

Potential Uses of MicroRNA in Lung Cancer Diagnosis, Prognosis, and Therapy

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Abstract: Lung cancer is the leading cause of death from cancer in the world. Although the molecular network of lung carcinogenesis has been partly known at the levels of genes and proteins, and personalized therapy based on the genetic changes has made considerable progress in the last decade, the high mortality rate is not markedly changed. MicroRNAs (miRNAs), a class of short endogenous RNAs, acting as post-transcriptional regulators of gene expression, are similar with siRNAs in both the biosynthesis and the function steps. While, miRNAs mostly silence gene expression by binding imperfectly matched sequences in the 3' UTR of target mRNA, which is different with siRNAs by targeting ORF of mRNA with a perfectly complementary manner. miRNAs have multiple functions in lung development, and abnormal expression of miRNAs could lead to lung tumorigenesis. The different expression profiles of miRNAs in lung cancer, and the stability of miRNAs in serum, all together make them as new potentially clinical biomarkers for diagnosis and prognosis. Moreover, miRNAs may serve as either novel potential targets acting directly as oncogenes (e.g. *miR-17-92* cluster) or directly therapeutic molecules working as tumor suppressor genes (e.g. *let-7* family). RNAi technology based on miRNAs has many advantages over siRNAs, such as *in vivo* stability, highly RNA promoter-compatibility and no overt toxicity. Eventually, it might overcome the present disadvantages and become a good candidate for lung cancer therapy.

Keywords: Lung cancer, microRNA, RNAi, *let-7*, *miR-17-92* cluster, oncogene, tumor suppressor gene.

INTRODUCTION

Lung cancer is the leading cause of death from cancer in the world. Its pathogenesis is closely associated with tobacco smoking. Lung cancer is divided into two main histological groups including non-small cell lung cancer (NSCLC, 85%) and small cell lung cancer (SCLC, 15%). NSCLC can be further subclassified into adenocarcinomas, squamous cell, large cell and bronchoalveolar carcinomas (BAC). It is well known that genetic alterations could occur at the chromosomal level (e.g. large gains and deletions), at the nucleotide level (e.g. nucleotide mutation), or at epigenetic level (e.g. DNA methylation). Such a change could result in the activation of oncogenes (e.g. *Ras*, *Myc*) and other growth promoting genes (e.g. *ERBB1*, *IGF-IR*), and the inactivation of tumor suppressor genes (e.g. *p53*, *p16INK4A*, *Rb*, *FHIT*). Ten or more genetic or epigenetic abnormalities are generally needed before lung tumor becomes clinically evident. The molecular network of lung carcinogenesis has been partly known at the levels of genes and proteins in the last decade (For reviews, see [1-3]). However, since 1970 the high mortality rate (80-85% within 5 years) has not been markedly improved. The lack of effective tools to diagnose at an early stage and the deficiency of therapeutic treatments to the late stage of the disease are the two major reasons.

Currently available diagnostics for lung cancer, including chest imaging (X-ray or low-dose CT) and sputum cytology, often lack the sensitivity and specificity that are necessary to identify early stage disease. Only 16% of lung cancer is discovered prior to spread of the disease so far. Previous reports

have shown that two blinded expert observers, when asked to give an independent histological classification of NSCLC, both agreed that the specificity of the disease was only 74.7 percent of the time, whereas sensitivity for squamous cell carcinoma is only 70.9 percent (For reviews, see [4]). And even 40 percent of samples diagnosed as SCLC at regional labs were later reclassified as other lung cancer at central labs. Surgery only partly cured lung cancer patients at early stages [5]. Experimental treatment approaches, including small molecule targeted therapeutics, gene modified tumor vaccines, and viral-based gene therapy induced tumor regression, are still only used in a small proportion of patients. Currently, lung-cancer staging rests on histopathological and clinical criteria have only limited power to predict relapse and survival. A major effort to improve the control of lung cancer entails the use of molecular profiling to characterize tumors and to provide accurate predictions of the outcome after standard or novel treatments [6].

MicroRNAs (miRNAs), a class of small, single-stranded endogenous RNAs, act as post-transcriptional regulators of gene expression. Some miRNAs may have as many as a few thousand targets, and that between 74% and 92% of the gene transcripts in four model genomes are likely under miRNA control. In general, the definition of miRNA requires mRNA that can be processed by Dicer (DCR) from the stem of a hairpin precursor to a ~22-nt RNA [7]. The first miRNA, *lin-4* was found in 1984 by Ambros and Horvitz [8]. While, the second 22-nt RNA gene *let-7* was found until the year of 2000, after the discovery of the RNAi phenomenon [9-12]. Until to Sept, 2008, the Sanger center in England had already contained 8619 entries representing hairpin precursor miRNAs (pre-miRNAs), expressing 8273 mature miRNA (mature miRNA) products in primates, rodents, birds, fishes, worms, flies, plants and viruses (<http://microrna.sanger.ac.uk/>).

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In lung, expression patterns of miRNAs vary from fetal to adult, from normal to lung cancer. Specific miRNAs may have multi-functions in lung development and aberrant expression of miRNAs could induce lung tumorigenesis [13, 14]. The difference in expression of miRNAs between the normal lung and lung cancer leads to emerge as a novel type of biomarkers, which may not only helpful to diagnosis, but also could be novel therapeutic targets and good candidates for lung cancer therapy [15, 16].

MECHANISM RELATIONSHIPS BETWEEN miRNAs AND siRNA

The phenomenon of RNAi was discovered in 1998 [12]. And now, both biochemical and genetic approaches have led to the current models of RNAi (For reviews, see [17-20]). RNAi is first initiated by dsRNAs, which are cleaved by the ribonuclease III-type enzyme DCR into siRNAs-short 21-23 nt duplexes with a symmetric 2 nt overhang at the 3'-end and a 5'-phosphate and 3'-hydroxy group. This siRNA molecule is incorporated into a nuclease-containing multi-protein complex called RNA-induced silencing complex (RISC). Then, RISC is activated by RNA helicase activity upon the loss of one strand of the siRNA duplex. In the effector steps, the single-stranded siRNA guides post-transcriptional gene silencing by degradation of their target mRNA (see Fig. 1).

In animal cells, miRNAs are first transcribed from genes by the RNA polymerase, most likely mediating by pol II [21, 22] (see Fig. 1). A majority of miRNA loci is found in intronic regions of protein-coding (intronic miRNAs), whereas the others are found in exonic regions of non-coding transcription units (exonic miRNAs) [23, 24]. MiRNA genes are transcribed to generate long primary transcripts (pri-miRNAs), then cleaved by RNase III enzyme (Drosha/DGCR8) and/or spliceosomal components into hairpin-like pre-miRNAs in the nucleus [23-25]. The pre-miRNAs are transported into the cytoplasm with the help of a protein called Exportin 5, which is a member of the Ran-dependent nuclear transport receptor family [26]. In the cytoplasm, the pre-miRNAs are also cut into double stranded RNA duplexes by DCR [27]. Typically, one of the strands becomes mat-miRNA (the other counterpart is named miRNA*) and is incorporated into a RISC with other components for target recognition [28]. The RISC then binds to its target mRNA through base pairing, and carries out its functions similar with siRNAs.

Many evidences point to a tight connection between miRNAs and siRNAs molecular machineries. The protein families that are most closely associated miRNAs with siRNAs are argonautes (AGOs) and DCR. The AGO1-4 is involved in miRNA biogenesis, while AGO2 is an essential component for siRNA-directed RNAi [29, 30]. Only one

