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Viral microRNA genomics and target validation

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Abstract

A subset of viruses express their own microRNAs (miRNAs) and one way to understand the functions of these microRNAs is to identify the targets of these miRNAs. Sequence analysis and mRNA expression profiling were some of the first techniques to identify targets of viral miRNAs. More recently, proteomics and sequencing of RNA by crosslinking and immunoprecipitation (CLIP) methods have been insightful and discovered many miRNA targets that may be missed using other methods. We are now at a point where numerous validated miRNA targets have been described and integration of these genomic datasets will provide a richer understanding of miRNA targeting and viral infection, persistence, and pathogenesis.

Introduction

MicroRNAs (miRNAs) are short single-stranded RNA molecules that are functional in the RNA-induced silencing complex (RISC) of proteins. They have largely been shown to repress gene expression by targeting mRNA transcripts and inducing mRNA destabilization and inhibiting protein translation [1]. A variety of viruses, but not all, have been shown to express their own microRNAs. The first discovery of viral miRNAs [2] presented the possibility that viruses could be manipulating cellular and viral gene expression without generating additional viral proteins which could be potentially detected by the host immune system. Currently, there are miRNAs from 27 different viruses described in the miRNA database, miRBase.org [3]. Herpesviruses are currently the only viral family that expresses multiple miRNAs, and some viral miRNAs are very abundantly expressed, with a single viral miRNA, KSHV-miR-K12-4-3p, representing 23% of all viral and cellular miRNAs [4]. In addition, three miRNAs from bovine foamy virus represent 70% of all miRNAs in infected cells [5]. The abundance of viral miRNAs, diseases associated with these viral infections, and the variety of viruses expressing miRNAs are some of the reasons why it is important to understand the targets and functions of these miRNAs. Viral infection can also alter the expression of host miRNAs [6,7]. Surprisingly little sequence conservation are found in miRNAs sequences between related viruses or between viral miRNAs and cellular

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miRNAs, although some examples of orthologs exist [8]. Many recent reviews [9–13] have described the expression profiles and targets of viral miRNAs. Specifically, this review is focused on miRNA genomics, target prediction strategies, and target validation. Ultimately, the goal is not a litany of miRNA target genes, but using the viral miRNAs as tools to enlighten us humans about the mechanisms of infection, pathogenesis, and perhaps discover novel therapeutic strategies.

Genomic organization and expression

Many viral miRNA are located in clusters within the viral genome and come from polycistronic transcripts. Despite coming from the same primary miRNA transcripts vast differences in mature miRNA levels have been reported as determined by RNA sequencing [4,14], suggesting substantial differences in miRNA biogenesis efficiency and degradation rates.

In addition to miRNA polymorphisms that can alter biogenesis [15,16], expression changes of miRNAs can occur during the viral cycle or by changes in the environment of the infected cell. The expression of viral miRNAs changes in different stages of the viral life cycle when comparing viruses. For example, HSV-1, EBV and KSHV miRNAs are predominately expressed during latency [17][18][19,20] and some EBV miRNAs displayed increased expression during the lytic phase [21]. HCMV miRNAs are mainly expressed during the early lytic stage [17,22,23]. SV40, JC, and BK miRNAs are expressed during the late stage of the viral cycle [24,25]. Indeed, viral miRNA expression expression has been shown to be influenced by the host cell type [26]. Many miRNA expression profiles have been determined from infected cell lines in culture, but fewer measurements have been performed using clinical samples of infection [27][28–30]. Finally, recent data [31] suggests that the amount of RISC-incorporated miRNAs is a more important measurement than total levels of mature miRNAs. Unfortunately, measuring RISC-association of miRNAs in patient samples will be difficult, given the limited amount of sample material.

