For all analysis, p value < 0.05 was considered as statistically significant.

4. Results

4.1 Infection optimization

To be able to compare the apoptosis pathways induced by ZIKV-15 and ZIKV-MU1 in A549 cells, the infection level was first optimized to gain equal percentage of infection in A549 by the two strains of virus. Flow cytometry was performed to determine the percentage of infection. A549 cells were first mock infected or infected with ZIVK-15 and ZIKV-MU1 separately with multiplicity of infection 1, 2, 5 and 10. All experiments were undertaken independently in triplicate. To check the percentage of infection in ZIKV-15 and ZIKV-MU1 infected cells at day 1, 2 and 3 post infection, mouse monoclonal pan flavivirus antibody (HB112) diluted in 1% BSA and FITC conjugated goat anti-mouse IgG antibody was used. The cells were analyzed by flow cytometry using mock infected cells as a negative control and results are shown in Figure 1A. Markedly, the levels of infection seen between ZIKV-15 and ZIKV-MU1 at a common MOI were quite different. To obtain a common level of infection between the two viruses, ZIKV-15 was used at MOI 5, while ZIKV-MU1 was used at MOI 1. As can be seen in Figure 1B, the use of unequal MOI resulted in equal levels of infection, so this condition was used in further experiments.

As shown in Figure 1A, A549 cells were mock infected or infected with ZIKV-15 and ZIKV-MU1 at MOI 1, 2, 5 and 10 were incubated for 1, 2- and 3-days post infection. The percentage of infection was determined by flow cytometry at multiple time points post-infection. Higher percentage of infection is seen in the cells incubated with both the virus for 1 day. In Figure 1B, quantitation of infection in A549 cells infected ZIKV-15 with MOI 5 and ZIKV-MU1 with MOI 1. ZIKV-15 with multiplicity of infection 5 and ZIK-MU1 with multiplicity of infection 1 shows same level of infection in 3 days post infected cells.