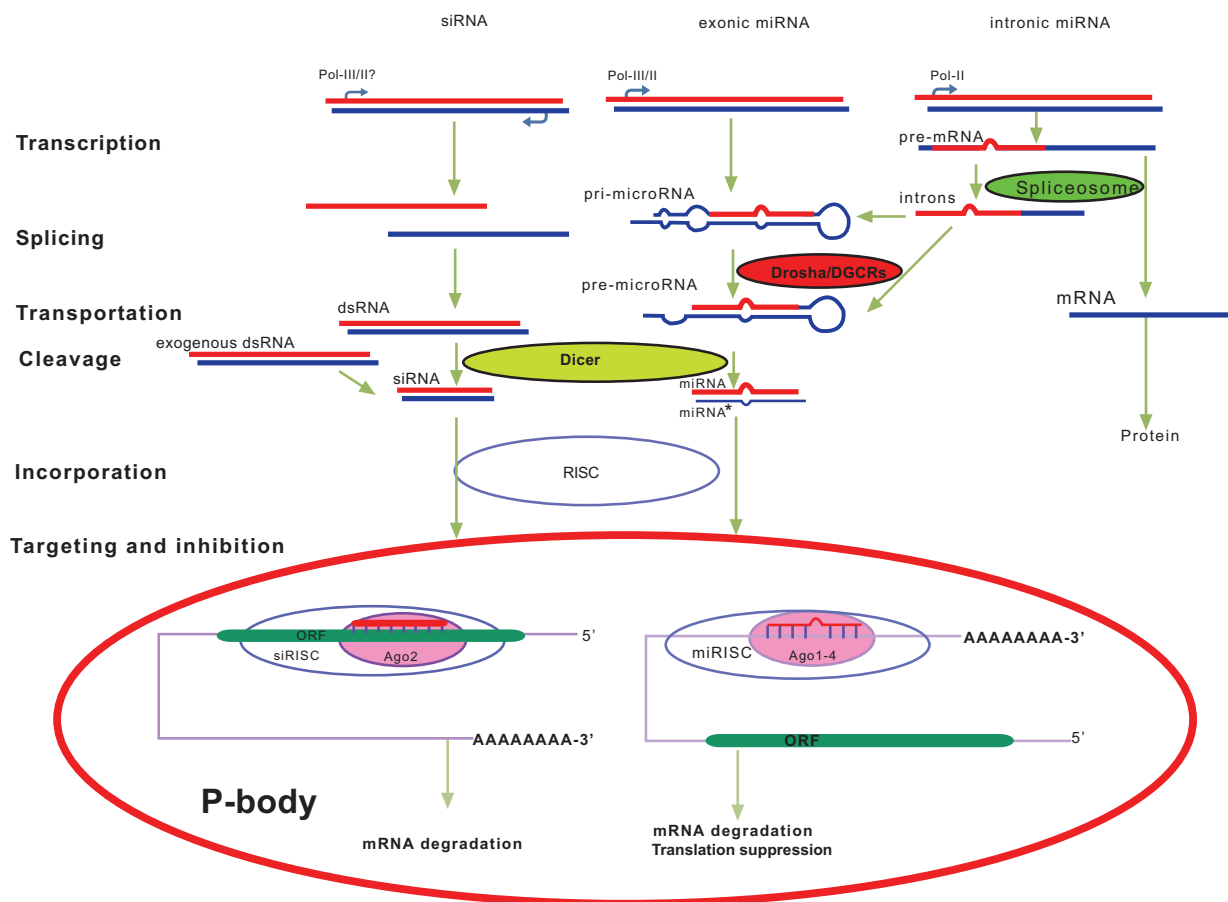


Fig. (1). Connection between miRNAs and siRNAs molecular machineries.

DCR enzyme is found in mouse and human, indicating that the same DCR serve for both miRNA-programmed RISC (miRISC) and siRNA-programmed RISC (siRISC). While, siRISC silences gene expression by cleaving a perfectly complementary target mRNA, and miRISC inhibits translation mostly by binding imperfectly matched sequences in the 3' UTR of target mRNA [31]. Regardless of the maturation process, once the small RNA is loaded, the RISC uses it to degrade or inhibit translation depending on the degree of complementarity between the small RNA and its mRNA target [29]. Components of the RNAi machinery, AGO1 and AGO2 are localized in mRNA processing bodies (P-bodies, also known as "cytoplasmic bodies" and "GW182 bodies") that provide a possible linkage between siRNA-directed RNAi and miRNA-directed RNAi [32-39]. Previous studies have also shown that miRNAs are crucial components in P-bodies formation, and the formation of P-bodies is a consequence of miRNA, but not the result of RNA-mediated gene silencing [40, 41]. Proteins that are involved in mRNA degradation, translational repression, mRNA surveillance and RNA-mediated gene silencing, together with their mRNA targets, colocalize within P-bodies [42].

LUNG-SPECIFIC miRNAs

The expanding inventory of human miRNAs along with their highly diverse expression patterns and high number of potential target mRNAs suggest that miRNAs were involved in a wide variety of human organ development and diseases progress [43, 44]. For mammals, miRNAs are specifically expressed or greatly enriched in a particular organ such as lung, implying an organ- or tissue-specific function. First, it is found that lung is one of the tissues with the most abundant expression of *let-7* [11], which is reconfirmed by others [14, 45]. Then, six lung-specific miRNAs are identified in the adult mice by using northern analysis [46]. High throughput platforms further expand the lung-specific miRNAs [47, 48]. A rat miRNA microarray containing 216 miRNA probes revealed that two miRNAs were expressed specifically in rat lung and nine miRNAs co-expressed in lung and heart [47].

The overall expression profile of miRNAs is similar in mouse and human lung [49, 50], indicating evolutionary conservation of miRNA expression. Twenty-three of the 30 most highly expressed miRNAs shared in adult lungs from mice and human [49]. The *miR-26*, *let-7*, *miR-29*, *miR-30* and *miR-99* were expressed highly in both species. Similarly, *miR-154*, *-134*, *-214*, *-296*, *-299*, *-323*, and *-337* were highly expressed in both the neonatal mouse and the fetal human lung [49]. However, the expression of miRNAs in the post-natal and adult lung was different. When compared with mouse neonatal lung (1d) and the adult lung (60d), 14 miRNAs were highly expressed in the neonatal lung, while 30 miRNAs were up-regulated in adult lung. Similarly, 13 miRNAs were expressed more in human fetal lung than the adult lung, and 8 miRNAs were up-regulated in the adult lung. Most miRNAs that were highly expressed both in the neonatal mouse and the fetal human lung were subsequently down-regulated in the adult lung. In contrast, miRNAs such as *miR-26b*, *-29a*, *-29b*, *-146-3p* and *-187* were up-regulated in the adult tissue of both mice and human. It was the same

when compared E11.5 stage mouse lung with E17.5 lung. Although the 20 most abundant miRNAs were very similar, the amount of each miRNA varied significantly [13]. We have systematically analyzed above data, and found that *miR-15*, *126-3p*, *30* and *let-7* are up-regulated for both the E11.5 lung and the E17.5 lung, and for both the neonatal and the adult [13] (Table 1). *MiR-214* and *miR-199* are both down-regulated in either mouse embryonic lung development process or human lung development from the neonatal to the adult lung [13, 49]. However, no significant changes are found for the expression of any individual miRNA in the aged lung (18 month) compared with the adult lung (6 month) [51]. The similar phenomenon also existed in the liver [52]. The dramatic change in miRNAs expression during embryonic and neonatal lung development indicates their crucial roles in these processes. NIH has therefore paid great attention to these molecules that may be of function in the embryonic and neonatal lung developmental process and lung tumorigenesis (<http://researchresources.bumc.bu.edu/abstract/1R01HL081800-01A1.htm>).

miRNA EXPRESSION PROFILE IN LUNG CANCER

Many studies have showed that miRNAs could mutate or poorly/over express in human cancer [53, 54]. Interestingly, miRNAs not only control the expression of known protein-coding oncogenes and tumor suppressors, but also act as oncogenes and tumor suppressors directly. It provides a high linkage between the altered expression of one or a few miRNAs and cancer development. Now, new clear evidences reveal that miRNA-expression profile of human tumors is closely associated with diagnosis, staging, progression, prognosis, and response to treatment [55-57]. A report named "micorRNA-based methods and compositions for the diagnosis and treatment of solid cancers" illustrated 123 miRNAs that may have potential roles in lung cancer (International publication number, WO2007/081740 A2). By comparing miRNA expression among lung cancer tissues versus the corresponding non-cancerous lung tissues, 43 miRNAs had statistical differences with 15 miRNAs up-regulation (Table 1). Volinia's studies also showed that 35 miRNAs were up-regulated, only 3 miRNAs were down-regulated [53]. In contrast to these reports, Ambion demonstrated that three miRNAs were also expressed at significantly higher levels in the lung tumors, while six miRNAs were expressed at significantly lower levels (<http://www.ambion.com/techlib/tn/121/4.html>). Nevertheless, not so many miRNAs presented the same change pattern among these reports. It may be due to the different samples, the different technical platforms, or even to the different analytical methods. Peltier and Latham used stable pair of miRNAs to normalize different miRNAs expressed in Luca and normal samples [58]. They found that 7 miRNAs were up-regulated and 5 miRNAs were down-regulated in lung cancer (Table 1). Again, these miRNAs are mostly different from the others [58].

A number of dys-regulated miRNAs in lung cancer are located at the frequently deleted or amplified regions. For example, *miR-21* and *miR-205* are both located at the region amplified in lung cancer, whereas *miR-126** and *miR-126* are located at 9q34.3, a region deleted in lung cancer. These data

Table 1. miRNA Dysregulation in Development Lung and Lung Cancer

Name		E11.5-E17.5	1d-60d	Lung-tumor dysregulation					High expression with survival outcome	WO2007/081740 A2
		Lu (2008)[13]	Williams (2007)[49]	Yanaihara (2006)[55]	Volinia (2006)[53]	Peltier (2008)[58]	Ambion	Serum copy number[65]		
let-7		up		down					protective	
	let-7a					down		down	protective	
	let-7a-1									y
	let-7a-2				up				protective	y
	let-7-a2									y
	let-7-a2-prec			down						
	let-7b		up					up	protective	
	let-7c							up		y
	let-7d		up					up		y
	let-7e							up		
	Let-7f							down		
	let-7f-1									y
	let-g		up		up			down		y
	let-i							down		
miR-7								down		
	miR-7-2				up					y
	miR-7-3									y
miR-8		up								
miR-9	miR-9-1				up					y
miR-10	miR-10a				up			up		y
miR-15										
	miR-15a	up	up					down		y
	miR-15b	up						=		y
miR-16		up				down		down		
	miR-16-1									y
	miR-16-2				up					y
miR-17-92		down								y
	miR-17							down		
	miR-17-3p			up					risky	
	miR-17-5p	down			up	up				y
	miR-18a/b	down						down		
	miR-19a/b	down						down		
	miR-20a/b	down						down		
	miR-92	down								
miR-21		up		up	up		up	up	risky	y
miR-22								up		
miR-23	miR-23a	down	up							
	miR-23b	down						up		y

(Table 1). Contd.....

