

A Computational Analysis to Construct a Potential Post-Exposure Therapy against Pox Epidemic Using miRNAs *in Silico*

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Abstract

Background: Smallpox was caused by Variola Virus (VARV) and even though it was eradicated in 1979, there exists the possibility of a zoonotic epidemic from Ortho-pox that may be pathogenic to humans. Moreover, VARV may still be used as a bioterrorist weapon. Monkey Pox Virus (MPXV), which is closely related to smallpox, is endemic to certain parts of Africa where sporadic outbreaks in humans are reported. In 2003, an outbreak of human MPXV occurred in the US after the importation of infected African rodents. Since the eradication of smallpox caused by an Ortho Pox Virus (OPXV) related to MPXV, and cessation of routine smallpox vaccination with the live Vaccinia Virus (VACV), there is an increasing population of people susceptible to VARV and perhaps certain other zoonotic OPXV diseases. Were OPXV to be employed as a bioterrorist weapon, there is distinct possibility that it would be deployed as a chimera pox, with OPXV genes being combined with those of other poxviruses (e.g., MPXV, Tatera pox, etc). In such a case, the currently approved smallpox vaccine VACV, may be ineffective. In recent years the antiviral potential of microRNAs (miRNAs) has been documented and there are numerous miRNAs approved for clinical trials. Most notably, miRNA-122 is in Phase III clinical trials against Hepatitis C virus (HCV). Our laboratory is investigating the potential use of human miRNAs that can be used as post-exposure silencing vehicles against pathogenic poxviruses. Specifically, our goal is to uncover miRNAs that can significantly silence pathogenic pox viruses. Our aim is to use VACV expressing the appropriate anti-poxviruses genetic fragments.

Methods: We computationally analysed human miRNAs (hsa-miRNAs) that displayed near perfect homology to VARV and all major potential poxviruses genomes, including MPXV, Camel Pox (CAXV) and Molluscum Contagiosum Pox Virus (MCPV), using gene alignment tools, and determined which of these miRNAs may silence a pathogenic poxvirus. Then, we designed a VACV-based vector, expressing all of the anti-pox miRNAs, with an ability to silence any chimera or naturally-occurring serious zoonotic event.

Results: We identified 26 hsa-miRNAs for VARV and 22 miRNAs for VACV, seven for CPXV, 11 for MPXV and 12 for MCPV that showed >90% homology with human miRNAs. We propose a design of a recombinant VACV that expresses all the anti-pox viruses that can quell, essentially, any pathogenic poxvirus that can be a threat to humans.

Conclusion: We present evidence, using bioinformatics tools, that a new recombinant VACV can be constructed and used as a post-exposure therapy in the case of a zoonotic outbreak or a bioweapon chimera pox created by genetic engineering.

Keywords: Smallpox; Micro-RNA; Chimera; Cytoplasmic factories; HCV; Post-transcriptional silencing; Mutual homology; Seed region; Variola virus; Vaccinia virus; Viral genome; Post-exposure; Zoonotic; Bioweapon; Genetic engineering

Abbreviations: miRNA: Micro-RNA; HCV: Hepatitis C virus; hsa-miR: Human micro-RNA; VARV : Variola virus; VACV : Vaccinia Virus; IMV: Intracellular Mature Virion; EEV: Extracellular Enveloped Virion; RNase III: Ribonuclease; Kbp: Kilo base pair; KDa: Kilo Dalton; ITRs: Inverted Terminal Repeats; VLTf: Viral Late Transcription Factor; RAP94: RNA Polymerase Associated Protein 94; CDS: Coding Sequence; MPXV: Monkey Pox Virus; CPXV: Cowpox Virus; CAXV: Camel pox Virus; MCPV: Molluscum Contagiosum Virus

Introduction

Since its appearance during the Neolithic approximately 12,000 years ago [1], the pox, red plague, or smallpox is said to have slaughtered more people than all other diseases combined. It has not respected geography, having inflicted its terror on all inhabited continents [2]; nor has it valued position or wealth, being responsible for the death and mutilation of Pharaohs, emperors, monarchs, and presidents alike. So frightening and devastating has this disease been throughout history that at least seven religions had idols dedicated to it [3]. Smallpox has been used deliberately and unwittingly as a weapon of biological warfare. Its arrival via an infected slave from the Narváez 1520 expedition is believed responsible for the massive post-Columbian depopulation of Central and South America and so played an influential role in

the conquest of Mexico and Peru [2]. The British are known to have employed smallpox as an agent of death during the French and Indian Wars (1754–1763) [4,5] and, in Australia, it has been suggested that the British used smallpox purposefully in the 1789 defence of fragile military positions [6]. Throughout the last century American, Japanese, Soviet and British researchers examined the potential for weaponizing smallpox but this was thwarted mainly due to the efficacy of vaccines.

It was long realized that survivors of smallpox were subsequently immune to reinfection and that individuals who were infected by smallpox by a small scratch to the skin experienced a less severe form of the disease. These observations led to the practice of variolation where people were inoculated artificially using pus from a pock or dried scab. This practice, which in all likelihood originated in China during the 10th century, spread at first to India and, by the 13th century appeared

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in Egypt [2]. By the 17th century variolation was widely used, being introduced to England from the Ottoman territories by Lady Mary Wortley Montagu in 1721. Interestingly, Edward Jenner was variolized prior to his discovery of vaccination in 1796. Remarkably, it is not known for certain which virus Jenner used for the first vaccinations although it is generally accepted that it was the cowpox virus, CPXV.

There are two principal forms of the smallpox virus, *Variola major* (VAVR), the most pathogenic species for humans, and the milder *V. minor* (VACV) or *Vaccinia virus* upon which modern smallpox vaccines are based. Global use of the latter led to the eventual eradication of smallpox in 1979. Remarkably, VARV expresses the smallest genome of all ortho pox viruses [7] and, with the potential for intermolecular and intra molecular recombination, genomic insertions and deletions, it has been implied that a VARV-like variant of existing zoonotic ortho pox viruses might emerge naturally [8]. The most prominent amongst these are MPXV and Tatera Pox Virus (TPXV). MPXV is endemic within Africa where sporadic zoonotic outbreaks are reported in humans [9]. In 2003, an outbreak of human MPXV occurred in the US from infected rodents imported from Africa [10]. Since the eradication of smallpox and the secession of routine smallpox vaccination using live VACV virus, there is an increase in the susceptible population who have no protection against ortho pox viruses. Even though the airborne particles are not considered the primary route of infection from monkeys to man, this potential route for transmission exists [11]. Recently, Hutson et al. [12] have shown that the Congo Basin clade of MPXV is more readily transmitted via the respiratory route than the West African clade. Together with the potential for the use of smallpox for bioterrorism, interest in the mechanisms of infection and pathogenesis of this virus persist. For this reason smallpox has recently been listed as one of the top bio-warfare weapons by the Centre for Disease Control and Prevention (CDC) [13].

All poxviruses belong to the family poxviridae replicate in the cytoplasm within distinct cytoplasmic factories (CFs) [14]. Replication is initiated by entry of two types of viral particles into the host cell: either extracellular enveloped virion (EEV) or Intracellular Mature Virion (IMV) [15]. Pox viral genes are expressed in a biphasic manner during the early phase of replication; the early genes encode non-structural proteins that initiate genome replication and late phase of replication while late genes encode structural proteins that involve formation of new viral particles. *Vaccinia* has 95% sequence identity with the VARV genome [16,17] (Figure 1). Although, pox viral immunology is still unknown, it has been suggested that VACV produces highly potent antibodies which completely neutralize VARV when it infects humans [18]. However, we propose that in addition to antibodies VACV infection stimulates intracellular immunity or microRNA (miRNAs) that share homologies to VARV and expressions of these miRNAs can silence the gene functions of the virus. The reasoning underlying our hypothesis is based on the well documented data that demonstrates that even the most avid antibodies cannot reach inside host cell CFs and it is unlikely that infection with VACV would completely block any of the intracellular events after the host cells have been infected [14].

