BMB
Reports

BMB Rep. 2013; 46(7): 338-345
www.bmbreports.org

Invited Mini Review

microRNA biomarkers in cystic diseases

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microRNAs (miRNAs) are small non-coding RNAs that regulate gene expression by targeting the 3'-untranslated region of multiple target genes. Pathogenesis results from defects in several gene sets; therefore, disease progression could be prevented using miRNAs targeting multiple genes. Moreover, recent studies suggest that miRNAs reflect the stage of the specific disease, such as carcinogenesis. Cystic diseases, including polycystic kidney disease, polycystic liver disease, pancreatic cystic disease, and ovarian cystic disease, have common processes of cyst formation in the specific organ. Specifically, epithelial cells initiate abnormal cell proliferation and apoptosis as a result of alterations to key genes. Cysts are caused by fluid accumulation in the lumen. However, the molecular mechanisms underlying cyst formation and progression remain unclear. This review aims to introduce the key miRNAs related to cyst formation, and we suggest that miRNAs could be useful biomarkers and potential therapeutic targets in several cystic diseases. [BMB Reports 2013; 46(7): 338-345]

INTRODUCTION

microRNAs (miRNAs) composed of 21-23 nucleotides are endogenous small non-coding RNAs that negatively regulate gene expression levels (1). Recent studies showed the presence of more than 800 known mammalian miRNA genes that are conserved across species. Computational analyses indicate that a single miRNA has the potential to regulate a wide range of mRNA transcripts (2, 3). In addition, one transcript may be regulated by multiple miRNAs (4). Therefore, miRNAs regulate the expression of at least 30% of human genes (3, 5). Furthermore, miRNAs are closely associated with a broad range of cellular processes, such as cell proliferation, differentiation, and apoptosis (6, 7). Importantly, these cellular processes contribute to cyst formation in several cystic diseases (8-12).

Cysts refer to an abnormal sac in the body that contains fluid. Although causative genes of each cystic disease are different, deregulation of key molecular signaling induces initiation of cyst formation. Cystic epithelial cells are characterized as abnormal proliferation and apoptosis. Progression factors promote cyst expansion with fluid secretion in the lumen (13). Cystic diseases in visceral organs include polycystic kidney disease (PKD), polycystic liver disease (PCLD), pancreatic cystic disease, and ovarian cystic disease (polycystic ovarian syndrome, PCOS) and commonly have a defect of ciliogenesis (8, 12). The mechanosensory function of the cilia plays an important role in sensing fluid flow and signal transduction into intracellular calcium signaling responses in many visceral organs such as kidney, liver, and pancreas (12). Primary cilia are antenna-shaped organelles that present on the epithelial cells of these organs. Polycystin-1 (PC-1) and polycystin-2 (PC-2), which are major causative genes of autosomal dominant PKD, are located on the primary cilia in renal epithelial cells, and dysfunction of these genes disrupts fluid flow sensing and decreases intracellular calcium levels (14). Low levels of intracellular calcium affect diverse cellular processes such as cell proliferation by stimulating activation of various proliferative pathways including cAMP, ERK, AKT/mTOR pathways (15-19). However, to date, the regulatory mechanism of cystogenesis remains unclear, and suitable diagnostic biomarkers have not yet been developed. Furthermore, the diagnostic significance of miRNAs has not been thoroughly examined, because the current literature regarding miRNA-associated regulatory mechanisms has been focused on tumors but not cystic diseases.

Herein, we review the possible miRNA biomarkers associated with cystic diseases including PKD, PCLD, pancreatic cystic disease, and polycystic ovarian syndrome (PCOS).

BIOGENESIS AND ACTION OF miRNAs

miRNAs are a large class of small non-coding RNAs that regulate target gene expression levels (1, 6). Although more than 1,000 miRNAs have been identified in animal genomes, only a few have been elucidated (20). It is now clear that miRNAs regulate every cellular process, including cell proliferation, apoptosis, differentiation, development, and tumorigenesis (6, 7). All miRNAs are processed and matured through a complex biogenesis process following a coordinated series of events (Fig. 1). At first, miRNA genes are transcribed into long primary transcripts (pri-miRNAs) with one or more stem-loop structure by RNA polymerase II. These pri-miRNAs are processed by RNase III Drosha complexes, to generate 70-100-nucleotide long pre-miRNAs that have a hair-

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http://dx.doi.org/10.5483/BMBRep.2013.46.7.151

Received 28 June 2013

Keywords: Biomarker, Cyst formation, Cystic disease, microRNA

ISSN: 1976-670X (electronic edition)

