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4. HHV8-encoded and HHV8-regulated microRNAs

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Summary. MicroRNAs (miRNAs) are noncoding, small RNAs able to inhibit protein expression at the post-transcriptional level and are encoded by a variety of species including plants, animals and even viruses. HHV8 encodes a cluster of 12 viral miRNAs expressed from within the latent region of the viral genome. Although the specific functions of these miRNAs have not been fully elucidated, several candidate target genes have been found. In addition, HHV8 has also been found to modulate several cellular miRNAs. Further study of the HHV8-encoded miRNAs and virally-regulated miRNAs may reveal new insight for progression of tumorigenesis and HHV8 pathogenesis.

1. Introduction

MicroRNAs (miRNAs) are small, noncoding RNAs which regulate protein expression at the post-transcriptional level [1]. They are generally well-conserved and encoded by a wide variety of species from plants to animals, as well as viruses. These small RNAs are processed into a 19-24 nucleotide (nt) mature form which can bind target mRNAs in a sequence-specific manner and inhibit mRNA translation through use of the RNA silencing machinery [1]. A schematic of miRNA processing is shown in Figure 1.

MiRNAs are first transcribed by RNA polymerase II as a longer primary miRNA (pri-miRNA) transcript that can encode one to several miRNAs [2,3]. As a result of sequence complementarity of inverted repeats within the pri-miRNA transcript, a hairpin

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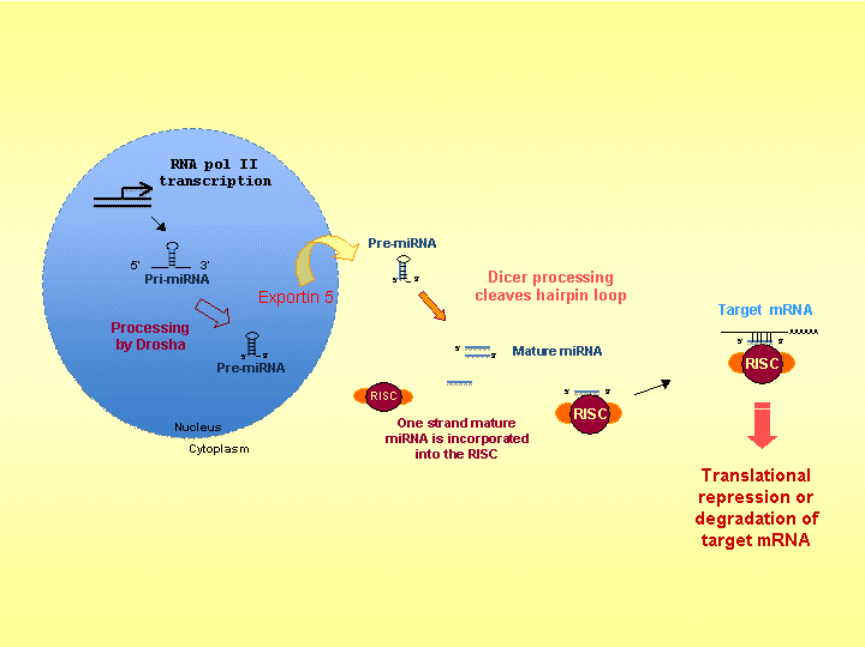


Figure 1. MicroRNA transcription, export and processing. MicroRNAs are transcribed by RNA polymerase II, yielding a primary (Pri-miRNA) transcript. This form is then processed by the enzyme Drosha to produce the pre-miRNA. The pre-miRNA is then exported from the nucleus by exportin 5 where it is further processed. The enzyme Dicer cleaves the hairpin loop of the pre-miRNA resulting in the formation of the mature miRNA. One of the mature miRNA strands is then incorporated into the RISC, allowing for translational repression or degradation of the target mRNA.

stem-loop structure known as the pre-miRNA is formed co-transcriptionally [4]. The pre-miRNA is recognized by the nuclear enzymes Drosha and DGCR8, which cleave the stem-loop structure from the pri-miRNA transcript [5-8]. The ~ 50 nt pre-miRNA then undergoes nuclear export into the cytoplasm. This is mediated by Exportin 5 [9-11]. Following export, the cytoplasmic enzyme Dicer binds to the pre-miRNA and cleaves the loop of the hairpin structure, yielding a RNA duplex of approximately 21-23bp [1,4,12,13]. One of the strands of the mature miRNA is then incorporated into the RNA-induced silencing complex (RISC) which can now function on its target mRNA, while the remaining mature miRNA strand is usually degraded [12,14-16]. Selection of the incorporated strand can depend on the relative stability of the base pairing at the 5' end of each strand. The mature miRNA strand exhibiting weaker base pairing at its 5' end is usually selected for incorporation into the RISC [17,18]. However, if there is little difference in the base pairing stability, each strand may be incorporated into the complex, thus increasing the number of potential mRNA targets.

