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Review

A challenge for miRNA: multiple isomiRs in miRNAomics

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ABSTRACT

Accumulating evidence suggests that a single microRNA (miRNA) locus can generate a series of sequences during miRNA maturation process. These multiple sequences, called miRNA variants, or isomiRs, have different lengths and different 5′ and 3′ ends. Some of these isomiRs are detected as varied nucleotides and 3′ additional non-template nucleotides. As physiological miRNA isoforms, they have drawn attention for possible regulatory biological roles. The present work mainly reviews miRNA/isomiR biogenesis, isomiR expression patterns, and functional and evolutionary implications, especially between isomiRs from homologous and clustered miRNA loci. The phenomenon of multiple isomiRs and their biological roles indicates that analysis performed at the miRNA and isomiR levels should be included in miRNA studies. This may enrich and complicate miRNA biogenesis and coding–non-coding RNA regulatory networks.

Contents

1. Introduction-miRNA/isomiR biogenesis

MicroRNAs (miRNAs) are a class of small non-coding RNAs (≈22 nt) that repress gene expression at the post-transcriptional level (Bartel, 2009; Huntzinger and Izaurralde, 2011; Krol et al., 2010). In animals, miRNA is first transcribed from the genomes as a primary miRNA transcript (pri-miRNA). The longer pri-miRNA molecules are then cleaved into a precursor miRNA (pre-miRNA) by a member of the RNase III family (such as Drosha) (Han et al., 2004, 2006; Lee et al., 2003). The pre-miRNA is about 70 nt with a stem-loop structure, which is then exported to the cytoplasm (Okada et al., 2009). The hairpin generates a miRNA:miRNA* duplex (now also called miR-#-5p:miR-#-3p or miR-#-3p:miR-#-5p duplex) using another member of the RNase III family (such as Dicer) (Gregory et al., 2005; Han et al., 2006). Increasing numbers of miRNA studies suggest that both miR-#-5p and miR-#-3p may be abundantly expressed and contribute to coding–non-coding RNA regulatory network by acting as negative regulatory molecules (Czech et al., 2009; Guo and Lu, 2010a; Jagadeeswaran et al., 2010; Okamura et al., 2008, 2009). In this way, miR-#-5p and miR-#-3p generated from pre-miRNA are simultaneously annotated in the miRBase database, and the word of "miRNA*" (miRNA star, also named miRNA passenger strand) is gradually reduced in the miRNA studies. In animals, miRNAs regulate target mRNAs primarily through complementary binding of seed sequences of 2–8 nucleotides of miRNAs and 3′ untranslated regions (UTRs) of mRNAs (Bartel, 2009; Huntzinger and Izaurralde, 2011; Krol et al., 2010). They play important roles in various biological pathways, acting as negative regulators by repressing gene expression (Alvarez-Garcia and Miska, 2005; Bartel,

Abbreviations: miRNA, microRNA; pri-miRNA, primary miRNA; pre-miRNA, precursor miRNA; miRNA*, miRNA star; UTRs, untranslated regions; SNPs, single nucleotide polymorphisms; RISC, RNA-induced silencing complex; aca, *Anolis carolinensis*; bta, *Bos taurus*; cfa, *Canis familiaris*; cgr, *Cricetulus griseus*; dre, *Danio rerio*; gga, *Gallus gallus*; eca, *Equus caballus*; ggo, *Gorilla gorilla*; hsa, *Homo sapiens*; mdo, *Monodelphis domestica*; mml, *Macaca mulatta*; mmu, *Mus musculus*; oan, *Ornithorhynchus anatinus*; oar, *Ovis aries*; ola, *Oryzias latipes*; pma, *Petromyzon marinus*; ppa, *Pan paniscus*; ppy, *Pongo pygmaeus*; ptr, *Pan troglodytes*; rno, *Rattus norvegicus*; ssc, *Sus scrofa*; tgu, *Taeniopygia guttata*; xtr, *Xenopus tropicalis*.

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2004). A series of specific miRNAs have been identified as crucial regulators in the oncogenic pathways, including human oncogenes, tumor suppressors, and tumor-associated and cancer-related genes (Koralov et al., 2008; Mendell, 2008).