miRNA target prediction strategies

One way to identify the functions of miRNAs is to discover the direct and indirect targets of miRNAs (Figure 1). Then, knowing the functions of the miRNA targets can reveal functions of the miRNAs. An alternative strategy is to perform functional assays to determine which viral miRNA can affect a certain process, then determine what miRNA target genes are responsible for the phenotype. The fastest and most economical method for predicting direct miRNA targets is to use sequence comparison and focus on mRNA sequences that contain complementary sequences to the 5' end of the miRNAs of interest. This is called seed-matching analysis which primarily focuses complementarity to miRNA positions 2 through 8. One of the most widely used seed-matching tools is TargetScan, which focuses on the 5' end of the miRNA to find mRNA targets [32]. Other parameters like mRNA sequence context, location in 3' untranslated region, and conservation among other species can be used to prioritize likely targets. Other related software tools consider additional sequences in the miRNA and will also consider imperfect seed-matching targets [33,34]. Many miRNA target prediction websites have miRNA prediction tools for miRNAs of model organisms,

but not for viral miRNAs. However, these same software applications can be downloaded and work with any miRNAs, including viral and custom miRNAs, with a little use of the command line applications. Additionally, these command line applications can be used without filtering for conserved target sequences, since mRNA sequence conservation is unlikely important for viral miRNA interactions within a specific host. While these related methods have discovered numerous targets that were eventually validated, these methods are also accompanied by significant false-positive and false-negative target predictions. Multiple miRNA target prediction programs look for perfect complimentary bases in the seed region (usually bases 2–8 of the miRNA). However, some validated sites similar to the miRNA let-7 and a target site in the mRNA lin-41 do not have a perfect complimentary site and related non-canonical sites are missed with prevalent miRNA target prediction software [35]. These strategies also operate under the assumption that a specific 3'UTR is in fact expressed in the cell type of interest. Others have recently shown that 3'UTR length can change dramatically with different developmental stages or growth environments [36,37]. Additionally, these methods assume that sequences are uniformly accessible to miRNAs in the RISC machinery. Some target prediction methods also attempt to filter miRNA target prediction sites based on secondary structure predictions [38]. Those sites with strongly secondary RNA structures may be less likely to be targeted by miRNAs. Other RNA-binding proteins can also inhibit miRNAs from interacting with a specific target.

Other methods for identifying microRNA targets include expression analysis at either the mRNA level using microarrays/RNA sequencing or at the protein level using proteomic methods. These methods typically ectopically express miRNA or inhibit miRNAs and measure changes in gene expression focusing on genes that are repressed in the presence of the viral miRNAs. However repression of gene expression will operate at different rates for different gene products. For example, expression changes for one targeted transcript may occur much faster than for another transcript. Additionally, expression profiling methods will show repressed genes that are both direct miRNA targets and also indirect miRNA targets. However, both direct and indirect targets may have significant phenotypic consequences for the virus. Furthermore, some miRNA targets are repressed at the level of translation and display little or no change at the mRNA level. Recently, a report have shown that using proteomic screening has been successful to identify viral miRNA targets that would be missed looking at the mRNA level [39][40]. Ideally, it is best to search for gene expression changes when showing a gain or loss of a single miRNA function. However, due to the possible redundancy of multiple miRNA targeting the same gene, inhibition of the single miRNA may not result in complete derepression of the target gene (Figure 2).

Some of the most recent methods for miRNA target identification include biochemical enrichment of microRNA:mRNA complexes followed by deep sequencing. Cross-linking microRNAs, mRNAs, and RISC protein complexes is followed by a immunoprecipitation for a RISC protein. RNA sequences associated with the RISC immunoprecipitate are then enriched for sequences targeted by microRNAs. This is typically done in infected cells [4,41,42], which benefits from endogenous expression of the miRNAs, but the immunoprecipitated mRNAs could be associated with both cellular and viral miRNAs. Careful execution should be used as some have reported that associations between microRNAs and RISC components can occur after cell lysis during sample preparation [43].

The specific association of a potential mRNA to an individual miRNA usually relies on seed-matching, which has the limitations that were discussed earlier (non-canonical seed matches are difficult to analyze). Some elegant approaches have included PAR-CLIP, in which, specific viral miRNAs are deleted. By comparing to wild type infections, sequences no longer in the immunoprecipitate in the mutant-infected cells are predicted to be targets of those miRNAs [14][44]. Another version of the biochemical strategy that has been used for cellular miRNAs was the transfection of biotinylated miRNA mimics, followed by purification of mRNA targets [45]. This approach has the advantage of specifically probing for targets of a single miRNA. In conclusion, a variety of sequence analysis, expression analysis and biochemical purification methods are ideal ways to enrich for microRNA targets.

