

# Non-Coding RNA

## *A New Tool for the Diagnosis, Prognosis, and Therapy of Small Cell Lung Cancer*

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**Abstract:** In recent years, novel classes of noncoding RNAs (ncRNAs) have been discovered, which are implicated in diverse functional and regulatory activities. Growing evidence indicates that deregulated ncRNAs play crucial roles in the onset and progression of cancer, including small-cell lung cancer. In this review, we highlight nearly all of the findings regarding the roles and the possible mechanisms of ncRNAs as oncogenes or tumor suppressors in small-cell lung cancer. Furthermore, we discuss the possible role of ncRNAs as diagnostic biomarkers, their significant contribution to the prognosis, and their functions in regulating the response to therapy.

**Key Words:** Non-coding RNA, Small cell lung cancer.

(*J Thorac Oncol.* 2015;10: 28–37)

Lung cancer is the most common cancer worldwide, inflicting over 1,090,000 men and 515,000 women annually. In addition, it is the leading cause of cancer mortality, responsible for 18.2% of all cancer deaths (World Health Organization. Cancer. 2013; <http://globocan.iarc.fr>).<sup>1</sup> Small-cell lung cancer (SCLC), accounting for approximately 15% of all lung cancers, is the most aggressive lung cancer type, with a 2-year survival rate of less than 5%.<sup>2</sup> Despite advances in chemotherapy, radiotherapy and our understanding of the molecular mechanisms of SCLC, most SCLC patients still succumb to the disease within 1 year.<sup>3</sup> This poor prognosis is largely due to the late stage at diagnosis and the lack of effective therapeutic regimens.<sup>4,5</sup> Thus, considerable effort is warranted to improve early diagnosis and targeted therapies for SCLC.

Noncoding RNAs (ncRNAs) are typically classified into two groups according to their size: small ncRNAs of less than 200 nucleotides (nt), which include microRNAs (miRNAs), Piwi-interacting RNAs and small nucleolar RNAs, and long

ncRNAs (lncRNAs, >200 nt).<sup>6,7</sup> In recent years, it has become increasingly apparent that ncRNAs are of crucial functional importance for both normal development and physiology and for disease.<sup>8</sup> They participate in target gene silencing, as well as transcriptional, post-transcriptional, and epigenetic processes. The latter events include chromatin remodeling and nuclear reorganization, which, in turn, involves the formation of silent compartments and the fine-tuning of gene recruitment into these compartments.<sup>7</sup> Recent evidence has implicated several ncRNAs in SCLC tumorigenesis and tumor progression, which has opened the door to ncRNA-based molecular diagnosis and therapy of SCLC.

In the present review, we summarize the primary findings regarding the biogenesis, regulation, and function of ncRNAs in SCLC, and discuss their diagnostic and prognostic value and potential clinical utility.

### MIRNAS IN SCLC

#### Biogenesis and Modification of miRNAs in SCLC

miRNAs, the most extensively studied ncRNAs, are small ncRNAs of approximately 22 nt with a role in post-transcriptional gene silencing.<sup>9</sup> They are transcribed as independent genes into 1 to 3 kb long precursors (pri-miRNAs) by RNA polymerase II or III. The pri-miRNAs undergo processing by Drosha-DGCR8 (microprocessor), giving rise to precursor miRNAs (pre-miRNAs) that are exported to the cytoplasm by the nuclear transport factor exportin-5 (*XPO5*). The pre-miRNAs are then cleaved by Dicer to mature imperfect RNA duplexes. Then the passenger strand is degraded, and the other guide stranded mature miRNA associates with Argonaute (AGO) proteins to form a miRNA-induced silencing complex (RISC), which leads to mRNA degradation or translational inhibition by binding to the 3'-untranslated region (3'-UTR) of target mRNA.<sup>10,11</sup> Alternatively, miRNAs can also be derived from the introns of protein-coding genes through splicing, after which they are cleaved into lariats by Dicer and enter the canonical pathway.<sup>12,13</sup> Some miRNAs are released as cell-free miRNAs from the cytoplasm into the extracellular environment. This release occurs through the packaging of miRNAs into various membrane-bound vesicles, such as exosomes, microvesicles, and apoptotic bodies.<sup>14–16</sup>

Aberrant miRNAs expression has been reported in many cancers including SCLC and non-small-cell lung cancer (NSCLC). Genetic and epigenetic alterations are implicated

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Disclosure: The authors declare no conflict of interest.

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DOI: 10.1097/JTO.0000000000000394

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ISSN: 1556-0864/15/1001-0028

in the aberrant expression of miRNAs. Nearly 50% of miRNAs are located in deleted regions, amplified regions, and breakpoint regions involved in human cancers.<sup>17</sup> The miR-17–92 family resides in 13q31.3, and the chromosome fragment frequently amplified in SCLC.<sup>18,19</sup> Defects in the miRNA biogenesis machinery may also be closely related to aberrant miRNAs expression. Lin-28 inhibits the biogenesis of let-7 miRNAs by blocking both Drosha- and Dicer-mediated cleavage and accelerating the decay of let-7 pre-miRNAs.<sup>20–25</sup> In addition, the SNP in pre-miRNA is another factor that affects the expression of mature miRNAs and miRNA target genes. miR-196a2 and miR-146a are modified in this manner.<sup>26,27</sup> Besides, the aberrant miRNAs expression could also be induced by changes in the miRNA promoter. DNA methylation of the promoter occurs for miR-34b/c and miR-886-3p.<sup>28,29</sup> miR-375 is activated through the binding of ASH1 to the three E-box elements in the promoter.<sup>30,31</sup>

Overall, the complex process of miRNA biogenesis has nearly been fully understood, and the modification mechanisms of miRNA in SCLC have also been extensively investigated in

recent years. Considering the increasing interest in translating miRNAs into the cancer clinic, clearly elucidating the biogenesis and modification of miRNA is a significant prerequisite not only for fully understanding the functions of miRNAs in SCLC but also to integrate miRNA into clinical applications.

