Abstract: Lung cancer is the leading cause of cancer related deaths in the United States. It is estimated that in 2008 there were 215,000 new diagnoses of lung cancer and 163,000 deaths. Despite emerging technologies for potential early diagnosis and discovery of novel targeted therapies, the overall 5-year survival remains a disappointing 15%. Explanations for the poor survival include late presentation of disease, a lack of markers for early detection, and both phenotypic and genotypic heterogeneity within patients of similar histologic classification. To further understand this heterogeneity and thus complexity of lung cancer, investigators have applied various technologies including high throughput analysis of both the genome and proteome. Such approaches have been successful in identifying signatures that may clarify molecular differences in tumors, identify new targets, and improve prognostication. In the last decade, investigators have identified a new mode of gene regulation in the form of noncoding RNAs termed microRNAs (miRNAs or miRs). First determined to be of importance in larval development, microRNAs are approximately 19–22 nucleotide single stranded RNAs that regulate genes by either inducing mRNA degradation or inhibiting translation. MiRNAs have been implicated in several cellular processes including apoptosis, development, proliferation, and differentiation. By regulating hundreds of genes simultaneously, miRNAs have the capacity for regulation of biologic networks. Global alterations in miRNA expression in both solid organ and hematological malignancies suggest their importance in the pathogenesis of disease. To date, both in vivo and in vitro studies in lung cancer demonstrate a dysregulation of miRNA expression. Furthermore, investigators are beginning to identify individual targets and pathways of miRNAs relevant to lung tumorigenesis. Thus, miRNAs may identify critical targets and be important in the pathogenesis of lung cancer.

Key Words: microRNA, Epigenetics, Lung cancer, Biomarkers.

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miRNA biogenesis, targeting and regulation and offer a perspective on the emerging role of miRNAs in lung cancer.

**miRNA Biogenesis/Targeting**

Generation of mature miRNAs requires multiple steps (Figure 1). To our knowledge, miRNA biogenesis and targeting derives from invertebrate models; however, many of the proteins are evolutionary conserved throughout evolution and have similar functions in mammals. Initially, a large primary (pri)-miRNA approximately 100 nucleotides (nt) in length is transcribed by RNA polymerase II. Within the nucleus, the pri-miRNAs are bound to double-stranded RNA-binding domain (dsRBD) protein. In invertebrates, Pasha or Partner of Drosha, whereas in humans DiGeorge syndrome critical region gene 8 (DGC8) binds to the pri-miRNA. A complex containing DGC8, the pri-miRNA and an universal RNase III endonuclease, RNASEN, (Drosha in invertebrates) facilitates the processing of the pri-miRNA to smaller approximately 70 nt precursor miRNA (pre-miRNA) molecules. The pre-miRNAs form an imperfect stem loop structure. These are then transported to the cytoplasm by a protein complex consisting of Exportin 5 and Ran GTPase. Once in the cytoplasm, a premiRNA processing complex containing a second RNase III endonuclease, Dicer, and transactivating RNA-binding protein is formed to cleave the pre-miRNA to double stranded approximately 22 nt miRNA molecules. The miRNA is then separated into two single stranded molecules; the antisense strand is incorporated in the RNA-induced silencing complex (RISC) through its interaction with Argonaute (AGO) proteins, while the other strand is degraded. Ago proteins bind to the 3' end of the RNA and serve to align and stabilize the miRNA:mRNA duplex. It seems that there is a strong bias for the association of specific AGO proteins and small RNA subclasses. In plants, the asymmetry of the 5' end of the miRNA dictates which Ago protein associates with the small RNA molecule, whereas in invertebrates structural features of the pre-miRNA dictate the binding of specific AGO proteins.