Target Validation

With any genomic-scale screening, it is important to validate predicted miRNA targets. Fortunately, studying viral miRNAs usually allows for utilizing the perfect negative control: the uninfected cell. These cells obviously lack any viral miRNAs and individual or combinations of viral miRNAs can be delivered usually into relevant primary cells. Predicted miRNA target gene expression should be repressed in the presence of the miRNA of interest. This has been demonstrated at the mRNA and the protein levels using qPCR and immunoblotting, respectively. These gene expression measurements are performed after introducing viral miRNAs into uninfected cells or inhibiting or deleting miRNAs in infected cells. There have been concerns about the expression levels of miRNA mimics, but recent data demonstrates [40,46][47] that the miRNA mimic levels incorporated into RISC are similar to amounts of endogenous miRNAs. Validation assays consisting of qPCR or immunoblotting assays are difficult to accomplish for the hundreds or thousands or predicted miRNA targets.

It is also important to map the sequence that is responsive to the viral miRNA. This is carried out using standard luciferase reporter assays. The potential region of interest, typically the full 3' untranslated region, is cloned downstream of a luciferase reporter. Ideally, an internal control, such as a separate luciferase reporter, is also co-transfected along with miRNA mimics or plasmids containing the miRNA gene. If the luciferase reporter contains a miRNA target sequence, then luciferase expression will be reduced in the presence of the miRNA of interest compared to a non-targeting miRNA control. However, it is also important to control for non-specific effects by normalizing luciferase activity to a control luciferase reporter that lacks the cloned potential target (3'UTR). Finally, the specific sequence targeted by the miRNA can be determined by using site-directed mutagenesis to demonstrate that the mutation of a small number of bases in the 3'UTR reporter renders the luciferase insensitive to the miRNA.

If viral miRNAs are expressed during a specific phase of infection, then it is reasonable to expect that miRNA targets will be repressed in infected cells compared to uninfected cells. This is easier to conduct and interpret with *de novo* latent infections. However, viral miRNAs may also suppress increased expression induced by the host [48]. In these cases, inhibition of the viral miRNA will still show derepression. A related validation assay is

determining miRNA target gene expression in normal versus infected clinical samples, if available. Clinical samples of real infections may be difficult to obtain and analyze at the protein level due to limited material. Furthermore, the appropriate normal control sample may be difficult to determine since viral infections may induce multiple changes in the types of host cells at the site of infection.

Since each viral miRNA is likely to have multiple targets, it is also important to address whether a specific phenotype is due to a specific miRNA target. One method is to use siRNAs targeting a specific miRNA target and determine whether the siRNA and miRNA display similar phenotypes. Furthermore, ectopic expression of a miRNA-resistant miRNA target is expected to reverse the phenotype caused by the miRNA, but delivering the relevant amount of ectopic target expression in primary cells can be complicated.

Genomic dangers

The ability to measure thousands of transcripts, proteins, immunoprecipitated sequence, and so forth produces an avalanche of data. However, it is best to carefully describe the specific questions you seek to answer before designing and conducting a genomic-scale search for miRNA targets. Many researchers are confronted with a long list of genes with various metrics (target prediction score, gene expression fold change, CLIP reads), but how to prioritize specific hits for validation and follow-up experiments can be a challenge. Integrating these various types of data is one way of filtering and prioritizing which potential hits to further study. Without a predetermined strategy, researchers can be sentenced to follow many paths, wasting time with false positives. Due to financial costs or other issues, researchers sometimes do not include negative and positive controls in genomic experiments that could be very useful for filtering out false positive hits in the screen. Biological replicates is a recommended requirement and having two different types of negative controls would not add significantly to the resource costs, but can be useful for filtering data and not relying on a single negative control. It is also important to consider the context of gene expression changes. Rather than to focus only of fold changes, consider where those fold change values are in context of the other gene products queried. Using a variety of methods to modulate viral miRNA activity followed by integration of these datasets can be useful for separating biological noise from subtle, but reproducible gene expression changes. With the explosion of online tools for analysis of genomics data comes the risk that researchers could upload their expression data and click a couple of buttons to generate a data analysis report that does not accurately reflect the analysis the researcher planned or understood.