Many miRNA genes have been characterized as homologous miRNAs and classified in specific miRNA gene families. Simultaneously, the small non-coding RNAs are well conserved phylogenetically across the animal kingdom, and this is why miRNA gene families are always composed of miRNAs from different animal species and homologous miRNAs in specific species. These homologous miRNAs, including multi-copy pre-miRNAs, are mainly derived from complex duplication evolutionary histories with varying nucleotides (Grimson et al., 2008; Guo and Lu, 2010a; Hertel et al., 2006; Sempere et al., 2006). Some of these sequence-related miRNAs are clustered in specific region on chromosomes, and they are composed of miRNA gene clusters with other miRNA members based on short physical distances (such as less than 10 kb). These close physical relationships allow that they can be co-transcribed from genomic DNA as a single polycistronic transcript (Kim and Nam, 2006; Lagos-Quintana et al., 2003; Lim et al., 2003; Xu and Wong, 2008). The miRNA gene families and miRNA gene clusters are widespread in metazoan genomes, and these homologous and clustered miRNAs have pivotal roles in multiple biological pathways by co-regulating or coordinately regulating biological processes. Some miRNA gene families (such as miR-103–107 gene family) and clusters (such as miR-17–92 gene cluster) have been found to be crucial and potential biomarkers of various human diseases (Chen et al., 2012; Khan et al., 2013; Patel et al., 2013; Polster et al., 2010; Trajkovski et al., 2011; Wang et al., 2013).

miRNA is ever detected and annotated as a single sequence based on traditional experimental validation and bioinformatic prediction methods. However, accumulating amounts of evidence suggest that a many different sequences can be generated from a single miRNA locus through the miRNA processing and maturation process (Guo et al., 2011c; Landgraf et al., 2007; Lee et al., 2010; Morin et al., 2008; Neilsen et al., 2012) (Fig. 1A). The similar phenomenon of multiple miRNA isoforms is also detected in plant miRNAs (Shao et al., 2013; Zhang et al., 2013). High-throughput sequencing techniques have been widely applied in miRNA studies, and multiple sequences from small RNA sequencing data are mapped to pre-miRNAs and to genome sequences. The cross-mapping and multiple-mapping events are common during mapping process (de Hoon et al., 2010; Guo and Lu, 2010b; Guo et al., 2011a, 2011b). Compared to the annotated and canonical miRNA sequences in the miRBase database, these shorter sequencing sequences may have various 5′ and 3′ ends (especially various 3′ ends) and lengths. These are called miRNA variants, or isomiRs (Fig. 1A). They are mainly derived from imprecise cleavage of Drosha and Dicer, 3′ addition events, RNA editing, and single nucleotide polymorphisms (SNPs) (Neilsen et al., 2012). The annotated or canonical miRNA sequence is only one type of multiple isomiRs. Some isomiRs are involved in non-template nucleotides in internal areas, 5′ ends, and especially 3′ ends. Although isomiRs are ever considered experimental artifacts or by-products, they have been found in many different

