Review

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MicroRNA-21 versus microRNA-34: Lung cancer promoting and inhibitory microRNAs analysed in silico and in vitro and their clinical impact

Meik Kunz[1], Claudia Göttlich[2], Thorsten Walles[3], Sarah Nietzer[2], Gudrun Dandekar[2,4] and Thomas Dandekar[1]

Abstract

MicroRNAs are well-known strong RNA regulators modulating whole functional units in complex signaling networks. Regarding clinical application, they have potential as biomarkers for prognosis, diagnosis, and therapy. In this review, we focus on two microRNAs centrally involved in lung cancer progression. MicroRNA-21 promotes and microRNA-34 inhibits cancer progression. We elucidate here involved pathways and imbed these antagonistic microRNAs in a network of interactions, stressing their cancer microRNA biology, followed by experimental and bioinformatics analysis of such microRNAs and their targets. This background is then illuminated from a clinical perspective on microRNA-21 and microRNA-34 as general examples for the complex microRNA biology in lung cancer and its diagnostic value. Moreover, we discuss the immense potential that microRNAs such as microRNA-21 and microRNA-34 imply by their broad regulatory effects. These should be explored for novel therapeutic strategies in the clinic.

Keywords

MicroRNAs, lung cancer, therapeutic strategy, biomarker, bioinformatics, microRNA–target interaction

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Introduction

Lung cancer is a wide-spread cancer disease with high mortality claiming 1.59 million deaths, that is, 19.4% of total cancer-related deaths annually.[1,2] It is divided into two major types: non-small-cell lung cancer (NSCLC) and small-cell lung cancer (SCLC). NSCLC represents 85% of the clinically diagnosed lung cancer, in which adenocarcinoma (AC) and squamous cell carcinoma (SQ) are the major histologic sub-categories.[2–5] Diagnosis is currently based on imaging methods, for example, computer tomography (CT)- or PET-CT scan, supplemented by biopsy and bronchoscopy. Molecular biomarkers include the epidermal growth factor receptor (EGFR) and ALK-EML4 which both became part of clinical guidelines for targeted therapy applications.[6] However, other diagnostic tools such as cell-cycle regulators are not validated by randomized trials, and due to a lack of blood tests, they are presently not useful for clinical primary diagnosis.[7,8]

MicroRNAs (miRNAs) have emerged as promising diagnostic and therapeutic tools due to their association

[1]Functional Genomics and Systems Biology Group, Department of Bioinformatics, Biocenter, Würzburg, Germany
[2]Department of Tissue Engineering and Regenerative Medicine (TERM), University Hospital Würzburg, Würzburg, Germany
[3]Department of Cardiothoracic Surgery, University Hospital Magdeburg, Magdeburg, Germany
[4]Translational Center Würzburg "Regenerative Therapies in Oncology and Musculoskeletal Disease", Branch of the Fraunhofer Institute for Interfacial Engineering and Biotechnology (IGB), Würzburg, Germany

Corresponding authors:
Gudrun Dandekar, Department of Tissue Engineering and Regenerative Medicine (TERM), University Hospital Würzburg, Röntgenring 11, Würzburg 97074, Germany.
Email: gudrun.dandekar@uni-wuerzburg.de

Thomas Dandekar, Functional Genomics and Systems Biology Group, Department of Bioinformatics, Biocenter, Würzburg 97074, Germany.
Email: dandekar@biozentrum.uni-wuerzburg.de