Recent Complexities

Data from deep sequencing has revealed complexities in miRNA analysis. First, multiple miRNAs have been found to contain slight sequences variations. Frequently, an miRNA will display a variety of bases, usually at the 3' end of the miRNA [4]. These sequence modifications may have less of an effect on miRNA targeting since they are more distal from the seed region, but some examples have also shown an additional base at the 5' end of a viral miRNA [49].

Many 3'UTR luciferase assays have been shown to contain multiple functional miRNA target sites; only when multiple sites are mutated is the reporter no longer inhibited by a specific miRNA [50,51]. However, when analyzing the number of PAR-CLIP hits [4] for each viral miRNA, only a small fraction of targets have multiple sites that are predicted to be bound by the same viral miRNA. Specifically, in BC3 cells of the 3760 hits predicted to be KSHV targets, 98% of transcripts contained only one PAR-CLIP per viral miRNA and 2% contained 2 or 3 hits. This may suggest that while many transcripts may contain multiple functional sites for the same miRNA, one site is preferred over others.

Multiple reports have demonstrated that 3'UTR length can change during development or growth condition [36,37]. Therefore, it remains an important consideration to be aware that the 3'UTR in a miRNA target database may not be expressed in the cell type growing in laboratory culture settings. Additionally, the mRNA of interest may not be expressed at all when comparing one cell type to another. We have recently studied a miRNA target that expresses multiple splice variants, which generate three different proteins. These splice variants are differentially targeted by viral miRNAs and are differentially expressed between different cell types (unpublished). More detailed miRNA target datasets refer to specific mRNA transcripts in their analysis, but it may be difficult to determine which short RNA reads from CLIP assays came for which specific alternatively spliced transcript. In conclusion, caution is advised about assumptions about specific 3'UTR expression in a specific cell type.

Direct miRNA targets and their associated functions have been a top priority, but indirect targets may also have interesting and relevant phenotypes. Redundancy of miRNA targeting of the same gene or genes within a pathway can suggest a higher priority of targets when considering the perspective of the virus (Figure 2). However, overlapping redundancy of multiple miRNAs can also hamper efforts of experimentalists because multiple miRNAs may need to be deleted or inhibited simultaneously in order to observe a significant phenotypic change.

Historically, many viral miRNA studies have focused on viral miRNAs expressed in cell culture. Recently, multiple groups have shown that viruses can spread their miRNAs to uninfected cells by incorporating viral miRNAs into exosomes [52,53]. Secreted exosomes with viral miRNAs may be enriched for specific viral miRNAs [54]. This could be the result of different rates of degradation of miRNAs in exosomes and in some cases cellular miRNAs are enriched in different classes of exosomes [55], though the mechanisms of selectivity are currently unclear. Thus, adjacent uninfected cells may actually contain viral miRNAs and display repression of a subset of miRNA targets as compared to infected cells.

Conclusions and Future Directions

It is a worthy pursuit to determine if a miRNA target is in fact displaying altered expression in the context of real infection in the natural host. This is complicated when studying human viruses or when current animal models of infection may lack the proper qualities of natural infections. Many new discoveries remain as animal models [56–60] and technology to inhibit miRNAs during infections continue to improve. In the future, we may be able to

exploit the unique aspects of viral miRNAs for targeted therapy and understand additional virus-host interactions.

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Highlights

- Most viral microRNAs show little or no sequence similarity to other viral microRNAs or cellular microRNAs.
- There is no single quintessential target prediction method, integration of various methods is key to filter and prioritize potential miRNA targets for further analysis.
- Secondary target validation assays are difficult to perform in a high-throughput fashion.

Figure 1.
Summary of common miRNA target prediction and validation methods.

Figure 2.

Complexities of redundant targeting by miRNAs. **A.** A single miRNA can target two sites in a transcript. **B.** Multiple miRNAs can target different sites in the same transcript. **C.** Multiple miRNAs can inhibit multiple nodes of an activation network. Proteins shown as ovals activate other activity/expression of other proteins (green arrows). Various miRNAs can repress the signal transduction pathway by targeting the transcripts giving rise to the depicted proteins.