Name		E11.5-E17.5	1d-60d	Lung-tumor dysregulation				High expression with survival outcome	WO2007/081740 A2
		Lu (2008)[13]	Williams (2007)[49]	Yanaihara (2006)[55]	Volinia (2006)[53]	Peltier (2008)[58]	Ambion		
miR-100		=	up					down	y
miR-101								down	
	miR-101-1			down					y
miR-103						up		up	y
miR-105	miR-105-1								y
miR-106				up					
	miR-106a	down				up		down	risky
	miR-106b	down						down	y
miR-107									y
miR-122			down					up	
miR-123									y
miR-124	miR-124a-1			down	up				y
	miR-124a-3			down					y
miR-125									
	miR-125a	down		down					y
	miR-125a-5p							up	
	miR-125a-prec			down					
	miR-125b	down						up	
	miR-125b-1								y
	miR-125b-2								y
miR-126		up	up	down			down	down	y
	miR-126-3p								
	miR-126*			down	down				y
miR-127					up				y
	miR-127-3p							up	
miR-128	miR-128a							up	
	miR-128b							up	risky
miR-129									y
	miR-129-1/2 prec				up				y
miR-130									y
	miR-130a	down						down	
	miR-130b	down						=	
miR-131		down							
miR-132					up				
miR-133	miR-133a							up	
miR-134			down					up	y
miR-135	miR-135-1								
	miR-135b		dpwn						y
miR-136		down			up				
miR-137								risky	y

(Table 1). Contd.....

Name	E11.5-E17.5	1d-60d	Lung-tumor dysregulation					High expression with survival outcome	WO2007/081740 A2
	Lu (2008)[13]	Williams (2007)[49]	Yanaiharu (2006)[55]	Volinia (2006)[53]	Peltier (2008)[58]	Ambion	Serum copy number[65]		
miR-139									y
	miR-139-3p						up		
	miR-139-5p						up		
miR-140			down						y
	miR-140-3p						up		
miR-141	=			up					y
miR-142	miR-142-as			up					y
	miR-142-3p	up	up				down		y
	miR-142-5p		up				down		
miR-143			down		down	down	up		
miR-144							down		
miR-145			down			down	up	protective	y
miR-146		up	up						y
	miR-146a						up		y
	miR-146b-5p						down		
miR-148				up					
	miR-148a						down		
	miR-148b						down		y
miR-149				up					y
miR-150		up	up				up		
miR-151		up							
	miR-151-3p						up		
	miR-151-5p						up		
miR-152							up		
miR-153	miR-153-1								y
	miR-153-2								y
miR-154		down							y
miR-155			up	up				risky	
miR-159	miR-159-1								y
miR-175									y
miR-181	miR-181a						up		
	miR-181b						up		y
	miR-181c								y
	miR-181c-prec		down						y
	miR-181d						up		
miR-182	miR-182a						down		y
	miR-182as							risky	y
miR-183							down		y
miR-184							down		
miR-185							down		
miR-186							up		y

(Table 1). Contd.....

Name	E11.5-E17.5 Lu (2008)[13]	1d-60d Williams (2007)[49]	Lung-tumor dysregulation					High expression with survival outcome	WO2007/ 081740 A2
			Yanaihara (2006)[55]	Volinia (2006)[53]	Peltier (2008)[58]	Ambion	Serum copy number[65]		
miR-187		up							y
miR-188							down		
miR-189							up		
miR-190		up					down		
miR-191				up			up		y
miR-192	miR-192-prec		down						y
	miR-192		up				down		
miR-193		up							y
	miR-193a-5p						up		
miR-194							down		
miR-195		up		up	down				y
miR-196									y
	miR-196-1			up					y
	miR-196-1-prec			up					
	miR-196-2			up					y
miR-197			up				up		y
miR-198			down						y
miR-199	miR-199a	down							y
	miR-199a-3p						up		
	miR-199a-5p						up		
	miR-199a-1			up					
	miR-199a-2			up					y
	miR-199b	down	down						y
	miR-199b-3p						up		
	miR-199b-prec		down						y
	miR-199a*	down							
miR-200	miR-200a	=							
	miR-200b	=		up		up			
	miR-200c	=							
miR-202				up					
miR-203		up	up						y
miR-204			up						y
miR-205				up			up		y
miR-206							up		
miR-210			up	up			down		y
miR-211									y
miR-212			up						y
miR-214		down	down						y
	miR-214a-1			up					y
miR-216									y
	miR-216-prec		down						y

(Table 1). Contd.....

Name		E11.5-E17.5	1d-60d	Lung-tumor dysregulation					High expression with survival outcome	WO2007/081740 A2
		Lu (2008)[13]	Williams (2007)[49]	Yanaihara (2006)[55]	Volinia (2006)[53]	Peltier (2008)[58]	Ambion	Serum copy number[65]		
miR-217										
miR-218	miR-218b				up					y
	miR-218-2			down						y
miR-219	miR-219-1			down						y
miR-220				down						y
miR-221						up		up	protective	
miR-222			up							
miR-223			up							
miR-224				down				up		
miR-296			down							y
miR-299			down							
miR-301	miR-301a	down	down							
	miR-301b	down								
miR-302	miR-302c		down							y
miR-322		up								
miR-323			down							
	miR-323-3p							up		
miR-328								up		
miR-330	miR-330-3p							up		
miR-331						down				
	miR-331-3p							down		
miR-335			down					up		
miR-337			down							
miR-338			up							
miR-351		up								
miR-362	miR-362-5p							down		
miR-368			down							
miR-370			down							
miR-372									risky	
miR-374	miR-374a							down		
miR-424								down		
miR-449		up								
miR-454								down		
miR-576	miR-576-5p							down		
miR-590	miR-590-5p							down		
miR-923								down		

are in agreement with the earlier reports that miRNA genes are frequently located at fragile sites (FRAs), as well as in minimal regions of loss of heterozygosity, minimal regions of amplification, or common breakpoint regions [59-61]. Moreover, approximately 30% of the 43 miRNAs is located within exons or introns of known protein-coding genes [55].

Indeed, the expression of many miRNAs are transcriptionally linked to the expression of other genes, coding for both proteins and noncoding RNAs [48, 62]. For example, the well-known *miR-17-92* cluster resided in intron 3 of the *CI3orf25* gene at 13q31.3, within 800 base-pair region [60, 63] (See below for details).

miRNAs AS BIOMARKERS FOR LUNG CANCER DIAGNOSIS

The expression patterns of miRNAs between the normal lung and the cancer lung, the SCLC and the NSCLC, and the lung cancer and the other solid tumors, even other diseases are different. Such a difference not only exists in solid tumors, but also appears at plasma and serum of the patients. Particularly, serum miRNAs are resistant to RNase digestion, suggesting that miRNAs in serum are sufficiently stable to serve as clinical biomarkers [64-66]. These findings contribute to blood-based measurement of tumor-derived miRNAs as a potential new approach for the detection of human cancer. Twenty-eight miRNAs were missed and 63 new miRNAs were detected in the lung cancer serum when compared with the healthy subjects [65]. And 8 serum miRNAs were uniquely detected in lung cancer patients. Furthermore, the differential miRNA expression between serum and blood cells of lung cancer patients is a striking contrast to that of healthy subjects, in which serum and blood cells essentially share the same miRNA profile. Because miRNAs have some advantages over protein-based early-detection systems, including that they can be detected potentially in smaller quantities with a high throughput manner. Therefore, many companies, such as Rosetta Genomics Ltd., a famous company in the development of miRNA-based diagnostics, have paid great attention to the blood-based, non-invasive early detection. In 2006, Rosetta Genomics Ltd. announced to develop the technology for lung cancer together with U.S Genomics Inc. Their reports showed that miRNAs could be used as clinical biomarkers for a wide range of indications [66]. Mitchell *et al.* have shown serum levels of *miR-141* can distinguish patients with prostate cancer from the healthy controls [64]. It is expected that these new biomarkers could be using in clinics successfully in near future.