Humoral and cell mediated immunity (classical immunity) has powerful effects on the viral-producing cells that express viral antigens on their cellular surfaces and can be cytopathic for the viral-producing cells by antibody-mediated cytotoxic effects or by cell mediated immunity (i.e. CTL). Antibodies can also neutralize viral particles in the vascular and extravascular spaces by binding to them and enhancing phagocytic function [19]. In the case of smallpox infection, the host cells are epithelial cells and intracellular inner linings of the

human host places where humoral immunity does not readily reach. Accordingly, our notion that intracellular miRNAs play a pivotal role in quelling VARV replication has biological merit. At the time when the majority of immunological research was carried out (i.e., before the 1980s) miRNA-based immunity was unknown.

MicroRNAs are genomically encoded, small non-coding double stranded RNA molecules (~22 bp) and play a major role in regulation of diverse biological process including gene expression at the post-transcriptional level, and in anti-viral and anti-tumor immunity [20,21]. MicroRNA was discovered around two decades ago and are currently being explored as novel therapeutic agents against several viral diseases [22]. So far, 2,578 mature human miRNAs have been identified from 1,872 precursor sequences (Sanger database version 20) [23]. By utilizing a computational genetic sequence alignment method, we propose the potential use of a recombinant VACV vaccine expressing multiple anti-pox miRNAs as therapeutic agents in case of a zoonotic event or bioterrorist attack. Of course, it will be very difficult to predict the type and nature of pathogens that could be used as bioweapons, but it is essential that we explore a wide range of possible miRNAs that can quell numerous potential human pathogenic poxviruses. It is logical to assume that in the case of bioterrorism the possibility of a chimera poxvirus being used would be high. The basic purpose of bioterrorism is to create “fear”. And, reappearance of one of history’s most dreaded viruses could readily achieve this goal. If traditional methods of vaccination are utilized, it may take years before an appropriate vaccine or therapy could be developed. Even though we have stockpiled VACV that can be injected into naive individuals, it is unlikely, in case of bioterrorism, that the infectious agent would be a classical VARV. We assume it would be a chimera where a typical VACV would be either ineffective or much less effective. Therefore, it is reasonable to predict that a new deadly bioweapon may be a chimera virus of highly pathogenic ortho pox virus genus family members. In a situation like this, currently available VACV may not be fully protective. However, we believe that we can custom design a specific anti-pox miRNA-expressing VACV-based vector that will carry the information for a wide range of potential pathogenic ortho pox viruses. This sort of post-exposure therapy can be mass-produced ahead of time in cell culture bioreactors, and the susceptible population could be vaccinated easily whenever the need arose. The goal of this research was to develop a novel post-exposure therapy against the Pox viral chimera or natural pathogen. The research utilized a VACV backbone-based vector that will express miRNAs in the host cells that would target all the ortho pox viral genes responsible for virulence and pathogenicity.

In order to decipher the mechanisms by which the human host can protect itself against a new chimera pox infection, or zoonotic event, and how a recombinant VACV that contains and expresses mature anti-pox miRNAs sequences may impart intracellular immunity against a chimera pox, we first analyzed the human miRNAs that showed identities to all major potential pathogenic ortho pox viruses. For this, we utilized multiple gene tools and then designed a recombinant vaccine that could quell any potentially pathogenic pox virus or a deadly zoonotic event.

Materials and Methods

Mature miRNA database and gene alignment

At the time of our studies (Sept 2013) 2,578 mature human miRNAs (hsa-miR) were listed in the Sanger database. By utilizing the human miR Base sequences database (<http://www.mirbase.org>, version 20.0), the hsa-miR sequences were downloaded from the database and aligned with each of the five viral genomes individually (i.e.

VARV: Accession# X69198; VACV: Accession # NC_006998; CPXV: Accession# NC_003391; MPXV: Accession# JX878428 and MOCV: Accession# U60315) using a free online gene alignment tool, EMBOSS EXPLORER. Before analysis, each U of hsa-miRs was converted to a T. In addition, all the genomic sequences were annotated in FASTA format before the alignment process since the alignment tools are generally programmed in the FASTA format. The reference genome sequences of five viruses were obtained from <http://www.ncbi.nlm.nih.gov/>. Following this, we utilized multiple alignment tools to search for miRNA that shared identities with both viruses as described previously [24].

Determination of miRNA alignment to viral sequences

To determine the suitability of each of the hsa-miRNAs as a potential therapeutic agent, we developed and refined the algorithm that incorporated the two critical elements that increases the suitability of a miRNA as a successful silencing agent. These included: 1) the length of the complementary pairing of human miRNAs. In this case, we downloaded the available miRNAs from miRbase and aligned with five viral genomes, as miRNA silencing requires a minimum of 19 bp; 2) a perfect or near-perfect alignment at miRNA seed sequences located at the 3'-untranslated region (UTR) base pair 2 to 8 that signals a successful silencing match [24,25] and, 3) an 80%-90% level of identity of the sequence of miRNAs with each of the Ortho pox viral genomes was considered as highly significant ($p < 0.001$) and reported to significantly reduce the "off-target" silencing of other genes [26].

Results

Human miRNA alignment

The target genes for the hsa-miRNAs were identified by retrieving the genome for VARV, VACV, MPXC, CPXC and MCV individually from PubMed. Each human miRNA (hsa-miR) found within the Sanger database (version 20.0) was determined to be identical to the Coding DNA Sequence (CDS) of the target gene by matching with 19-26 bp. The lengths of the 19-26 bp hsa-miRs were identical to those of the target genes of five viruses, as identified by alignments, according to the full length coding sequences (CDS) of the two viral genes using the specific alignment tools (Table 1).

Human miRNAs alignment with VARV genome

First, we analysed the numbers of human miRNAs (hsa-miRs) that showed significant identity to VARV genomic sequences. As shown in (Figure 1), by utilizing bioinformatics tools, we identified 26 miRNAs i.e. hsa-miR-32-5p, hsa-miR-599, hsa-miR-103a-3p, hsa-miR-876-3p, hsa-miR-488-3p, hsa-miR-4647, hsa-miR-1264, hsa-miR-5186, hsa-miR-198, hsa-miR-6781-3p, hsa-miR-3128, hsa-miR-7161-5p, hsa-miR-3668, hsa-miR-338-3p, hsa-miR-3121-5p, hsa-miR-1205, hsa-miR-4789-3p, hsa-miR-548a-5p, hsa-miR-4528, hsa-miR-337-3p, hsa-miR-6824-3p, hsa-miR-545-5p, hsa-miR-4719, hsa-miR-3921, hsa-miR-33a-3p and hsa-miR-514b-3p that exhibited ~90% sequence identity with the VARV genome.

Three miRNAs i.e. hsa-miR-103a-3p, hsa-miR-5186 and hsa-miR-4789-3p, exhibited identity to B gene (combined) of the virus and their specific target genes encode hypothetical proteins. Hsa-miR-876-3p and hsa-miR-3668 exhibited identity to E2L gene of the virus that encodes a hypothetical protein. Both hsa-miR-514b-3p and hsa-miR-548a-5p also showed identity to the M3L gene of the viral genome that also encodes hypothetical protein.