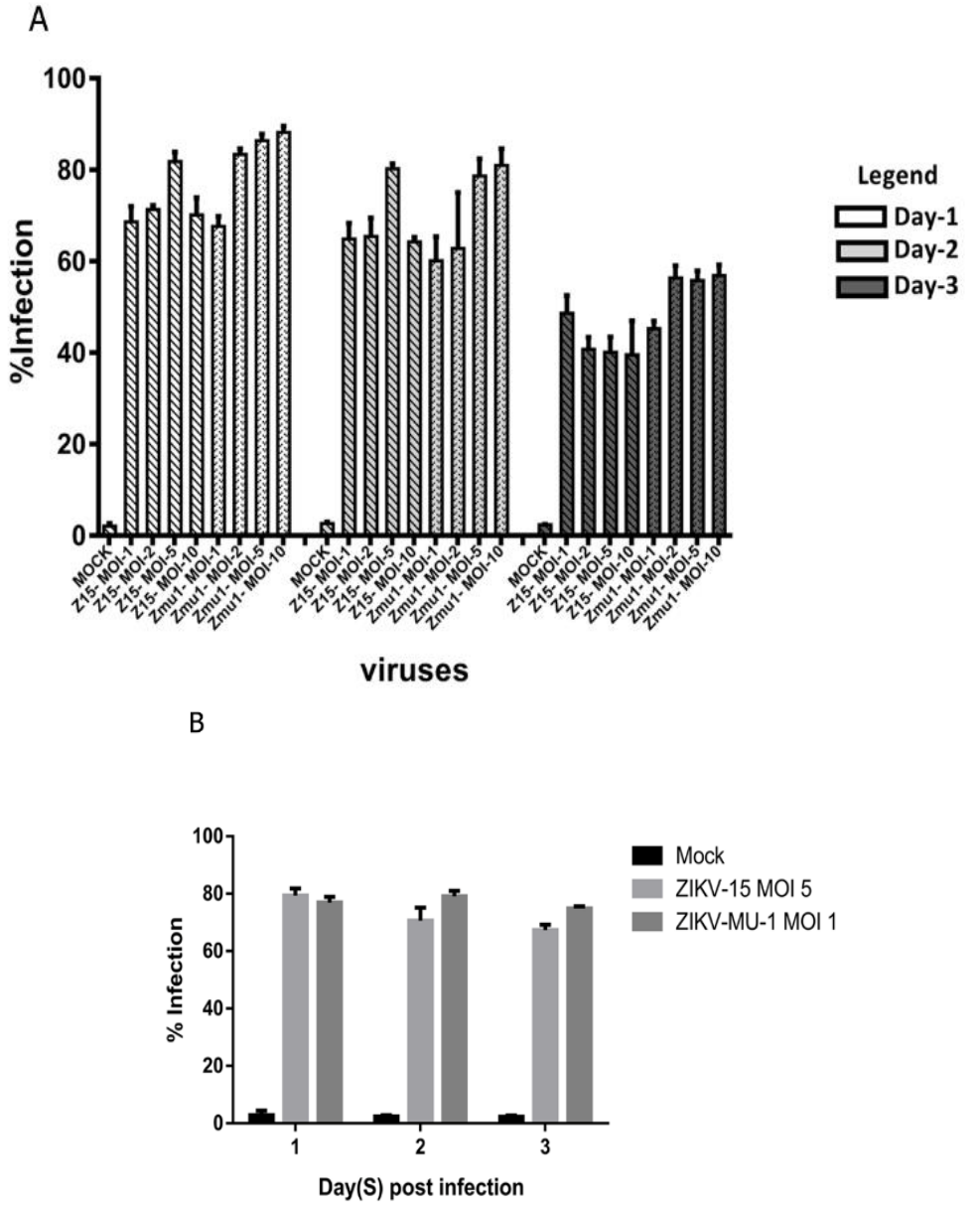

Figure 1 Quantitative analysis of ZIKV-15 and ZIKV-MU1 infection

4.2 Cell morphology

A549 cells were either mock infected or infected with ZIKV-15 and ZIKV-MU1 at m.o.i 5 and 1, respectively and incubated for 1, 2 and 3days post infection. Mock was used as control without infection. ZIKV-15 infected cells showed significant levels of cell detachment from day 1, reaching the highest on day 3 post infection. However, very little cell detachment in day 1 post infected cells when infected with ZIKV-MU1. Cell detachment was higher when post infection time was longer for both strains of virus. This result suggests that these viruses may have different replication strategies, with ZIKV-15 having a fast replication strategy, and ZIKV-MU1 having a slow replication strategy, as has been observed with some dengue viruses (Goh et al., 2016).

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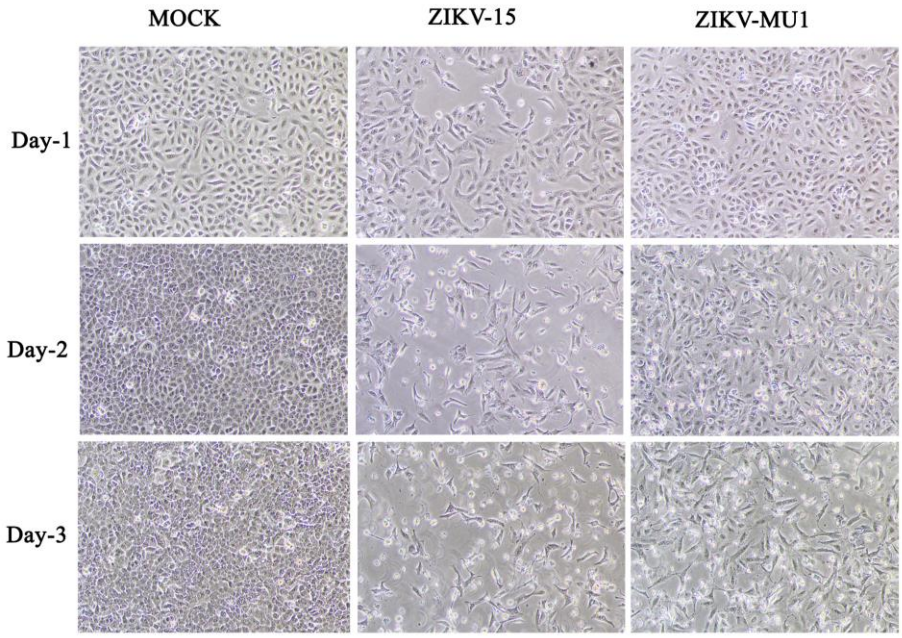

Figure 2 Morphology of ZIKV infected A549 cells

Morphology of A549 cells infected with ZIKV-15 and ZIKV-MU1 with MOI 5 and 1, respectively for 1, 2 and 3days post infection. Higher cell detachment is seen in ZIKV-15 infected cells when compared with ZIK-MU1 infected cells. Cell detachment is higher when duration of infection is longer. Each image from each panel is the representative image of three independent replicates taken under an inverted microscope with a magnification 100X.