Although simplistic in concept that the AGO proteins and additional components of RISC are important in the formation of miRNA:mRNA duplex, this alone is not sufficient to predict whether translational repression or degradation of mRNA targets occur. Conserved base-pairing between the 3' untranslated region (UTR) of the mRNA and the "seed sequence" located in the 5' end of the miRNA also have an important function. Through evaluation of the minimal requirements for miRNA:mRNA interactions, Brennecke et al. identified two categories of target sites; the 5'-dominant sites that require little to no support from the 3' miRNA end for function because of a high degree of complimentary and the 3'-compensatory sites that generally have sufficient complementarity to the 5' end of the miRNA. Interestingly, 3'-compensatory sites may not necessarily increase efficacy of interaction and function. There are four types of 5'-dominant interaction sites: 6mer, 7mer-m8, 7mer-A1, and 8mer. The 6-mer site matches perfectly to the seed sequence (2-7 nt), while the 7mer-m8 also matches these nucleotides with additional nucleotide in the 8th position. The 7mer-A1 matches the seed sequence which is augmented by an Ala in the first position of the miRNA. Lastly, the 8mer is a combination of the two 7mer sites and is comprised of a match seed sequence with the Ala in the first position and the matched 8th position of the miRNA. When the 8mer is the sole site present in the 3' UTR of an mRNA target, deregul-

**FIGURE 1.** miRNA Biogenesis: miRNA are transcribed as a large primary (pri)-miRNA which are approximately 100 nucleotides (nt) in length. Pri-miRNAs are bound to double-stranded RNA-binding domain (dsRBD) protein. In invertebrates, Pasha or Partner of Drosha, whereas in humans DiGeorge syndrome critical region gene 8 (DGC8) binds to the pri-miRNA. DGC8, the pri-miRNA and a universal RNase III endonuclease, RNASEN, (Drosha in invertebrates) facilitates the processing of the pri-miRNA to a smaller approximate 70 nt precursor miRNA (pre-miRNA) molecules. These are then transported to the cytoplasm by the nuclear transmembrane Ran GTP-dependent transported Exportin 5. Once in the cytoplasm, a premiRNA processing complex containing a second RNase III endonuclease, Dicer, and a transactivating response RNA-binding protein (TRBP) is formed to cleave the premiRNA to double stranded approximately 22 nt miRNA molecules. The miRNA is then separated into two single stranded molecules; the antisense strand is incorporated in the RNA-induced silencing complex (RISC) through its interaction with Argonaute (AGO) proteins, while the other strand is degraded. Ago proteins bind to the 3' end of the RNA and serve to align and stabilize the miRNA:mRNA duplex. The mature miRNA then targets either the 5' or 3' UTR based on complimentarity.
loration of the mRNA target is optimal. Notably, multiple sites in the 3’ UTR of the targeted mRNA are better than one. However, cooperation and spacing between these sites are also important factors. The number of mismatches between the miRNA and it target dictate the end result. If perfect complementarity exists, then generally the target is degraded through ubiquitination. Imperfect matches mainly predict inhibition of protein translation. In some cases, the perfect match of one miRNA is not sufficient to degrade its target but rather multiple miRNAs are required to bind the 3’ UTR for a full effect. Given the ubiquitous nature of miRNA/mRNA targeting, several prediction databases have been developed based on in silico analysis. Many of these score the free-energy generated between 5’ and 3’ base-pairing, consider phylogenetic conservation, and/or limit target identification by specific 5’–interaction sites. For example, TargetScan (http://www.targetscan.org/) considers matches in the 3’ UTR for only 7mer and 8mer interactions sites. The Sanger Database (http://microrna.sanger.ac.uk/targets/v5) uses the miRanda algorithm which weighs 5’ miRNA complementary in the seed sequence, thermodynamic calculations for the RNA duplex, and conservation across species. Recently, the miRDB database/MirTarget2 (http://mirdb.org/miRDB) was developed using a machine learning approach to analyze miRNA targets that are conserved and nonconserved across species. Comparison of these databases using a specific dataset indicated that the MirTarget2 performed similarly to TargetScan. These bioinformatic tools are very useful in providing cues on the function of a particular miRNA. The predicted targets generated from these or other databases should then be validated experimentally. Using luciferase assays, by cloning the 3’-UTR of target gene into a luciferase reporter gene downstream and cotransfection with miRNA to -UTR of target gene into a luciferase assays, by cloning the 3’/H11032 should then be validated experimentally. Using luciferase predicted targets generated from these or other databases providing cues on the function of a particular miRNA. The rather multiple miRNAs are required to bind the 3’/H11032 match of one miRNA is not sufficient to degrade its target but through ubiquitination. Imperfect matches mainly predict complimentarity exists, then generally the target is degraded (http://www.targetscan.org/) considers matches in the 3’ UTR for only 7mer and 8mer interactions sites. The Sanger Database (http://microrna.sanger.ac.uk/targets/v5) uses the miRanda algorithm which weighs 5’ miRNA complementary in the seed sequence, thermodynamic calculations for the RNA duplex, and conservation across species. Recently, the miRDB database/MirTarget2 (http://mirdb.org/miRDB) was developed using a machine learning approach to analyze miRNA targets that are conserved and nonconserved across species. Comparison of these databases using a specific dataset indicated that the MirTarget2 performed similarly to TargetScan. These bioinformatic tools are very useful in providing cues on the function of a particular miRNA. The predicted targets generated from these or other databases should then be validated experimentally. Using luciferase assays, by cloning the 3’-UTR of target gene into a luciferase reporter gene downstream and cotransfection with miRNA to observe the inhibition, is the standard method to confirm the direct interaction between miRNA and target genes. Western blotting or in situ hybridization can be used to confirm the interaction at protein expression level for miRNA and target protein localization.