Current diagnostics available for lung cancer often lack the sensitivity and specificity between NSCLC and SCLC and between adenocarcinoma and squamous cell carcinoma [4, 5]. The discovery of the new miRNA-based biomarkers may improve methodology for both sensitivity and specificity. It has been found that the expression of *miR-29a*, *-29b*, and *-29c* is down-regulated in NSCLCs [55, 67]. Six miRNAs were expressed differently in adenocarcinoma and squamous cell carcinoma, whereas six miRNAs shared both histological types of NSCLCs with higher expression levels of *miR-99b* and *-102* in adenocarcinoma [55]. A decrease in precursor *let-7a-2* and *let-7f-1* expression was also found in adenocarcinoma and squamous cell carcinoma, respectively. These data strongly support miRNAs working as biomarkers for differentiating lung cancer. Although miRNAs have been identified as no different expression when classified by age, gender, or race [55], their expression profiles in serum should be explored in more details. Recently, Rosetta Genomics Ltd, together with Columbia University Medical Center, has developed a cancer diagnostic test based on miRNA technology for differentiating squamous cell from non-squamous NSCLC. In their reports, miRNA signatures could classify squamous cell carcinoma of the lung at 90 percent of specificity and 96 percent of sensitivity. This is the first test utilizing miRNAs' unique sensitivity and specificity as biomarkers that may offer a standardized and objec-

tive method for cancer classification. Moreover, Lebanony *et al.* presented an abstract in 2008 ASCO Annual Meeting, showing that a single miRNA was sufficient to discriminate squamous cell carcinoma from other types of NSCLC. Studies also showed that miRNAs expression was very efficient to predict the histological classification between low and high grade lesions and between *in situ* and invasive squamous cell carcinoma [68]. In addition, miRNAs may also be powerful diagnostic tools for distinguishing difference between the primary lung tumors and lung metastases. For example, lung cancer shared a portion of its signature with breast cancer [53, 55], but the loss of *miR-335* and *-126* expression was associated with poor distal lung metastasis-free survival in breast cancer [69], and the suppression of *miR-21* in metastatic breast cancer MDA-MB-231 cells significantly reduced invasion and lung metastasis [70]. Therefore, the lung cancer with low *miR-335* and *-126* levels and high *miR-21* level may be metastasized from breast cancer.

CORRELATION BETWEEN miRNA AND LUNG CANCER SURVIVAL OUTCOME

The expression of individual miRNA and miRNA signatures has been linked to the prognosis of a number of human cancers. For lung cancer, firstly, it has been found that NSCLC had undergone potentially curative resection, which could be classified into two major groups according to *let-7* expression levels [71]. A decrease in *let-7* was associated with shortened postoperative survival and this prognostic impact was independent of disease stage, despite it was reported that reduction of *let-7* expression was not correlated with the prognosis of BAC [72]. Secondly, eight miRNAs are found to be related to the patient's survival with the adenocarcinoma. Patients with high expression of *miR-155*, *-17-3p*, *-106a*, *-93*, *-21* and low expression of *let-7a-2*, *let-7b*, *miR-145* have had a significantly worse prognosis [55]. In particular, high *miR-155* and low *let-7a-2* expression appeared with poor survival. Yu *et al.* further identified a five-miRNA signatures, including *let-7a*, *miR-221*, *-137*, *-372*, and *-182**, were closely associated with survival and cancer relapse in NSCLC patients [73]. *let-7a* and *miR-221* were protective and the other three miRNAs were risky. *miR-128b*, a regulator of *Egfr*, its heterozygosity (LOH) was frequent in tumor samples and correlated significantly with clinical response and survival following gefitinib [74]. There is a strong correlation between miRNA expression and lung cancer patients' prognosis, suggesting that miRNAs may ultimately prove to be valuable cancer diagnostic and therapeutic analysis. Interestingly, most of protective miRNAs (e.g. *let-7* family and *miR-145*) are down-regulated in lung cancer, while the risky miRNAs (e.g. *miR-17-3p*, *-21*, *-93*, *-106a*, and *-155*) are up-regulated in lung cancer (Table 1).

TUMOR SUPPRESSOR miRNAs AND ONCOGENIC miRNAs

In Table 1, two out of 8 lung cancer reports show the miRNA expression profiles during embryonic lung and neonatal lung developmental process. There are four reports focusing on the dys-regulation of miRNAs from the normal lung to lung cancer, one report describes the different

Table 2. The Scores of Each miRNA and miRNA Family. miRNAs with Higher Scores are Potential Tumor Suppressor Genes. In Turn, miRNAs with Lower Scores are Potential Oncogenes

miRNA scores									Down-regulated miRNA in the lungs of rats exposed to cigarette smoke
≥ 3	2	1	0	-1	-2	≤ -3			
let-7	miR-16	miR-8	miR-219	miR-24	miR-7	miR-186	miR-10	miR-17-92	let-7
miR-15	miR-100	miR-33	miR-220	miR-31	miR-9	miR-189	miR-23	miR-21	miR-10
miR-26	miR-101	miR-96	miR-222	miR-32	miR-25	miR-202	miR-24	miR-27	miR-26
miR-29	miR-143	miR-98	miR-223	miR-34	miR-95	miR-204	miR-28	miR-93	miR-30
miR-30	miR-145	miR-124	miR-322	miR-99	miR-129	miR-206	miR-103	miR-106	miR-34
miR-126	miR-190	miR-144	miR-338	miR-107	miR-130	miR-212	miR-122	miR-128	miR-99
miR-142	miR-331	miR-148	miR-351	miR-123	miR-131	miR-221	miR-125	miR-155	miR-122
		miR-183	miR-362	miR-140	miR-132	miR-229	miR-127	miR-181	miR-123
		miR-184	miR-374	miR-146	miR-133	miR-296	miR-134	miR-196	miR-124
		miR-185	miR-424	miR-153	miR-135	miR-299	miR-136	miR-199	miR-125
		miR-187	miR-449	miR-159	miR-137	miR-302	miR-139	miR-210	miR-140
		miR-188	miR-454	miR-193	miR-141	miR-328	miR-191	miR-214	miR-145
		miR-192	miR-576	miR-203	miR-149	miR-330	miR-197	miR-301	miR-146
		miR-194	miR-590	miR-211	miR-150	miR-337	miR-200		miR-191
		miR-195	miR-923	miR-217	miR-151	miR-368	miR-205		miR-192
		miR-198		miR-218	miR-152	miR-370	miR-224		miR-219
					miR-154	miR-372	miR-323		miR-222
							miR-335		miR-223

miRNA expression in the serum between lung cancer patients and healthy population, while the last one enumerates the miRNAs that have been linked with the survival of lung cancer patients. The report, WO2007/081740 A2, in which miRNAs have been paid much attention, is also included herewith (Table 1). In order to analyze the potential activity of miRNAs in lung cancer, we defined that the miRNA, either up-regulated during lung development process, or down-regulated from normal lung to lung tumor transition, either down-expressed in lung tumor patients' serum, or had protective roles in lung cancer patients, obtained 1 point for each report. In turn, the miRNA will be subtracted 1 point.

Accordingly, the total scores of each miRNA or miRNA family are shown in Table 2. The total scores of miRNAs at 3 or over include *let-7*, *miR-15*, *-26*, *-29*, *-30*, *-126*, and *-142*. These miRNAs are lower expression in lung cancer when compared with the normal lung, and an increase during embryonic and/or neonatal lung developmental process. Also, the expression of these miRNAs decreases in the serum of lung cancer patients. Among those miRNAs, *let-7* family becomes the most famous, and its tumor suppressor activities have been demonstrated by several groups [See details below]. *miR-15a*, together with *miR-16-1*, locating at the FRAs in 13q14.3, were lost or down-regulated in cancer with a tumor suppressor function partly by targeting the *BCL2* oncogene [59, 75, 76]. *miR-26a* possessed the ability to attenuate proliferation in MYC-dependent cells, and was one of the 20 most abundant in E11.5 lung [13, 77]. *miRNA-29* family reverted aberrant methylation in lung cancer by targeting 3'-UTRs of DNA methyltransferases 3A and 3B [67], whereas *miR-148* repressed methyltransferases 3B gene expression through a region in its ORF [78]. *miR-126* and *miR-126** could inhibit cancer metastasis and invasiveness not only in lung cancer, but also in breast cancer and prostate cancer

[69, 79-81]. *miR-142* has been found to play an important role in both bronchoalveolar stem cell and bronchial squamous carcinogenesis [82, 68]. Among the scores of those miRNAs at 2, *miR-16*, *-100*, *-101*, *-143*, *-145* and *-331* have been identified to down-regulate in lung cancer, squamous cell carcinoma of tongue, prostate tumor, cervical cancer, colorectal neoplasia, B-cell malignancies, and breast cancer [83-89]. Besides targeting *BCL-2*, *miR-16* induces cell cycle arrest in A549 cells by regulating multiple cell cycle genes, including *CCND1*, *CCND3*, *CCNE1* and *CDK6* [83]. In addition, *miR-101* expression decreases during cancer progression, paralleling an increase in *EZH2*, a mammalian histone methyltransferase that contributes to the epigenetic silencing of target genes and that regulates the survival and metastasis of cancer cells [85]. The down-expression of *miR-143* and *miR-145* has a consistency in many types of cancer, including lung cancer, B-cell malignancies, colorectal neoplasia, ACTH-secreting pituitary tumors [55, 88-91].