Hsa-miR-1264 and hsa-miR-338-3p exhibited identity to the Q1L

gene of the virus where hsa-miR-4528 and hsa-miR-33a-3p exhibited identity to a gene (combined) of the virus. Hsa-miR-198, hsa-miR-6781 and hsa-miR-7161-5p showed alignment to D1L, I4L and F5R genes of the viral genome respectively. Hsa-miR-545-5p exhibited identity to the N2L gene of the virus whereas hsa-miR-3921 showed near perfect identity to the J2L gene of the viral genome. Hsa-miR-4647 aligned to the B gene at VARV genome but its specific targets are unknown.

Human miRNAs alignment with VACV genome

We carried sequence alignments between human miRNAs and VACV genomic sequences as an important step toward determining which miRNAs can be induced by utilizing VACV-based vaccination and to determine which miRNAs are needed in order to design a broad spectrum anti-ortho pox vaccine. As shown in (Figure 2) we identified 22 hsa-miRs by computational analyses and alignment tools. These were hsa-miRs: hsa-miR-6748-3p, hsa-miR-599, hsa-miR-548aq-3p, hsa-miR-876-3p, hsa-miR-542-3p, hsa-miR-488-3p, hsa-miR-6755-5p, hsa-miR-3128, hsa-miR-3668, hsa-miR-338-3p, hsa-miR-1205, hsa-miR-3121-5p, hsa-miR-6809-3p, hsa-miR-548f-3p, hsa-miR-7111-3p, hsa-miR-337-3p, hsa-miR-6824-3p, hsa-miR-4719, hsa-miR-4774-5p, hsa-miR-6873-3p, hsa-miR-33a-3p and hsa-miR-514b-3p. All of them showed ~90% sequence identity with VACV.

Both hsa-miR-876-3p and hsa-miR-3668 exhibited identity to the E2L gene of the virus whereas hsa-miR-599 showed identity to the B2R gene of the viral genome. Hsa-miR-876-3p aligned to the O1L gene of the virus and hsa-miR-7111-3p exhibited identity with the F7L gene of the viral genome. Hsa-miR-514b-3p was aligned to the VACV genome's L3L gene.

Hsa-miR-548aq-3p, hsa-miR-6809-3p, hsa-miR-548f-3p and hsa-miR-6873-3p were aligned to the VACV genome but their specific target genes remain unknown.

Hsa-miR-542-3p and hsa-miR-6748-3p were aligned with B8R and B13R VACV genes respectively. B8R counteracts the antiviral effects of host IFN-gamma and acts as a soluble IFN-gamma receptor and thus inhibits the interaction between host IFN-gamma and its receptor. The B13R gene, expressed in the early phase of the viral replicative cycle, inhibits the proteolytic activity of interleukin 1-beta converting enzyme (ICE) and ICE-like enzymes. It can also block apoptosis through host tumour necrosis factor (TNF) receptor.

Hsa-miR-33a-3p targets the A41L gene that is responsible for host defence modulation where hsa-miR-6755-3p targets the K2L gene which encodes a serine protease inhibitor-like protein that irreversibly inhibits specific peptidases and blocks the activity of one or more proteins.

Hsa-miR-4774-5p targets J6R genes at VACV genome that are responsible for enzyme DNA-dependent RNA polymerase subunit rpo147 which plays an important role in DNA replication and repair.

Mutually homologous human miRNAs alignment with both VARV and VACV viral genomes

In order to evaluate which miRNAs have mutual homologies to both VARV and VACV we carried out an extensive analyses of the Hsa-miRs that illustrated mutual identities to both of the viruses. By utilizing various gene alignment tools, we identified 13 miRNAs i.e. hsa-miR-599, hsa-miR-876-3p, hsa-miR-488-3p, hsa-miR-3128, hsa-miR-3668, hsa-miR-338-3p, hsa-miR-3121-5p, hsa-miR-1205, hsa-miR-337-3p, hsa-miR-6824-3p, hsa-miR-4719, hsa-miR-33a-3p, and hsa-miR-514b-3p that showed ~90% sequence identity with both

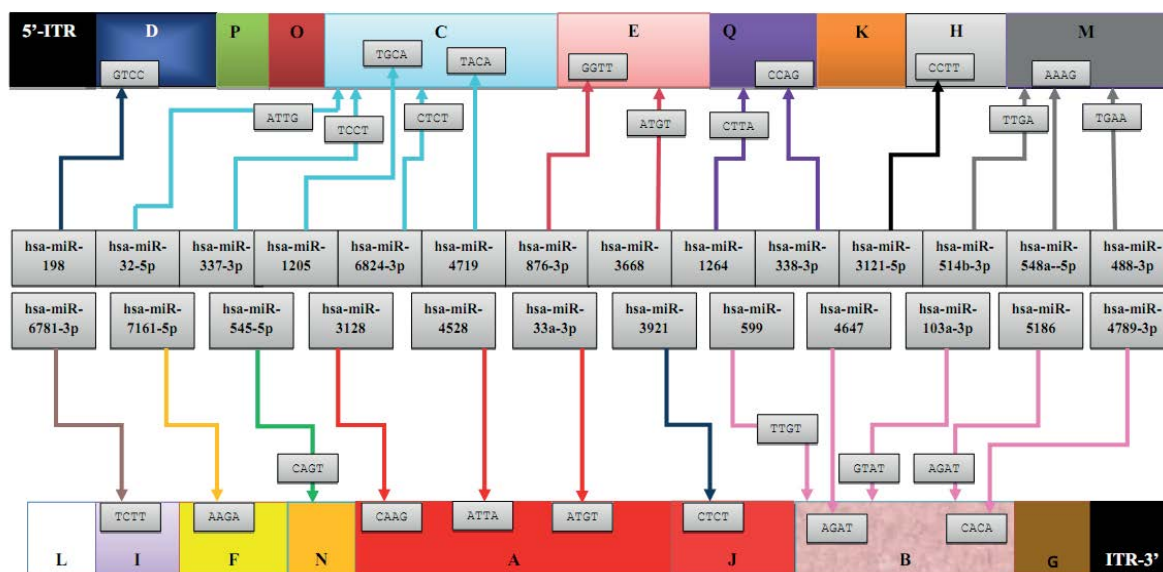


Figure 1: Twenty-six human miRNAs targeting sequences of VARV genetic motifs. Genomic fragments of VARV showing the locations of the human miRNAs with ~90% identities, carried out by multiple sequence alignment tools. Only the seed sequences of each of the miRNA are shown.

the VARV and VACV viral genome. Hsa-miR-33a-3p targets the A46L gene (VARV) and A41L gene (VACV) where the A46L gene encodes a hypothetical protein and A41L gene acts as a host defence modulator. Five miRNAs i.e. hsa-miR-599, hsa-miR-876-3p, hsa-miR-3668, hsa-miR-338-3p, and hsa-miR-514b-3p target specific genes in both VARV and VACV genomes. (Figure 3) illustrates these mutually homologous miRNAs.

Hsa-miR-337-3p

Hsa-miR-337-3p was aligned both to the VARV and VACV viral genome and it targets C6L gene (VARV) and F2L gene (VACV). Hsa-miR-337-3p showed ~90% sequence identity with specific target genes in both viral genomes (Figure 4). The VARV C6L gene and VACV F2L gene encodes an essential enzyme dUTPase. It has been reported that dUTPase is exquisitely specific for dUTP and is crucial for the fidelity of DNA replication and repair [27]. Amino acid sequence alignments (protein blast) of both enzymes demonstrated 98% identity to each other (Supplementary Figure 1). The enzyme dUTPase not only hydrolyses dUTP to dUMP and pyrophosphate, but also simultaneously reduces dUTP levels and provides the dUMP for dTTP biosynthesis [27]. Our computational analysis indicated that hsa-miR-337-3p will be a potential antiviral agent for blocking the VARV C6L gene and VACV F2L gene as well as both viral replications.