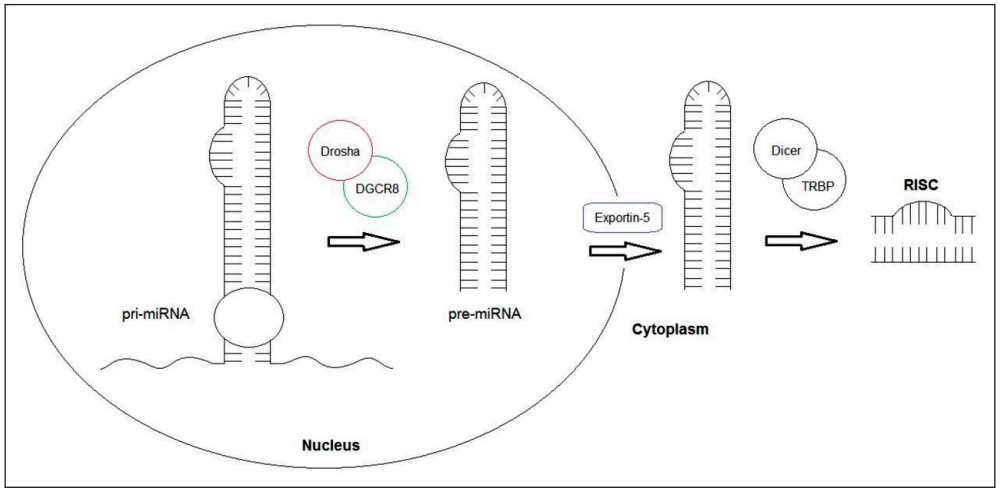

Figure 1. miRNA biogenesis. A long miRNA transcript is processed in the nucleus by RNA-Polymerase II (RNA-Pol II; not shown) as a primary-miRNA transcript (pri-miRNA; left one in nucleus). The pri-miRNA is cleaved by the RNase III enzyme Drosha (red circle) and its cofactor DGCR8 (green circle) into the ~70 nt long precursor-miRNA (pre-miRNA; right one in nucleus) which is further transported into the cytoplasm by the export protein Exportin-5 (blue square). There, the enzyme Dicer (black circle) and its RNA binding partner TRBP (black circle) cleave the pre-miRNA into the ~22 nt mature miRNA duplex which integrates into the multi-protein complex RISC to post-transcriptionally regulate gene expression (right one in cytoplasm; single guide strand, passenger strand is removed; here not shown).

with tumorigenesis, survival, and prognosis. They are highly conserved small non-coding RNAs (~22 nucleotides; nt) regulating genes associated with several biological functions and signaling pathways implying lung cancer pathogenesis and treatment resistance.[9,10] Interestingly, miRNAs show altered expression profiles in lung cancer. For instance, miRNA-21 and miRNA-34 display different expressions between NSCLC and SCLC,[11] whereas miRNA-205 and miRNA-375 are differentially expressed in AC and SQ.[5,12–15] Many studies highlight the diagnostic and therapeutic potential of miRNAs with the EGFR-regulated miRNA-21 and p53-regulated miRNA-34 as most promising candidates for a better clinical management of lung cancer.[5,7,8,16–18] An important role of selected miRNAs in lung cancer is derived from observations that low miRNA-21 levels suggest to be predictive for beneficial adjuvant chemotherapy after lung tumor resection,[19] and induction of miRNA-34 shows a preventive role in cancer initiation, as well as slows down progression in therapeutically resistant *KRAS*/*p53* mutant lung AC tumors.[20]

Notably, miRNA levels allow fast and systemic regulation of cell growth in culture as the imperfect seed region pairing of miRNAs regulates several targets and pathways. Even though delivery of miRNAs to cell culture is simple, this remains challenging in intact animal or human patient. Thus, targeting the tightly connected proteins modulated by these miRNAs is a powerful alternative. Moreover, miRNA expression profiles might be helpful as molecular biomarkers for early identification and categorization of NSCLC, especially for the detection of cancer disease in early stages.[7,21–23] Late diagnosis is one of the main reasons why survival stayed low over decades in this tumor entity (5-year survival: stage I = 60%–70%, stage II = only 33%).[2,24] Further clinical studies underline miRNAs' value as attractive diagnostic and therapeutic markers.[20,25–27]

In this review, starting from the observation that miRNA-21 acts as an oncogenic (oncomiR) and miRNA-34 as an onco-suppressor miRNA with an intimate connection to key cancer pathways, we elucidate miRNA interaction networks involved in this antagonism. In addition, we present how experimental and bioinformatic approaches can help in understanding the function of miRNAs in lung cancer and embed this analysis in general considerations. Subsequently, we discuss the high potential of both miRNAs as diagnostic markers and resulting new potential therapeutic targets as well as implied treatment strategies for lung cancer.