4.3 Noxa and PUMA in ZIKV infection

To determine the induction of Noxa and PUMA in ZIKV infected cells, semi-quantitative PCR was undertaken on RNA from A549 cells infected with ZIKV-15 and ZIKV-MU1 at MOI 5 and 1. Results showed significant increases in expression of Noxa and PUMA in A549 cells infected by both viruses, ZIKV-15 and ZIKV-MU1, when compared with mock infected cells. However, no significant difference in expression of Noxa and PUMA was seen when comparing between the two viruses, ZIKV-15 and ZIKV-MU1.

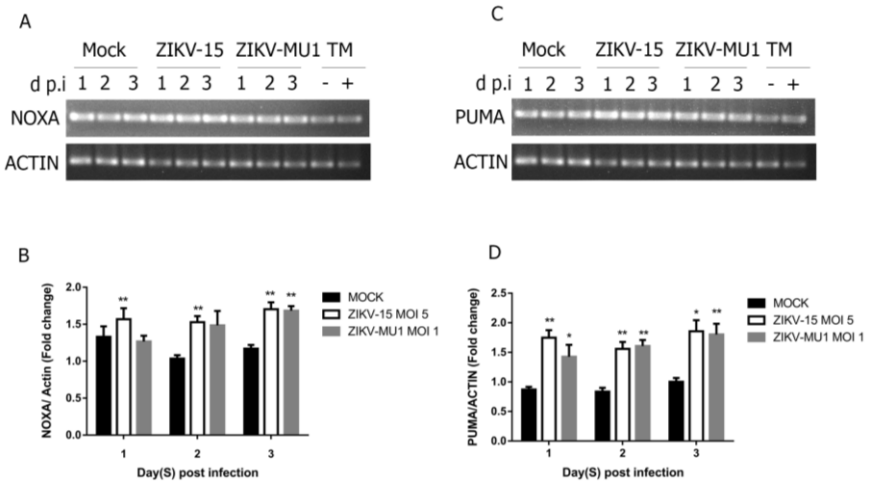

Figure 3 NOXA and PUMA in ZIKV infection

A549 cells either mock infected or infected with ZIKV-15 at m.o.i 5 or ZIKV-MU1 at MOI 1 were examined on days 1-3 p.i to determine the expression of ER stress response genes, Noxa (A, B) and PUMA (C, D) by semi- quantitative PCR. The PCR product was run on 2% agarose gel. The band intensity of Noxa and PUMA was calculated by using ImageJ analyzing software and normalized against β-actin. Error bars represents SEM (*; p value < 0.05)

5. Discussion

ZIKV, in common with other flavivirus replicates in the endoplasmic reticulum of host cells which can generate high polyproteins that can contribute to ER stress and eventually leading to apoptosis. ZIKV infection has been shown to induce ER-stress and finally leading to apoptosis in ZIKV infected neuronal progenitor cells (NPCs) as well as in mouse embryos (Gladwyn-Ng et al., 2018). ZIKV infection leading to apoptosis has also shown in human umbilical vein endothelial cells (HUVECs) (Anfasa et al., 2019). Similarly, Brazilian ZIKV strain H/PF/2013 (Asian lineage) has been shown to causes apoptosis of NPC (Benazzato et al., 2016). Apoptosis has been shown to occur in different cell lines by ZIKV infection as well as by other flavivirus infection. Dengue virus 2 infection of HepG2 liver cells has been shown to increase expression of Noxa and PUMA (Thepparit et al., 2013). Likewise, Chikungunya virus infection of both Hela and HepG2 cells has also shown to increase expression of Noxa and PUMA (Khongwichit et al., 2016).

In this study, expression of Noxa and PUMA was analyzed in A549 cells after infecting with two different strains of ZIKV, Zika SV0010/15 (ZIKV-15) isolated from the serum of Thai with self-limiting Zika fever, and Zika MU1-2017 (ZIKV-MU1) isolated from an aborted fetus with ZIKV associated abnormalities (Thai strain). To be able to compare the expression of Noxa and PUMA in A549 cells after infection with two different strains of ZIKV, infection condition was optimized first in order to gain same percentage of infection. Our result here also shows the upregulation of Noxa and PUMA in A549 cells infected with both strains of ZIKV. Noxa and PUMA are transcriptionally regulated by p53 gene under ER-stress condition which might contribute to apoptosis.

6. Conclusion

This study demonstrates that both (Thai) strains of ZIKV induce expression of Noxa and PUMA by p53 mediated pathways. This suggests that both virus strains can induce ER-stress in cells after infection. Further study is needed to understand the mechanism of ZIKV induced ER stress and apoptosis.

7. Acknowledgement

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