Hsa-miR-1205, hsa-miR-6824-3p and hsa-miR-4719

Three miRNAs (hsa-miR-1205, hsa-miR-6824-3p and hsa-miR-4719) were aligned with the C gene (combined) of the VARV genome and F gene (combined) of the VACV genome. These 3 miRNAs target the C16L gene (VARV) and F12L gene (VACV) where both genes encode EEV maturation protein that is a core protein and plays important roles in the infection of a new host. VARV C16L gene encoded EEV maturation protein exhibited 96% identity with VACV F12L gene encoded EEV maturation protein (Figure 5). The EEV mature protein is 65 kDa in mass which facilitates the transport of intracellular viral particles to the cell membrane. Several reports have suggested that EEV maturation protein is expressed during the late phase of infection and plays a crucial role including plaque formation, EEV production

and virulence [28]. Although, some earlier evidence suggested that both IMV and EEV infectivity could be inhibited by antibodies two other studies reported that EEV was resistant to neutralization [29].

Hsa-miR-488-3p

Hsa-miR-488-3p was aligned with both the VARV and VACV viral genome (Figure 6) and it targets M4R (VARV) and L4R (VACV) where both genes encode DNA-binding virion core proteins which play crucial roles in DNA replication and/or repair. The amino acid sequence alignment of both the VARV M4R gene encoded DNA-binding virion core protein and VACV L4R gene encoded DNA-binding virion core protein displayed 99% identity to each other (Supplementary Figure 2). DNA-binding virion core protein is a 25-kD protein that comprises about 6.5% of the total viral polypeptides by mass [30]. It has been reported that DNA-binding virion core protein is proteolytically processed during virion morphogenesis by removal of 32 amino-terminal amino acids from the 28-kD primary translation product encoded by the L4R gene at VACV genome [31,32]. DNA-binding virion core protein is also considered a major core protein that possesses DNA-binding activity. It has been hypothesized that the L4R protein stabilizes a subset of the genomic sequences in a single-stranded conformation [33]. Although, absence of the L4R protein doesn't have any impact on the virion's formation, it severely influences infectivity [34].

Hsa-miR-3128

Hsa-miR-3128 was aligned with both the VARV and VACV viral genome (Figure 7) and it targets the A2L gene in both viral genomes that encodes a late transcription factor VLTF-3. VLTF-3 interacts with the late transcription elongation factor H5/VLTF-4. It is expressed during intermediate stages of infection and acts with RNA polymerase to initiate transcription from late gene promoters. Transient transfection assays indicated that A2L is required for the transcriptional transactivation of viral late genes. Activation of late VACV gene transcription is not only dependent on DNA replication, but also the expression of the A2L gene [35]. Amino acid sequences of both the VARV and VACV VLTF-3

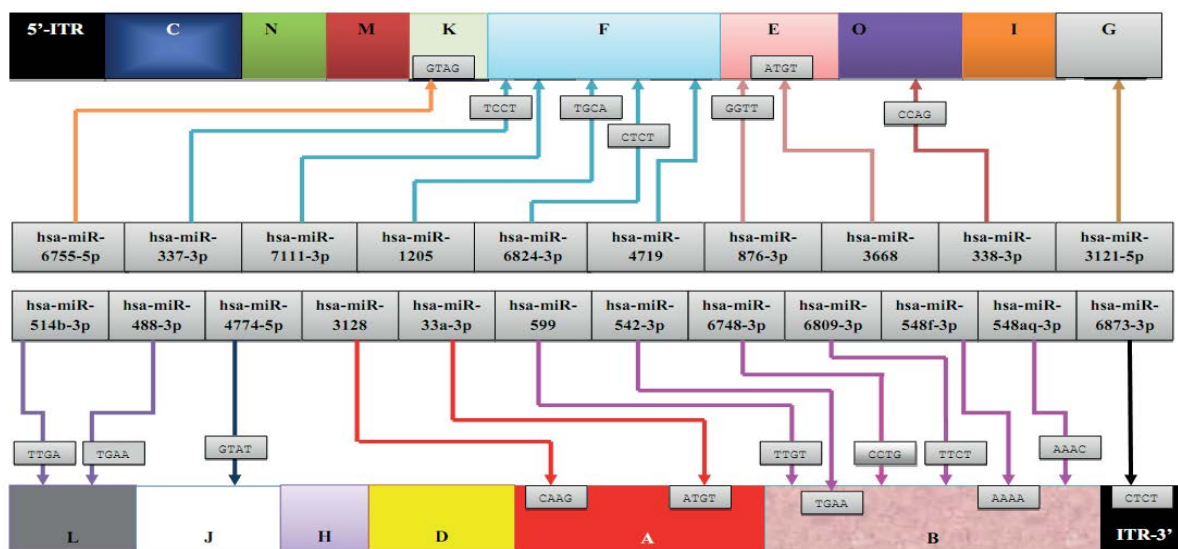


Figure 2: hsa-miRNAs targeting sequences of VACV genomic motifs. Genomic fragments of VACV showing the location of the human miRNAs with >90% identities, carried out by multiple alignment tools. Only the seed sequences of each of the miRNAs are shown.

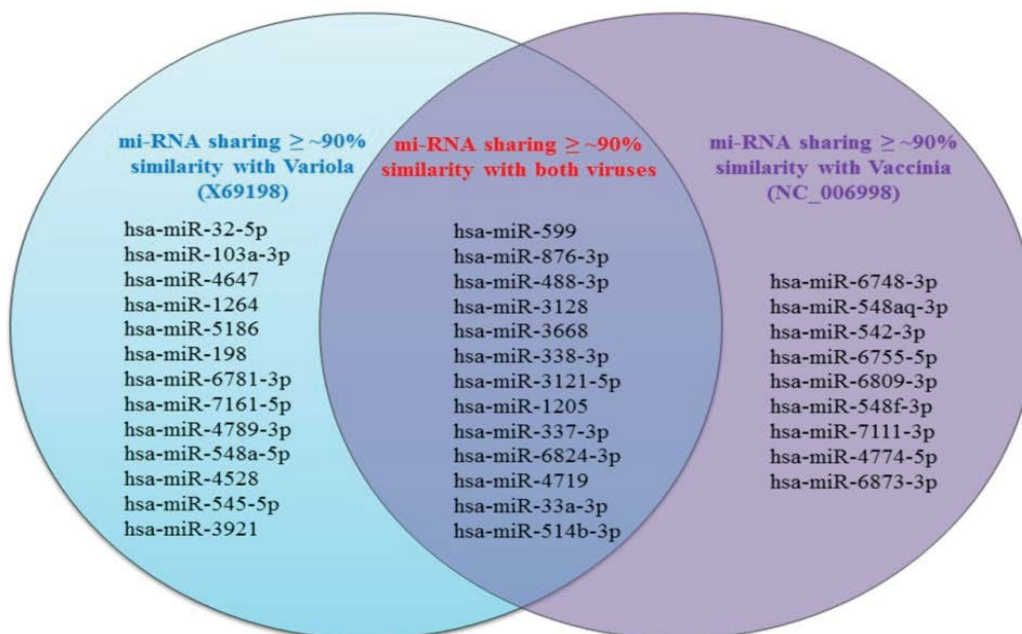


Figure 3: Identical *hsa-miRNAs* between both VARV and VACV viruses. By utilizing gene alignment tools, 26 *hsa-miRNAs* showed >90% identities to VARV (Left circle, light blue) and 22 *hsa-miRNAs* (right circle, purple) exhibited >90% identities to VACV. The middle circle (central, dark blue) shows 13 human miRNAs with mutual homologies to the both viral genome.

illustrated 100% homology (Supplementary Figure 3).