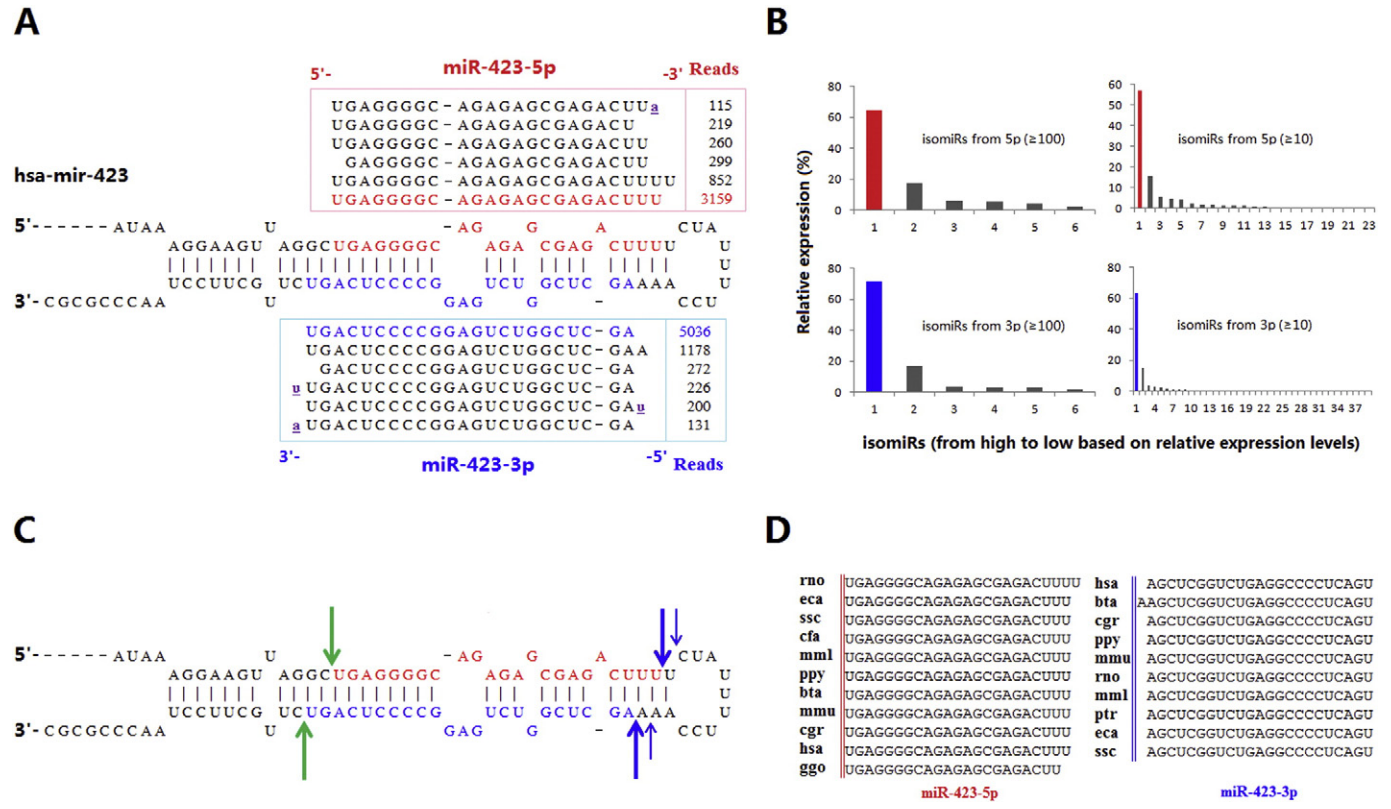

Fig. 1. Multiple isomiRs and their characteristics are demonstrated using the miR-423 example. (A) Both arms of hsa-mir-423 can generate abundant mature miRNAs (these isomiRs and their relative expression levels can be found in box with different colors, and the canonical miR-423-5p is highlighted in red and the canonical miR-423-3p is highlighted in blue). Various isomiR sequences can be detected from the two miRNA loci with various sequences and expression levels. Generally, 5′ isomiRs (with the novel 5′ ends and seed sequences) and isomiRs with 3′ additions are not dominant isomiR species. (B) Distribution of the expression of isomiRs based on relative expression levels (expression percentage in the miRNA locus). Different isomiRs are analyzed according to their normalized sequence counts (≥100 or ≥10). (C) According to differences in expression between different isomiRs, dominant cleavage sites of Drosha and Dicer are identified. The green arrow indicates the dominant cleavage site of Drosha, and the blue arrow indicates the dominant and secondary dominant cleavage sites of Dicer. (D) Diversity of miRNA sequences across different animal species as indicated by the annotated and canonical miRNA sequences in the miRBase database (Release 20.0, http://www.mirbase.org/) (Kozomara and Griffiths-Jones, 2011). These annotated miRNA sequences only indicate the specific sequences through experimental validation or bioinformatic prediction. The data of isomiR expression patterns are obtained from the L02 cells according to the published literature (Guo et al., 2014b). The small RNA sequencing dataset was profiled using Genome Analyzer IIx platform.