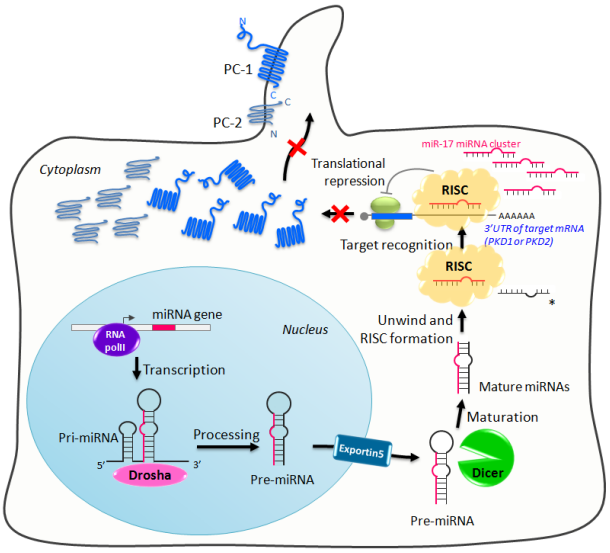

Fig. 1. miRNA biogenesis and targeting of PKD genes by miR-17-92 miRNA cluster in PKD. Primary miRNA (Pri-miRNA) is transcribed from miRNA gene and processed by the enzymes Drosha to produce precursor miRNA (Pre-miRNA). Pre-miRNA is then transported into cytoplasm through Exportin 5 and processed into mature miRNA duplex by Dicer enzyme. Finally, the guide strand of mature RNAs recognizes and negatively regulates the target mRNAs after composing a complex with RISC. In PKD, overexpressed miR-17-92 miRNA cluster directly targets *PKD1* or *PKD2* and leads to inhibit their localization on the cilia as well as expression levels, which finally induce cyst formation. *miRNA indicates passenger strand.

pin structure with a 3′-overhang. Pre-miRNAs are then transported into the cytosol by exportin 5 and further processed by RNase III Dicer into mature miRNAs. Finally, the mature single-stranded miRNA, which is called the guide strand and exhibits a much higher level than the other strand, enters the RNA-induced silencing complex (RISC) (21). The other strand which is called the passenger strand and denoted with an asterisk (*) at the end of the name is degraded. In some cases, both strands, which are named 5p and 3p, are functional and detected at significant levels (22). miRNA-RISC complexes bind to the 3′-untranslated region (3′-UTR) of the target mRNA by imperfect base-pairing (23, 24). This miRNA-mRNA interaction suppresses either translational initiation or induces endonucleolytic cleavage of the target mRNA (1, 23). Animal miRNAs usually have only partial homology with 7-8 bases of conserved seed sequence of their target genes and may induce translational repression (25).

It has been reported that miRNA expression patterns are tissue-specific. miRNA expression patterns have been investigated in the kidneys of humans and mice (26-29). miR-192, miR-194, miR-215, miR-204, and miR-216 are abundant in the kidney tissue compared with other organs. On the other hand, miR-122, miR-1, miR-16, miR-27b, miR-30d, miR-126, miR-133, and the let-7 family are enriched in adult liver tissues (30). Therefore, the deregulation of these tissue-specific miRNAs may contribute to the expression of target proteins that are important for kidney or liver functions.

miRNAs AS POTENTIAL BIOMARKERS

Renal diseases, including PKD, are associated with high mortality and morbidity, and very few suitable therapies are available (31). Therefore, there is an urgent need to develop more sensitive and specific biomarkers for early diagnosis of these diseases.

The ideal biomarkers for diagnosis of renal diseases should satisfy the following several criteria. First, the biomarker should be specific to the diseased organ or tissue and should be able to differentiate pathologies. Second, the biomarker should be sensitive to alterations in the pathology to allow the analysis of the disease progression or therapeutic response. Third, biomarkers should be predictive to reflect the degree of pathology severity. Fourth, biomarkers should be translatable to enable its application in linking pre-clinical and clinical data. Finally, biomarkers should be easily accessible. For example, biomarkers that present in body fluid samples such as serum or urine are desirable (32).

A recent study suggests that miRNAs can be used as high potential biomarkers for a wide range of diseases including tumors (31-34). Unfortunately, to date, although much research has been completed involving miRNAs expression patterns and functions related to cancer, few studies have analyzed the association between miRNAs and cystic diseases for the early detection of cystic diseases. miRNA expression profiles have been shown to reflect precancerous and cancerous conditions. Therefore, miRNAs might be a powerful source of diagnostic, prognostic, and predictive information as biomarkers in cancer (33). Moreover, as pathogenesis typically includes the deregulation of regulatory networks of different genes and proteins, targeting disease-specific miRNAs may simultaneously and more efficiently control gene sets associated with pathogenesis than targeting one gene or protein.

