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Review

Briefing in family characteristics of microRNAs and their applications in cancer research☆

Qicong Wang [a], Leyi Wei [a], Xinjun Guan [a], Yunfeng Wu [a], Quan Zou [a], ZhiLiang Ji [b,c,*]

[a] School of Information Science and Technology, Xiamen University, Xiamen, 361005 Fujian, PR China
[b] State Key Laboratory of Stress Cell Biology, School of Life Sciences, Xiamen University, Xiamen, 361005 Fujian, PR China
[c] The Key Laboratory for Chemical Biology of Fujian Province, Xiamen University, Xiamen, 361005 Fujian, PR China

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ABSTRACT

MicroRNAs (miRNAs) are endogenous, short, non-coding RNA molecules that are directly involved in the post-transcriptional regulation of gene expression. Dysregulation of miRNAs is usually associated with diseases. Since miRNAs in a family intend to have common functional characteristics, proper assignment of miRNA family becomes heuristic for better understanding of miRNA nature and their potentials in clinic. In this review, we will briefly discuss the recent progress in miRNA research, particularly its impact on protein and its clinical application in cancer research in a view of miRNA family. This article is part of a Special Issue entitled: Computational Proteomics, Systems Biology & Clinical Implications. Guest Editor: Yudong Cai.

1. Introduction

It is estimated that about 97% of the transcriptional outputs in eukaryotes are non-protein coding [1,2]. These so-called non-coding RNAs are far more abundant and important than what we thought earlier. Recent progress in non-coding RNA research mainly focuses on the discovery and functional annotation of microRNA (miRNA), one of the fundamental members of non-coding RNAs.

miRNAs are a class of small single-stranded non-coding RNAs of approximately 22 nucleotides that can regulate gene expression in the post-transcriptional stage through interaction with target messenger RNAs (mRNAs), thus leading to either translational inhibition or gene silencing [3,4]. The first miRNA was discovered by Ambros and his colleagues in *Caenorhabditis elegans* wherein a short 22 nucleotide RNA fragment inhibited translation of lin-14 mRNA into its protein product [5]. It was first thought to be an idiosyncratic phenomenon in nematodes until the 2000s when the second miRNA let-7 and its homologs were characterized to repress expressions of multiple genes like lin-14, lin-28, and lin-41 in *C. elegans* and many other species. By that time, miRNA was eventually recognized as a novel transcriptional regulator that hasn't been paid enough attention to. Meanwhile, the first miRNA family, let-7s, came into sight.

Generally, the miRNA family is a group of miRNAs that originates from a common ancestor. In a miRNA family, miRNAs are normally common in biology but may be diverse in sequence between distant lineages. However, it was also observed that the family members of different lineages are well conserved in the seed region, a strict at least six-nucleotide region at the 5′-end of miRNA [6,7]. Within an organism, a family normally contains one or more precursor miRNAs (pre-miRNAs), whose genes are often clustered in a genome. These pre-miRNAs later produce similar miRNAs that have co-related functions through targeting either different genes in the same pathway or the same gene.

2. Identification of miRNAs

In the past decade, many efforts have been made to identify novel miRNAs both experimentally and theoretically [8]. Techniques like in situ hybridization, Northern blotting, and other PCR-based molecular methods that are commonly used for RNA study can be easily applied in miRNA identification. To monitor hundreds of miRNAs (so-called miRNA profiling), platforms like quantitative real-time PCR (qPCR), microarrays and next-generation sequencing (miRNA–Seq) are often adopted; any of them is able to screen thousands of miRNAs and their expressions simultaneously in a high throughput manner. Besides, algorithms were proposed for computational identification of miRNAs, including miRScan [9], miRSeeker [10], miRGator [11], miRank [12], miRDeep2 [13], etc. Most of these methods attempt to distinguish miRNA sequences from protein-coding or other non-protein-coding sequences [1,2]; however, few attention has been paid for miRNA family classification.

Up to date, a number of miRNAs and their pre-miRNAs have been identified. At the time of this writing, 21,264 miRNAs have been

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* Corresponding author at: School of Information Science and Technology, Xiamen University, Xiamen, 361005 Fujian, PR China. Tel.: +86 592 2182897.
E-mail address: appo@xmu.edu.cn (Z. Ji).