Biogenesis and miRNA biology

miRNAs are expressed by an RNA polymerase II as primary transcript (pri-miRNA; see Figure 1).[10,28,29] The nuclease enzyme Drosha (RNase III) and the cofactor DiGeorge syndrome critical region gene-8 (DGCR8) build then the ~70-nt-long stem-loop precursor-miRNA (pre-miRNA).[10,28,29] Exportin-5 (XPO5) transports it to cytoplasm where ribonuclease enzyme Dicer and RNA binding partner HIV-1 trans-activating responsive element (TRBP) process it to the mature miRNA (guide strand, miRNA)[28–31] which is bound to Argonaute protein AGO2 to degrade mRNA in the RISC complex.[10,28,31] Passenger miRNA (miRNA*) is removed or tissue-specific replaces miRNA in RISC when more stable with AGO2.[28,31,32] The miRNA seed region (~8 nt) binds mRNA 3′-untranslated region perfectly for translational inhibition or imperfectly

Table 1. Key deregulated miRNAs associated with miRNA-21 and miRNA-34 and their important targets in lung cancer. miRNA-21 and miRNA-34 are connected to EGFR- and p53-signaling and are associated with several cancer-deregulated miRNAs, thus forming a miRNA network (see Figure 2). The table lists the miRNAs, its altered expression in lung cancer (tumor) with important targets and references.

MiRNA	Tumor	Targets	References
MiRNA-21	Up	PDCD4, PTEN, Spry1, SMAD7	[29, 30, 56, 67–71]
MiRNA-155	Up	TCF4, APAF-1	[23, 72–77]
MiRNA-221/222	Up	p27 (Kip1), PTEN, CDKN1C, TIMP3, APAF-1	[27, 78–80]
MiRNA-130a	Down	c-MET	[78, 81, 82]
MiRNA-27a	Down	c-MET, EGFR, Sprouty2	[44, 49, 56, 83]
MiRNA-143	Down	HK2, CD44v3	[84–86]
MiRNA-145	Down	c-Myc, CDK4, EGFR, NUDT1, OCT4, MUC1	[78, 87–90]
Let-7	Down	KRAS, c-Myc, HMGA2	[67, 91–94]
MiRNA-34/449	Down	c-Myc, c-MET, E2F, Sirt1, RB, AXL, SNAIL1, CDC25A, HDAC1, HMGA2, SERPINE1	[20, 29, 67, 78, 95–103]

for down-regulation of multiple mRNAs[10,26,28,31] in differentiation and cancer-related signaling pathways.[10,28,29,33] 5′-UTR binding affects protein synthesis or post-transcriptional silencing in the nucleus.[5,10,28,31] Lin28 can inhibit processing of tumor-suppressor pri-miRNA lethal-7 (pri-let-7 miRNA).[5,28,34]

Lung cancer: key genes and signaling pathways

Lung cancer is often characterized by alterations of key genes such as *EGFR* and *p53* and downstream signaling pathways associated with tumor growth, differentiation, and survival.[2,4,24,35] These pathways are intimately associated with miRNAs.

EGFR is a receptor tyrosine kinase (RTK) overexpressed in 15%– 30% of NSCLC patients activating signaling pathways such as RAS/RAF/MEK/ERK and PI3K/AKT. This leads to tumor initiation, metastasis, angiogenesis and cell survival, reduced patient survival rate, and tumor resistance to chemotherapy.[2,35–37] The EGFR protein is already investigated as a therapeutic target in lung cancer.[38,39] For this, tyrosine kinase inhibitors (TKIs) such as gefitinib (Iressa®), erlotinib (Tarceva®), or afatinib (Giotrif®) are used in clinic. These are standard therapies in patients with *EGFR* mutations and used for additional treatment or after failure of the initial treatment in patients developing resistance to other chemotherapeutics.[40–43] However, after some time of TKI treatment, resistance occurs. For instance, the EGFR T790M mutation or *c-MET* gene amplification are important mechanisms for primary and acquired resistance to EGFR-TKI treatment in lung cancer.[44–46] Moreover, co-mutations in *KRAS* and *HER2* (*ERBB2*) are reported.[25,40,47,48] Notably, EGFR and also c-MET are known to regulate miRNA expression to influence metastasis and gefitinib resistance of NSCLC.[44,49]