tissues and species, including animals and plants (Burroughs et al., 2010; Cloonan et al., 2011; Ebhardt et al., 2009; Fernandez et al., 2009; Guo et al., 2011c; Kuchenbauer et al., 2008; Landgraf et al., 2007; Lee et al., 2010; Lu et al., 2009; Shao et al., 2013; Zhang et al., 2013). They may be potential regulatory molecules and they may act by associating with mRNAs (Cloonan et al., 2011; Lee et al., 2010). For example, isomiRs can increase miRNA stability (Fernandez-Valverde et al., 2010), load into RISC (RNA-induced silencing complex) (Ebhardt et al., 2009; Seitz et al., 2008), and affect the effectiveness of miRNAs (Burroughs et al., 2010; Lu et al., 2009). These findings suggest that the multiple isomiRs from a single miRNA locus are not random events, but are rather physiological miRNA isoforms. However, most public databases only list the canonical miRNA sequences, and several database record the detailed information for isomiRs (such as YM500 (Cheng et al., 2013) and isomiRex (Sablok et al., 2013). miRNA studies, especially for those studies involved in

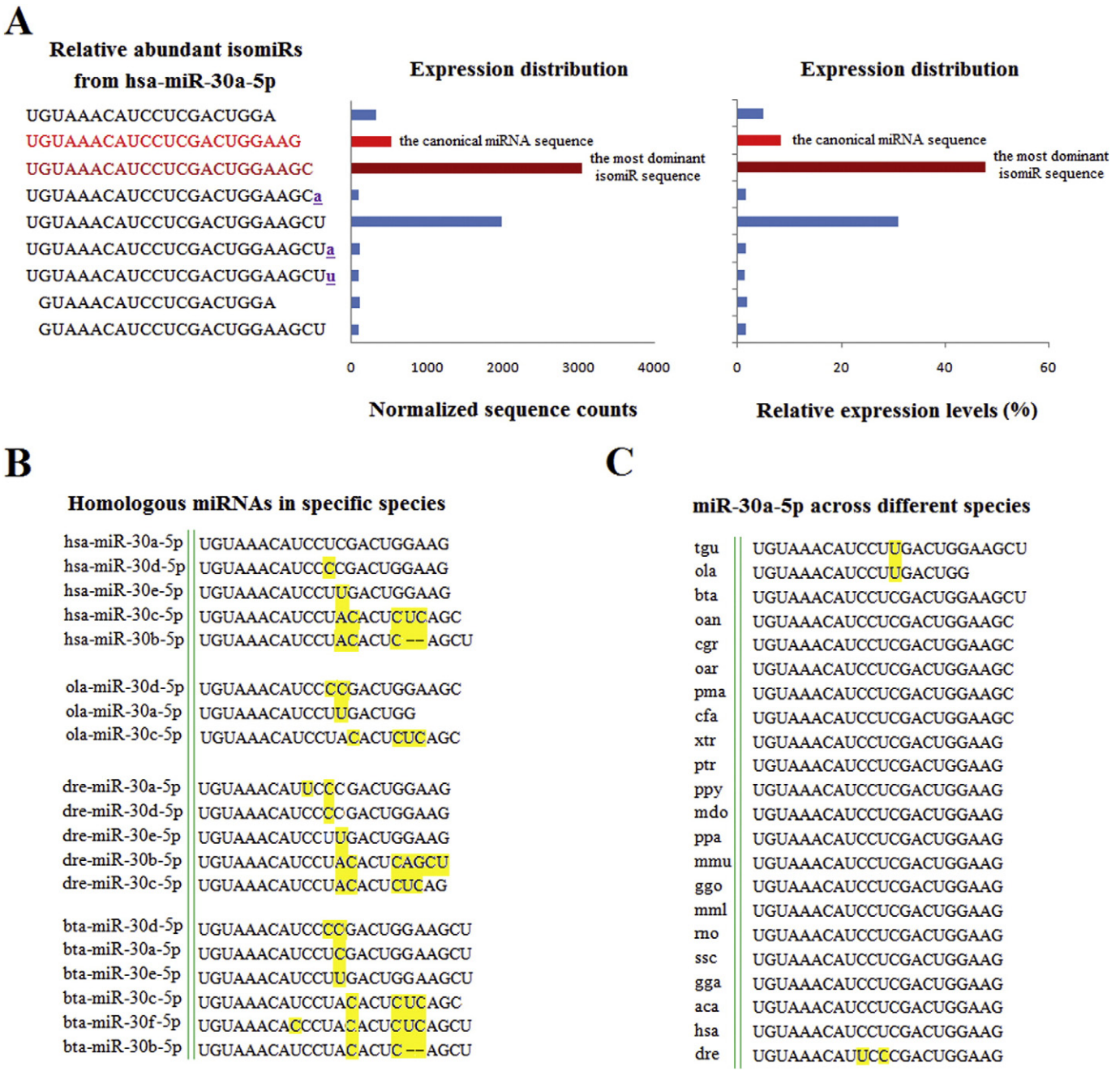

Fig. 2. Sequence diversity between multiple isomiRs, homologous miRNAs, and miRNAs across different animal species are demonstrated using the miR-30a and its homologous miRNA examples. (A) Sequence diversity of multiple isomiRs from the hsa-miR-30a-5p locus, and their expression patterns as indicated on the normalized sequence counts and relative expression levels, respectively. The red sequence is the annotated canonical miRNA sequence as recorded in the miRBase database, and the deep purple sequence is the most dominant isomiR sequence. The canonical miRNA sequence is not always the most abundant species, and abundant isomiRs are 3′ isomiRs with the same seed sequences. Abundant isomiRs are not involved in 3′ additional non-template nucleotides. The data are from the L02 cells from the published literature (Guo et al., 2014b). (B) Sequence diversity of homologous miRNAs in four specific species according to annotated miRNA sequences in the miRBase database. These homologous miRNAs have the same 5′ ends and seed sequences. However, they contain various differences in nucleotide sequence, including nucleotide substitutions (transition and transversion) and nucleotide insertions and deletions. These homologous miRNAs, which are in the miR-30 gene family, are obtained from the miRBase database They are aligned with Clustal X 2.0 software (Larkin et al., 2007). (C) These miRNA sequences show diversity across different animal species. miR-30a-5p is well conserved; the same sequence is shared by many different species. The same 5′ ends and seed sequences are detected across the 22 animal species evaluated here, and only a few of them are found to contain divergent nucleotide sequences and 3′ ends.

experimental validation, only focus on the single miRNA sequences. The present study mainly addresses the isomiR expression patterns and evolutionary and functional implication between isomiRs from homologous and clustered miRNA loci. IsomiR repertoires and expression patterns from a specific miRNA locus, or across different miRNA loci, especially between related miRNA loci (homologous miRNAs and clustered miRNAs), may provide insight into the study of miRNA and isomiRs.