There are several high-throughput approaches to quantify miRNAs in tissue samples but the gold standard is unclear. cDNA oligonucleotide arrays have become a standard global scale technique for miRNA profiling. Microarrays hybridize at a lower melting temperature compared with traditional cDNA arrays, therefore, probe design is critical. Polymerase chain reaction (PCR) arrays also provide another high-throughput approach to detect miRNA in a plate form. Cloning of all miRNAs is another technique mainly used to identify miRNAs and counting the number of sequenced miRNAs in a library also provides estimation of miRNA distribution in a specific tissue. Northern blotting using high percentage of urea-acrylamide gels was initially one of the primary modalities used to detect individual small RNAs. An advantage of Northern blotting is that it can simultaneously identify both observe precursor and mature miRNA. Real time PCR has been successfully used for detecting low copy number precursor and mature miRNA with high sensitivity and specificity. Stem-loop reverse transcription (RT-PCR) which provides primers and probe binding sites by incorporating elongated stem-loop RT primer can be used for accurate miRNA quantification. In situ hybridization can provide the quantification and the distribution of miRNA simultaneously (Figure 2). Alternatively, bead-based flow cytometric miRNA expression profiling methods combining xMAP with locked nucleic acids technology is just emerging. The principle in this approach is that biotin label total RNA is hybridized with locked nucleic acid-modified capture probes coupled to xMAP beads, then a streptavidin-phycocerythrin reporter molecule is for detection on the Luminex analyser.

**miRNA Regulation**

MiRNAs are often conserved across species. Not only do miRNAs serve as important regulators of gene expression, but their expression is also regulated by multiple factors. The genomic organization of miRNAs often dictates translational control. MiRNAs tend to be located in fragile chromosomal regions that are susceptible to translocations, microdeletions and amplifications. Allelic loss is a frequent genetic alteration in human lung cancers. Cytogenetic and molecular analyses have revealed chromosomal deletion on 3p, 8p, 9p, 11p, 13q, 17p, 18q, and 22q. In fact, several miRNAs located in these regions are currently being studied. Most miRNAs are located in genomic regions distant from annotated genes and encoded in independent transcription units. Approximately one quarter of human miRNAs are within introns of miRNAs in the same orientation as these miRNAs, suggesting that they are not transcribed from their own promoters but processed from introns. Other miRNAs are clustered in the genome and transcribed as a polycistronic primary transcript, suggesting their functional relationships. To date, 148 miRNAs have been identified within 51 clusters. Each of these clusters contains an upstream promoter region. For example, the miR-17–92 cluster encodes 7 miRNAs and is regulated by promoter elements for c-Myc, and E2F1-3 transcription factors. Dews et al. reported that the c-
Myc transcription factor up-regulates this cluster leading to the promotion of angiogenesis in solid tumors. This finding has led to the hypothesis that increased expression of c-Myc in malignancies would augment the expression of miRNAs. Surprisingly, over-expression of c-Myc in B-cell lymphomas leads to global decrease in miRNAs expression.36 It is predicted that c-Myc binds promoter elements to inhibit translation. Another important cluster in tumorigenesis is the let-7 family which is decreased in many malignancies.37 Despite these clusters being regulated by a promoter, not all the miRNAs contained within the cluster are similarly expressed, suggesting a second level of posttranscriptional regulation.38 During embryogenesis, the pri-miRNA for the let-7 cluster is abundant whereas the premiRNAs and mature miRNAs from this cluster are absent.39 Newman et al.40 identified highly conserved nucleotides in the loop regions of the primary transcript for let-7 cluster to which a Drosha inhibitor binds preventing the processing of the pri-miRNA.