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deposited in the miRBase 19.0 release (http://www.mirbase.org/), the most comprehensive repository for experimentally validated miRNAs and their pre-miRNAs [14]. This number is about 2000 more than that of its last release, and it grows steeply with the widespread applications of next generation sequencing (NGS) in transcriptome determination.

3. Classification of miRNA family

In the miRBase 19.0 release, the 12,264 miRNAs were properly assigned into 1543 miRNA families. The family annotation in miRBase follows the primary criteria in which identical or nearly identical miRNAs produced from the paralogous miRNA loci in a definite organism are grouped together into a family. Homologous miRNAs from different organisms are often classified into the same family. Manual adjustment sometimes is undertaken to assign miRNAs of similar sequences into a distinct family as they might derive from different ancestors. Of these miRNA families, 69% are composed of less than five members, accounting for about 17% of the total miRNAs (Fig. 1). Comparatively, about 8% of miRNA families consist of 21–100 members per family; and 2% of families are very large families, each of which contains more than 100 members. These large and very large families (about 10% of total families) together cover about 65% of miRNAs. The detailed information of family can also be acquired from the attached Supplementary Table 1.

The non-even distribution of miRNAs in families could be the result of: (1) some miRNA families like let-7 family, one of the largest miRNA families, are involved in some fundamental cellular processes, e.g. cell cycle and apoptosis [15], so that they are required and conserved in many species. On the other hand, some miRNA family members participate in post-transcriptional regulation of phenotype-specific functions that only exist in some organisms. (2) Many miRNA family members haven't been fully identified yet. It looks like the miRNAs in a family are more preserved in their functions or target genes compared to their sequences [16]. For instance, rno-let-7b-3p (*Rattus norvegicus*) and sme-let-7d (*Schmidtea mediterranea*) are two members of the let-7 family, which share no significant similarity in the whole sequence. Therefore, conventional sequence alignment-based approaches may be insufficient to detect all miRNAs.

Besides the miRBase, the Rfam (http://rfam.sanger.ac.uk/) also serves as a good reference of miRNA family [17]. The Rfam database is a collection of RNA families, which were pre-assigned by criteria of sequence conservation, consensus secondary structures and covariance models. Its latest release includes 523 miRNA families, and most of the families are exactly same as what they are in the miRBase. The protocol of miRBase and Rfam for miRNA family assignment has been generally adopted in identification and annotation of novel miRNAs from a sequenced genome. However, such homology-based method sometimes is unable to detect those sequence-unrelated miRNAs, although in many cases members of the same miRNA family are well conserved across species both in sequence or hairpin structure. Manual adjustment of family assignment also hinders its application in high throughput identification of novel miRNAs.

Recently, Ding and his colleagues presented an efficient alignment free model miRFam for miRNA family classification based on a multiclass support vector machine (SVM) model [18]. The miRFam model was constructed on sub-datasets derived from the miRBase families. In miRFam, the authors first extracted k-grams, i.e. k consecutive nucleotides for a given sequence, as their features. For example, if k is equal to 4, the features have the forms as "NNNN", which N ∈ {A,C,G,U}. On the basis of these features, a multiclass SVM classifier was trained, which can properly classify novel miRNAs into families from their primary sequences regardless of sequence/structure similarity. It is the first advanced server that can conduct miRNA family classification on-line. A high prediction accuracy of 90.66% was achieved by miRFam on an uneven dataset of 1056 miRNA families. However, like most of machine learning applications, the performance of miRFam is highly dependent on the selection of training set which would probably lead to unreliable prediction results for generalized datasets.

Besides miRFam, there is another advanced machine learning based server miRClassify, which employed a hierarchical random forest (RF) classifier to assign either known or novel miRNA sequences into families (Fig. 2). In this hierarchical model, three independent layers (classifiers) were defined. The first layer was composed of 20 classes, corresponding to the 19 largest miRNA families from miRBase and one integrated from the remaining families. The second layer was composed of 100 classes, corresponding to the 99 largest miRNA families, excluding the 19 miRNA families in the first layer, and one integrated from the remaining families. In the third layer, all remaining miRNA families were incorporated for model construction. An independent RF model was constructed for each layer respectively. For a query nucleotide sequence, a pre-miRNA prediction is normally demonstrated. Subsequently, the putative pre-miRNA sequence is further passed through the three-layer classifier one by one until it is properly assigned into a miRNA family. In this way, the unbalanced dataset problem is, to some extent, solved and the method becomes more robust.