### miRNAs Regulate SCLC Cell Viability

Previous studies demonstrated that miRNAs are always involved in the tumorigenesis of SCLC by regulating oncogenes and tumor suppressor genes that play crucial roles in cell proliferation, apoptosis, migration, and invasion (see Table 1).

### miRNAs Function as Oncogenes in SCLC

The members of the miR-17–92 cluster (miR-17, miR-18a, miR-19a, miR-20a, miR-19b-1, and miR-92-1), which resides in the 13q31.3, are the first miRNAs to be recognized as oncogenes. The miR-17–92 cluster is significantly overexpressed in SCLC, which enhances cell proliferation and inhibits apoptosis.<sup>18,19</sup> Ebi et al.<sup>32</sup> found that miR-17–92

**TABLE 1.** ncRNAs Regulate Cell Viability in SCLC

ncRNA	Direction of Expression in SCLC	Material	Function	Effector	Ref.
<b>Oncogenic miRNAs</b>					
miR-17–92 cluster	Up	Lung cancer cell lines, immortalized lung epithelial cell lines, lung cancer tissues, and normal lung tissues	Enhances cell proliferation, inhibits apoptosis and protects against DNA damage.	miR-20a targets E2F1 and downregulates the cyclin-dependent kinase inhibitor p21 and the pro-apoptotic protein Bim, but not the PP2A catalytic subunit (PP2Ac).	18, 19, 32
miR-93, miR-98, miR-197	Up	Lung cancer cell lines and FFPE lung tumor tissue specimens	Inhibit tumor suppressor gene <i>FUS1</i> expression.	FUS1	33
miR-375	Up	Human lung cancer cell lines and specimens and mouse SCLC-like model tumors	Induces NE differentiation.	ASH1-miR-375-YAP1 pathway	30, 31
<b>Oncogenic lncRNA</b>					
HOTAIR	Up	SCLC cell lines and surgical SCLC samples	Promotes proliferation and invasion	NS	34
<b>Tumor suppressive miRNAs</b>					
let-7 family	Down	A SCLC cell line	Suppresses cell proliferation by inducing cell cycle arrest at the G1 phase	Repressed by Lin-28 and targets CDC25	24
miR-34b/c	Down	Resected lung cancer tumor specimens, malignant pleural effusions from SCLC patients, and lung cancer cell lines	Inhibits cell growth, migration, and invasion.	NS	28
miR-126	Down	SCLC cell lines and primary tumors	Inhibits cell proliferation by arresting the cells in the G1 phase.	SLC7A5	3, 35
miR-27a	Down	SCLC cell lines	Decreases cell proliferation, self renewal, and promotes differentiation.	NS	36
miR-886-3p	Down	SCLC cell lines and FFPE specimens	Inhibits cell proliferation, migration, invasion, tumor growth, and lung metastasis.	PLK1 and TGF-β1	29
miR-335	Down	SCLC cell lines and mouse SCLC skeletal metastases model	Reduces cell migration, invasion, proliferation, and metastasis.	IGF-1R and RANKL pathways	37

FFPE, formalin-fixed paraffin-embedded; ncRNAs, noncoding RNAs; NE, neuroendocrine; NS, not specified; SCLC, small cell lung carcinoma.

overexpression counterbalances the generation of DNA damage in RB-inactivated SCLC cells, thus reducing excessive DNA damage to a tolerable level and leading to genetic instability.

MiR-93, miR-98, and miR-197, which target the tumor suppressor gene *FUS1/TUSC2*, are additional examples of miRNAs with oncogenic properties in SCLC. As early as 1982, deletions in the short arm of chromosome 3 (3p) were identified and characterized in SCLC cell lines and cultures. *FUS1/TUSC2*, located on 3p21.3, was reduced in or completely deleted from 82% and 100% of NSCLC and SCLC cell lines, respectively. In contrast to *FUS1/TUSC2*, miR-93 and miR-98 are expressed at higher levels in SCLC cell lines than in NSCLC cell lines and immortalized human bronchial epithelial cell (HBE) line, whereas miR-197 is expressed at higher levels in SCLC and in NSCLC cell lines than in the HBE line. In addition, miR-93, miR-98, and miR-197 function as oncomirs by directly inhibiting the expression of *FUS1/TUSC2*. Furthermore, elevated miR-93 and miR-197 expression correlates with reduced *Fus1/TUSC2* expression in NSCLC tumor specimens.<sup>33,38,39</sup>

The pro-tumor mechanism of another miRNA, miR-375, in SCLC is also relatively well identified. Most SCLC cell lines display overexpression of miR-375, whereas miR-375 is only weakly expressed in the lung adenocarcinoma (AD) and lung squamous cell carcinoma (SQ) cell lines. MiR-375, a direct transcriptional target for achaete-scute complex homolog 1 (*ASH1/ASCL1*), is upregulated by the direct interaction between *ASH1* and the E-box elements in the miR-375 promoter. In addition, miR-375 mediates signals required for the *ASH1*-mediated induction of neuroendocrine features in lung cancers, the majority of which are SCLCs. Furthermore, miR-375 is highly expressed in the cell lines generated from mouse SCLC-like tumors, and its expression correlates with *ASH1/ASCL1*.<sup>31</sup> The transcriptional coactivator *YAP1*, which negatively correlates with miR-375, has been found to be a direct target of miR-375 and showed growth inhibitory activity in SCLC cell lines. Taken together, the downregulation of *YAP1* by *ASH1*-transactivated miR-375 promotes the growth of SCLC cells, and the *ASH1*-miR-375-*YAP1* pathway plays a key role in lung cancers exhibiting neuroendocrine features.<sup>30</sup>