Take together, these miRNAs play tumor suppressor roles through inhibiting tumor proliferation, repressing the activity of oncogenes, reverting aberrant methylation, regulating the invasiveness, disturbing endothelial cell activities. In light of these data, we suppose that miRNAs with high scores could be potential tumor suppressor genes. Interestingly, the most remarkably down-regulated miRNAs of smoke rat lung are among the miRNAs with score ≥ 1 (Table 2) [92]. Thus, cigarette smoke may change miRNA expression, especially tumor suppressor miRNAs at an early phase. In fact, treatment of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), a carcinogen present in tobacco products, reduced the expression of a number of miRNAs, such as *miR-101*, *miR-126**, *miR-34* and *miR-199* [93]. NNK treatment in rats repressed *miR-126** but induced CYP2A3

Table 3. Human let-7 miRNA Family

name	pre-miRNA	mat-miRNA
let-7a-1	<pre> u gu uuagggucacac uggga gag aguagguuguauaguu c c auccu uuc ucaucuaacauaucaa a - ug uagaggucacc </pre>	6 -ugagguaguagguuguauaguu-27
let-7a-2	<pre> uu g u uagaa ua a agg gag uag agguuguauaguu u c u c ucc uuc auc uccgacaugucaa a g a -u g c --uag gg a </pre>	5 -ugagguaguagguuguauaguu-26
let-7a-3	<pre> u gu ----- ggg gag aguagguuguauaguu uggggcu c ucc uuc ucaucuaacauaucaa gucccg u ug uaggguauc </pre>	4 -ugagguaguagguuguauaguu-25
let-7b	<pre> u ucagggcagugaug cgggg gagguaguagguuguguguu u guccc uuccgucauccaacaauaucaa u - uagaaggcuccccg </pre>	6 - ugagguaguagguuguguguu-27
let-7c	<pre> a uu g u ua g ua a gc uccggg gag uag agguuguauaguu ga u c c c cg agguuc uuc auc uccaacaugucaa uu a g c - cu g u -- g gg u </pre>	11-ugagguaguagguuguauaguu-32
let-7d	<pre> a c uuagggcagggauu ccuagga gagguaguagguug auaguu u ggauuc uuccgucguccagc uaucaa u - a uggaggaacacccg </pre>	8 - agagguaguagguugcauaguu-29
let-7e	<pre> c cu g u ----gga a cc ggg gag uaggagguuguauagu ga gg c gg ccc uuc auccuccggcauauca cu cc a a cu g - agaggaa c </pre>	8 -ugagguaggagguuguauaguu-29
let-7f-1	<pre> a ug ----- u ucag g agguaguagauuguauaguuu gggguag g a aguc c uccguuaucaacaauaucaua ucccuu u - cu gaggacuug u </pre>	7-ugagguaguagauuguauaguu-28
let-7f-2	<pre> u u gu uuagggucauac guggga gag aguagauuguauaguu c caccu uuc ucaucugacauaucaa c g - ug uagagguucuc </pre>	8 -ugagguaguagauuguauaguu-29
let-7g	<pre> a u a ugagg -a a a ggc gagguagu guuugacaguu gucu ug uacc c ccg uuccguca cggacaugucaa uaga ac augg c a - c ----- gg - c </pre>	5 -ugagguaguaguuguacaguu-26
let-7i	<pre> c u u ----- u ugu uggc gagguaguaguuguc guu gg cgggu g a aucg uuccgucaucgaacgcg caa uc gcccg c - - u uagaggug - uua </pre>	6 -ugagguaguaguugucuguu-27
miR-98	<pre> a uc - u u ----- aggg ggau ugcu caugccaggg gagguaguaguuguau guug ugggu a u cuua acgg guguguccc uuucauaucaacaau caau accccg a a -u u - u agaagaua gauu </pre>	22-ugagguaguaguuguauugu-43

expression, a mechanism that may potentiate the oncogenic effects of NNK.

For those miRNAs with lower scores, such as *miR-17-92*, *-21*, *-27*, *-93*, *-106*, *-128*, *-155*, *-181*, *-196*, *-199*, *-210*, *-214*, and *-301* may have high potential as oncogenes in lung cancer. The oncogenic activities of *miR-17-92* cluster have been demonstrated extensively (see details below). It has been reported that the *miR-21* is over-expressed in a wide variety of cancers, and has been causally linked to cellular proliferation, apoptosis, and migration, invasion with prognostic significance in NSCLC [70, 94]. It targets genes including *PDCD4*, *RECK* and *TIMPs*, *TPM1*, *SPRY2*, *Maspin* [95-99]. Human *CYP1B1*, a protein abundant in cancerous tissues, is post-transcriptionally regulated by *miR-27b* [100]. The oncogenic activity of *miR-27a* in MDA-MB-231 cells is due, in part, to suppression of *ZBTB10* and *Myt-1* [101]. Transfection of lung carcinoma cells line H1299 with *miR-93* reduces TP53INP1, leading to cell cycle arrest and apoptosis [102], while oncogenic potential of *miR-106* has been found in human T-cell leukemias [103], which contributes to tumor cell proliferation in part by regulating cell cycle progression (p21/CDKN1A), and by modulating checkpoint functions [104]. *miR-128* expression could significantly reduced glioma cell proliferation *in vitro* and glioma xenograft growth *in vivo*, through inducing a decrease in the *Bmi-1* oncogene expression [105]. *miR-155* is another marked miRNA [For reviews, see 106], in both the cancer cell [107-109] and the stem cell [110], even in Epstein-Barr virus infected cells [111]. *miR-196*, a miRNA encoded at three paralogous locations in the A, B, and C mammalian *HOX* clusters, has an extensive, and evolutionarily conserved complementarity to the messages of *HOXB8*, *HOXC8*, and *HOXD8* [112]. Levels of *miR-210* expression showed an inverse correlation with disease-free and overall survival in breast cancer [113]. Further studies demonstrated that it could modulate cancer cell, endothelial cell, and erythrocytic cells response to hypoxia [113-115], whereas *miR-214* induced cell survival and cisplatin resistance by targeting *Pten* (phosphatase and tensin homolog) in human ovarian cancer [116]. Many of miRNAs with score 2, also have been proved as potential oncogenes. *miR-136*, *-191*, *-196* and *-210* were also up-regulated in breast cancer [117], and *miR-28* was up-regulated in renal cell carcinoma significantly [118]. High expression of *miR-103*, often together with *miR-107*, correlated with poor survival of cancer patients [119, 120]. *miR-122*, a liver specific miRNA, enhanced the colony formation efficiency of the HCV replicon and increased the steady-state level of HCV RNA in HEK-293 cells [121], while *miR-125b* acted as an oncogene, contributing to the pathogenesis of prostate cancer [122]. An increase in *miR-127* expression was significantly associated with lymph node metastasis in cervical carcinomas [123]. The colon cancer patients with higher *miR-200c* expression had a shorter survival timing when compared to the patients with lower expression [124]. Moreover, the *miR-200* family also determined the epithelial phenotype of cancer cells by targeting the E-cadherin repressors *ZEB1* and *ZEB2*.

But these are not always the cases. *miR-199* (Score \leq 3) family members have demonstrated down-regulation in lung development and up-regulation in tumor in two reports. *MiR-199a* showed down-regulation in NNK treated lung, but

*miR-199a** presented as a tumor suppressor gene, regulating the *MET* proto-oncogene and the downstream extracellular signal-regulated kinase 2 (*ERK2*) [93, 123, 125]. Moreover, *miR-34* family (Score=0) has been found to join the p53 network [126, 127]. The expression of *miR-34a*, *-34b*, and *-34c* is robustly induced by DNA damage and oncogenic stress in a p53-dependent manner. Tumor-suppressive *miR-34* has been studied by several groups, but their linking with p53 still remains for further investigation.

miRNAs AS LUNG TUMOR SUPPRESSOR GENES-*LET-7* FAMILY

let-7, as a tumor suppressor gene, might provide a prospective therapeutic strategy for the curing of lung cancer. *let-7* is selected to study in details due to that (1) *let-7* is the second miRNA identified; (2) It is the first miRNA found to be down-regulated in lung cancer; and (3) It is one of miRNAs well-studied. Till now, 154 *pre-let-7* family members have been identified, including 11 in humans, *hsa-let-7a-1*, *-7a-2*, *-7a-3*, *-7b*, *-7c*, *-7d*, *-7e*, *-7f-1*, *-7f-2*, *-7g*, and *-7i* (Table 3), that finally generate 8 mature *let-7* miRNAs with the same seed sequence(GAGGUA). Another miRNA, *miR-98*, being the same seed sequence, might also be included in *let-7* family. Frequently, there was a decrease in the *let-7* expression levels in lung cancer for both *in vitro* and *in vivo* [71, 72, 128], but only a sporadic decrease in *let-7* in breast and colon cancer samples. Northern analysis showed that the down-regulation of *let-7c* could change from 25 to 75%, and *let-7g* had an average 30% less expression in the lung tumor samples [128]. The down-regulation of *let-7* has been associated with poor survival of lung patients [55, 71]. Moreover, work has been mapped *let-7* family members to human chromosomal sites implicated in a variety of cancer [60]. *let-7a-2*, *let-7c* and *let-7g*, in particular, have been linked to small chromosomal intervals which are deleted in lung cancers. These studies together showed that *let-7* may have potential function in lung tumorigenesis. *let-7* family may take effect through paring with 3'UTR of the targeting mRNAs. The first group as a target by *let-7* is *Ras* genes (*K-ras*, *N-ras* and *H-ras*). The 3'UTR of the human *Ras* genes contains multiple *let-7* complementary sites (LCSs) (Fig. 2), allowing *let-7* to regulate *Ras* expression [128, 129].