Tumor suppressors within lung cancer are often susceptible to epigenetic silencing by methylation.41 The miR-29 family functionally targets DNA methyltransferases 3A and -3B, two key enzymes involved in DNA methylation, and often up-regulated in several malignancies including lung cancer.42 Reexpression of miR-29s in lung cancer cells restores the normal DNA methylation patterns and reduced tumor development.43 MiRNA expression profiling comparing DNA methylation deficient cells with normal HCT116 cells found that miR-124a underwent inactivation by CpG island hypermethylation in human tumors including lung cancers.44

Investigators have determined that polymorphisms within the 3’UTR of a target mRNA may affect miRNA functional targeting as well. MiRNAs may also be regulated by other miRNAs and environmental factors such as hypoxia and cigarette smoke. For example, miRNAs seem to be globally altered following cigarette smoke exposure.55,56 However, the mechanisms by which this occurs and biologic implications for lung tumorigenesis remain to be explored.

miRNA and Lung Cancer Cell Phenotype

Several miRNAs have been implicated in lung cancer by altering fundamental cellular processes (Table 1) thus making them attractive candidates as therapeutic targets. Both miR-221 and miR-222 are up-regulated in TNF-alpha related apoptosis-inducing ligand cell lines. Silencing of these miRNAs sensitized resistant cell lines to TRAIL agents.47 MiR-200c may enhance the initiation of an invasive phenotype through decreasing the expression of zinc finger transcription factor transcription factor translation factor 8, which is an inhibitor of E-cadherin that is a crucial event in epithelial-to-mesenchymal transition.48 Nasser et al. recently showed that the muscle enriched miR-1 inhibited both in vitro growth and survival in non-mall cell lung cancer (NSCLC) cell lines through functional targeting of several oncogenes including MET, Pim-1, FoxP1, and HDAC4. In addition, miR-1 sensitized cells to chemotherapeutic mediated apoptosis.49 MiR-21 is overexpressed in several solid malignancies including glioblastoma, breast cancer, and lung cancer. MiR-21 targets several tumor suppressors including PDCD4 and has anti-apoptotic properties.50 In lung cancer, increased tumor expression of miR-21 carried a poor overall survival but did not correlate with histologic features.51 MiR-126 alters both tumor growth and metastatic potential in breast cancer.52 A recent investigation suggested that miR-126 may alter lung cancer cell migratory and invasive capacity through functional targeting of Crk.53

### TABLE 1. MiRNA Implicated in Lung Cancer

<table>
<thead>
<tr>
<th>miRNA</th>
<th>Function</th>
<th>Target Gene</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>miR-1</td>
<td>Proliferation and apoptosis</td>
<td>MET, Pim-1, FoxP1, and HDAC4</td>
<td>50</td>
</tr>
<tr>
<td>miR-7</td>
<td></td>
<td>EGFR</td>
<td>72</td>
</tr>
<tr>
<td>Let-7</td>
<td>Proliferation</td>
<td>Cdk6, CDC25,NRAS, KRAS, HMGA2</td>
<td>61–64</td>
</tr>
<tr>
<td>miR-17–92</td>
<td>Angiogenesis and proliferation</td>
<td>Tsp1, CTGF, HIF1a</td>
<td>36</td>
</tr>
<tr>
<td>miR-21</td>
<td>Proliferation</td>
<td>PDCD4</td>
<td>52</td>
</tr>
<tr>
<td>miR-29</td>
<td>Demethylation</td>
<td>DNMT 3A and 3B</td>
<td>45</td>
</tr>
<tr>
<td>miR-126</td>
<td>Invasion</td>
<td>CRK</td>
<td>55</td>
</tr>
<tr>
<td>miR-128b</td>
<td></td>
<td>EGFR</td>
<td>71</td>
</tr>
<tr>
<td>miR-200c</td>
<td>Epithelial and mesenchymal transition</td>
<td>TCF 8</td>
<td>50</td>
</tr>
<tr>
<td>miR-221, miR-222</td>
<td>Apoptosis</td>
<td>p27 (kip1), Kit</td>
<td>49</td>
</tr>
</tbody>
</table>