## miRNAs Function as Tumor Suppressors in SCLC

The let-7 family is a cluster of miRNAs whose genes map to different chromosomal regions that are frequently deleted in lung cancer.<sup>17</sup> The downregulation of let-7 in SCLC is induced by the RNA-binding protein Lin-28, which blocks both Drosha- and Dicer-mediated cleavage and promotes cell cycle progression and cell proliferation.<sup>24</sup> Microarray analysis identified many cell cycle-associated genes that are directly or indirectly repressed by let-7, and further reporter assays validated that cell cycle regulators *CDK6* and *CDC25A* are direct targets of let-7.<sup>40</sup> In SCLC, it was found that let-7 knockdown results in the upregulation of *CDC25A*, which is necessary for the progression from the G1 phase to the S phase of the cell cycle. Taken together, the reduced expression of let-7 in SCLC, induced by Lin-28, enhances cell proliferation by promoting cell cycle progression at the G1 phase through upregulation of *CDC25A*.

Members of the miR-34 family, another important group of miRNAs, function as tumor suppressors and are down-expressed in a variety of cancers, including SCLC.<sup>41–43</sup> Methylation of miR-34 family has been detected in SCLC cell lines and clinical specimens and is responsible for the reduced expression of miR-34 family in SCLC. Furthermore, the frequency of miR-34b/c methylation is significantly higher in SCLC than in NSCLC. The transfection of miR-34b/c into SCLC cell lines resulted in significant inhibition of cell growth, migration, and invasion compared with control treatment, which indicates that the aberrant methylation of miR-34b/c plays an important role in the pathogenesis of SCLC.<sup>28</sup> Although miR-34a is known to play a key role in p53-mediated apoptosis in NSCLC, thereby affecting NSCLC cell survival,<sup>44</sup> Lee et al. showed that expression of miR-34 family members is not related to P53 protein expression in SCLC tumors. In addition, neither increased nor decreased expression of miR-34a in SCLCs affects cell viability, malignant behavior, or the endogenous expression of the miR-34a target genes *cMET* and *Axl* in NSCLC cell lines.<sup>43</sup> These findings further validate the specificity of miRNA function in the context of different lung cancer subtypes.

Additional miRNAs have been showed to act as tumor suppressors in SCLC. Microarray and real-time reverse transcription polymerase chain reaction (RT-PCR) analyses validated that miR-126 expression is uniformly decreased in SCLC cell lines and primary tumors.<sup>35</sup> Transfection of the SCLC cell lines H69 and HTB192 with miR-126 inhibited cell proliferation by arresting the cells in the G1 phase. Short interfering RNA-mediated suppression of *SLC7A5*, a direct target of miR-126 in SCLC, causes the same effects on H69 cells, suggesting that miR-126 performs tumor suppressor function that is mediated by the downregulation of its target *SLC7A5*.<sup>35</sup> Miao et al.<sup>36</sup> showed that miR-27a is consistently downregulated in sphere-forming cells of all three SCLC cell lines (H446, H209, and H69), and that antagonizing miR-27a using an inhibitor in parental cells enhances the proliferation, self renewal, and proportion of undifferentiated cells in vitro.

Recent evidence suggests that miR-886-3p, regulated by DNA hypermethylation of its promoter, is another important tumor suppressor in SCLC.<sup>29</sup> MiR-886-3p has been reported to negatively regulate the proliferation, migration, and invasion of NCI-H446 cells in vitro, as well as tumor growth, bone/muscle invasion, and lung metastasis in vivo, by repressing the expression of its targets: *PLK1* and *TGF-β1*, two well-known active oncogenic genes.

The antitumor activity of miR-335 in SCLC has also been characterized. Human SCLC cells SBC-5 cells localize to skeletal and nonskeletal sites, whereas SBC-3 cells only invade nonskeletal sites. The expression of miR-335 is reduced in SBC-5 compared with SBC-3 cells. In contrast, the expression of *RANKL* and *IGF-1R*, two key cytokines, are elevated in SBC-5 as compared with SBC-3 cells. Lentiviral overexpression of miR-335 in SBC-5 cells not only significantly reduces in vitro carcinogenesis, cell migration, and invasion but also inhibits osteolytic lesions formation in vivo, which is mediated by upregulation of the *IGF-1R* and *RANKL* pathways.<sup>37</sup>



Taken together, the above miRNAs have been indicated to function as tumor suppressors or oncogenes in SCLC and in many other cancer types. There are other miRNAs that have been characterized to promote or inhibit tumorigenesis in a variety of tumors including NSCLC, but whose functions have not been studied in SCLC. Therefore, there are more miRNAs with significance, which are worth evaluation in the context of SCLC. Moreover, among the above miRNAs, some have been only studied in cancer cells and consequently require further validation in tumor specimens and SCLC patients.

## Clinical Application of miRNAs to the Diagnosis, Prognosis, and Therapy of SCLC

### miRNAs as Diagnostic Biomarkers of SCLC

Because of the aggressiveness of SCLC, timely diagnosis is expected to improve the prognosis of SCLC patients. Unfortunately, no specific biomarker currently identifies early stage SCLC. Recently, several studies reported that a specific miRNA profile may serve as a reliable, noninvasive diagnostic tool for SCLC (see Table 2).