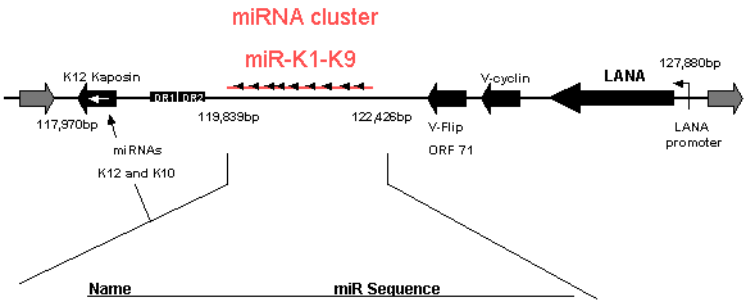
Once the miRNA/RISC complex is formed, the mature miRNA directs the RISC to a complementary mRNA target species [19]. Target specificity is conferred by the miRNA seed sequence, which refers to the 5' nucleotides 2-7 of the miRNA sequence [20]. In the case of a perfect match between the miRNA seed sequence and the target mRNA, the mRNA is cleaved and degraded by Argonaute-2, the catalytically active component within the RISC complex [21-24]. If the sequence is only partially complementary, the

binding of the miRNA to its target can induce translational repression of the target mRNA [25-27]. Since miRNAs are able to regulate mRNA targets with either perfect or imperfect complementarity, this enables them to exert their inhibitory effects on a larger number of potential mRNA targets. Furthermore, this allows miRNAs to function in a wide variety of cellular processes, including cellular proliferation, differentiation, apoptosis, metabolism, immunity and cancer.

MiRNAs have been identified in several herpesviruses including the gamma-herpesviruses Epstein-Barr Virus (EBV) and Human Herpesvirus-8/Kaposi's sarcoma-associated herpesvirus (HHV8/KSHV) [28-31]. Given the role of these viruses in solid organ and lymphoproliferative neoplasms, it is not surprising that virally-encoded miRNAs play a role in the dysregulation of cellular processes which result in these malignancies.

2. HHV8 pathogenesis and virally-encoded miRNAs

HHV8 is the etiological agent of Kaposi sarcoma (KS) and also associated with lymphoproliferative diseases including primary effusion lymphoma (PEL) and multicentric Castleman's disease (MCD) [32]. This virus primarily infects B cells and endothelial cells. It is known to use its viral genes to manipulate host cell signaling to induce angiogenesis, proliferation and tumorigenesis. In addition to its many viral genes, HHV8 also encodes several viral miRNAs [28,30,33,34, see Figure 2). Thus far, a total of



Name	miR Sequence
miR-K12-1	ATTACAGGAAACTGGGTGTAAG
miR-K12-2	TCACATTCTGAGGACGGCAGCGA
miR-K12-3	AGCTAAACCGCAGTACTCTAGG
miR-K12-4	TAGGATGCCTGGAACTTGCCGGT
miR-K12-5	TGATGGTTTTCGGGCTGTTGAG
miR-K12-6	TTAATGCTTAGCCTGTGTCCGAT
miR-K12-7	TGATCCCATGTTGCTGGCGCTC
miR-K12-8	CTAGGCGCGACTGAGAGAGCAC
miR-K12-9	GCTGGGTATACGCAGCTGCGT
miR-K12-10	TTAGTGTTGTCCCCCCGAGTGGC
miR-K12-11	TTAATGCTTAGCCTGTGTCCGA
miR-K12-12	ACCAGGCCACCATTCCTCTCCG

Figure 2. HHV8-encoded microRNA cluster and sequence of individual viral miRs. The latent region of the HHV8 genome between 117,970 and 127,880bps is shown. A cluster of virally-encoded miRNAs is found between the K12 and V-Flip ORFs (denoted by the small arrowheads between 119,839 and 122,426bps). Two miRNAs are also found within the K12 ORF (shown as a white arrow within K12). Together, these comprise the 12 known HHV8-encoded miRNAs, each of whose sequence is located below.