The tumor-suppressor p53 is found to be deregulated in 50%–70% of NSCLC patients and correlate with reduced apoptosis and higher cancer survival rate.[2,24,35,50] Over 50% of lung cancers show mutation in the p53 gene on chromosome 17p13.1, however, p53 mutations are obtained more commonly in smokers than in never smokers.[4,51,52] p53 mediates a complex signaling network and regulates several downstream genes such as *p21, BAX*, and *PTEN* which influence cell division, cell death, and genomic integrity.[2,24,35,50,53] p53 also mediates miRNAs to regulate the downstream p53 pathway.[53,54] Most prominently, p53 functions in a feedback loop with miRNA-34 and regulates c-MET which promotes tumorigenesis.[53,55,56] Targeting the p53 tumor-suppressor pathway is an interesting strategy for lung cancer treatment. For instance, treatment with small molecules such as PRIMA-1MET (p53 reactivator)[57] and heat shock protein 90 inhibitor Ganetespib (depletion of mutant p53)[58,59] but also an adenovirus gene therapy (Ad-p53) combined with chemotherapeutic drug cisplatin[60] or radiotherapy[61] are under clinical trials.[62,63]

For additional information including clinical implications about other genes which are deregulated in lung cancer, for example, *KRAS, HER2* (*ERBB2*), and *c-MET*, see the studies of Herbst et al.,[2] Sun et al.,[4] Minna et al.,[24] Sekido et al.,[35] and Fong et al.[50]

Key miRNAs in lung cancer form a network around EGFR and p53

MiRNA signatures associated with the above-described pathways become apparent from high-throughput experiments.[64–66] Several miRNA signatures correlated with patient relapse and shortened survival in NSCLC.[22,23] As several miRNAs are correlated to lung cancer, we highlight in the following section a clinical network of selected key miRNAs based on their link to EGFR and p53 signaling. We review their importance in literature, focusing on the well-studied miRNA-21 and miRNA-34 and their connected miRNAs and signaling pathways as promising targets in NSCLC (see Table 1 and Figure 2). In the following, we only report few targets for the connected miRNAs, additional targets and references are listed in Table 1.