In an effort to investigate the contributions of miRNAs to the pathogenesis of SCLC and the possible use of miRNAs for its diagnosis, Du et al. performed microarray analysis to compare 136 miRNAs in a set of SCLC cell lines to that in a group of NSCLC cell lines and the normal immortalized HBEC line. They found that the miRNA profiles consistently distinguished the SCLC cell lines from NSCLC and HBEC cell lines, suggesting that the miRNA profile may serve as a diagnostic marker of SCLC.<sup>45</sup> Gilad et al. developed an miR-based assay using pathologic and cytologic samples to differentiate between the four primary types of lung cancers: SCLC, cacinoid, and squamous and nonsquamous NSCLC. This assay included eight miRNAs (miR-106a, miR-125a-5p, miR-129-3p, miR-205, miR-21, miR-29b, miR-375, and miR-7) and displayed an overall accuracy of 94% in the validation cohort.<sup>46</sup> More recently, Huang et al.<sup>47</sup> confirmed the high diagnostic accuracy of miR-205 to distinguish SQ from AC and SCLC in Chinese patients.

Apart from surgical and needle biopsy specimens, miRNAs can also be detected in bronchial brushing specimens. Two miRNA panels are constructed and validated, which distinguish SCLC from NSCLC and SQ from AC using not only FFPE surgical lung specimens but also bronchial brushing specimens, indicating that these panels have considerable clinical value for differential diagnosis.<sup>48</sup>

Considering the high stability of miRNAs in body fluids and in tissues, these molecules also represent potential biomarkers for the early, noninvasive detection of SCLC. According to published microarray data, Zheng et al. selected the 15 most frequently overexpressed miRNAs in lung cancer tissues and examined them in the plasma of lung cancer patients. They found that the levels of miR-155, miR-197, and miR-182 were significantly elevated compared with the controls, allowing for the discrimination between lung cancer patients and cancer-free controls with high specificity and sensitivity.<sup>49</sup> This result demonstrates that only a subset of circulating miRNAs is likely derived from the secretion/release of overexpressed miRNAs by tumor cells. Roth et

al.<sup>50</sup> provided evidence that a signature of miR-10b, miR-34a, miR-141, and miR-155 expression in serum discriminated lung cancer patients from healthy individuals. Although miRNAs are generally stable for several years, Keller et al. found that most obvious changes in the miRNA expression pattern appears to occur near the time of lung cancer diagnosis. This finding supports the concept that developing lung cancer might be detectable for years preceding traditional diagnosis based on a specific miRNA expression profile and that this profile changes during tumor development.<sup>51</sup>

### miRNAs as Prognostic Biomarkers of SCLC

The prognosis always exerts a great effect on the selection of treatment. As a result, robust prognostic biomarkers are needed to improve clinical treatment selection. Growing evidence suggests that changes in miRNA expression can significantly influence the prognosis of SCLC, which raises the possibility that miRNAs could serve as prognostic biomarkers in SCLC patients (see Table 3).

Ranade et al. suggested that a high level of miR-92a-2\* expression is associated with a poor prognosis and shorter survival in SCLC patients. Moreover, stepwise multivariate analysis revealed that miR-92a-2\* is an independent prognostic factor of SCLC.<sup>52</sup> Bi et al. developed and validated that an miR-150/miR-886-3P expression profile that significantly correlated to the overall survival (OS) of SCLC patients. And the risk score for each SCLC patient was calculated according to the expressions of miR-150 and miR-886-3p. Patients with high-risk miRNA expression profile experienced poor OS and progression-free survival compared with those with low-risk expression profiles, suggesting that this miRNA profile may represent an independent predictor of survival.<sup>53</sup> However, negative results have also been found. Lee et al.<sup>43</sup> investigated the expression of a panel of seven miRNAs in SCLC tumors and found that these miRNAs are unrelated to the clinical characteristics of SCLC patients and are neither prognostic of OS or progression-free survival nor predictive of the response of these patients to treatment.

In addition to polymorphisms in miRNAs themselves, a growing number of miRNA-related polymorphisms have been demonstrated to be associated with the prognosis of SCLC. On the basis of multivariate analysis, Ding et al.<sup>54</sup> found that a polymorphism (rs16917496) in the miR-502 binding site of the 3' UTR of the SET8 gene is associated with SCLC patient survival. Similar phenomena were also detected for miR-1827 and miR-629. An SNP in an miRNA-1827 binding site of MYCL1 results in the altered regulation of MYCL1 expression and may increase the susceptibility of these individuals to SCLC.<sup>55</sup> In southern and eastern Chinese populations, a tagSNP in the miR-629 binding site of the NBS1 3'UTR confers an increased risk of lung cancer by diminishing the expression of this gene.<sup>56</sup> It is proposed that SNPs located in the miRNA seed region of the 3'UTR of target genes might affect miRNA-mediated gene regulation and are thus associated with cancer susceptibility.<sup>57</sup> Besides miRNA seed regions, SNPs in genes that encode the miRNA processing machinery as well as in pre-miRNAs, also affect cancer risk and patient prognosis. For example, Guo et al.<sup>58</sup> reported that an miR-SNP located in the 3'UTR of the