On the other hand, circulating nucleic acids provide unique opportunities for efficient early diagnosis using clinical samples (35). Secreted miRNAs have many the following characteristics of good biomarkers: (i) they are unexpectedly stable in body fluids, likely due to incorporation into microparticles and exosomes; (ii) miRNA expressions are specific for tissue or pathogenic stage; and (iii) their expression levels can be easily detected by various methods such as TaqMan real-time reverse transcription-polymerase chain reaction (RT-PCR) (32, 33). Therefore, information regarding differential miRNA signatures, especially those obtained from cyst fluid, might be useful for the improvement of cystic disease diagnosis by enabling healthy tissues to be distinguished from cystic tissues.

THE ROLE OF miRNAs IN CYSTIC DISEASES

PKD

PKD is a common genetic disorder in which clusters of cysts devel-

op to contain fluid in the renal epithelial cells. There are 2 major types of hereditary PKD, namely, autosomal dominant (ADPKD) and autosomal recessive (ARPKD) (36, 37). ADPKD is 20-fold more common than ARPKD and the mutations or dysregulated expression of *PKD1* or *PKD2* induces cyst formation in both kidneys. In contrast, mutation of the *polycystic kidney and hepatic disease 1* (*PKHD1*) gene causes ARPKD, which has a high mortality rate (38, 39). Despite the differences between the 2 types of renal cystic disease, they share common features. First, their clinical features overlap and are indistinguishable. Second, although ADPKD is an autosomal dominant disorder, it is recessive on a molecular level, which is explained by the "two hit" model. Third, the proteins of both PC-1 and PC-2, which are encoded by the *PKD1* and *PKD2* genes, and fibrocystin/polyductin (FPC), which is encoded by *PKHD1* have been co-localized to primary cilia. Finally, ADPKD and ARPKD cystic epithelial cells have similar abnormalities with respect to cAMP-mediated signaling (40, 41).

Most of the ADPKD patients are heterozygotes but studies of cyst lining epithelial cells isolated from individual cysts of ADPKD patients have indicated the loss of heterozygosity (LOH), thereby leading to the hypothesis that cyst initiation is a "two-hit" process. However, the two-hit model has been challenged by studies that showed a low frequency of second hits within individual cysts, as well as the continuous expression of germline wild-type *PKD1* in most of the cystic epithelial cells derived from ADPKD patients (42, 43). Taken together, these data suggest that *PKD1* inactivation alone in renal tubular epithelial cells is not sufficient to initiate the cyst formation and additional renal injury may be required for initiation and progression of ADPKD pathogenesis as a "third hit" (44, 45).

The contribution of miRNA in PKD pathogenesis was researched in several studies. miRNAs can affect ADPKD cystogenesis by regulating multiple target genes or directly inhibiting PKD gene expressions (46-50). A recent study demonstrated that defects in the miRNA-processing enzyme Dicer from maturing renal tubules induces tubular and glomerular cysts in *Dicer* mutant mouse models such as Hoxb7/cre;*Dicer*$^{f/f}$ and ksp/cre;*Dicer*$^{f/f}$ (46, 47). Inactivation of the Dicer enzyme causes abnormal processing of miRNAs including miR-200, which directly represses *Pkd1* expression levels by binding to the 3′-UTR of *Pkd1*. Upregulation of *Pkd1* by inhibition of miR-200 in renal epithelial cells is sufficient to impair the tubulogenesis and induce cystogenesis (47).

Several studies established the direct correlation between miRNAs and PKD genes. Some of the putative miRNA binding sites in the 3′-UTR of *PKD1* and *PKD2* were predicted by *in silico* analysis such as TargetScan (51). The study by Sun *et al*. investigating the interactions between miR-17 and its putative target *PKD2 in vitro* suggested that miR-17 can directly bind the *PKD2* 3′-UTR. Overexpression of miR-17 in the human embryonic kidney cell line HEK293T can repress *PKD2* translational activity but not transcriptional activity. They also demonstrated that this interaction is associated with cell proliferation (52). Tran *et al*. also demonstrated that mutation to the *RNA-binding protein Bicaudal C* (*Bicc1*), which is a key regulator of embryonic development, induces fluid-filled cyst formation in the kidney. Bicc1 acts as a post-transcriptional regulator of *Pkd2* by antagonizing the repressive activity of the miR-17 family at the 3′-UTR of *Pkd2* mRNA (53). They suggested that the PKD phenotype of *Bicc1* mutant mice could be explained by the abnormal control of this miRNA-based translational mechanism. Patel *et al*. further demonstrated that members of miR-17-92 miRNA cluster (miR-17, miR-18, and miR-20a) were upregulated in *Kif3a*-knockout mice, which are an animal model of PKD, and promoted renal cyst growth. miR-17-92 may be associated with cystogenesis by promoting proliferation and posttranscriptional repression of PKD genes *Pkd1*, *Pkd2*, and *hepatocyte nuclear factor-1β* (*Hnf-1β*) (54).