12 HHV8 miRNAs have been identified using latently infected PEL cell lines [34,35]. Further detail of the HHV8 miRNAs, their regulation, processing and what little is currently known about their targets and function will be the focus of this chapter.

2.1. Discovery of HHV8 miRNA cluster and miRNA expression

Four groups independently identified the same HHV8-encoded miRNAs. Three through cloning of small RNA libraries from PEL cell lines and alignment of these sequences to the HHV8 genome [28,33,34], and one through a viral genome-spanning array [30]. All 12 miRNAs identified are expressed in latently infected PEL cells, although the relative abundance of each miRNA differed. All virally-encoded miRNAs were found within the latency region of the HHV8 genome, within a contiguous miRNA cluster [28, 33,34]. This miRNA cluster mapped to the intergenic region between the latency genes Kaposin (K12) and v-FLIP (orf71) [28,33-35]. Only two of the 12 identified viral miRNAs, mir-K12-12 and mir-K12-10, are located outside of this miRNA cluster (Figure 2). Mir-K12-12 is found in the 3'UTR of the K12 gene whilst miR-K12-10 is located in the middle of the K12 open reading frame [28,33,34].

Since all of the HHV8 miRNAs are within this small latency region of the genome, and since they are transcribed in the same direction, it is speculated that they are all processed from a single promoter [28,36]. Because the HHV8 miRNAs are expressed within the latency region of the genome, the function of these miRNAs may be important in establishing latency. However, since they are also expressed during lytic viral replication, miRNA functions relating to other lytic processes cannot be ruled out. Several studies have shown that the expression of most HHV8 miRNAs is unchanged when latently infected cells are stimulated with 12-O-tetradecanoylphorbol-13-acetate (TPA) to undergo lytic replication [28,33,34]. This would be expected if these HHV8 microRNAs were processed out of an intron of the HHV8 latency associated nuclear antigen (LANA) mRNA, which is under control of a viral latent promoter that also does not change upon TPA stimulation [37]. The exception is mir-K12-10, the expression of which is upregulated upon induction of lytic replication. This would be expected, since mir-K12-10 is derived from the kaposin mRNA, which is under control of the distal LANA promoter during latency and under control if its own proximal promoter during lytic replication. This suggests that mir-K12-10 and mir-K12-12 may play a role during the HHV8 lytic phase as well as during viral latency [28,33].

2.2. HHV8 miRNAs function

The microRNAs of several species have been demonstrated to be highly conserved, suggesting that they might play an important role in evolutionarily conserved cellular processes [1,38]. Interestingly, sequencing analysis of the HHV8 viral genes has also indicated that there is a high level of conservation among the HHV8 miRNA sequences in latently infected cell lines and in KS, MCD and PEL patient samples [39, O'Hara and Chugh, unpublished observations]. The observation that the HHV8 miRNAs may be highly conserved suggests that there is a tight level of selection on these miRNAs, which may be attributed to the importance of their function in all HHV8-associated malignancies.

Although the functions of the HHV8 miRNAs remain largely unknown, several viral and cellular genes have been implicated as targets. Computational predictions have implicated a role for the HHV8 miRNAs in regulation of the viral genes ORF 23, 27, 31,

52, 49, 61, 68, K7, K13 and K14 [28], though none of the viral targets have been verified. While further studies validating these genes as targets remain to be performed, it has been speculated that these miRNAs may play a role in viral replication and pathogenesis.

Since expression of the viral miRNAs are detectable in KS tumors, it is likely that their functions may contribute to tumor progression and the dysregulation of cellular processes associated with oncogenesis. Several studies have identified cellular genes that are drastically decreased following expression of the HHV8 miRNAs. Some of the proposed targets include genes involved in regulation of proliferation, immune modulation, angiogenesis and apoptosis [40,41]. Of particular interest is thrombospondin 1 (THBS1), a gene that inhibits many of these activities and is known to be downregulated in KS as well as other cancers. THBS1 has been confirmed as a target of several of the HHV8 miRNAs including mir-K1-1, mir-K12-3-3p, mir-K12-6-3p and mir-K12-11 [40]. The resulting inhibition of THBS1 translation leads to decreased activity of TGF-β, a cytokine important in proliferation and apoptosis, which may ultimately contribute to KS tumorigenesis [40]. BCLAF1, or Bcl2-associated transcription factor, was identified as a cellular mRNA target of miR-K12-5 and miR-K12-9 and -10 through a series of array-based expression screens [42]. Expression of these HHV8 miRNAs resulted in decreased BCLAF1 mRNA levels and led to decreased PARP cleavage and caspase activation, two hallmarks of apoptosis induction [42]. Additional studies examining the consequences of BCLAF1 inhibition may reveal important functions for the HHV8 miRNAs that target this gene.