**TABLE 2.** miRNAs as Diagnostic Biomarkers of SCLC

Experimental Setting	Material	Candidate miRNA	Diagnostic Value	Ref.
miRNA microarray analysis of 136 miRNAs in nine SCLC cell lines, seven NSCLC cell lines, and three HBEC lines	Cell lines	miRNA panel 1: 30 miRNAs including miR-101, miR-1, etc. miRNA panel 2: miR-31 and miR-205. miRNA panel 3: 29 miRNAs including miR-16, miR-21, etc.	All miRNAs of miRNA panel 1 are significantly differentially expressed between the SCLC and HBEC cell lines; two miRNAs of miRNA panel 2 are significantly differentially expressed between the NSCLC and HBEC cell lines; all miRNAs of miRNA panel 3 are significantly differentially expressed between the SCLC and NSCLC cell lines.	45
Discovery: Microarray and PCR analyses of 94 primary resection samples from four histologic types of lung cancer (SCLC, SQ, nonsquamous NSCLC, and carcinoid). Training: Eight miRNAs were selected to be tested in 216 resection, biopsy, and FNA samples. Validation: Assessing the levels of these eight miRNAs in 451 resection, biopsy, FNA, and bronchial brushing and washing samples.	Pathologic and cytologic samples	miRNA panel: miR-106a, miR-125a-5p, miR-129-3p, miR-205, miR-21, miR-29b, miR-375, and miR-7	The miRNA panel displays an overall accuracy of 93.7% and a sensitivity of >90% for classification of the four main types of lung cancer (SCLC, SQ, nonsquamous NSCLC, and carcinoid).	46
Discovery: qRT-PCR analysis of miR-205 expression in 197 macrodissected surgical lung tissues. Validation: The expression profiles of miR-205 were assessed in an independent cohort of 44 snap-frozen surgical lung tissues	Macrodissected surgical lung tissues and snap-frozen surgical lung tissues	miR-205	miR-205 discriminates SQ from AC and SCLC with an AUC of 0.985 and 0.978, respectively.	47
Discovery: Microarray analyses of 723 miRNAs in 82 snap-frozen surgical lung specimens. Training: qRT-PCR was performed on a training cohort of 85 macrodissected FFPE surgical lung specimens to evaluate seven miRNA candidates based on the microarrays analyses and to establish two miRNA panels. Validation: Evaluation of the two miRNA panels in an independent cohort of 68 FFPE surgical lung specimens. Application: The effectiveness of the miRNA panels as differentiators of 207 bronchial brushing specimens.	Snap-frozen surgical lung specimens, macrodissected FFPE surgical lung specimens, and bronchial brushing specimens	miRNA panel 1: miR-29a and miR-375; miRNA panel 2: miR-205 and miR-34a.	miRNA panel 1 discriminates SCLC from NSCLC with an AUC of 0.947 (Sp 94%, Sn 79%); miRNA panel 2 discriminates SQ from AC with an AUC of 0.962 (Sp 88%, Sn 92%).	48
Analysis of 15 miRNAs frequently upregulated in lung cancer tissues from the plasma of 74 lung cancer patients (23 SQ, 18 AC, 17 SCLC, 7 large cell carcinoma, and 9 others) and 68 age-matched cancer-free controls.	Total plasma RNA	MiRNA panel: miR-155, miR-197 and miR-182	The miRNA panel displays 81.33% sensitivity and 86.76% specificity for discriminating the lung cancer patients from the controls.	49
Measurement of the concentrations of four circulating miRNAs in 35 lung cancer patients (19 NSCLC, 8 SCLC, and 8 indefinite type), 7 patients with benign lung tumors, and 28 healthy individuals	Total cell-free RNA in blood serum	miR-10b, miR-34a, miR-141, and miR-155	The median expression levels of miR-10b, miR-34a, miR-141, and miR-155 are significantly higher in lung cancer patients than in healthy individuals, and the AUC values exceed 0.899 for each miRNA in distinguishing between lung cancer patients and healthy individuals; the levels of miR-10b, miR-141, and miR-155 are significantly higher in lung cancer patients than in patients with benign lung tumor, and the AUC values exceed 0.963 for each miRNA in distinguishing between lung cancer patients and patients with benign lung tumor.	50

AC, adenocarcinoma; AUC, area under the curve; CI, confidence interval; FFPE, formalin-fixed paraffin-embedded; FNA, fine-needle aspiration; HBEC, human bronchial epithelial cell; miRNAs, micro RNAs; NSCLC, non-small cell lung carcinoma; SCLC, small-cell lung carcinoma; Sn, Sensitivity; Sp, Specificity; SQ, squamous cell carcinoma; qRT-PCR, quantitative real-time reverse transcription-polymerase chain reaction.

miRNA processing gene *xpo5* is associated with the OS of SCLC patients. They also found that the AA genotype correlates to improved survival duration. Moreover, functional polymorphisms in pre-miR-146a and pre-miR-196a2 are associated with increased risk of lung cancer in Korea and Chinese individuals, respectively.<sup>26,27</sup>

### miRNAs as Therapeutic Biomarkers of SCLC

Chemotherapy and radiotherapy are currently considered as the optimal treatment options for SCLC patients. However, drug and radiotherapy resistance have caused great clinical challenges. Therefore, identifying the molecular mechanisms that regulate drug resistance responses is