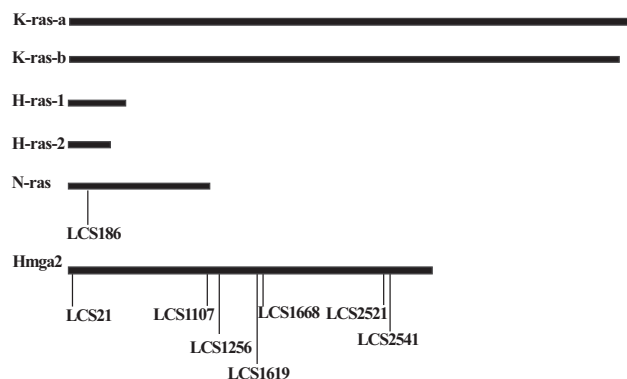


Fig. (2). *Let-7* complementary sites (LCSs) in 3'UTR of *ras* family and *Hmga2* mRNA.

Note: Only *N-ras* gene, but not *K-ras* and *H-ras*, has a LCS in its 3'UTR by using TargetScan and miRndna analysis, while *Hmga2* has 7 conserved LCSs in its 3'UTR.

The combined observations have revealed that (1) There is a decrease in *let-7* expression in lung cancer; (2) Several *let-7* genes map to genomic patients; (3) Over-expression of *let-7* can inhibit lung tumor cell line growth; (4) The expression of the *Ras* oncogene is regulated by *let-7*, and (5) Significant over-expression of RAS in the lung tumor samples strongly implicates that *let-7* is a tumor suppressor in the lung tissue [128]. Indeed, over-expression of *let-7* in A549 lung adenocarcinoma cell line inhibited lung cancer cell growth *in vitro*. This discovery firstly indicated that *let-7* may have potential clinical value in treating lung cancers [71]. Further studies reconfirmed that the *let-7* miRNA could directly represses cancer growth in multiple lung cancer cell lines *in vitro*, as well as lung cancer cell xenografts in immunodeficient mice. Ectopic expression of *let-7g* in *K-Ras^{G12D}*-expressing murine lung cancer cells induced both cell cycle arrest and cell death [130], and inhibiting *let-7* function, led to increase cell division in A549 lung cancer cells. Over-expression of *let-7* in the cancer cell lines altered cell cycle progression and reduced cell division [14]. *let-7* could also be applied as an intranasal drug to reduce tumor formation in a *Ras* mouse model lung cancer [131]. Besides acting as tumor suppressor directly, *let-7* may also be a viable tool to augment current cancer therapies. Over-expression of *let-7* in lung cancer cells could suppress radioresistance in those cells [132]. Nevertheless, re-expressing ectopic *K-Ras^{G12D}* led to substantial, but not complete rescue of tumor growth in the face of elevated *let-7g* levels, suggesting that *let-7* could suppress tumor growth by silencing more than one target.

Hmga2, which expressed a high-mobility protein, was also targeted by *let-7* and the expression was inversely associated with survival [133, 134]. Similarly, ectopic expression of *let-7* reduced HMGA2 and cell proliferation in a lung cancer cell [135]. The effect of *let-7* on *Hmga2* was also dependent on multiple LCSs in the 3' UTR. The growth-suppressive effect of *let-7* on lung cancer cells was rescued by over-expression of the *Hmga2* ORF without a 3'UTR [135]. However, in the case of expressing ectopic *Hmga2*, there was a less robust rescue of tumor growth when compared with re-expressing of *K-Ras^{G12D}* [130]. It may be due to that the expression of *Hmga2* represents an early event during cancer progression. Enforced *let-7* expression has been found to be associated with a decrease in self-renewal in breast tumor-initiating cells and mouse mammary epithelial progenitor cells [136, 137]. An increase of *let-7* in breast tumor-initiating cells was paralleled with a decrease of H-RAS and HMGA2. However, silencing *H-Ras* resulted in a decline in self renewal but causes no effect on differentiation, while silencing *Hmga2* enhanced differentiation but produces no effect on self renewal [137].

Hmga2 was more efficiently targeted by *let-7* than *Ras* in NCI60 cell lines. Expression of *Ras* did not change when amounts of *let-7* efficiently silences expression of *Hmga2* in the tumor cells [138]. Indeed, using TargetScan, and miRndna, the two famous miRNA target prediction softwares (<http://www.targetscan.org/index.html>; <http://www.microna.org/microna/home.do>), we did not found any LCS in *K-ras* and *H-ras*. One and 7 conserved LCSs were found in *N-ras* and *Hmga2* 3'UTR, respectively (Fig. 2). However, over-expression of *let-7a* could indeed suppress *N-ras*, *K-ras*

and *H-ras* [128, 130, 137]. Both TargetScan and miRndna predict biological targets of miRNAs mostly by searching for the presence of conserved sites that match the seed region of each miRNA. Nevertheless, a seed site is neither necessary nor sufficient for miRNA down-regulation. miRNA target sites can tolerate G:U wobble base pairs within the seed region. The extensive base pairing between the 3'UTR and the remainder of the miRNA may offset missing complementarity of the 5' seed [139]. Multiple target sites in the same 3'UTR can potentially increase the degree of translational suppression, thus the degree of repression corresponded to the number of intact sites [140]. Among the seven LCSs at 3'UTR of *Hmga2*, Lee and Dutta showed that LCS2541 did not respond to *let-7* [135], while Shell *et al.* found both LCS2521 and LCS2541 were targets for *let-7*, and targeting these two LCSs was sufficient to suppress expression of HMGA2 [141]. Except for *let-7*, *K-ras* and *Hmga2* 3'UTR have other potential targets for miRNAs, such as *miR-155*, *143* and *-30*. It still remains unclear whether these miRNAs can really regulate *Ras* and *Hmga* families, and whether they can cooperate with *let-7* or not.

It is reported that *let-7* negatively regulates expression of MYC directly by targeting their mRNAs for translation repression [128, 142]. However, inhibition of MYC-MAX transcription factor results in an increase in levels of members of the *let-7* family (*let-7a*, *let-7b*, and *miR-98*) [142]. IMP-1, having growth-promoting activities through stabilization of *c-Myc* mRNA, is also a direct *let-7* target [143]. *let-7* also up-regulates MXI1, a transcription regulator which antagonizes MYC function [14]. These findings with *let-7* family add to the complexity of MYC regulation, implying that dysregulation of these miRNAs may participate in the genesis and maintenance of cancer phenotype in MYC-dysregulated cancers [142].

Multiple genes involved in cell cycle and cell division functions are also directly or indirectly repressed by *let-7* in both A549 lung cancer cells and HepG2 hepatic cancer cells, including *CCNA2*, *CDC34*, *ASK/DBF4*, *AURKA/STK6*, *AURKB/STK12*, *E2F5*, *CDK8*, *PLAGL1* and *PLAGL2* [14]. Combination with microarray analysis and miRNA predication program, PicTar, the putative genes targeted by *let-7* include *CDK6*, *PLAGL2*, *2-PDE*, *CDC25A*, *DZIPI*, *OPRS1*, *DLC1*, *NXT2*, *E2F6*, *GPX7*, *SSR1*, *YAPI*, *ZNF336*, *SOX9*, *RRM2*, *STK6*, *FARP1*, *CBFB*, *DSCR1*, *CDCA7*, *MAP3K7IP2*, *GTF2I/GTF2IP1*, *ARID3A*, *DMD*, *CCNF*, *CCNJ*, *EIF2C3*, *CDC34*, *E2F5* and *LIN28* [14]. *CDK4*, *CDK6*, *CDC25A*, *CCND1*, *CCND3* and *CCNA*, have been identified as *let-7* targets by 3'UTR reporter assays [14, 144].

Besides direct anti-cancer activities, *let-7* may also have functions related with angiogenesis. The mature form of *let-7* miRNA was readily detectable in immortalized lung epithelial cell lines at a level comparable with that in the normal lung tissues [71]. Against *let-7f* reduced sprout formation, indicating that *let-7f* promote angiogenesis by targeting antiangiogenic genes, may be *thrombospondin-1*, and endogenous angiogenesis inhibitor [145]. In addition, *let-7* is down-regulated in mouse mammary epithelial progenitor cells [136]. However, it remains to be ascertained whether such a function could suppress tumor growth or not?