2. IsomiR repertoires and expression patterns

Each miRNA locus can be detected as multiple isomiRs unless the locus is not expressed. These isomiRs have different 5′ and 3′ ends and different length distributions (Fig. 1A). The types of isomiRs, isomiR repertoires, and expression patterns may be similar or different across different miRNA loci. Similar to the annotated or canonical miRNA sequences, most of isomiRs are 3′ isomiRs with the same 5′ ends and seed sequences, and fewer 5′ isomiRs are detected (Fig. 1A). For those 5′ isomiRs with the new seed sequences, the "seed shifting" events can be detected. Despite with "seed shifting", these 5′ isomiRs may have novel function by regulating novel mRNAs, but they still share many target mRNAs through binding different regions of UTR. 3′ isomiRs with the same seeds ensure that these isomiRs are associated with the same target mRNAs and contribute to the same biological pathways as the canonical miRNA sequence. Among these multiple isomiRs, the canonical miRNA sequence is not always the most dominant isomiR, even at low level of expression (Fig. 2A). Many pre-miRNAs have been detected in two clusters of isomiRs from miR-#-5p and miR-#-3p loci (Fig. 1A) (Guo et al., 2014b). Generally, the two loci are different considerably in level of expression, but no strict correlation is detected between isomiR expression patterns in the miR-#-5p and miR-#-3p loci. Except for the effect of expression levels, diverse isomiR expression profiles may be also derived from the phenomenon of arm switching (Guo et al., 2014b). Specifically, arm switching may show that dominant product (miR-#-5p or miR-#-3p) is dynamically selected, or expression ratio between miR-#-5p and miR-#-3p is changed across different tissues, developmental stages, and species (Cheng et al., 2013; Cloonan et al., 2011; Griffiths-Jones et al., 2011; Li et al., 2010, 2011, 2012; Marco et al., 2010). Generally, isomiR expression profiles are conserved across different samples and animal species so long as they involve in the same miRNA locus (Burroughs et al., 2010; Guo et al., 2011c). These findings indicate that isomiRs are not randomly expressed at the miRNA locus, which should be strictly regulated. These non-random distributions of expression suggest functional and evolutionary implications. However, inconsistent isomiR expression patterns can also be detected across different developmental stages and tissues (Fernandez-Valverde et al., 2010; Guo et al., 2011c). This is especially true of isomiRs from abnormally expressed miRNA loci (deregulated miRNAs are accessed at the miRNA levels). These results may have functional implications, although no evidence suggests that abnormal isomiR expression patterns have biological roles, and more evidences particularly in experimental validation is necessary to validate their roles.