Pandey *et al*. identified upregulation of miR-21 as well as downregulation of miR-31 and miR-217 in the kidney of the ADPKD rat model PKD/mhm (cy/+) compared to controls by parallel profiling of transcripts and miRNAs (50). Moreover, Pandey *et al*. examined the possible involvement of miRNAs in the Pkd1-/- mouse model based on a computational approach involving an extensive mRNA microarray. They predicted and verified several miRNAs that include miRs-10a, -30a-5p, -96, -126-5p, -182, -200a, -204, -429, and -488, which may be important players in cellular signaling pathways, thereby leading to PKD by targeting differentially regulated genes (55). Another group performed parallel mRNA and miRNA microarray profiling in PKD/Mhm (cy/+), which has been used as a rat model for PKD, thereby resulting in cystogenesis and slowly progressive chronic renal failure (56, 57). They found 3,333 abnormally expressed genes and 8 upregulated miRNAs including rno-miR-214, -31, -199a-5p, -21, -34a, -132, -146b and -503 in PKD. Several potential binding sites between miRNAs and target mRNAs were predicted using the miR Walk database (58).

Therefore, elucidating the regulatory mechanisms of gene dosage associated with miRNAs will provide novel biomarkers and new therapeutic targets in PKD.

PCLD

A liver cyst is a fluid-filled, epithelial lined lumen that can vary in volume from a few milliliters to several liters. ADPKD patients often have polycystic livers as well as polycystic kidneys. The prevalence of liver cysts in ADPKD is high at about 67-83% (59, 60). Both ADPKD and PCLD are autosomal dominant disorders and 2 gene mutations induce cyst initiation and progression. The mutations in *PRKCSH* or *SEC63* lead to PCLD and *PKD1* or *PKD2* mutations cause ADPKD (61-63). In addition, like cystogenesis in ADPKD, abnormalities in biliary cell proliferation and apoptosis initiate and develop cystogenesis in PCLD (64, 65). Activation of ERK and AKT/mTOR pathways, which are well-known pathways that are deregulated in ADPKD, is found in hepatic cysts (66). Increased cAMP levels promote cholangiocytes proliferation and cyst expansion in both ADPKD and PCLD (67, 68). Mouse models with defects in the *Prkcsh*, *Sec63*, *Pkd1*, *Pkd2*, or *Pkhd1* genes showed cyst formation with alterations to the expression of *Pkd1*. It indicates that *Pkd1* is central

in cystogenesis in both PKD and PLD (12). Furthermore, it implies that PKD and PLD may share a common pathogenic pathway even in cyst formation in different organs.

Importantly, Lee *et al*. identified that downregulation of miR-15a in the cystic tissues was related to upregulation of its target gene Cdc25A, which is known as a cell cycle regulator, and affected acceleration of cell proliferation and cyst growth. *In situ* hybridization indicated that miR-15a was significantly decreased in the liver tissues of ADPKD and ARPKD patients as well as the PKD/Mhm (cy/+) rat model (38). Therefore, these findings indicate that alterations in miRNA expression contribute to the molecular pathogenesis of PCLD as well as PKD.

Pancreatic cysts

Pancreatic cysts are frequent with a prevalence of 2% in patients without known pancreatic disease. In contrast to earlier reports, a recent study observed that most pancreatic cystic lesions are neoplastic cystic lesions, not pseudocysts (69). There are 3 major types of cyst: serous cystadenoma (SCA), mucinous cystic neoplasm (MCN), and intraductal papillary mucinous neoplasm (IPMN). MCN and IPMN lesions are mucinous cysts and can develop into pancreatic cancer, whereas pseudocysts and SCA lesions are nonmucinous (70). However, it is difficult to determine if the detected cyst has malignant potential or not. Therefore, valid and cost-effective biomarkers are urgently needed.

