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Virus-encoded miR-155 ortholog is an important potential regulator but not essential for the development of lymphomas induced by very virulent Marek's disease virus

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ABSTRACT

The microRNA (miRNA) mdv1-miR-M4, a functional miR-155 ortholog encoded by oncogenic Marek's disease virus (MDV), has previously been suggested to be involved in MDV pathogenesis. Using the technique of bacterial artificial chromosome mutagenesis, we have presently evaluated the potential role of mdv1-miR-M4 in the oncogenesis of the very virulent (vv) MDV strain GX0101. Unexpectedly, deletions of the Meq-cluster or mdv1-miR-M4 alone from the viral genome strongly decreased rather than abolished its oncogenicity. Compared to GX0101, mortalities of mutants GXΔmiR-M4 and GXΔMeq-miRs were reduced from 100% to 18% and 4%, coupled with the gross tumor incidence reduction from 28% to 22% and 8%, respectively. Our data suggests that the mdv1-miR-M4 is possibly an important regulator in the development of Marek's disease (MD) lymphomas but is not essential for the oncogenicity of vvMDV. In addition, some of the other Meq-clustered miRNAs may also play potentially critical roles in vvMDV induction of lymphomas.

Introduction

The discovery of microRNA (miRNA) can be seen as one of the more important discoveries in the life sciences during the past 2 decades. These small non-coding RNAs (sncRNAs) play important post-transcriptional regulatory roles in various cellular processes, including development, differentiation, and all aspects of cancer biology (Bartel, 2004; Filipowicz et al., 2008; Lee and Dutta, 2009). Thousands of miRNAs have been identified in animals and plants, and even in viruses. MiRNAs encoded by diverse virus families, such as Epstein–Barr virus (EBV), Kaposi's sarcoma-associated herpesvirus (KSHV), and Marek's disease virus (MDV), are found to be involved in their pathogenicity, especially in the tumorigenicity (Boss et al., 2009; Morgan and Burnside, 2011; Grundhoff and Sullivan, 2011; Kincaid and Sullivan, 2012). Phylogenetically, MDV is classified into the subfamily *Alphaherpesvirinae* (Davison et al., 2009). As one of the most potent oncogenic herpesvirus, the infection of MDV can cause a highly contagious, lymphoproliferative disorder, and neoplastic disease of poultry named as Marek's disease (MD) (Witter and Schat, 2003). Since it was identified as a pathogen, three distinct serotypes, serotype 1 (MDV-1), serotype 2 (MDV-2), and serotype 3 (herpesvirus of turkeys, HVT), have been characterized but recently reclassified as species of *Gallid herpesvirus* 2 (GaHV2), *Gallid herpesvirus* 3 (GaHV3), and *Meleagrid herpesvirus* 1 (MeHV1), respectively (Witter and Schat, 2003; Davison et al., 2009). The virulent strains of GaHV2 establish and maintain latent infections in their natural hosts and may finally

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cause a rapid onset aggressive T-cell lymphoma (Nair and Kung, 2004). MD is considered to be an excellent biomedical model for the study of virus-induced lymphoma (Osterrieder et al., 2006) and provided the first demonstration of the efficacy of anti-viral vaccination in the control of cancer. The molecular mechanisms of MDV pathogenicity and particularly with respect to the oncogenesis have historically been an attractive focus of research.

In recent years, it has been suggested that among the more than 100 viral genes, the GaHV2-specific gene Meq (MDV EcoRI-Q), which encodes the basic leucine zipper protein, is the major oncogene while the other genes, including the BamH I-H family of transcripts, phosphoprotein 38 (pp38), viral interleukin-8 (vIL-8), ICP4-related transcripts (R-ICP4), and MDV-encoded telomerase RNA, are associated with MDV pathogenicity (Burgess, 2004). However a large number of viral miRNAs have been found to be encoded in the genomes of all three serotypes of MDV (Burnside et al., 2006; Yao et al., 2007, 2008, 2009; Waidner et al., 2009). Although MDV-encoded miRNAs do not show conservation in sequence, their genomic location in the long or short repeat (R_L/R_S) regions is conserved in all three serotypes of MDV. MDV genomes are primarily composed of a 'unique long region' (UL) and a 'unique short region' (US), flanked by inverted repeats namely as 'terminal and internal repeat long regions' (TRL/IRL) and 'internal and terminal repeat short regions' (IRS/TRS) (Cebrian et al., 1982). The genes encoded by the UL and US regions are highly conserved among herpesviruses whereas virus-specific genes are mainly located in the inverted repeat regions (Osterrieder et al., 2006). Thus the highly conserved genomic location of the viral miRNA implies that they have important functions.