Hsa-miR-3121-5p

Hsa-miR-3121-5p was aligned with both the VARV and VACV viral genome (Figure 8) and it targets the H1L gene of the VARV genome and the G1L gene of the VACV genome. Both genes encode an enzyme putative metallo protease where metalloendopeptidase likely plays a role in the maturation of viral proteins. Amino acid sequence alignment of both VARV putative metalloprotease and VACV putative

metalloprotease showed 98% identity (Supplementary Figure 4). It has been reported that metalloprotease may be involved in maturation of some poxviral proteins by processing them preferentially at Ala-Gly-Ser/Thr/Lys motifs at VACV genome [36,37]. The putative metallo peptidase is proteolytically processed during the course of infection by removal of some amino-terminal amino acids from the 46 kDa primary translation product encoded by the H1L gene in VACV.

Human miRNAs that aligned with monkey pox virus (MPXC)

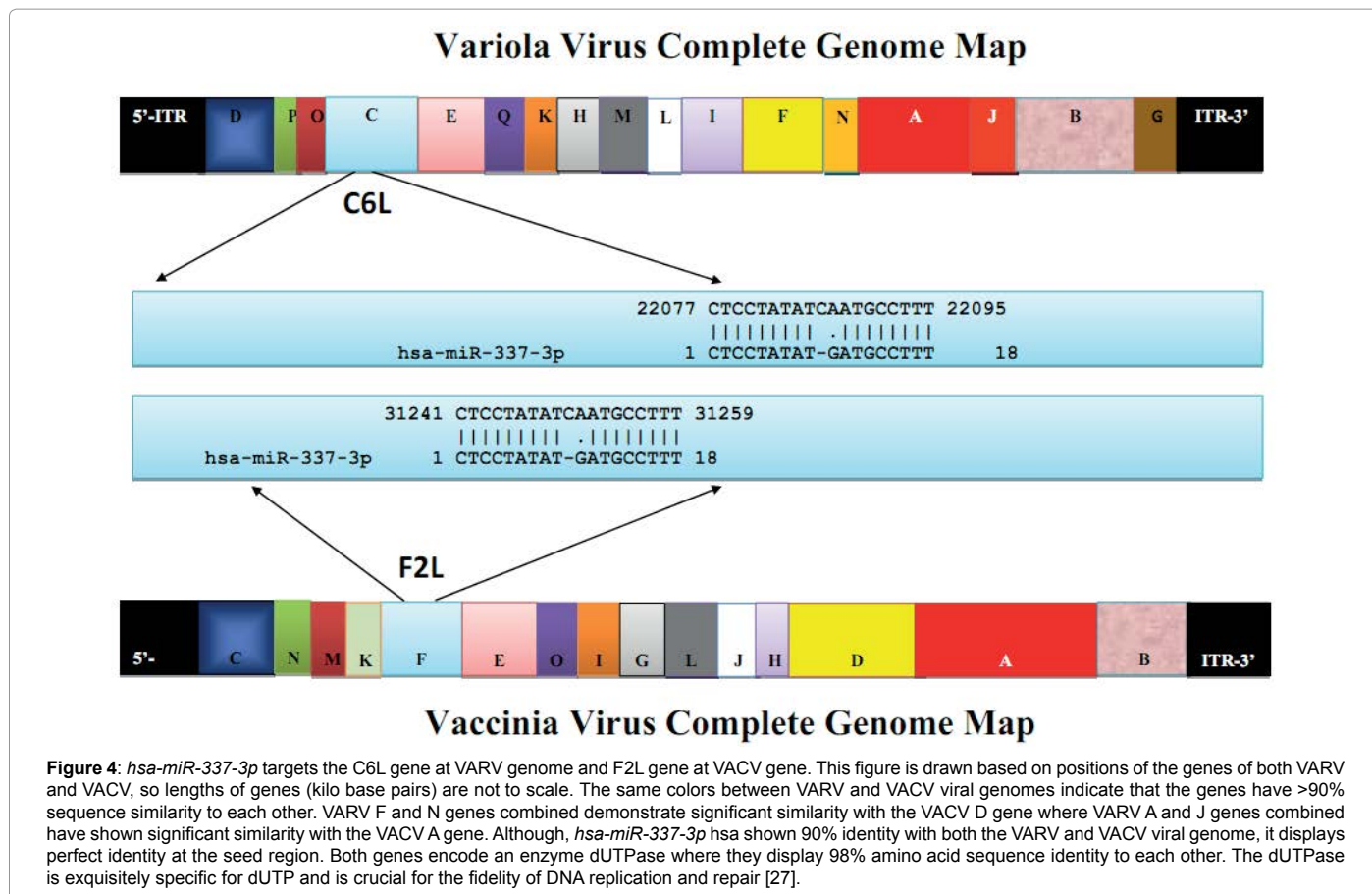


Figure 4: *hsa-miR-337-3p* targets the C6L gene at VARV genome and F2L gene at VACV gene. This figure is drawn based on positions of the genes of both VARV and VACV, so lengths of genes (kilo base pairs) are not to scale. The same colors between VARV and VACV viral genomes indicate that the genes have >90% sequence similarity to each other. VARV F and N genes combined demonstrate significant similarity with the VACV D gene where VARV A and J genes combined have shown significant similarity with the VACV A gene. Although, *hsa-miR-337-3p* hsa shown 90% identity with both the VARV and VACV viral genome, it displays perfect identity at the seed region. Both genes encode an enzyme dUTPase where they display 98% amino acid sequence identity to each other. The dUTPase is exquisitely specific for dUTP and is crucial for the fidelity of DNA replication and repair [27].

Similar to that described in the preceding section, we carried out miRNA alignment with MPXC genomes. As shown in (Supplementary Figure 5), we discovered 19 miRNAs that showed near perfect identity with MPXC genetic sequences, targeting genes in 5' ITR (*hsa-miR-6739*), gene D (*hsa-miR-122-3p*), gene C (*hsa-miR337-3p* and *hsa-miR-1205*), gene F (*hsa-miR-876-3p*, *hsa-miR-3668*, *hsa-miR-613*, and *hsa-miR-4719*), gene Q (*hsa-miR-338-3p*), gene G (*hsa-miR-3121*), gene M (*hsa-miR-548a-5p*, and *hsa-miR488-3p*), gene H (*hsa-miR548-ab*), gene A (*hsa-miR-3128*), gene B (*hsa-miR-1251-5p*, *hsa-miR-599*, *hsa-miR-4647* and *hsa-miR-6748-3p*), and gene J (*hsa-miR-517-5p*). Most interestingly, 9/19 exhibited significant identity to VARV genome. However, there were an additional 10 miRNAs that were unique and did not align with VARV and VACV. Of note, all 19 miRNAs showed perfect alignments at the seed sequences.