**TABLE 3.** microRNAs and miRNA-Related Polymorphisms as Prognostic Biomarkers of SCLC

Experimental Setting	Material	Candidate miRNA	Prognostic Value	Ref.
<b>miRNA polymorphisms</b>				
Discovery: miRNA profiling of 34 diagnostic SCLC tumor samples analyzed using XenoBaseValidation: Assessing the top 16 miRNAs in 25 samples from the 34 SCLC tumor samples by qRT-PCR and their correlation to clinicopathological characteristics, normalized to 5S-rRNA and RNU6	FFPE specimens	miR-92a-a*	High miR-92a-a* expression correlates with decreased median survival.	52
Discovery: Survey of the expression of 924 miRNAs from 42 SCLC patients through miRNA profiling to develop prognostic models. Validation: assessing these models in an independent cohort of 40 FFPE specimens through qRT-PCR	FFPE specimens	The miR-150/ miR-886-3p profile	High expression levels of the miRNA profile correlates with poor OS and PFS.	53
Investigating the expression of a panel of 7 miRNAs in 31 SCLC tumors, 14 SCLC cell lines, and 26 NSCLC cell lines and their correlation to clinical characteristics and sensitivity to cisplatin or etoposide.	FFPE specimens and cell lines	miRNA panel: miR-21, miR-29b, miR-34a/b/c, miR-155, and let-7a	The expression of the miRNA panel is neither prognostic of OS or PFS nor predictive of the response of the patients to treatment.	43
<b>miRNA-related polymorphisms</b>				
Analyzing an SNP (rs16917496) within the miR-502 miRNA seed region in the 3' UTR of <i>SET8</i> in blood samples from 44 SCLC patients and 44 healthy female controls and correlation of this SNP to clinicopathological characteristics.	Blood samples	The rs16917496 polymorphism within the miR-502 seed region of the 3' UTR of <i>SET8</i>	The <i>SET8</i> CC + CT genotype is independently associated with longer survival.	54
Discovery: Identification of 26 genes that are markedly deregulated in SCLC using Web-based NextBio software, detection of 53 putative SNPs located in the 3' UTR of these 26 genes that might generate or destroy miRNA-binding sites using Patrocles database and selection of two SNPs (rs3134615 G > T in the 3'UTR of <i>MYCL1</i> and rs2291854 C > T in the 3'UTR of <i>ASCL1</i> ) displaying a MAF of 5% or greater from the putative SNPs. Validation: analysis of the genotype distributions of these two SNPs and their association with SCLC susceptibility in 666 SCLC patients and 758 controls.	Peripheral blood samples	rs3134615 G > T located within the miR-1827 seed region of the 3' UTR of <i>MYCL1</i> and rs2291854 C > T located in the 3'UTR of <i>ASCL1</i>	The rs3134615T allele is associated with a significantly increased risk of SCLC compared with the GG genotype. No significant differences are detected in the risk of SCLC among the rs2291854 genotypes.	55
Discovery: Identification of seven SNPs displaying a MAF of >5% in the 3'UTR of the <i>NBS1</i> gene using the dbSNP database, analysis of the haplotype block based on the Chinese Han Beijing population data of HapMap, and selection of three haplotype-tagged SNPs (rs14448, rs13312986 and rs2735383) that might cover the genetic information of common SNPs in the 3'UTR of the <i>NBS1</i> gene using Stram's tagSNPs program. Validation: Genotyping three tagSNPs (rs14448, rs13312986 and rs2735383) in southern Chinese individuals (1056 SCLC cases and 1056 controls) and then validating the discovered association in eastern Chinese individuals (503 SCLC cases and 623 controls).	Peripheral blood samples, lung cancer tissues and their adjacent normal tissues	Three tagSNPs (rs14448, rs13312986 and rs2735383) within the miR-629 seed region of the 3'UTR of the <i>NBS1</i> gene	The rs2735383CC genotype is associated with a significantly increased risk of lung cancer compared with the GG or GC genotype.	56
Analysis of an miRNA-related SNP (rs11077) in the 3' UTR of <i>XPO5</i> in blood samples from 42 SCLC patients and its correlation to cancer survival.	Blood samples	The rs11077 polymorphism in the 3' UTR of the miRNA processing machinery gene <i>XPO5</i>	The rs11077 AA genotype is significantly associated with longer survival time and better OS compared with the AC and CC genotypes.	58
Examination of the rs2910164 C > G genotypes in 1094 lung cancer patients and 1100 healthy controls and their association with lung cancer susceptibility.	Peripheral blood samples, cancer tissues and paired non-malignant lung tissues	rs2910164 C > G in pre-miR-146a	The rs2910164 CG or GG genotype is associated with a significantly decreased risk for lung cancer compared with the CC genotype in never smokers.	27
Discovery: Identification of 273 common genetic variants (MAF > 5%) in pre-miRNAs and their surrounding regions in all ethnic groups using the dbSNP database and selection of four pre-miRNA SNPs (rs2910164 C/G, rs2292832 C/T, rs11614913 T/C, and rs3746444 A/G) located in the pre-miRNA regions. Validation: evaluation of the associations between these four SNPs in pre-miRNAs and lung cancer susceptibility in a case-control study of 1058 incident lung cancer patients and 1035 cancer-free controls.	Blood samples	rs11614913 T/C in pre-miR-196a2	The rs11614913 CC genotype is associated with a significantly increased risk of lung cancer compared with the TT and TC genotypes.	26

CI, confidence interval; FFPE, formalin-fixed paraffin-embedded; MAF, minor allele frequency; miRNAs, micro RNAs; OR, odds ratio; OS, overall survival; PFS, progression-free survival; SNP, single nucleotide polymorphism; qRT-PCR, quantitative real-time reverse transcription-polymerase chain reaction.

crucial for developing effective approaches to overcome such resistance and further improve patient prognosis. As reviewed here, our group and others have found that several miRNAs are closely associated with the response of patients to therapy and that some of these miRNAs are implicated in regulating chemosensitivity and radiosensitivity in SCLC (see Table 4).

Previously, we reported that overexpression of miR-134, miR-379, and miR-495 increased the sensitivity of drug-resistant H69AR SCLC cells to multiple drugs, including doxorubicin, cisplatin, and etoposide, whereas knockdown of these miRNAs decreased this sensitivity. Further investigation revealed that miR-134 increases drug sensitivity in part through downregulation of MRP1/ABCC1, which contributes to chemoresistance and decreased cell survival by attenuating G1 phase arrest in H69AR cells.<sup>58</sup> Recently, we found that another miRNA, miR-100, modulates chemosensitivity in H69 and H69AR cells by directly targeting HOXA1.<sup>59</sup> Furthermore, Li et al.<sup>60</sup> demonstrated that miR-137 is closely associated with SCLC multidrug resistance, and that interfering with miR-137 expression may decrease cisplatin resistance in drug-resistant H446/CDDP SCLC cells partly by the regulation of KIT expression.