Ryu *et al*. showed that increased miR-21 levels in pancreatic cyst fluid are predictive of mucinous pancreatic cancer (71). They used quantitative RT-PCR to show that the expression levels of miR-21, miR-221, and miR-17-3p were significantly elevated in cyst fluid samples obtained from mucinous (n = 24) compared nonmucinous (n = 16) cysts of the pancreas. In particular, miR-21 had the best performance criteria in receiver operating characteristic (ROC) curves with a median specificity of 76%, at a sensitivity of 80%. These results suggest that miR-21 has the potential to be a promising biomarker for the distinction of mucinous and nonmucinous cysts of the pancreas. In addition to miR-21, miR-221 may be useful to identify benign, premalignant, or malignant characteristics in pancreatic cysts (72).

A more recent study showed that miRNAs have potential as biomarkers for diagnosis and management of pancreatic cyst fluid (73). Matthaei *et al*. identified differential signatures of 9 miRNAs, including miR-24, miR-30a-3p, miR-18a, miR-92a, miR-342-3p, and miR-21, between pancreatic cyst fluids from high-grade and low-grade IPMNs with a significant degree of accuracy. Although this unique signature needs to be validated, this result may prove valuable for the appropriate management of patients with pancreatic cysts.

On the other hand, primary cilia expressed in pancreatic epithelial cells play a role as a mechanosensor of luminal flow (74, 75). *Hepatocyte nuclear factor-6* gene (*HNF-6*)-deficient mice showed significantly repressed expression levels of ciliary proteins (76), thereby suggesting that *HNF-6* is important in the activation of genes that maintain appropriate epithelial cell polarity, primary ciliogenesis in pancreatic cells, and pancreatic cystic disease (12, 76). Intriguingly, Simion *et al*. found that miR-495 and miR-218 are expressed in the developing liver and pancreas and suppressed the endogenous *HNF-6* mRNA levels by interacting with 3'-UTR of HNF-6 (77). Taken together, these findings suggest that miRNAs may be tightly involved in pancreatic cystic disease and further miRNA-based research of pancreatic cysts is needed to enable its use as a diagnostic biomarker.

Ovarian cysts

An ovarian cyst is filled with fluid secreted from the local microenvironment, tumor cells, and stroma and is surrounded by a very thin wall (78). Ovarian cysts can be classified into 2 groups: functional cysts and non-functional cysts. Most ovarian cysts are non-functional and asymptomatic (79), which includes polycystic ovary syndrome (PCOS) (80).

PCOS is the main cause of chronic anovulation and affects about 30% of women of reproductive age (80). Although dysregulation of many genes were observed in PCOS, the mechanisms by which these genes are regulated transcriptionally and post-transcriptionally remain unknown. miRNAs have emerged as a mediator of post-transcriptional gene regulation. However, there have been a few studies analyzing miRNAs in human follicular fluids of PCOS.

Several studies showed that miRNAs are associated with the regulation of steroidogenesis, cell proliferation, and apoptosis in human granulosa cells (81, 82). Differential expression of miR-23a, miR-23b, miR-542-3p, miR-211, and miR-17-5p, and few of their predicted target genes, *namely*, *COX-2*, *IL-1β*, *StAR*, *CYP-19A1*, and *ER-β* in the PCOS group, suggested abnormal miRNA expression may be related to PCOS pathogenesis (83). Another group treated a rat model with dihydrotestosterone (DHT), which is increased in PCOS patients, to show that PCOS is associated with the dysregulation of ovarian miRNAs. Three hundred forty-nine miRNAs showed different expression levels in the DHT-treated and control rats (84). Recently, Sang *et al*. investigated the role of miRNAs in human follicular fluids by performing genome-wide deep sequencing and TaqMan miRNA assays. They found that miR-132, miR-320, miR-520c-3p, miR-24, and miR-222 are involved in controlling estradiol concentrations and that miR-24, miR-193b, and miR-483-5p are associated with the regulation of progesterone concentrations. Interestingly, miR-132 and miR-320 are significantly decreased in the follicular fluid of PCOS patients compared with healthy controls (85). Furthermore, dysregulation of the miR-29 family and its specific target genes that are associated with folliculogenesis in PCOS could lead to ovarian malfunction related to this patient population (86). These studies indicate that a number of miRNAs are present in human follicular fluids and some of them are related with steroidogenesis and PCOS.

CONCLUSION

miRNAs have several potential advantages because they can be sensitively and reproducibly detected in clinical samples, includ-

ing body fluids. Therefore, miRNAs are emerging as a possible novel class of biomarkers for diagnostic applications based on previous studies of cystic diseases. However, for the clinical application of miRNA biomarkers, future studies are required, as well as analysis of the common features and networks among the several cystic diseases, to better understand the function of miRNAs related with cystic diseases.

Acknowledgements
This work was supported by the Sookmyung Women's University Research Grants 2013.

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