Human miRNAs that aligned with camel pox virus (CAXC)

The computational analyses of CPXC revealed 16 human miRNAs that have near perfect identity to CPXC. Nine of these miRNAs were the same as those that showed identity to VARV. As shown in (Supplementary Figure 6), these miRNAs targeted various vital elements of CPXC genetic motif and include gene gp047 (*hsa-miR-1205*, *hsa-miR-6824-3p*, and *hsa-miR-4719*), gene gp054 (*hsa-miR-3668*, an unknown gene * (*hsa-miR-338-3p*), gene Vgp088 (*hsa-miR-514-3p*), gene gp089 (*hsa-miR-488-3p*), gene gp108 (*hsa-miR-7161-5p*), gene gp118 (*hsa-miR-3128*), gene gp154 (*hsa-miR-4528*), gene gp179 (*hsa-miR-599*, *hsa-miR-4647*), gene gp184 (*hsa-miR-103a-3p*), gene gp211 (*hsa-miR-123*) and two yet to be named genes # and ** where *hsa-miR-3914* and *hsa-miR-877-3p* respectively showed identity.

Human miRNAs that aligned with molluscum contagiosum virus subtype 1 (MC)

Our computational studies unveiled 21 human miRNAs that targeted a wide variety of MCV genetic motifs (Supplementary Figure 7). These included the gene MC003L (*hsa-miR-3960*), MC018L (*hsa-miR-3130-3p*), MC019L (*hsa-miR-764*), gene MC 028L (*hsa-miR-6729-5p*), gene MC035R (*hsa-miR-4798-3p* and *hsa-miR-6885-5p*), gene MC072L (*hsa-miR-595*), gene MC079R (*hsa-miR-3130*), gene MC090R (*hsa-miR-4782-5p*), gene 094R (*hsa-miR-6789-3p*), gene MC115L (*hsa-miR-3135b*), gene MC129R (*hsa-miR-4254*), MC131R (*hsa-miR-3160-3p*), gene MC140L (*hsa-miR-6872-3p*), gene MC159L (*hsa-miR-208a-3p*), gene MC161R (*hsa-miR-4520a-3p*), gene MC163 (*hsa-miR-3663-3p* and *hsa-miR-6069*), gene ITR-3' (*hsa-miR-4668-5p*) and a unknown gene designated* (*hsa-miR-3912-5p*).

Discussion

Although smallpox was eradicated over 35 years ago, the disease still remains a threat. High mortality, high infectivity and low resistance of the contemporary population due to absence of vaccination against smallpox for over three decades make the virus very attractive to bioterrorists. The potential presence of stocks of the virus as a bioweapon, the possibility of creating genetic chimeras of deadly poxviruses that may bypass even VACV based immunization and spread of the disease by zoonosis are matters of serious concern (Figure 9). One example of such a potential event is seen with the ectromelia virus, a mouse pox virus. Jackson et al. [38-41] reported that expression of the murine IL-4 gene by a recombinant ectromelia virus caused enhanced lethality for inherently resistant C57BL/6 mice, and overcame protective immune

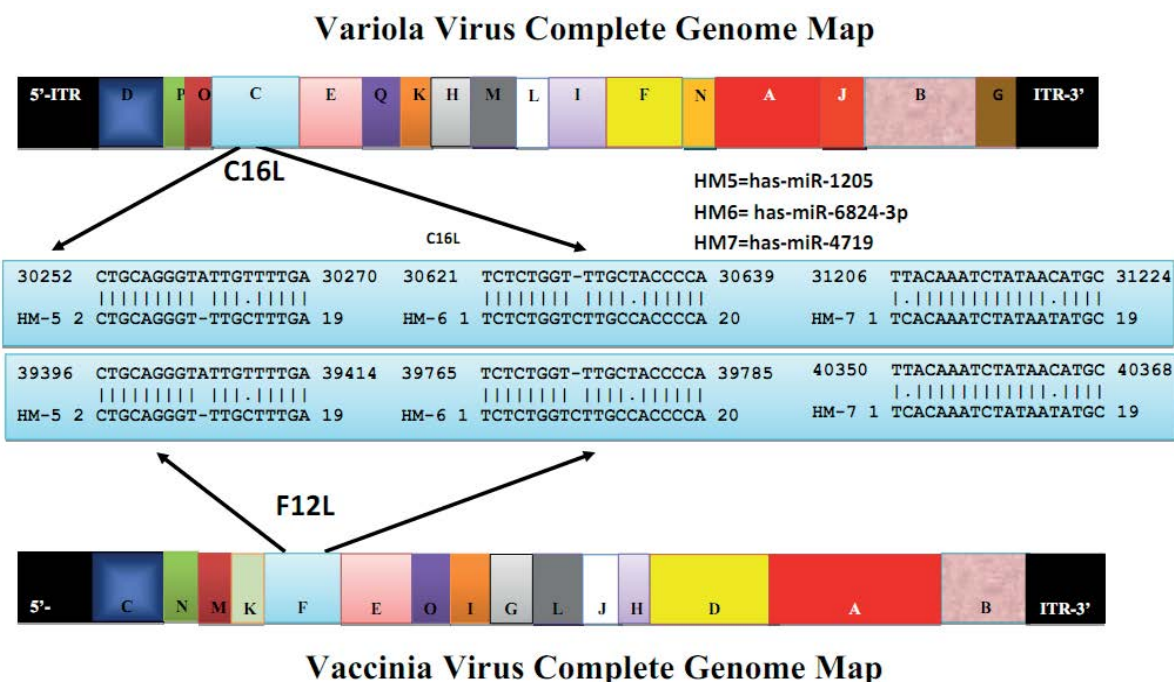


Figure 5: *hsa-miR-1205*, *hsa-miR-6824-3p* and *hsa-miR-4719* target C16L gene at VARV genome and F12L gene at VACV gene. This figure is drawn based on the gene's position of both VARV and VACV, so lengths of genes (kilo base pairs) are not to scale. The same colors between VARV and VACV viral genomes indicate that those genes are genetically similar. VARV F and N genes (combined) exposed significant similarity with the VACV D gene whereas VARV A and J genes (combined) have shown significant similarity with the VACV A gene. Although, all 3 miRNAs showed ~90% identity with both VARV and VACV viral genomic motifs, most importantly, they showed perfect identity at the seed region. Both genes encoded EEV maturation protein where they presented 98% amino acid sequence identity with each other. The EEV maturation protein facilitates the transport of intracellular viral particles to the cell membrane and absence of EEV maturation protein hsa the inverse effect to infect new cell membrane as well as viral replication.