Other miRNAs are also associated with drug resistance in SCLC, although the mechanisms remain unclear. MiRNA microarray analysis revealed that miR-92a-2\*, miR-147, and miR-574-5p are significantly associated with chemoresistance in SCLC tumor samples.<sup>52</sup> However, not all miRNAs affect response to chemotherapy. Lee et al.<sup>43</sup> found that the expression of a panel of seven miRNAs predicts neither the treatment response of SCLC patients nor the sensitivity of SCLC cell lines cisplatin or etoposide in vitro.

In addition to chemosensitivity, miRNAs are also related to radiotherapy responses. MiRNA microarray analysis showed higher expression of miR-324-5p in SCLC radio-resistant cells than in radiosensitive cells, which was validated by qRT-PCR analysis.<sup>61</sup> In addition, the effect of core miRNA biogenesis machinery proteins on the response of lung cancer cell lines to treatment with ionizing radiation was assessed.

Neither knockdown of these core proteins involved in miRNA biogenesis nor downregulation of the primary components of the RISC sensitized these cells to irradiation.<sup>62</sup>

Recent studies have shown that miRNA signature is a rising star that may provide new resolutions for SCLC. miRNAs are much more stable in blood and tissues, raising the exciting prospect that miRNAs might be used as diagnostic, prognostic, and therapeutic biomarkers for SCLC.

## LNCRNAS IN SCLC

LncRNAs, a heterogeneous group of ncRNAs that are more than 200 nt in length, comprise the largest portion of the mammalian noncoding transcriptome.<sup>6</sup> LncRNAs are known to mediate epigenetic modifications of DNA by recruiting chromatin-remodeling complexes to specific loci in either the nucleus or the cytoplasm. However, the signals that drive their localization have yet to be established.<sup>6,63–65</sup> The involvement of lncRNA in SCLC is not as well understood as that of miRNA. To date, only one study has reported a role of lncRNA in SCLC, and the specific alteration of the lncRNA was not elucidated. However, lncRNA has become an important topic of research, and its functional role in SCLC cannot be discounted.

Hox transcript antisense intergenic RNA (HOTAIR) is one of the few well-studied lncRNAs. HOTAIR is transcribed from the HoxC gene and binds as a scaffold to polycomb repressive complex 2 (PRC2) and the LSD1-CoREST-REST complex, leading to the catalysis of H3K27 trimethylation and the spontaneous demethylation of H3K4, as well as to the repression of HoxD genes transcription.<sup>66,67</sup> Ono et al. assessed HOTAIR expression in SCLC cell lines and surgical SCLC samples through qRT-PCR analysis. They found that HOTAIR is more strongly expressed in SCLC cell lines and tumor tissues than in normal cells and tissues. Knocking down HOTAIR in SBC-3 SCLC cells inhibits their proliferative activity and invasiveness in vitro. Moreover, high expression of HOTAIR is associated with an aggressive phenotype and poor prognosis, suggesting that HOTAIR could be used as a prognostic biomarker.<sup>34</sup>

**TABLE 4.** miRNAs and miRNA-Related Molecules as Therapeutic Biomarkers of SCLC

miRNA	Material	Main Finding	Mediator	Ref.
miR-134, miR-379, and miR-495	A SCLC cell line and its drug-resistant subline	Increase sensitivity to adriamycin, cisplatin and etoposide	miR-134 downregulates MRP1/ABCC1	58
miR-100	A SCLC cell line and its drug-resistant subline	Induces resistance to adriamycin, cisplatin and etoposide	Directly targets <i>HOXA1</i>	59
miR-137	A SCLC cell line and its drug-resistant subline	Sensitizes SCLC cells to cisplatin	Downregulates KIT	60
miR-92a-2*, miR-147, and miR-574-5p	FFPE samples	Positively correlates with chemoresistance	NS	52
miRNA panel: miR-21, miR-29b, miR-34a/b/c, miR-155, and let-7a	FFPE tumor samples and cell lines	No effect on the sensitivity to cisplatin or etoposide	NS	43
miR-324-5p	SCLC cell lines	upregulated in SCLC radio-resistant cells	NS	61
Core proteins involved in miRNA biogenesis	SCLC cell lines	No effect on the sensitivity to ionizing irradiation	NS	62

FFPE, formalin-fixed paraffin-embedded; miRNAs, micro RNAs; NS, not specified; SCLC, small-cell lung carcinoma.