Compared to the annotated miRNA sequence, some isomiRs can be detected as variant nucleotides (non-template nucleotides based on pre-miRNAs), but additional nucleotides at the 3′ ends are more popular (Fig. 1A). These non-template additional nucleotides are added after Dicer processing through miRNA maturation (Katoh et al., 2009), and therefore the phenomenon is also named 3′ addition event (herein mainly aim at 3′ additional non-template nucleotides). Although many isomiRs can be found in the 3′ additional non-template nucleotides, most of these additional nucleotides are located in the 3′ ends of the canonical miRNA sequences (Fernandez-Valverde et al., 2010). These 3′ additional nucleotides tend to be nucleotides of adenine (A) and uracil (U) (Burroughs et al., 2010; Guo et al., 2011c; Lu et al., 2009; Reid et al., 2008), also including unambiguous double-nucleotide additions at the 3′ ends, most notably AA additions (Burroughs et al., 2010; Guo et al., 2011c). Indeed, similar nucleotide bias can be also found at the 5′ ends. The phenomenon of 3′ addition may facilitate many biological functions: they may affect miRNA stability and target selection and contribute to interactions between miRNA and mRNA (Burroughs et al., 2010; Fernandez-Valverde et al., 2010). These modified isomiRs might have very high levels of enrichment, but generally, they are not dominantly expressed species at the miRNA locus (Guo et al., 2011c, 2012b). Dominantly expressed isomiRs are almost always those 3′ isomiRs without varied nucleotides. There are a few rare cases of isomiRs with 3′ additions that are dominant species at some miRNA loci.

Although many isomiRs have been detected at the same miRNA locus, only a few of these (always 1–3 kinds) isomiR sequences are dominantly expressed (Fig. 1B) (Guo and Lu, 2010b; Guo et al., 2011c, 2012b, 2013b). According to the relative expression rates, many of the dominantly expressed isomiRs are expressed at either much higher levels (the rate more than 90%) or moderately higher levels (the rate under 60%). There are several dominant isomiR species, but others are rarely expressed and have lower expression rates, although they may also have higher relative expression levels (Fig. 1A and B). Several dominant isomiRs always have the same 5′ ends with the canonical miRNA sequence, and they are only involved in various 3′ ends (divergence of lengths). Even though they are characterized as 3′ isomiRs, fewer of them have been found to have variant or additional nucleotides, including 3′ additional non-template nucleotides. These isomiR expression patterns suggest that the phenomenon of multiple isomiRs is not random through pre-miRNA processing and miRNA maturation processes. According to the relative expression levels, dominant cleavage sites of Drosha and Dicer can be preferred (Fig. 1C). The cleavage bias leads to pronounced differences in levels of expression of different isomiRs. However, although isomiR expression distributions are mainly derived from imprecise and alternative cleavage of Dicer and Drosha, degradation of hairpins may also partially contribute to isomiR expression patterns, especially for those of rare isomiRs (Friedlander et al., 2008).