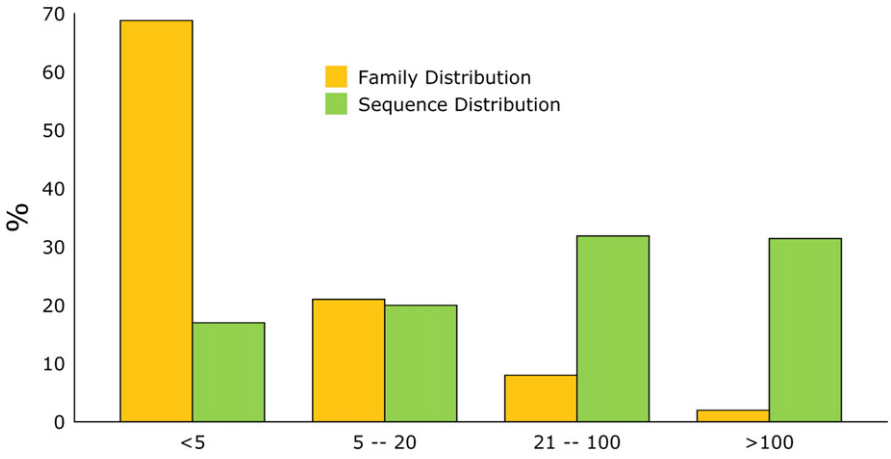

Fig. 1. The statistics of miRNA families in the miRBase 19.0 release. In this figure, "<5" denotes that the miRNA family consists of less than 5 members; "5–20" denotes that the family consists of more than 5 but less than 20 members; "21–100" denotes that the family consists of more than 21 but less than 100 members; ">100" denotes that the family consists of more than 100 members.

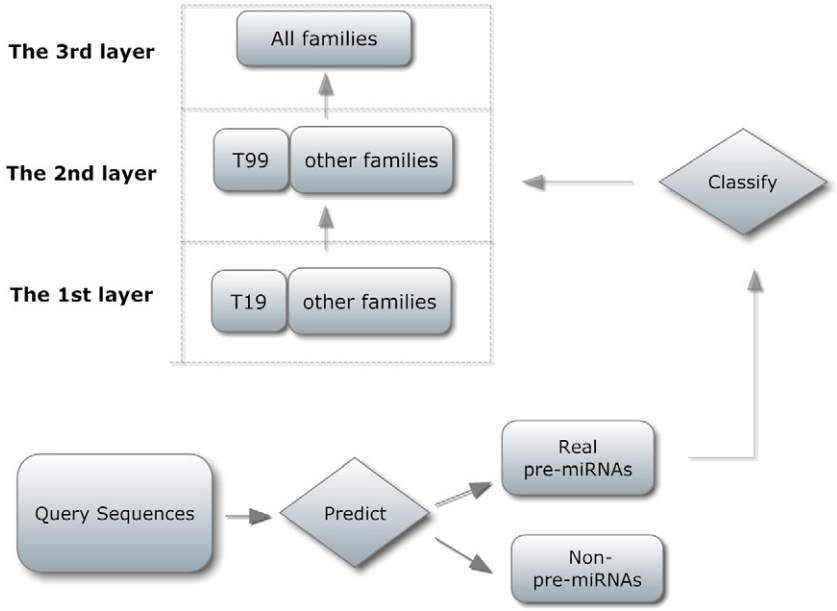

Fig. 2. The hierarchical model of miRClassify for miRNA family classification. Given a query nucleotide sequence, the model will first classify the query sequence into pre-miRNA or not. For a putative pre-miRNA, the miRClassify classifier will go through a three-layer classifier until the sequence has been properly assigned into a definite miRNA family. Remarks: T19 denotes the 19 largest families in the miRBase 19.0; T99 denotes the 99 largest families in the miRBase 19.0, excluding those 19 families adopted in the last layer classifier.

These four repositories for miRNA family classification were also summarized in Table 1. Generally, the investigation of miRNA family classification hasn't received enough attention yet. One of the major reasons lies in the ambiguous definition of miRNA family, which is based on evolutionary consideration of miRNAs other than their conservation in sequence, structure or function. Moreover, the diversity in sequence and promiscuity in function sometimes makes it difficult to achieve distinct characteristics of miRNA family.