miRNA, microRNA; EGFR, epidermal growth factor receptor; CTGF, connective tissue growth factor; TCF 8, transcription factor transcription factor 8.

miRNA Biogenesis and Lung Cancer

Global repression of miRNA maturation by targeting three key components of miRNA processing machinery decreased miRNA expression in steady-state miRNA levels and caused a pronounced transformed phenotype in lung cancer cells. Impaired processing through in vitro targeting of Drosha, DGCR8 and Dicer resulted in accelerated colony formation and growth in soft agar and in vivo tumor development.54 Karube et al.,55 demonstrated in a cohort of 67 lung cancer patients that reduced tumor expression of Dicer predicted a poor post resection prognosis independent of disease stage. Chiosea et al.56 reported a transient increase in Dicer expression in both atypical adenomatous hyperplasia and bronchoalveolar carcinoma followed by a steady decrease in expression with more advanced invasive adenocarcinoma. This would indicate that abrogation of global miRNA processing promotes tumorigenesis.

Let-7

Let-7 was the first miRNA reported to be aberrantly expressed in human lung cancer.57 The RAS 3’-UTR contains multiple let-7 complementary sites making it a functional target.58 HMGA2, another oncogene in a variety of tumors including lung cancers and mesenchymal tumors, is also a predicted target of let-7.59 Although both RAS and HMGA2 are let-7 targets, K-Ras seems to be stronger than HMGA2 in rescuing let-7g mediated tumor suppression.60 Overexpression of let-7 in the A549 cell line inhibited cell growth and reduced cell cycle progression and division.61 In a murine model of K-Ras(G12D) induced lung cancer let-7g signifi-
cantly reduced the number, size and volume of murine tumors and human non-small cell lung xenografts. In addition, intranasal let-7 administration reduced tumor formation in a similar K-Ras (G12D) model. These findings have translated to human application. For example, analysis of a small cohort of early in situ bronchioloalveolar carcinomas, well-differentiated adenocarcinomas and invasive adenocarcinomas revealed that let-7 is important to early occurrence in carcinogenesis but is not related to prognosis. Furthermore, these observations are supported clinically by the correlation between reduced let-7 expression in 143 resected lung cancer cases and poor clinical outcome.

**miR-17–92 Cluster**

Although Dicer affects miRNA maturation, the miRNA cluster miR-17–92 is important in lung development and homeostasis. MiR-17–92 cluster expression is high in embryonic development and steadily declines throughout development and into adulthood. The miR-17–92 cluster was first identified as a potential oncogene in B-cell lymphoma. This Myc-activated miRNA cluster can increase tumor vasculature and form larger, better-perfused tumors by functionally targeting antiangiogenic thrombospondin-1 (Tsp1) and connective tissue growth factor genes. In human lung cancers, particularly small cell carcinoma, miR-17–92 is also overexpressed and in vitro introduction enhanced cell proliferation. Antisense oligonucleotide mediated inhibition of both miR-17-5p and miR-20a induced apoptosis selectively in lung cancer cells over-expressing miR-17–92, suggesting oncogenic potential.