Recently, researches about epigenetics of *let-7* gene impels to pay more attention to the potential therapy function of those family. Several reports showed that the human *let-7a-3* gene on chromosome 22q13.31 was associated with a CpG island, and it was heavily methylated in normal human tissues and hypomethylated in some lung adenocarcinomas. *let-7a-3* hypomethylation facilitated epigenetic reactivation of the gene and elevated expression of *let-7a-3* in a human lung cancer cell line resulted in enhanced tumor phenotypes and oncogenic changes in transcription profiles [146]. Ovarian cancer patients with methylated *let-7a-3* seemed to reduce risk from death compared with those without, and the association was independent of patient's age at surgery, tumor grade, disease stage, and IGF-II or IGFBP-3 expression, but the expression of *let-7a* was slightly affected by the methylation [147]. In addition, *let-7a-2* and *let-7g* were found up-regulated in Volinia's reports [53]. And the serum level of *let-7b*, *let-7c*, *let-7d* and *let-7e* increased in lung cancer patients compared with healthy [65]. Therefore, the individual function of the *let-7* family members requires further investigation. Indeed, we found that mature *let-7* members had different ability to suppress A549 cell growth in our recently *in vitro* study (see Fig. 3, n=6). *let-7i* was the highest repression effect, while *let-7d* and *let-7e* had a little least repression when compared with other *let-7* family. The similar phenomenon was found by Johnson and colleagues [14]. Another question is whether *let-7* would repress tumor-suppressor genes which append the *Hmga2* 3' UTR. *Hmga2* translocations frequently append the *Hmga2* 3' UTR to the 3' end of known tumor-suppressor genes, including *FHIT*, *RAD51L1*, or *HEI10*. Repression of these translocation partners with *let-7* might cooperate with *Hmga2* to promote tumorigenesis [140].

miRNAs AS ONCOGENES-*miR-17-92* CLUSTER

In the *miR-17-92* cluster, containing the *miR-17-18-19-20-92* and residing in intron 3 of the *C13orf25* gene at 13q31.3 (within a 800bp region), the up-regulation in lymphoma cell lines was first discovered (Fig. 4) [63]. *miR-17-92* cluster was markedly over-expressed in lung cancers, especially with SCLC histology [148]. However, over-

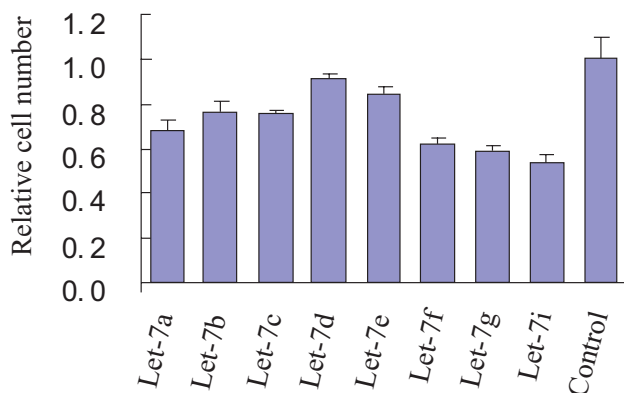


Fig. (3). Impact of *let-7* on proliferation of A549 lung cancer cell line.

Mature *let-7* duplex RNAs (100nM) was transfected into A549 by lipofectamine-2000.

expression of the *C13orf25* ORF did not enhance lung cancer cell growth. Furthermore, the vast majority of *C13orf25* transcripts were detected as Drosha-processed cleavage products [149, 150]. Thus, the *C13orf25* gene may only be serving as a vehicle for *miR-17-92* expression. Indeed, a novel polyadenylation site is present 3' to the *miR-17-92* cluster, and 5' to the C2 region (C2 region is resided 3' to *miR-17-92* in the intron 3 of *C13orf25*) [150]. Enforced expression of C2 led to a marked tumor growth inhibition in association with double stranded RNA-dependent protein kinase activation. Supporting evidences came from a direct experiment that the *miR-17-92* cluster had oncogenic activity [149]. In the lung cancer cell lines, SK-LC-2 and Calu6 exhibited a marked increase in gene copy numbers of *miR-17-92* cluster, while ACC-LC-94, PC-1, PC-10, ACC-LC-97 and ACC-LC-76 showed a moderate increase in copy number. An increase in expression of *miR-20* *in vivo* was observed in all five primary SCLC specimens. The observation revealed that the expression of the *miR-17-92* cluster could enhance lung cancer cell growth [148]. Linkage between the *miR-17-92* cluster and the *Myc* oncogenes has been proposed [151], and concomitant *Myc* and *miR-17-92* cluster amplification has been discovered in multiple human cancers including lung cancer [148, 151-154]. The transcription of the *miR-17-92* cluster was directly up-regulated by C-MYC [151, 154, 155], in contrast, inhibition of MYC-MAX transcription factor with 10058-F4 reduced the level of *miR-17-5p* [142]. The members of the *Myc* gene family (*c-Myc*, *l-Myc* and *n-Myc*) have been shown to be frequently amplified and/or over-expressed in SCLC [148]. It has been shown that *Eμ-Myc* mice enforced by over-expression of the *miR-17-19b*, could develop tumor significantly earlier than those without expression [149]. Furthermore, the *miR-17-92* cluster and C-MYC could synergistically contribute to cancer development [154]. Up-regulation of *miR-17-92* cluster and *c-Myc* might contribute to tumorigenesis via the down-regulation of both the TBR1-Smads and the C-MYC-Miz-1-p15^{INK4b} pathways, respectively [154]. In the lung, *n-Myc* is specifically expressed in the epithelium, while *c-Myc* is transcribed only in the mesenchyme. Over-expression of *n-Myc* in the lung epithelium under the control of human *Sftpc* regulatory elements, cause elevated cell proliferation and inhibition of differentiation, with a phenotype very similar to the phenotype of *miR-17-92* cluster transgenic mice. At least, it has been postulated that high level of the *miR-17-92* cluster might negatively regulate *n-Myc* expression [45].

In addition, both thrombospondin-1 (*Tsp1*) (targeted by *miR-19*) and connective tissue growth factor (*CTGF*) (targeted by *miR-18*) are predicted targets for repression by the *miR-17-92* cluster [155]. The angiogenic activity of C-MYC is partly due to downstream activation of the *miR-17-92* that has down-regulated those antiangiogenic proteins. The transcription factor *E2F1* is a target of two of the miRNAs encoded by *miR-17-92* (*miR-17-5p* and *-20a*), and the gene encoding *E2F1* is also a target of C-MYC [151, 156]. The correlation between *E2Fs* mRNA and the expression of the *miR-17-92* cluster is reconfirmed by two other groups [157, 158]. All 3'UTR of *E2F1*, *E2F2* and *E2F3* mRNA have *miR-20a* binding sites [157]. The core promoter region of *miR-17-92* cluster gene contains two functional E2F transcription factor binding sites. E2F3 is the primary E2F fam-

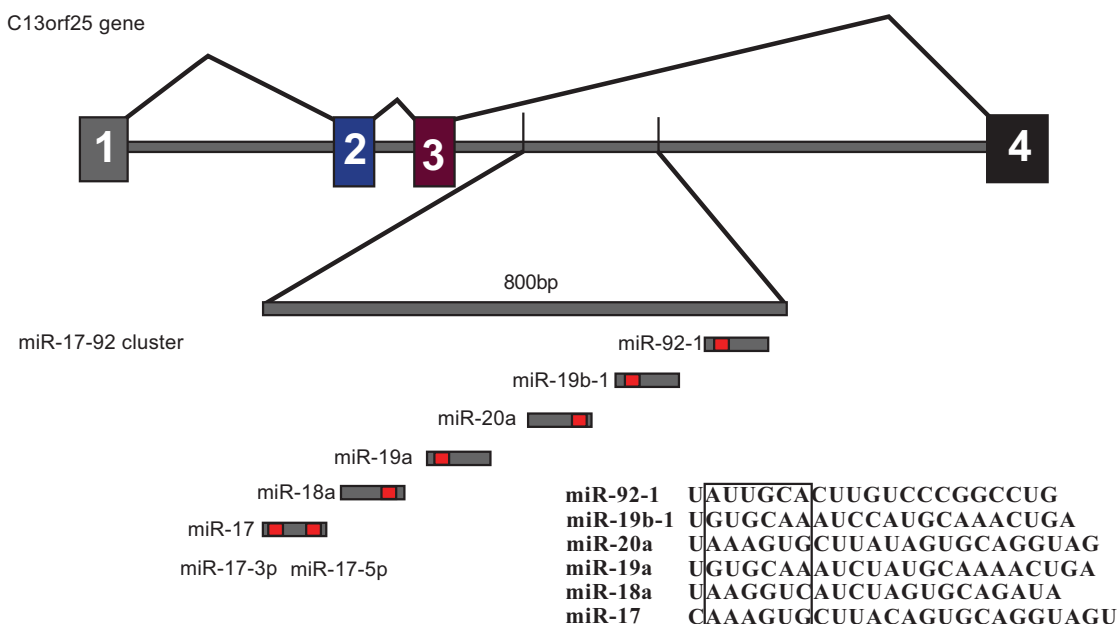


Fig. (4). *miR-17-92* cluster resides in *C13orf25* gene within 800bp of intron 3. The pane showed the seed sequences of miRNAs.

ily member that occupies the promoter [156, 158]. E2F1, E2F2, and E2F3 can directly activate transcription of *miR-17-92* by binding the promoters [158], and their effects similarly [157]. However, *miR-20a* that reduced *E2F1* expression is more significant than *E2F2* and *E2F3* [157]. The negative feedback loop established between transcription factor and *miR-17-92* may shift the E2F transcriptional balance away from the pro-apoptotic E2F1 and toward the proliferative E2F3 transcriptional network [158]. Such a balance shift may be also contributed by MYC, which could transactivate E2Fs. E2Fs, Myc and *miR-17-92* cluster together provide a complex regulatory for cell proliferation and/or apoptosis.