4. Functional characteristics of miRNA family

Compared to the progress in identification of miRNAs, the physiological functions of miRNAs are largely under-understood. According to current knowledge, the mature miRNA normally interacts with its target mRNA, resulting in mRNA cleavage or reduced protein translation when there is perfect or imperfect complementary binding respectively [19,20]. Occasionally, miRNAs modulate gene transcription by inducing histone modification and DNA methylation of promoter sites [21].

Generally, the miRNAs are relatively conserved in eukaryotic organisms, although their interaction modes against target genes may be different. For instance, perfect or nearly perfect complementarity is required for miRNA–target mRNA binding in plants, normally in the coding regions. In contrast, in animals as well as in humans, the binding sites are usually located in the three prime untranslated regions (3′ UTRs) of the mRNAs and mismatches are allowed outside the seed region [22,23]. The comparatively flexible binding mode supports the functional promiscuity of miRNAs wherein one miRNA can target different sites on the same mRNA or on different mRNAs. This definitely makes it tougher in fully elucidating physiological functions of miRNAs. On the other hand, genes that exhibit common cellular functions intend to have relatively fewer miRNA target sites to avoid multiple binding [24].

In most cases, miRNA family members show conservation in biology, thus proper assignment of miRNAs into a family would not only greatly aid in the understanding of miRNAs' nature but also provide clues for their further application in research and clinic. However, the family members don't always play the same physiological role. It is not often,

Table 1
A summary of repositories about miRNA family classification and miRNA–disease relationship.

Databases	Description	Websites
miRNA family		
miRBase [6]	A database that collects published miRNA entries and miRNA families. Its version 19 release includes 1543 miRNA families.	http://www.mirbase.org/
RFam [27]	A database that provides a collection of RNA families, which includes 523 miRNA families in its latest release.	http://rfam.sanger.ac.uk/
miRFam [28]	An alignment-free method to classify miRNA genes into families with *n*-grams and a multiclass SVM.	http://admis.fudan.edu.cn/projects/miRFam.htm
miRClassify (unpublished data)	A hierarchical (three-layer) RF based classifier method to categorize miRNAs into miRNA families	http://datamining.xmu.edu.cn/software/MIR/home.html
miRNA–disease relationship		
miR2Disease [30]	A database that collects comprehensive information of miRNA–disease relationship.	http://www.mir2disease.org/
PhenomiR [31]	A database that provides information about differentially regulated miRNA expression in diseases.	http://mips.helmholtz-muenchen.de/phenomir/
PolymiRTS [32]	A database that deposits naturally occurring DNA variations in miRNA target sites.	http://compbio.uthsc.edu/miRSNP/
miRdSNP [33]	A database of disease-related SNPs (single nucleotide polymorphisms) and miRNA target sites.	http://mirdsnp.ccr.buffalo.edu/

but it was observed, that some close miRNA family members execute absolutely opposite physiological functions. For instance, Jiang and his colleagues compared the expressions of hsa-miR-125a-5p and hsa-miR-125a-3p (two miR-125 family members derived from 3′ and 5′ ends of pre-miR-125a, respectively) in different pathological stages and lymph node metastasis. It was surprisingly found that these two miRNAs, hsa-miR-125a-3p and hsa-miR-125a-5p, play exactly opposite roles in suppressing or enhancing cell migration and invasion respectively in non-small cell lung cancer [25].

Sometimes, miRNAs from different families can together play a similar regulatory role. For instance, the miR-17–92 cluster is a superfamily cluster that contains seven miRNAs from different miRNA families (miR-17-3p, miR-17-5p, miR-18a, miR-19a, miR-19b, miR-20a, and miR-92). Members of this cluster were recognized as oncogenic genes by the Southern blot analysis. The marked over-expression of the miR-17–92 cluster promoted the development of lung cancers, particularly the small lung cancer [26]. Recent microarray data demonstrated that the over-expressions of the miR-17–92 cluster members were frequently observed in most acute myelogenous leukemia (AML) cases [26]. Additionally, the miR-17–92 cluster was found to be co-expressed with MYC, an oncogene, and inhibited the tumor suppressor PTEN in AML [27].