2.3. MIR-155 homolog

In addition to targeting several known cellular genes important in tumor progression, one HHV8 miRNA has been shown to target a host cellular microRNA network. This miRNA, mir-K12-11, encodes an ortholog of the human cellular miRNA miR-155 with which it shares 100% seed sequence homology [43,44]. Interestingly, miR-155 is upregulated in several human lymphomas and was identified as the first oncomiR. Mir-155 plays a role in B cell development and adaptive immunity by controlling cytokine production, which is dysregulated in both PEL and KS [45-48].

These findings led to further studies into the proposed shared functions of the two microRNAs. Mir-155 is processed from the B-cell integration cluster (BIC) mRNA, which itself is frequently overexpressed in B cell lymphomas and several other cancers [49,50]. When a subset of PEL cell lines were tested for BIC mRNA and miR-155 expression, the HHV8-positive PEL cell lines were found to lack both of these, but all highly expressed miR-K12-11 [44]. This suggests that the HHV8 miRNA miR-K12-11 may mimic the miR-155 pathway and therefore may also share similar targets.

Of particular interest is the shared target BACH-1, which is knocked down significantly in cells expressing miR-155 or miR-K12-11 [43,44]. BACH-1 is a transcriptional repressor that regulates genes involved in the response to hypoxia [51]. One of its regulatory genes HMOX1, displays increased levels associated with enhanced cellular survival and proliferation following HHV8 infection of endothelial cells [52]. Several other genes were identified as targets of both miRNAs. These include LDOC1 and several members of the Bcl-2 and Bcl-6 family, many of which are known to be differentially expressed in human cancers [43,44]. Undoubtedly, there are likely many more target mRNAs which could not be identified due to statistical limitations. Therefore, miR-K12-11 may regulate a similar set of target genes as miR-155 in order to

contribute to the dysregulation of B cell proliferation, apoptosis and lymphomagenesis associated with HHV8 malignancies.

3. HHV8 infection and alteration of cellular miRNAs

In addition to regulation of certain cellular genes by the HHV8 viral miRNAs, the cellular miRNA repertoire can also be significantly altered upon infection with HHV8. Recently, miRNA signatures that are unique for PEL samples and KS tumors have been described [53]. Following analysis of several PEL cell lines and patient samples by quantitative PCR array, 68 cellular miRNAs emerged as unique PEL-specific miRNAs [53]. The miRNA signature for PEL included several miRNAs specific to the B cell lineage and B cell lymphomas. Interestingly, there were similar trends among the pre-miRNA and mature miRNA profiles, suggesting that analysis of pre-miRNA levels may be good predictors of the abundance of mature miRNAs [53].

The pre-miRNA signatures for KS have also been described. Using a variety of cell lines representing stages from untransformed endothelial cells to KS tumor biopsies, a number of cellular miRNAs that play a role in transformation and tumorigenesis were identified (O'Hara in press). The increased expression of mir-15 and the subsequent loss of mir-221 correlated with the transition from immortalized to fully tumorigenic endothelial cells [53]. As expected, the expression of the HHV8 miRNAs increased linearly with the degree of cellular transformation. Mir-140 also exhibited a linear increase with progression through transformation while the cellular microRNA mir-24 emerged as a signature biomarker specific for KS [53]. These data suggest that different miRNAs may be expressed during specific stages of transformation following HHV8 infection, resulting in the formation of KS tumors.

4. Conclusion

MicroRNAs have emerged as a novel group of gene expression regulators with potential roles in viral infection and tumorigenesis. Virally-encoded miRNAs, such as those encoded by HHV8 have the ability to modulate their own viral gene expression, cellular gene expression as well as the cellular microRNA repertoire. This can ultimately interfere with the regulation of several key cellular processes including proliferation, apoptosis and immunity, resulting in the progression of HHV8-associated malignancies. Further studies focusing on the identification and regulation of potential targets will provide great insight and may reveal novel strategies for treatment of these diseases.

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