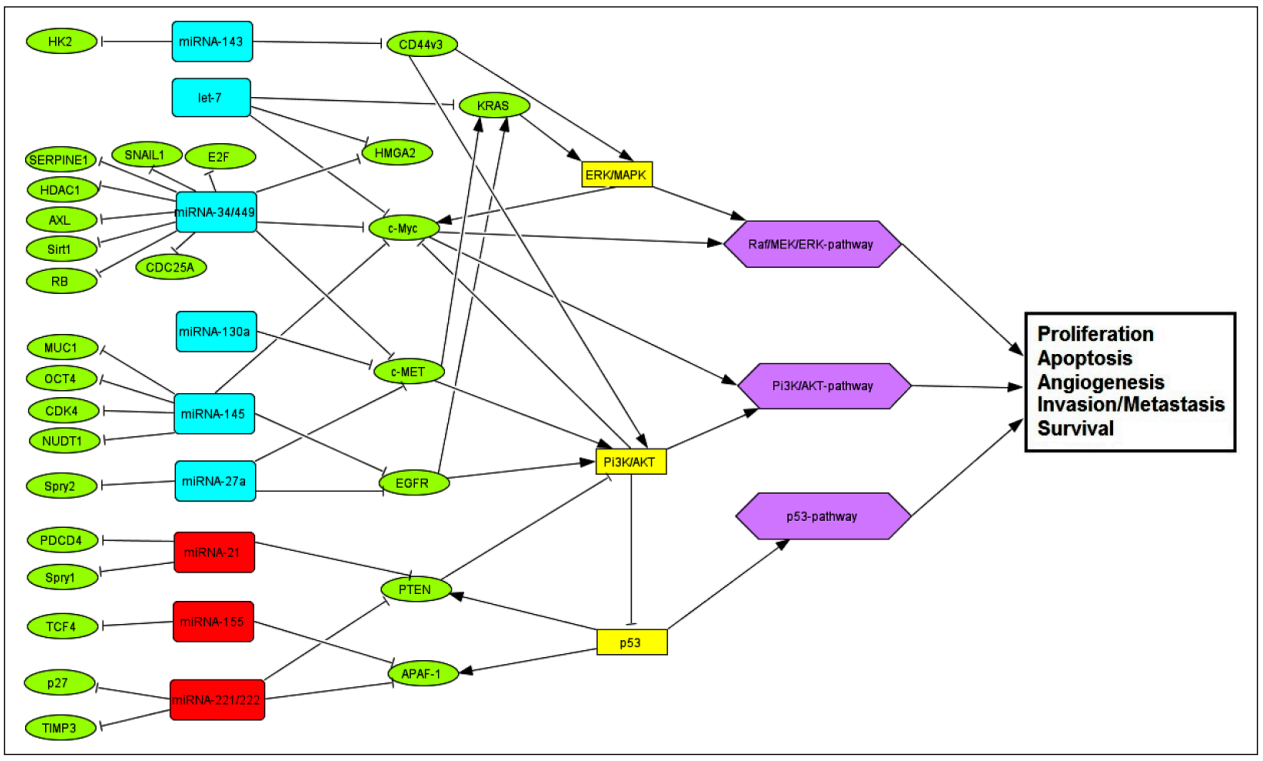

Figure 2. Connection of oncomiRs (red) and tumor-suppressor (blue) miRNAs to EGFR and p53 in lung cancer. Simplified network topology of key miRNAs (overexpressed in red, down-regulated in cyan) and their targets (in light green) implicated in lung cancer (see Table 1). Important EGFR- and p53-signaling pathways in lung cancer are represented as violet hexagons (e.g. Raf/MEK/ERK and p53), downstream nodes connecting miRNA targets as yellow rectangles (e.g. ERK/MAPK). Arrows represent activation, blunted arrows inhibition. miRNAs deregulation is associated with characteristic tumorigenic functions and lung cancer pathogenesis (black box, e.g. proliferation and angiogenesis).

Role of the oncomiR miRNA-21 as EGFR-regulated miRNA in lung cancer

The miRNA-21 is induced by EGFR and regulates the downstream EGFR signaling pathway, thus it can function as an indicator of cellular growth, tumor transformation, and progression.[19] It is overexpressed in lung cancer and known to be associated with cell-cycle and cancer progression, advanced tumor stage, poor survival rates, and chemotherapy sensitivity in NSCLC.[78,104–106] MiRNA-21 targets several genes among which Programmed Cell Death 4 (PDCD4) and PTEN indicate a pivotal role in regulating RAS/MEK/ERK and RAS/PI3K/AKT signaling pathways.[29,56,67,68] PTEN is a negative regulator of PI3K/AKT-signaling, playing a role in cell migration, cell-cycle progression, and survival, whereas PDCD4 activates p21 which regulates apoptosis by inhibition of CDK.[69,70,107] Studies have shown that inhibition of miRNA-21 expression through Foxo3a can induce apoptosis in NSCLC,[108] whereas post-transcriptional activation of miRNA-21 by collagen type I (Col-1) is associated with loss of polarity in epithelial cells and progression of tumors.[109]

Notably, miRNA-21 shows co-expression with additional miRNAs such as the cancer pathway promoting miRNA-155 and miRNA-221/222 and the tumor-suppressor miRNA-143/145.[110–112] Similarly, studies reported that miRNA-221/222 are co-expressed with miRNA-21 and miRNA-155 in resected stage I NSCLC patient tissues, highlighting a potential predictive role of these miRNAs in NSCLC.[113] Moreover, both miRNA-155 and miRNA-21 are up-regulated in a therapeutically resistant *KRAS*/*p53* lung cancer mouse which were deficient in p53-regulated miRNAs such as miRNA-34.[20]