5. miRNAs' impact on protein

Today, most of the interest and progress of miRNA research have been focused on transcriptional regulation of protein-coding genes; comparatively, its global impact on protein hasn't been well examined yet. It is reasonable to infer that miRNAs could affect protein output and activity via regulating its coding gene. However, the depth and the consistency of miRNA influence on protein remain to be determined. The miRNA–protein relationship has been largely underscored, although efforts have been made to monitor protein output and activity changes with/without miRNA treatment using large scale proteomics analyses as well as single protein analysis technologies like immunoblotting, crosslinking and immunoprecipitation (CLIP), and fluorescent reporter system. It was proposed that in most cases miRNAs likely acted as rheostats in fine-scale adjustment of protein output [28,29]. Regulatory promiscuity was also commonly observed wherein miRNAs can modulate the levels of many different proteins simultaneously [29]. Generally, the profile of miRNA–protein interactions is in agreement with that of miRNA–gene interactions; however, the degree of protein regulation is always consistent with that of transcriptional regulation. The gap between the miRNA regulated gene expression and protein output is the result of multiple factors. For instance, gene expression can be modulated in different levels of transcriptional initiation, RNA processing and the post-translational modification of a protein. The add-on or synergetic effects of multiple miRNAs on co-regulating the same target gene may amplify the protein output [30]. Hence, it is still necessary to profile the miRNA–protein interaction as protein is closest to the physiological or pathogenetic end point. Higher level studies on miRNA-regulated protein–protein interaction have also been undertaken in seeking the preference of miRNA regulation on cellular processes and some novel therapy clues for diseases [31,32]. It seems that miRNAs from the same or close miRNA family tend to have similar miRNA–protein profiles or be associated with the same diseases [26,30].

6. miRNA–disease association

The first evidence of pathogenesis-associated miRNA was discovered in 2004 in which down-regulations of miR-15a and miR-16 were linked with different lymphocytic leukemias [33]. To date, the connection between miRNA and disease has been well established with the availability of daily-increasing evidences. A shortcut to acquire information on miRNA–disease association is via searching related databases. The miR2Disease (http://www.mir2disease.org/) database is a comprehensive resource of miRNA deregulation in various human diseases [34]. By Nov. 2012, miR2Disease has collected 3273 literature-reported miRNA–disease associations. The PhenomiR database (http://mips.helmholtz-muenchen.de/phenomir/) provides information about differentially regulated miRNA expression in diseases and other biological processes [35]. In its last update, differential expressions of 675 distinct miRNAs in 145 diseases were recorded. The PolymiRTS (http://compbio.uthsc.edu/miRSNP/) is a database that collects single nucleotide polymorphisms (SNPs) in predicted and experimentally identified miRNA target sites and links them with diseases [36]. miRdSNP (http://mirdsnp.ccr.buffalo.edu/) is a novel database introduced in 2012. It incorporated 786 dSNP–disease associations for 630 unique dSNPs on the miRNA target sites and 204 diseases [37]. A list of databases on miRNA–disease relationship has been summarized in Table 1. These databases provide useful information for further association analysis like genome-wide association studies (GWAS) of human traits and diseases.

Most often, the miRNAs may intercept normal physiological processes via dysregulation of its target genes and thus result in diseases. The aberrant regulation of target genes is attributed to either down-expression, in most cases, or over-expression of miRNAs, which suggests two different interference modes: suppressor or enhancer. For example, synthetic let-7 mimic was used as a suppressor in lung cancer to compensate abnormal down-expression of let-7 [38]; while inhibition of miR-21 with antisense oligonucleotides was adopted in breast cancer therapy [39].

Reviewing currently known miRNA–disease relationships, it seems that miRNAs from the same family tend to share similar roles in cellar function and pathogenesis via similar mechanisms. Even members of the same miRNA family are often located in the same disease-related loci, have the same abnormal epigenetic modifications, and interact with the same enzymes [40]. Hence, it would be a plausible solution for a new miRNA to refer its physiological functions and medical implications from the other members of same family.

7. miRNAs in cancer research

Dysregulation of miRNAs has been acknowledged to link with various diseases like heart diseases, obesity, diabetes and especially different cancers. A list of recent miRNA applications in cancer research was summarized in Table 2. In the following sections, we will briefly survey recent applications of miRNA families in countermining cancers.