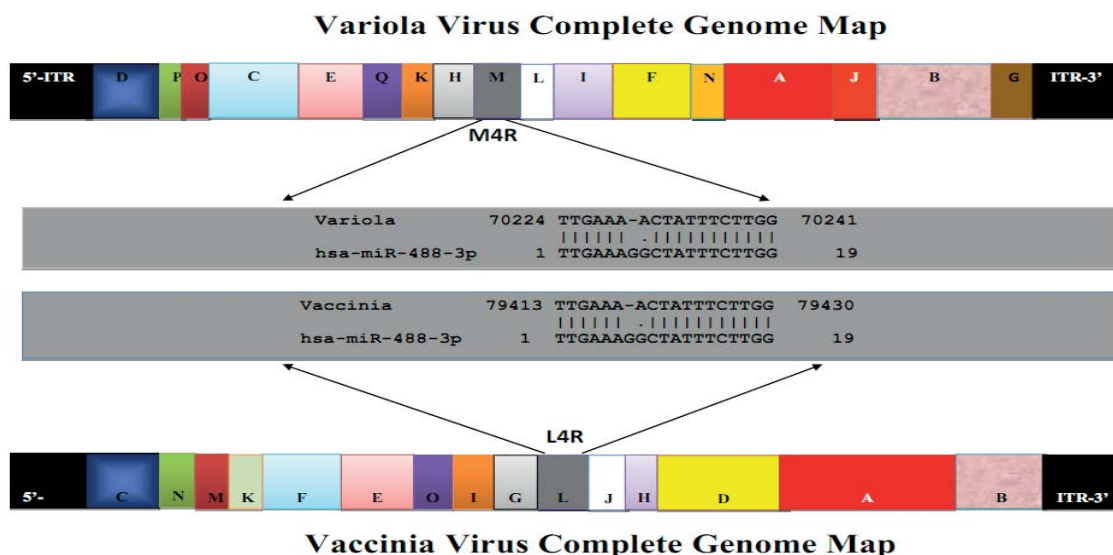


Figure 6: *hsa-miR-488-3p* targets M4R gene at VARV genome and L4R gene at VACV gene. This figure is drawn based on the gene's position of both VARV and VACV, so lengths of genes (kilo base pairs) are not to scale. Identical colors between VARV and VACV viral genome indicate that genes are genetically similar. VARV F and N genes (combined) have shown significant similarity with VACV D gene where VARV A and J genes (combined) have shown significant similarity with VACV A gene. Although, *hsa-miR-488-3p* hsa shown ~90% identity with both VARV and VACV viral genome it showed perfect identity at the seed region. Both genes encoded DNA binding virion core protein where they displayed 99% amino acid sequence identity to each other. The DNA binding virion core protein is one of the major core proteins which plays a crucial role in DNA replication and/or repair.

responses induced by vaccination. The enhanced lethality of the ECTV-IL-4 recombinant was likely due to immunosuppression, although the exact mechanism of action remains to be determined. Hence, it is reasonable to be alert to either genetically engineered virus or natural recombinant strains of POXV that may become lethal to humans and or food animals and to seek effective anti-pox agents that could be used in the event of a pox outbreak.

One may question the validity of carrying our miRNA analyses that can quell a man-made pox epidemic and even a need to develop an alternate VACV-based vaccine. It may be argued that VACV was an effective vaccine against VARV in part because VACV cross-reacted with other OPXV. Therefore, VACV would presumably be an effective vaccine against any chimera pox that can be harmful to humans. However, we consider that a deliberate bio-weaponization of pox viruses is not beyond the realms of possibility. Further, we argue that VACV would not be effective against chimeras if someone transfected multiple OPXVs in cells in the presence of VACV antibodies, creating a selective pressure such that only the VACV resistance chimeras would emerge and bypass the VACV-induced resistance. It would be similar to growing *E.coli* in the presence of penicillin and developing a penicillin resistant strain.

One of the most difficult aspects of poxviruses is their high degree of complexity. These are among the largest and most complex of animal viruses. VACV is the prototypical member of the Ortho pox virus genus and of the *Pox viridae* as a whole. The genomic length of the members of the Ortho pox viruses varies from 170-240 kbp and these members show substantial similarity to each other and are serologically cross-reactive [42]. VACV is notable in being not only the smallpox vaccine but also a very close evolutionary relative of the smallpox virus. It has over 200 genes and its replication occurs in a sequential manner with the early genes expressed immediately after entering the host cell, from the still encapsulated genome. The early phase is followed by genome replication, synthesis of intermediate- and late-class mRNAs, the complex processes of new virion assembly, and then cell exit [43].

Multiple studies have characterized the global kinetics of transcription and homeostasis during poxvirus infection. Hybridization studies have shown that by 2 hours and 7 hours post-infection of HeLa cells with VACV virus, 50%-60% and 80%-90% respectively, of the total poly (A)⁺RNA was virus specific [44]. More recently, deep sequencing of VACV and host cell transcriptomes showed that, at 4 hours post-infection of HeLa cells, 25 to 55% of poly(A)⁺ RNA sequences were viral mRNAs [45-47]. Although overall amounts of mRNA were comparable in infected and uninfected cells, the proportion attributable to the virus was shown to change significantly [45]. In A594 cells infected with a virus very similar to VACV virus, namely, rabbit pox virus, a decline in overall cellular mRNA levels was reported beginning at 2.5 hours post-infection, and, by 5 hours post-infection transcriptome levels were markedly lower for the majority of cellular genes [48].

The tools of molecular biology have provided numerous advances in understanding the basic biology and immunology of poxviruses [49-56]; however, there is much that remains undiscovered [57]. A number of unanswered questions relate to evolutionary biology, and an evolutionary perspective may provide important insights into the functional biology of these viruses, particularly in the area of immune evasion. Most interesting among the unknown is what role VARV homologous miRNAs play in quelling smallpox after exposure to VACV used as a vaccine. Until two decades ago, classical immunity was considered to be the major mode of defence against VARV after VACV immunization. However, since then important roles of innate immunity and miRNAs have been shown to play major roles in viral infections [58]. Therefore, classical immunity-

based interpretations may not be as comprehensive as it was thought previously [59-61].

Role of miRNA in intracellular defences

In the current study, we have utilized bioinformatics tools to analyse whether both VARV and VACV virus chimeras of pox can be silenced by miRNA targeting the specific genetic sequences of the two viruses.

While it has been suggested that host miRNAs may down-regulate viral gene expression as an antiviral defence mechanism, such a mechanism has not been explored in the pox viruses. As it is difficult to conduct such studies on VARV, which would be unavailable for this type of research, computational analyses represent an alternative method that can provide some insight.

We identify miRNAs which would target the key early, intermediate and late genes of any pox virus in the ortho pox genus. Historically, infections with these viruses have proven to be pathogenic to humans [8]. Since, VARV and VACV share significant genetic homology and the latter has successfully been developed as a vaccine against smallpox, we hypothesize that a recombinant vaccinia virus that expresses all the miRNAs that do not share mutual identity with VARV can be utilized as a post-exposure therapeutic vaccine and as a vaccine against any chimera pox-based bioweapon or in case of serious zoonotic events. This proposed vaccine would target all the closely-related, potentially pathogenic viruses (i.e. CAXV, CPXV, MPXV and Molluscum Contagiosum, etc.) Our study also provides an interesting hypothesis concerning the miRNA-based antiviral defence mechanism against poxvirus in humans. Among the effects, some studies showed the binding mode between human-encoded miRNAs and viruses. For example, a transcript in human foamy virus (PFV) can be used as the target site of human-encoded miR-32 [62]; hepatitis C virus (HCV) replication is regulated by miR-122 that is currently in Phase III clinical trial as an anti-HCV therapy [63]; and human-encoded miRNAs can target crucial HIV-1 genes [64].

Nucleic-acid based, RNAi provides immune defence when the body is faced with challenges from transgenes, viruses, transposons, and aberrant mRNAs. Short interfering RNAs (siRNA) or miRNA molecules trigger gene silencing in eukaryotic cells. Investigators have identified more than 3,000 different human miRNAs (hsa-miRs), and it has come to be regularly accepted that cellular gene regulation is significantly impacted by cellular miRNAs. Recent scholarship demonstrates that some viruses can actually encode miRNAs if these are processed through cellular RNAi. During ontogenesis, and in the development of certain tissues, miRNAs are differentially expressed. A single miRNA has the complex capacity to target multiple genes simultaneously [25,65-67]. During the last few years, several investigators have studied the possible role of miRNA in VACV-mediated protective mechanisms [68-77]. Therefore, Grinberg et al. [78] studied the effects of VACV viral infection on the expression of host-encoded miRNAs. A marked general suppression of most miRNAs in the infected cells was observed within 24 hours post-VACV infection of a number of cell types. They showed that this suppression was associated with abrogation of expression of the Dicer1 enzyme, which is a key enzyme in the generation of miRNAs. Hikichi et al. [79] have explored how miRNA regulates glycoprotein B5R in oncolytic VACV and how certain miRNA can reduce the viral pathogenicity without impairing anti-tumour efficacy. They showed that miRNA regulation also enables tumour-specific viral replication by altering the expression of targeted viral genes. Since the deletion of viral glycoprotein B5R not only decreases viral pathogenicity but also impairs the oncolytic activity of VACV virus, they used miRNA-based gene regulation to suppress B5R expression through let-7a, a