The miRNA-221/222 are both overexpressed in NSCLC and activated by a c-MET-dependent c-Jun stimulation, as well as EGFR.[27,56,78,79] They inhibit p27, CDKN1C, PTEN, and APAF-1 and are thus linked to lung tumorigenesis, invasion, and apoptosis.[27,78–80] The miRNA-155 is overexpressed in lung cancer and regulates cell division, immunity, and angiogenesis.[23,72–74] As it was shown that the miRNA-155 is up-regulated in EGFR/KRAS-negative cells and associated with bad prognosis, new therapeutic strategies focus on this miRNA.[75,114–116] For example, inhibition of miRNA-155 leads to reduction in tumor growth in EGFR mutant NSCLC tumors and enhances the sensitivity to cisplatin treatment as it regulates apoptotic peptidase activating factor 1 (APAF-1).[76]

Down-regulation of several other key miRNAs influences lung cancer progression and are connected to our miRNA-21 expression network. For instance, miRNA-130a

is weakly expressed in NSCLC cells, implying treatment resistance and cell migration.[81,82] MiRNA-130a modulates miRNA-221/222 expression[78,82] and is inversely correlated with c-MET: it is overexpressed in gefitinib-sensitive NSCLC cells, while down-regulated in gefitinib-resistant NSCLC cells.[81] Moreover, it was also demonstrated that miRNA-130a reverses gefitinib resistance in NSCLC cells through direct targeting of c-MET.[81]

Similarly, the onco-suppressor miRNA-27a is linked to EGFR and c-MET through direct and indirect targeting of c-MET, EGFR, and Sprouty2. Thus, the lack of miRNA-27a results in uncontrolled proliferation and tumor progression.[44,49,56,83] Moreover, miRNA-27a is associated with early NSCLC stages,[7,56] whereas Wang et al.[44] identified that miRNA-21 and miRNA-27a (and miRNA-218) are associated with primary resistance to EGFR-TKI in NSCLC patients.

The miRNA-143/145 is down-regulated in lung cancer which triggers cell growth and carcinogenesis.[110,117–119] MiRNA-145 directly targets EGFR, c-Myc, and OCT4 to regulate cell proliferation and cell-cycle arrest,[78,87–89] whereas inhibition of metastasis and invasion results from targeting MUC1.[90] However, the miRNA-143 inhibits migration and invasion in NSCLC through targeting the lung stem cell marker CD44v3[84,85] and Hexokinase 2 (HK2).[86] However, compared to miRNA-145, less is known about the role of miRNA-143 in lung tumorigenesis.[78]

Onco-suppressor miRNA-34 is a non-EGFR-regulated miRNA connected to other miRNAs

Beside *EGFR, p53* is frequently mutated in lung cancer. The miRNA-34 family (miRNA-34a, -34b, and -34c) functions as a tumor suppressor showing a reduced expression with poor prognosis in NSCLC patients.[20,29] MiRNA-34 family members are important downstream targets of p53 signaling, and p53 binding sites are also present in the miRNA-34 promoter.[95,96,120] MiRNA-34 targets c-Myc and E2F, and also inhibits c-MET activation and invasion.[29,67,97,98] There is an inverse tumorigenesis effect and induction of apoptosis, as well as cell-cycle arrest, through restoration of miRNA-34 expression in lung cancer cells.[20,120,121] Moreover, several studies report that expression of miRNA-34 stops the cell cycle and also reverses in lung cancer the epithelial to mesenchymal transition (EMT) through targeting RB, AXL, and SNAIL1.[99–101]