3. Functional and evolutionary relationships among isomiRs from different miRNA loci

Although isomiRs are rarely studied as small RNA regulatory molecules, some literatures have shown their versatile biological roles, including loading in RISC as functional silencing small RNAs and affecting the effectiveness of miRNAs, etc. (Baran-Gale et al., 2013; Burroughs et al., 2010; Chan et al., 2013; Fernandez-Valverde et al., 2010; Kozlowska et al., 2013; Llorens et al., 2013). Bioinformatic analysis indicates that most of 3′ isomiRs regulate the same target mRNAs with the same seed sequences, and even those 5′ isomiRs also can target the common targets despite they may have the novel seed sequences via "seed shifting" events. Generally, seed sequences of 5′ isomiRs may be shifted 1–2 nucleotides, and the shifted seeds may bind the same targets via shifting on target mRNAs. Thus, multiple isomiRs from an miRNA locus may coordinately regulate target mRNAs, although the sequences and lengths are slightly changed. Simultaneously, the additional novel target mRNAs of those dominant 5′ isomiRs should not be ignored, which implies that they have an opportunity to regulate other target mRNAs and contribute to biological pathways. The reported versatile biological roles of isomiRs strongly support that the phenomenon of isomiRs, especially those abundant isomiRs, are not endproducts destined for degradation. These heterogeneous length/sequence isomiRs should have important roles and functional/evolutionary implications, and further studies will reveal the versatile miRNA world.

The functions of isomiRs have been reviewed (Neilsen et al., 2012), and herein we mainly discuss the functional and evolutionary relationships among isomiRs from different miRNA loci. Many miRNAs can be classified as members of miRNA gene clusters or families based on their physical and sequence relationships. Some miRNA gene clusters,

such as the miR-17–92 gene cluster, have been investigated for their roles as regulatory molecules in human cancers (Bomben et al., 2012; Feuermann et al., 2012; Tong et al., 2012). These clustered miRNAs may show consistent or inconsistent expression patterns, although they are always co-transcribed as a single polycistronic transcript (Guo and Lu, 2010b; Guo et al., 2012a; Viswanathan et al., 2009; Yu et al., 2006). Collaborative interactions between homologous and clustered miRNAs enrich and complicate miRNA–miRNA interactions and the coding–non-coding RNA regulatory network. We found that dominant homologous or clustered miRNAs are always generated from the same arms, and even have the same length distributions, which may be derived from the possible functional and evolutionary relationship (Guo and Lu, 2010b). During the pre-miRNA processing and miRNA maturation processes, they always have the same isomiR repertoires and expression patterns, including expression distributions of 3′ additions (Guo et al., 2012a). The similarities in the miRNA maturation processes are unlikely to be random, and these results may be attributable to functional and evolutionary pressure. The evolutionary relationships among these clustered and homologous miRNAs indicate that they may have evolved from the same ancestral gene via duplication. Similar isomiR expression profiles are detected via similar pre-miRNA processing and miRNA maturation processes. Simultaneously, homologous and clustered miRNAs may co-regulate or coordinately regulate biological processes, and consistent isomiR expression profiles (including isomiRs with 3′ additions) may provide an opportunity for co-regulation of target mRNAs or contribute to the same biological pathways. Collectively, the isomiR expression profiles and patterns are not random. They may also have some evolutionary and functional implications. IsomiRs may be suitable for use as adaptive markers and the inference of relationships among miRNAs.

Further, annotated or canonical miRNA sequences from other animal species have been occasionally detected among human isomiRs (Figs. 1 and 2) (Guo et al., 2013a, 2013b). Similar phenomena have been detected in other species. Many isomiR sequences from the miRNA locus are similar to many other miRNA sequences from a wide range of animal species, including those isomiRs with various 5′ and 3′ ends and nucleotide substitutions (Fig. 2) (Guo et al., 2013a, 2013b). The same isomiR sequences can be detected among these homologous miRNA loci. The sequence diversity of multiple isomiRs, homologous miRNAs within the same species, and miRNAs across different species, indicates the dominant sequence selection. miRNAs in different animal species may have different sequences (miRNA sequence diversity across animal species), although many miRNAs are phylogenetically well-conserved and always have the same seed sequences (Fig. 2C). The canonical or annotated miRNA sequence is only one member of multiple isomiRs, and miRNA sequences from other species may be among these isomiRs (Guo et al., 2013b). The most dominant isomiR sequence is selected from multiple isomiRs with dominant expression rates, and the selection process may be dynamic. In this way, the phenomenon of multiple isomiRs provides an opportunity for selection of dominant sequences suitable for functional and evolutionary needs. Furthermore, the sequence diversity of homologous miRNAs is mainly derived from evolutionary divergence among and between species. Although the selection of dominant isomiR also contributes to sequence diversity, homologous and clustered miRNAs always have similar isomiR expression patterns (Guo et al., 2012a). Similar pre-miRNA processing and miRNA maturation processes may contribute to functional relationships among homologous and clustered miRNAs.