7.1. miRNA biomarkers

The association of disease with altered miRNA expressions can be used to generate “prediction algorithms” for assessing developmental

Table 2
A summary of recent findings of miRNA families in cancers. Remarks: TS: tumor suppressor; OG: oncogenes; CLL: chronic lymphocytic leukemia; ALL: acute lymphoblastic leukemia; AML: acute myelogenous leukemia.

miRNA family	Function	Cancer types	Confirmed targets	References
let-7	TS	Lung	RAS, HMGA2, C-MYC	[16,40,41,55]
miR-34	TS	Prostate, breast	P53, P21, BBC3, HuR, CD44, BCL2, SIRT1, E2F3	[56,57]
miR-200	TS	Lung	FLT1, VEGFR1	[58]
miR-29	TS	CLL, AML	MCL1, TCL1, CDK6	[43,44]
miR-9	TS	ALL	FGFR1, CDK6	[45]
miR-345	TS	Colorectal	BAG3	[46]
miR-365	TS	Colorectal	CCND1, BCL2	[42]
miR-21	OG	Breast, prostate, colorectal	PTEN, PDCD4, TPM1	[36,37,47–51]
miR-155	OG	Breast	FOXO3A, SOCS1, RHOA	[52]

stages of diseases. For instance, miRNA profiling has been tried in cancer prognosis and diagnosis. Compared to mRNA profiling, which has been adopted in cancer prognosis and diagnosis in clinical stages, miRNA profiling seems to be more sensitive in the identification of poorly differentiated cancers as they normally have a comparatively lower expression level in these cancer cells [41]. Previous trials of miRNA profiling in the prognosis of various cancer types have produced successful results. In lung cancers, Yu et al. [42] defined a miRNA signature that comprised five miRNAs (hsa-miR-372, hsa-miR-221, hsa-let-7a, hsa-miR-182*, and hsa-miR-137), which can be used in the prognosis of cancer relapse and survival of non-small cell lung cancer (NSCLC) patients. Similarly, Gao et al. reported that the low expression of miR-181a and high expression of miR-21, could serve as potential prognostic biomarkers for lung cancer [43]. Furthermore, Yan et al. identified a significantly up-regulated miRNA (miR-21) in 113 breast cancer patients. The enhanced miR-21 expression significantly shortened the survival period of patients, which suggests miR-21 as a potential indicator for the assessment of breast cancer [44]. Diaz et al. reported that down-regulation of miR-106a shortened the overall survival time of colorectal cancer patients [40]. In addition, Garzon's group found that high expression of two miRNAs, miR-191 and miR-199a, could reduce the survival rate of AML patients, which suggests their potential in the prognosis of AML [45]. The above examples and many other cases all suggest that miRNAs could serve as good markers for disease diagnosis.

7.2. miRNAs as tumor suppressors

Typically, miRNA exhibits its anti-tumor activities via deregulating expression of its downstream target genes. In most cases, these target genes are oncogenic genes that are directly associated with tumor growth or development. Synthetic oligonucleotides were thus designed as miRNA mimics to inhibit target genes. A typical example of miRNA suppressor is let-7, one of the largest and organism-conserved miRNA families. Via hierarchy classification of hundreds of lung cancer cases according to let-7 expression, Takamizawa's group observed that over-expression of let-7 hindered, likely through inhibition of the oncogenic RAS's expression, lung cancer cell growth in vitro [46], which indicated let-7 as a potential cancer suppressor. This result was later supported by a trial in which injection of synthetic let-7 in mouse models of NSCLC substantially reduced the tumor development [38]. Nie's group recently investigated the antitumor effects of miR-365 in colorectal cancer using a computational approach along with experimental validation. It was revealed that miR-365 inhibited cell cycle progression and promoted apoptosis by suppressing the expressions of two oncogenic genes, CCND1 and BCL2 [47]. Considering the tumor suppressor role of miRNAs, it is rational to apply synthetic miRNAs, as a novel strategy, in improving cancer therapy. For instance, miR-29 was considered as a general tumor suppressor by acting on the oncogenic targets including MCL1, TCL1, and CDK6, which are crucial in the pathogenesis of tumors like CLL by inducing cell survival and proliferation [48]. Recently, Garzon et al. used synthetic miR-29b (a miR-29 family member) oligonucleotides in improving AML treatment [49].