Other targets of *miR-17-92* cluster include both oncogenes and antiapoptotic genes (*Ncoa3*, targeted by *miR-17-5p*) and tumor suppress genes or proapoptotic genes (*Pten*, targeted by *miR-19*; *Rbl2*, targeted by *miR-17-5p*; *Bim*, targeted by *miR-20-92*) [153, 159-161]. Among them, BIM, a proapoptotic protein that regulated cell death, has been confirmed that it was up-regulated in the lungs of *miR-17-92* null late gestation embryos [162], and *Rbl2* was a direct target of *miR-17-5p* during normal lung development. It is still unknown that other genes are directly targeted by *miR-17-92* or not and whether their relationship would be functional in lung development and lung cancer. Nevertheless, significant distinctions among miRNAs of the *miR-17-92* cluster have been found, just as above enumerated, in terms of their roles in cancer cell growth. For example, mature *miR-19a* has a very low abundance when compared with other members, while *miR-17-5p*, *-20a*, and *-19b* are present in both the epithelium and mesenchyme of the embryonic lung [13]. Inhibition of *miR-17* and *miR-20a* with antisense oligonucleotides could induce apoptosis selectively in lung cancer cells by over-expressing *miR-17-92* [150]. In contrast, antisense oligonucleotides against *miR-18a* and *miR-19a* do not exhibit such inhibitory effects, whereas inhibition of *miR-92-1* results in only modest reduction of cell growth.

In addition to its up-regulation in cancer cells, *miR-17-92* is highly expressed in embryonic stem cells, with a decrease in expression levels during embryonic development in mice [13]. Expression of the *miR-17-92* cluster is considerably high at early stages, but declines as lung development proceeds [45]. Transgenic over-expression of the *miR-17-92* cluster by mouse surfactant protein C promoter promotes the high proliferation and undifferentiated phenotype of lung epithelial progenitor cells, and transgenic lungs have a very abnormal lethal phenotype [45]. Interestingly, deletion of *miR-17-92* leads to severely hypoplastic lungs [162]. These findings clearly suggest that marked over-expression of the *miR-17-92* cluster with occasional gene amplification may play a role in the development of lung cancers. Also, taken together, the present findings contribute towards better understanding of the oncogenic roles of *miR-17-92*, which might ultimately lead to the future translation into clinical applications.

miRNA BIOGENESIS AND LUNG CANCER

Differential miRNA expression has been described in lung and lung cancer as above, both in mouse and humans. But only one *Dcr* gene exists in the mouse and human genome, which presumably mediates the processing of all miRNAs and endogenous siRNAs. Several mutant alleles of *Dcr* have been generated in mouse, and analyses of their phenotypes demonstrate that *Dcr* functions in multiple developing tissues [163-169]. *Dcr* plays a specific role in regulating lung epithelial morphogenesis independent of its requirement in cell survival [170]. *Dcr* mutant lungs exhibit two major cellular defects: first, a disruption in epithelial morphogenesis and second an increase in epithelial cell death. In the mesenchyme of *Dcr* mutant lungs, the expression of *Fgf10*, a key gene involved in lung development, was found up-regulated and expanded. However, the mechanism by which DCR functions in the epithelium to influence

Fgf10 expression in the mesenchyme remains unclear [170]. It has been reported that *Ago2*-null mice die early in development, and show severe neural tube defects [171]. The embryo analysis of day 11.5 and 14.5 is expressed, which is the member of ago gene family, the analysis includes the brain, neural tube, limb, lungs, and hair follicles. In the developing lung, expression of *Ago1* and *Ago2* is localized to branching regions, in distal epithelium and mesenchyme, respectively. These are sites undergoing the most dynamic changes in gene expression and rapid remodeling [50].

In the multistep carcinogenesis model, peripheral adenocarcinoma of the lung develops from noninvasive precursor lesions known as atypical adenomatous hyperplasia (AAH) and BAC. The stoichiometry of miRNA machinery and RISC depends on histologic subtype of lung carcinoma, varies along the AAH-BAC-adenocarcinoma sequence. DCR is up-regulated in AAH and BAC and down-regulated in areas of invasion and in advanced adenocarcinoma [172]. Higher DCR level in SCLC is found when compared with adenocarcinoma. Other proteins of the RNA-induced silencing complex (RISC, SND1, PACT, and FXR1) and are also present at higher levels in a SCLC cell line when compared with an adenocarcinoma cell line. In 67 NSCLC cases, DCR expression levels are reduced in a fraction of lung cancers with a significant prognostic impact on the survival of surgically treated cases [173]. DCR expression is inversely correlated with expression levels of mature *let-7* in a panel of human cancer cell lines. Over-expression of *let-7* significantly reduces the expression of DCR at both the protein and mRNA levels, whereas reduction of *let-7* expression conversely increases DCR at both levels. There is possible existence of a novel regulatory loop, in which *let-7* may play a role as a key miRNA for implementing the tightly regulated, equilibrated state of DCR and various miRNAs [174].

Conditional deletion of *Dcr* enhances tumor development in a *K-Ras*-induced mouse model of lung cancer [175]. Cancer cells expressing siRNAs which target three different components of the miRNA processing machinery, show a substantial decrease in steady-state miRNA levels and a more pronounced transformed phenotype [175]. These results indicate the involvement of reduced DCR expression in the development of lung cancers. The up-regulation of DCR has also been observed in prostatic intraepithelial neoplasia, and in 81% of prostate adenocarcinoma [176]. However, the underlying mechanisms are still unclear. Loss of LOH on the long arm of chromosome 14, where *Dcr* resides, has been reported in lung cancers [177, 178], therefore the altered chromatin conformation and haploinsufficiency may be a possible explanation. Since *Dcr* is one of targets by *let-7* family, therefore down-regulation of *let-7* in lung cancer may promote tumorigenesis through up-regulation with DCR.

PROSPECTIVE AND CONCLUSION

miRNAs are frequently dysregulated in lung cancer and have shown to be a promise as tissue-based markers for cancer classification and prognostication. The 1st validated miRNA diagnostic assay developed by Asuragen has been used to improve diagnostics in pancreatic cancer. The finding that miRNAs originated from lung cancer are present in

human plasma in a remarkably stable form, supports them to serve as useful clinical biomarkers, for blood-based detection of human cancer and other diseases in particular.

It is important for that some miRNAs function as oncogenes while others act as tumor suppressors in pharmaceutical industry. For oncogenic miRNAs, miRNA inhibitors with single stranded PNA probe could be used to specifically inhibit each of endogenous miRNAs individually. As for tumor suppression miRNAs, such as *let-7* family, a desirable therapeutic strategy, enhance their functions in cells. The feasibility of miRNA-based therapeutics has been well supported by data from preclinical models and ongoing clinical trials. For example, antisense oligonucleotides corresponding to *miR-17* and *miR-20a* could specifically induce apoptosis in lung cancer cells over-expressing *miR-17-92* [150]. And chemically modified cholesterol-conjugated single strand RNA complementary to a mature miRNA, or called "antagomir", has been reported which could markedly inhibit miRNA expression and function [69]. *let-7* over-expressing by viral vector or transfected directly by pre-miRNAs could inhibit lung cancer growth both *in vitro* and *in vivo*. Encouragingly, the first miRNA drug, SPC3649, has been used for hepatitis C infection therapy.

Potential targeting genes in lung cancer for miRNA-based RNAi includes oncogenes (e.g. *Ras* family, *Myc* family), autocrine/paracrine loops (e.g. *EGBB* family, *IGF* axes), anti-apoptotic genes (e.g. *BCL-2* family, *XIAP*), genes that promote tumor vasculature development (e.g. *VEGF* family, *VEGFR* family), *MMP* family, cell cycle related genes (e.g. *Skp-2*, *Cyclin* family, *Cdk* family), and metastasis related genes (e.g. *E-cadherin*, *COX2*), chemoresistance genes (e.g. *FGF* family, *GSTP1*), and other genes (e.g. *CT120A*, *DDH*). In lung cancer, these genes targeted by artificial siRNAs have been partly studied [179]. And drug base-siRNAs has entered phase I/II trials and showed with good potential (e.g. ALN-RSV01, Cand5, Sirna-027) [180]. This oligo-based therapeutics should facilitate miRNAs to clinical use for their high comparability. The advantages of using miRNAs over siRNAs may be because of (1) long-term activity, (2) *in vivo* stability, (3) highly RNA promoter-compatibility and (4) no overt toxicity. Nevertheless, miRNAs are of multiple targets when compared with siRNAs. Balance and caution must be taken into consideration, as the systemic delivery of miRNA may lead to unwanted gene silence. An investigation on all possible targets and potential effects must be carried throughout prior to the initiation of any clinical trial. The potential of miRNAs in facilitating diagnosis, prognosis, and survival outcome for lung cancer should also be made feasible.

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ABBREVIATIONS

AAH = atypical adenomatous hyperplasia
AGO = argonaute

BAC	= bronchoalveolar carcinomas
DCR	= Dicer
FRAs	= fragile sites
LCS	= <i>let-7</i> complementary site
LOH	= heterozygosity
mat-miRNA	= mature miRNA
miRISC	= miRNA-programmed RISC
miRNA	= MicroRNA
NSCLC	= non- small cell lung cancer
P-body	= processing body
pre-miRNAs	= precursor miRNAs
RISC	= RNA-induced silencing complex
SCLC	= small cell lung cancer
siRISC	= siRNA-programmed RISC

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