4. Multiple isomiRs provide an opportunity to select dominant sequence

Based on isomiR expression patterns and functional and/or evolutionary relationships of different isomiRs, the phenomenon of multiple isomiRs is found to be non-random. There are several major causes of this: (1) Although different isomiRs can be come from the same miRNA locus, only a few of those isomiRs are dominantly expressed. (2) Dominantly expressed isomiRs are always 3′ isomiRs with constant seed sequences and no 3′ additions, although non-dominant isomiRs with 3′ additions may also have unexpectedly high levels of expression. (3) IsomiR expression profiles are always stable across different samples, tissues, and animal species (some abnormal isomiR expression profiles have also been detected in tumor or diseased samples). (4) Samples may differ with respect to the identity of the most dominant isomiR sequence. This is especially true of tumor and normal tissues and different animal species, even if they have the same 5′ ends and seed sequences. (5) Homologous and clustered miRNAs always have similar isomiR expression patterns, and these may have considerable evolutionary and functional implications. Similar isomiR expression profiles may contribute to co-regulation and coordinate regulation of biological processes. (6) The canonical miRNAs in other animal species are members of families that also are contained in human isomiRs. This indicates that species can select a specific miRNA sequence (usually the most dominant isomiR) from among several isomiRs. In this way, multiple isomiRs may have been produced by evolutionary pressure associated with miRNA processing and maturation processes. Although these various isomiRs may have biological roles that involve in co-regulating biological processes. They also provide the opportunity to select the most suitable functional isomiR sequence for specific stages of development and specific animal species. The phenomenon of multiple isomiRs largely contributes to dynamic miRNAome, and simultaneously presents a challenge for miRNA study.

5. Concluding remarks: a challenge for miRNA study

Although miRNA has always been studied in single-sequence form, multiple isomiRs from the miRNA locus have also drawn interest. The isomiRs have attracted a great deal of attention based on their biological characteristics and regulatory roles. The isomiR expression profiles may be suitable for use as markers in the tracking of miRNA processing and maturation mechanisms, especially between homologous and clustered miRNAs. The expression patterns of isomiRs from the miRNA locus, especially for several specific dominant isomiR species, which may be also suitable for use as tags to indicate the selection and switching of the most dominant isomiR sequence across different samples, tissues, and animal species. These various isomiR sequences address miRNA–miRNA interactions by coordinately regulating target mRNAs in coding–non-coding RNA regulatory networks. Only a few isomiRs have been found to be functional regulatory molecules, and more studies about isomiRs (especially for those 5′ isomiRs and isomiRs with 3′ additions, and abnormal isomiR expression profiles) should be performed and taken into consideration. Traditional analysis at the miRNA level is not enough to explore many biological roles of miRNAs, and we propose that miRNA studies should be simultaneously performed at the miRNA and isomiR levels (Guo et al., 2014a). Specifically, these factors should be preferentially considered: (1) expression patterns of isomiRs from the single miRNA locus; (2) functional relationships between multiple isomiRs from the single miRNA locus; (3) expression and function between isomiRs generated from homologous or clustered miRNA loci; (4) expression and function between isomiRs at the isomiR levels (across all the miRNA locus); (5) potential isomiR:isomiR and isomiR:mRNA interactions should be analyzed and studied at the isomiR levels. Novel analysis and study at the miRNA/isomiR levels will enrich understanding of miRNA based on the biological characteristics of miRNA and isomiR.

Conflict of interest

The authors declare no potential conflict of interests with respect to the authorship and/or publication of this paper.

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