Aberrant methylation of miRNA may connect with tumorigenesis. Rodriguez-Otero and his colleagues identified 13 miRNAs, including three members of the miR-9 family (miR-9-1, miR-9-2, and miR-9-3) that were epigenetically modified in the acute lymphoblastic leukemia (ALL) [50]. These miR-9 members were found to negatively regulate FGFR1 and CDK6 expressions in ALL patients. Via FGFR1 and CDK6, the over-expression of miR-9 could induce a decrease cell proliferation and increase apoptosis of ALL cells, thereby realizing tumor suppression. A similar regulation mode was also observed in aberrant methylation of miR-345 in colorectal cancer therapy. The miR-345 was hypermethylated at low expression in colorectal cancer cells compared with healthy tissue cells [51]. The enhanced expression of miR-345 inhibited the invasion and growth of colorectal cancer cells in vitro, likely via deregulation of some anti-apoptosis proteins like BCL2-associated athanogene 3 (BAG3) [51]. It seems that hyper-methylation could be a potential mechanism underlying miRNA disruption. In such cases, epigenetic modification of miRNA, e.g. demethylation of DNA, may suggest a novel therapeutic strategy for cancers. However, such strategy is currently immature for medical practice.

7.3. miRNAs as oncogenes

Compared to tumor suppressors, miRNAs as oncogenes has just started. The first two oncogenic miRNA families are miR-21 and miR-155, which have been well characterized in various cancers, including breast cancer, prostate cancer, colorectal cancer, etc. MiR-21 was initially recognized as an oncogene in a comparative analysis of miRNA expression between breast cancer and healthy breast tissues [39]. Further knockdown of miR-21 reduced the growth of breast cancer MCF-7 cells and increased apoptosis in a dose-dependent manner, which validated the role of miR-21 in tumorigenesis [52]. Several gene targets were involved in miR-21 regulation, including PDCD4, TPM1, and MARCKS [53–55]. Protein products of these gene targets are usually tumor suppressors wherein their activities are negatively regulated by miR-21. It was observed that over-expression of miR-21 promoted tumor invasion or metastasis, which indicated the oncogenic role of miR-21. Like miR-21, miR-155 was found to be up-regulated in breast cancer. It was reported that expression of cytokine signaling 1 (SOCS1), a tumor suppressive gene, was inversely correlated with miR-155 in breast cancer [56]. The tumorigenesis induced by over-expression of miR-155 (correspondingly, down-expression of SOCS1) can be obviously inhibited by restoration of SOCS1 expression.

Today, targeting the oncogenic miRNAs and miRNA-regulated pathways becomes a potentially powerful approach in countermining cancers. For instance, inhibition of miR-21 with antisense oligonucleotides generates an anti-proliferative response in vitro and reduces the tumor development as well as cancer metastasis in vivo [39]. Similarly, reducing miR-125b activity using the anti-miR-125b oligonucleotides can induce apoptosis in prostate cancer cells [57]. Furthermore, as an alternative solution to chemically modified antisense oligonucleotides, transgenes that can express miRNA inhibitors, or the so-called miRNA sponges, were constructed for specific inhibition of oncogenic miRNAs' expression so as to realize their anti-tumor activities [58].

8. Perspectives

Undoubtedly, miRNA is one of the hotspots in current biomedical research. A lot of attention has been set on miRNA as an effective tool in repressing or silencing the expression of a particular gene, as well as a "magic bullet" in blocking tumor progress. The new term "oncomers", miRNAs that are associated with cancers, is even brought up to describe the prosperous growth of this field [59,60]. However, a lot about miRNA remains unclear. For instance, how important an individual miRNA-medicated regulation is to cellular processes still remains uncertain. Are all target bindings of a miRNA equally important in post-transcriptional regulation? Whether and how these miRNA–target pairs, in a form of network, work together in a particular biological event? In which level of conservation are miRNAs in a family? What causes different sizes of miRNA families? All these mysteries still remain to be explored. Moreover, many current applications of miRNAs in cancer prognosis and therapy hardly produced a reliable and robust outcome due to the poor understanding of miRNA–target interactions and miRNA–disease associations. Till now, the nearest application to marketing of miRNA drugs is the locked nucleic acid (LNA)-modified antisense oligonucleotides "miravirsen", which was explicitly designed to target liver-expressed miRNA-122 in phase 2 clinical trials for the treatment of hepatitis C virus (HCV) infection [61,62]. Therefore, more efforts, both experimental and computational, are desired for better understanding of miRNAs. Proper assignment of miRNAs into families

just provides a shortcut to figure out the consensus nature of miRNAs. Nevertheless, the emergency of miRNAs reveals a new mode of gene regulation that many researchers haven't touched upon previously. It not only provides a powerful tool in studying gene regulation, but also starts a new paradigm of drug discovery [60].

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