## CELL-FREE RNA IN SCLC

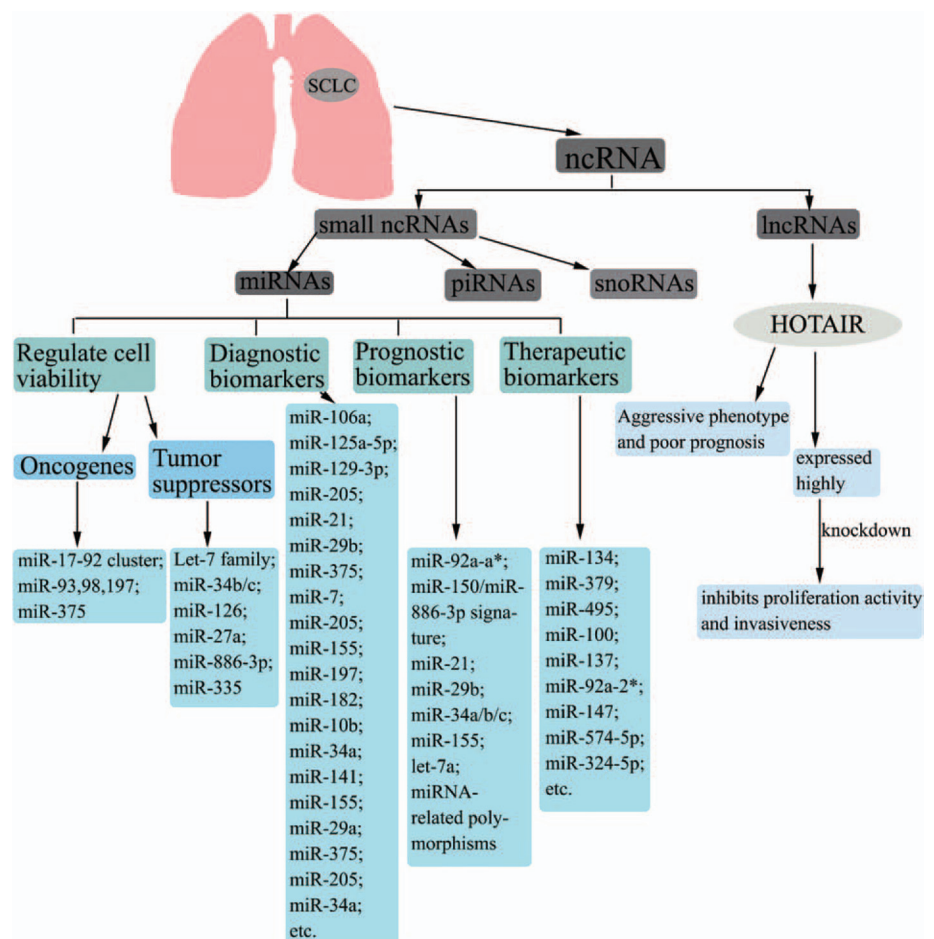
As recently as 10 years ago, Schmidt et al. investigated whether cell-free RNA could be detected in the supernatant of bronchial lavage fluid (BLF) and in serum. They successfully isolated and quantified cell-free RNA from serum and BLF supernatants of lung cancer patients, as well as patients with a benign lung disease. Furthermore, the cell-free RNA levels were higher in the BLF supernatant than in serum, and the RNA concentration in BLF from tumor patients was higher than in that from non-tumor patients, suggesting that the quantification of cell-free RNA levels in BLF supernatants and serum may represent a new diagnostic tool to differentiate between lung cancer patients and patients without tumors.<sup>68</sup>

## DISCUSSION

To date, a considerable number of studies have investigated the roles of ncRNAs in lung cancer. However, most of these studies have focused on NSCLC, whereas far fewer studies of SCLC, the most lethal lung cancer subtype. Because the same ncRNAs might be expressed in different lung cancer subtypes but perform distinct functions, the results of studies of NSCLC or other subtypes cannot necessarily be applied to SCLC. For example, miR-34a, which is activated by P53, plays a pivotal role in regulating NSCLC

cell survival. However, in SCLC, neither upregulation nor downregulation of miR-34a influence cell viability or drug sensitivity, and its activity is unrelated to P53 expression.<sup>39</sup> Therefore, it is imperative to devote more effort to the study of ncRNAs in the context of SCLC. Furthermore, the identification of ncRNAs that specifically regulate cell survival and drug responses in SCLC may facilitate the development of specific therapeutic regimens.

Among the ncRNAs implicated in SCLC (Fig. 1), miRNAs are the most extensively studied. Cytological evidence indicates that numerous miRNAs are involved in tumorigenesis and function as oncogenes or tumor suppressors in SCLC. Several miRNAs have been identified as diagnostic or prognostic biomarkers of SCLC with high sensitivity and specificity. However, there remains a lack of clinical studies identifying the miRNAs that are altered during the early phases of carcinogenesis. Such studies will be critical for making early diagnoses and improving the management of SCLC. Although previous studies reported that cancer-related miRNAs can be detected in serum before clinical diagnosis, the prediagnostic circulating miRNA expression profiles have yet to be elucidated for SCLC. This lack of understanding is partially due to the aggressiveness of SCLC and the difficulty in collecting sufficient and appropriate samples to study this disease during its initial stages.



**FIGURE 1.** Structure of noncoding RNAs in small-cell lung cancer.



Besides, accumulating evidence suggests a role for miRNAs in regulating the response of a tumor to therapy, which may guide the design of personalized, novel therapeutic strategies based on specific miRNA expression levels. However, it is necessary to consider the nonspecificity of miRNA targets, as well as effective and nontoxic delivery systems. The issues must be addressed before translating basic studies into clinical applications.

Currently, as miRNA expression profiling reaches a plateau, lncRNA has opened an intriguing new area of study in cancer biology. Despite our currently limited understanding of the role of lncRNAs, increasing studies highlight the exciting prospect that these molecules may serve as new biomarkers of and therapeutic targets for human cancers.<sup>6,8</sup> Significant effort is required to discover additional ncRNAs that perform crucial biological functions and to determine the mechanisms and roles of these molecules in SCLC.

In conclusion, the studies of ncRNAs not only provide a new insight into SCLC biology but also broaden the library of valuable biomarkers that can be used for SCLC diagnosis, prognosis, and therapy. Clinical applications of ncRNAs for SCLC are expected in the future, although there remain major hurdles to overcome.

## ACKNOWLEDGMENTS

*This work has been supported by the National Natural Science Foundation of China (81172241).*

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