Interestingly, the miRNA-449 family (miRNA-449a, -449b, and -449c) possesses the same seed region as the miRNA-34 family and regulates the same targets, but are less well investigated.[29,78] Both miRNA-34 and miRNA-449 feedback on p53 and E2F transcription factors.[102] To close the feedback loop, miRNA-34 is activated by p53, while miRNA-449 is activated by E2F. Similar to miRNA-34, miRNA-449 directly inhibits E2F and up-regulates p53 by targeting deacetylase gene Sirt1.[78,102] In addition, other known targets of both miRNAs include CDC25A, HDAC1, HMGA2, and SERPINE1, thus regulating cell-cycle arrest, apoptosis, and migration/invasion.[78,103]

Furthermore, miRNA-34 shows association with the miRNA let-7. This tumor-suppressor miRNA family regulates cell proliferation and tumor development, in which low expression levels are associated with poor survival rate in lung tumors.[67,91,122] Let-7 targets KRAS, c-Myc, and HMGA2 signaling.[67,91–93] Studies already focus on therapeutic modulation: ectopic expression of let-7 induces cell death in lung cancer,[122,123] whereas combined treatment of miRNA-34 and let-7 inhibits tumor growth in aggressive *KRAS*/*p53* mutant NSCLC mouse models and cells.[94] In addition, let-7b and miRNA-34a also enhance the anti-proliferative effect of erlotinib, highlighting that both miRNAs influence tumor signaling pathways which are not suppressed by EGFR, indicating an effective therapeutic strategy to overcome EGFR-TKI resistance in lung cancer.[25]

MiRNA target identification

How can the above networks involving miRNA-21 and miRNA-34 be identified? We will review in the following section the experimental and bioinformatic methods for miRNA target identification. Our current understanding of miRNAs relies on a combination of extensive experimental methods (details in Hausser and Zavolan[124] and Thomas et al.[125]) and theoretical approaches (details in Kunz et al.[5,10]).

Experimental miRNA target identification

Initially, expression profiling of mRNAs after overexpression or knockdown of miRNAs was the method of choice. Following miRNA transfection, several techniques exist for measuring effects ranging from transcriptome analysis to proteomic-based approaches. Changes in the mRNA expression profile can be examined either by gene expression microarrays or RNA sequencing.[126] Concurrently, effects of miRNAs on protein expression can be detected using stable isotope labeling with amino acids in cell culture (SILAC)[127] which is a spectrometry-based method. Protein and mRNA expression analysis methods cannot distinguish direct and indirect miRNA targets. Direct miRNA targets are detected by biochemical isolation of the miRISC and the associated mRNAs by immunoprecipitation of the native RISC complex or components such as AGO proteins with microarrays or RNA sequencing.[128] To identify individual target sites down to single nucleotide resolutions, there exists the method of crosslinking and immunoprecipitation (CLIP). CLIP uses ultraviolet (UV) light to crosslink nucleic acids to miRISC components. The complex is immunoprecipitated, unbound RNA is digested, so that only the miRISC-protected RNA fragments are preserved and the AGO-associated miRNA recognition elements are identified using high-throughput

sequencing. The efficiency of miRNA target capture is further increased in photoactivatable ribonucleoside-enhanced CLIP (PAR-CLIP),[129] whereby photoreactive 4-thiouridine is incorporated into RNAs before the crosslinking is achieved by 365-nm UV-A light. In HITS-CLIP,[130] 254-nm UV-C light is used for crosslinking. Disadvantages of CLIP-based methods as discussed by Hausser and Zavolan[124] include the fact that low-abundance targets may not be captured and it is not clear that miRNA–target interaction is relevant for the phenotype. Furthermore, the procedure requires highly specific antibodies for the precipitation.