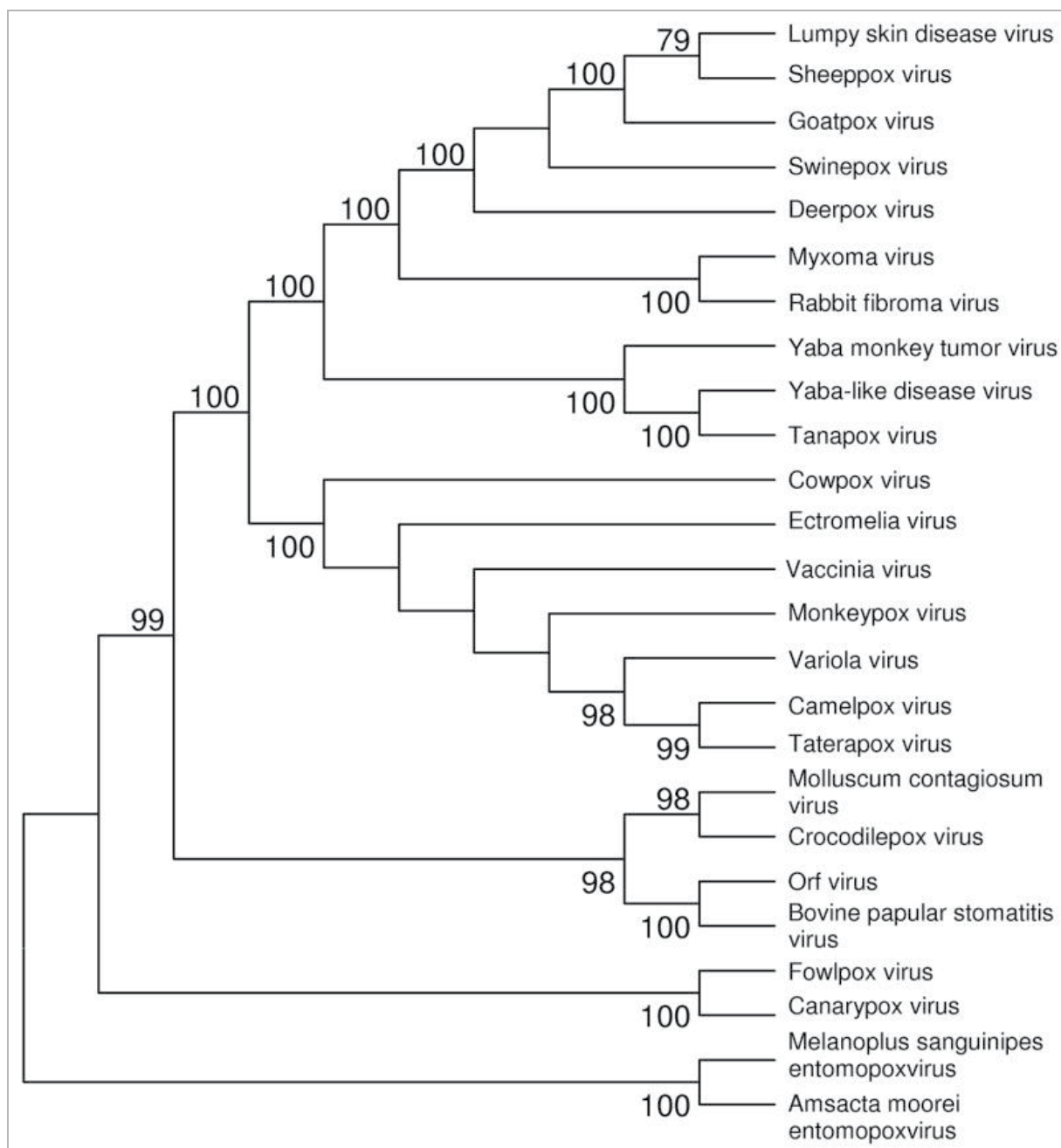


Figure 9: Phylogenetic analyses of poxviruses based on concatenated amino acid sequences from 29 conserved orthologous proteins (13, 475 aligned sites). The tree was constructed on the basis of the JTT amino acid distance, assuming that rate variation among sites follows a gamma distribution (shape parameter $\alpha = 0.86$). Numbers on the branches represent percentages of 1000 bootstrap samples supporting each branch; only values $\geq 50\%$ are shown [41].

miRNA that is down-regulated in many tumours. The expression of B5R and the replication of miRNA-regulated VACV (MRVV) with target sequences homologous to let-7a in the 3'-untranslated region (UTR) of the B5R gene depended on the endogenous expression level of let-7a in the infected cells. Intra-tumoural administration of MRVV in mice with human cancer xenografts that expressed low levels of let-7a resulted in tumour-specific viral replication and significant tumour regression without side-effects, which were observed in the control virus. Their results demonstrated that miRNA-based gene regulation is a potentially

novel and versatile platform for engineering VACV viruses for cancer biotherapy. Simon-Mateo and Garcia et al. [80] have analysed whether Plum pox virus (PPV) chimeras bearing miRNA target sequences (miR171, miR167, and miR159), which have been reported to be functional in Arabidopsis, were affected by miRNA function in three different host plants. They determined that some of these PPV chimeras had clearly impaired infectivity compared with those carrying non-functional miRNA target sequences. The behaviours of PPV chimeras were similar but not identical in all the plants tested, and the deleterious

effect on virus infectivity depended on the miRNA sequence cloned and on the site of insertion in the viral genome. The effect of the miRNA target sequence was drastically alleviated in transgenic plants expressing the silencing suppressor P1/HCP. Furthermore, they showed that virus chimeras readily escape RNA silencing interference through mutations within the miRNA target sequence, which mainly affected nucleotides matching the 5'-terminal region of the miRNA. From the above observations, we hypothesize that utility of the one or two anti-pox miRNAs may not be an entirely useful strategy but multiple miRNA expressing vectors based on VACV are very useful. Table 1 lists additional miRNA expression VACV vector that can be designed to quell any Pox viral epidemic, natural zoonotic or man-made.

One may question why virulent poxviruses infect humans despite the existence of human miRNAs that can quell them easily. We believe that this may be due to temporal tissue and cell specific expressions of miRNAs that express only certain type of cells at specific periods of cell differentiation and life cycle. Evolutionarily, a down-regulation or absence of certain anti-viral miRNAs makes these cells highly vulnerable to certain viral infections. This can be explained by the primary targets of VARV-the epithelial cells - but not other cell types. Similarly, the prime targets of poliovirus are cells that express CD155 or Necl-5 receptor positive cells and no other cell types. Therefore, we believe that VARV target cells are devoid of anti-VARV miRNAs [25,26].

We have approached the identity issue at the molecular (intracellular) level, a level at which potential interference would take place. Since there are 13 miRNAs that are mutually homologous with VARV and VARV and many of the miRNAs that also share identity also are mutually homologous to MPXV and CPXV, we suggest that addition of 26 miRNAs would complete a recombinant VARV that would be able to serve as a new universal vaccine in case of serious zoonotic events or a chimera pox bioweapon. These miRNAs would be processes and mature and reach their intended target by the natural process that is present in every nucleated cell [25].

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