**miRNA and Epidermal Growth Factor**

The presence of abnormal levels of growth factor and its receptor can deregulate the normal cell growth process and initiate cancer. Epidermal growth factor receptor (EGFR) has increased expression in several malignancies and has become the focus of targeted therapeutics. EGFR induces cancer in three ways: overexpression of EGFR ligands, amplification of EGFR, and mutational activation of EGFR. Mutations in the EGFR tyrosine kinase in NSCLC can cause oncogenic transformation and change the sensitivity to tyrosine kinase inhibitors such as gefitinib. Two recent studies demonstrated that EGFR may be a functional target of miRNAs. MiRNA-128b, located on chromosome 3p which is frequently deleted in lung cancers, directly down-regulated EGFR. However, EGFR expression and mutation status did not correlate with survival outcome, suggesting a minor effect of this miRNA on EGFR regulation. Webster et al. determined in vitro that EGFR was a functional target of let-7. Let-7 also altered expression patterns of several other signaling effectors including protein kinase B (Akt) and extracellular signal regulated kinase 1/2 (ERK 1/2).

**Polymorphisms and miRNA Expression**

Single-nucleotide polymorphisms (SNPs), are major determinants of variations in disease susceptibility, medication response and toxicity. Thirty-seven SNPs have been identified in the p53 gene alone. The presence of polymorphisms in the 3’UTR of the target gene may also compromise the miRNA binding and regulation. In addition, SNPs in premiRNAs can alter miRNA processing, expression, and binding to target mRNA. The homozygous CC at SNP rs11614913 in miR-196a2 correlated with survival in individuals with NSCLC. This mutation enhanced the processing of premiRNA to its mature form and affected the miRNA binding ability to its target mRNA. Sequence regions within the 3’UTR of K-RAS which is a functional target of let-7 contain SNPs as well. Variant alleles within this SNP caused a 2.3-fold increased risk for NSCLC and in vitro suppression of let-7 mediated regulation of K-RAS.

**miRNAs Signatures and Lung Cancer**

Platforms for global miRNA detection have been used to identify their involvement in lung carcinogenesis. Yanaihara et al. compared miRNA patterns of expression in tumor versus adjacent uninvolved lung in 104 cases of lung cancer. They identified 43 differentially expressed miRNAs between lung tumors and adjacent uninvolved lung. In addition, 5 miRNAs (miR-155, 17-3p, let-7a-2, 145, and 21) predicted prognosis among patients with lung cancer.

Using an RT-PCR platform for miRNA analysis, Yu et al. recently showed that a 5 miRNA signature correlated with overall disease free survival in a cohort of 122 patients with NSCLC. In vitro allied studies identified 3 high-risk miRNAs (miR-137, miR-182*, miR-372) that increased the invasive capacity in cancer cells while the protective miR-221 decreased invasion.

Another novel approach to identifying miRNA signatures involves examining target genes of lung enriched miRNAs. In one such study, investigators identified a 17 gene signature of genes targeted by miR-34b/34c/449 that accurately distinguished adenocarcinomas from squamous cell and carried a 90% sensitivity in detecting lung cancer in the airway when combined with cytopathology.

**CONCLUSION**

Lung cancer remains a deadly disease that carries a poor 5-year prognosis. However, studies focused on elucidating the molecular heterogeneity are proving successful in identifying novel targets and subcategorizing clinical phenotypes. MiRNAs represent newly identified molecules that have the capacity of multiple gene and thus pathways regulation. To date, several studies have suggested that miRNAs demonstrate discrete patterns of expression in lung cancer and are likely critical to the pathogenesis of lung cancer. Therefore, the study of miRNAs in lung cancer may complement other more established platforms. We still have only a preliminary understanding of miRNAs and how targeting both in vitro and murine studies may be translated to human disease. Several questions remain: What genes regulate miRNAs? Which miRNAs regulate which genes? What are the mechanisms of miRNA function? Given the multitude of genes that may be targeted by a single miRNA, how can miRNAs be applied as targeted therapies in vivo while avoiding deleterious effects? With the development of techniques of miRNA cloning, more functional miRNAs related to lung cancer will be discovered. In addition, the identification of miRNAs by noninvasive means (bronchoalveolar...
lavage and peripheral blood) are being studied. MiRNA offer a promising platform that has the potential to eventually influence the prevention, diagnosis, prognosis, and therapy for lung cancer.

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