In the viral genome of GaHV2 as shown in Fig. 1a and b, all of the virus-encoded miRNAs, each presenting as two identical copies, are focused in three gene clusters, namely Meq-cluster, mid-cluster, and LAT-cluster (Luo et al., 2010). The Meq-clustered miRNAs, upstream from the *meq* oncogene, are mdv1-miR-M9, mdv1-miR-M5, mdv1-miR-M12, mdv1-miR-M3, mdv1-miR-M2, and mdv1-miR-M4 (Burnside et al., 2006; Yao et al., 2008). Intervals between these miRNA precursors are short, less than 220 nt. A recent report has shown that these miRNAs are located in the first intron of the transcripts covering the IR_L/TR_L region, and their transcription is driven by a single promoter, prmiRM9M4, under two distinct transcriptional models during different infection phases (Coupeau et al., 2012). The mid-clustered miRNAs, including mdv1-miR-M11, mdv1-miR-M32, and mdv1-miR-M1, are embedded within the open reading frame (ORF) of the L1/LORF5a transcript as well as within the intron of the splice variant Meq-sp, located downstream of the Meq-cluster and upstream of the LAT-cluster. Other GaHV2-encoded miRNAs, such as mdv1-miR-M8, mdv1-miR-M13, mdv1-miR-M6, mdv1-miR-M7 and mdv1-miR M10, are located in the large intron of the latency-associated transcript (LAT) and comprise the LAT-cluster (Burnside et al., 2006; Yao et al., 2008). Compared to Meq-clustered miRNAs, intervals between LAT-clustered miRNA precursors are shorter, with some overlaps. A p53-dependent promoter, which has no consensus core promoter element but contains at least two 60-bp tandem repeats harboring a p53-response element, has been found to drive the transcription of LAT-clustered miRNAs (Stik et al., 2010).

MiRNA expression signatures in many cancers have been characterized and further increased our understanding of the connections between miRNA and tumorigenesis (Calin and Croce, 2006). For GaHV2-encoded miRNAs, studies focusing on the miRNA expressions in virus-infected chicken embryo fibroblast (CEF) cultures, virus-transformed cell lines, and virus-caused splenic tumors have shown

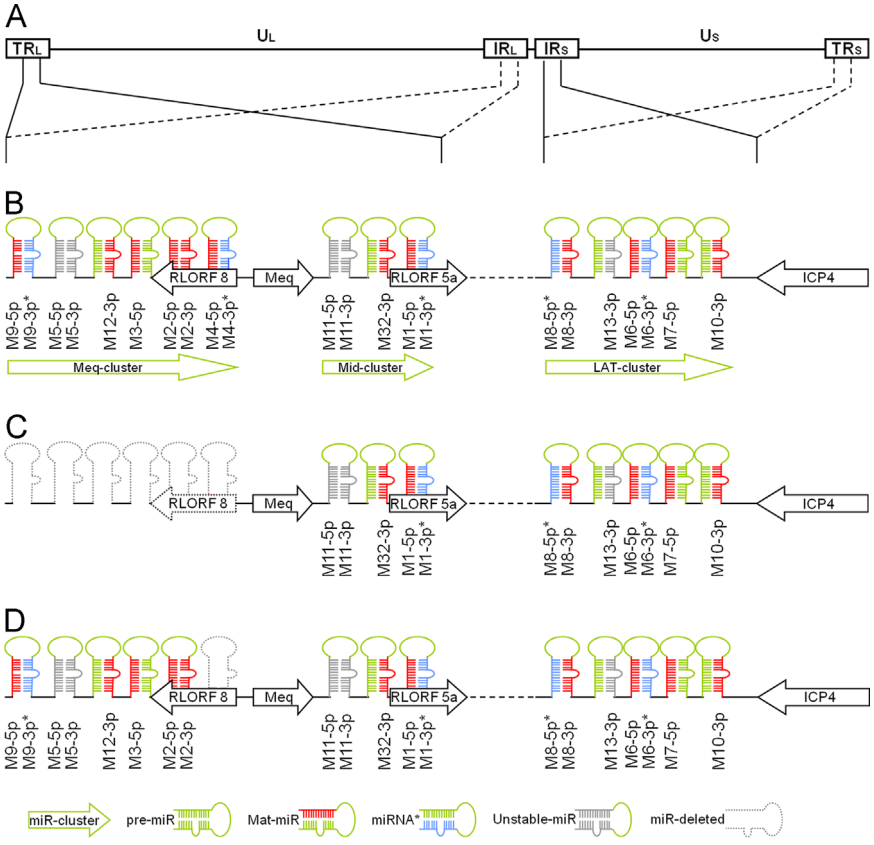

Fig. 1. Schematics of the viral genome of GaHV2 (A) and viral miRNAs located in MDV strains GX0101 (B), GXΔMeq-miRs (C), and GXΔmiR-M4 (D). Relative genomic locations of viral miRNAs at two identical genomic loci are shown by solid or dashed lines. MiRNA precursors (pre-miR) and miRNA gene clusters (miR-cluster) are shown by green hairpins or hollow arrows, respectively. Mature miRNAs (mat-miR), passenger miRNAs (miRNA*) and unstable miRNAs (unstable-miR) are shown by red, blue or gray strands in the stems of green hairpins, respectively. As for the deleted miRNAs (miR-deleted), they are shown by dashed hairpins without strands in the stems.