Bioinformatics and prediction algorithms

General databases such as RFAM (RNA family database) collect miRNAs including sequence and structure alignments among species, as well as seed region information, whereas the Gene Expression Omnibus (GEO) database consists of experimental datasets and specific regulatory effects. As miRNAs target mRNAs with their short about 8-nt conserved seed region, potential base pairing between a miRNA and a mRNA motif can be predicted applying bioinformatics. Computational miRNA target prediction is extensively reviewed in previous studies.[5,10,28,131,132]

Prediction algorithms combine miRNA seed region similarity, sequence conservation with structure, folding energy, and target site accessibility.[10,28,131,133] The popular TargetScan is based on a thermodynamically RNA duplex interaction modeling with comparative seed region analysis.[134] miRanda,[135] PicTar,[136] and DIANA-microT[137] algorithms include seed region mismatches and free-folding energy. The PITA algorithm even includes structural target site accessibility for seed matching.[138]

Drawbacks and limitations are the high false-positive prediction rate and low mRNA target overlap, as algorithms use different parameters, for example, seed matching and free energy.[10] Most algorithms are not based on experimental data or, furthermore, miRNAs often show tissue-specific expression.[131,133,139,140] However, they are quite helpful for pre-selection of targets for experimental validation. It was shown that prediction algorithms based on stringent seed region matching show the highest sensitivity and specificity compared to experimentally validated miRNA targets.[10,28,131,139] TargetScan (perfect complementarity) and PicTar have the highest sensitivity and overlap of predicted and experimentally validated targets, whereas the DIANA-microT algorithm shows low sensitivity. miRanda algorithm shows similar sensitivity to TargetScan and PicTar but has much higher number of total target predictions.[131] Thus, miRanda, TargetScan, and PicTar algorithms were all combined for the three miRNAs, miRNA-34b/34c/449, to reveal a diagnostic signature of 17 target genes that shows a high sensitivity for predicting lung cancer and distinguishing between NSCLC subtypes AC and SQ from microarray data sets.[141] Similarly, analysis using these algorithms identified conserved binding sites among different species for the miRNA-21 and the tumor-suppressor PDCD4 in colorectal and breast cancer cells,[142,143] as well as NUAK family kinase 1 (known oncogene in NSCLC) as new target of miRNA-96 in pancreatic cancer.[144] This was further validated by experiments. Moreover, computational prediction analysis using the TargetScan algorithm found that miRNA-21 targets SMAD7 which was experimentally validated in NSCLC.[71]

Thus, to avoid over-predictions and achieve the best biological targets, different prediction algorithms should be combined with different experiments such as tissue-specific gene expression microarray and proteome analysis.[5,10,28,131]

Clinical implications of the miRNA-21 and miRNA-34 network for lung cancer diagnosis and therapeutic strategies

Lung cancer is often detected late with poor treatment prognosis. MiRNAs as non-invasive biomarkers could complement CT for lung cancer diagnosis and monitoring to improve this situation.[16,111,145,146] Interestingly, recent studies report that miRNA expression methods are more accurate in defining cancer subtypes than protein-coding gene profiling.[22,116,147,148] Moreover, miRNA expression signatures correlate with lung cancer tumor stages, progression, and treatment response.[21–23,111] In a more general approach with several tumor entities, miRNA-21 was identified as a biomarker for poor prognosis including lung, breast, stomach, prostate, colon, and pancreatic tumors underlining its central role in cancer.[116]

Clinical diagnostics

MiRNA profiles revealed that besides miRNA-21, miRNA-155, miRNA-17, miRNA-143/145, miRNA-221/222, and let-7a are also strongly deregulated in lung cancer. This deregulation is linked to patient prognosis and survival, indicating a role of miRNAs as potential biomarkers for early lung cancer diagnosis.[110–112,118] Up-regulated miRNA-17, miRNA-21, and miRNA-155 demonstrated oncogenic potential,[23,111,116,122] whereas miRNA-155 shows high correlation with the angiogenic marker FGF2, as well as nodal metastasis.[115] Altered let-7a and miRNA-221 levels imply cancer relapse and bad prognosis for NSCLC patients.[22] Moreover, Izzotti et al.[118] demonstrated that altered expression of miRNAs occur in early events of healthy tissues as they found a deregulation of let-7, miRNA-34, miRNA-145, and miRNA-222 in healthy rat lungs exposed to cigarette smoke. MiRNA signatures in lung cancer have already been evaluated

by bioinformatic meta-analyses, highlighting that such analyses are very useful to identify best miRNA markers. For example, Vosa et al.[112] used a rank aggregation method to robustly identify seven up-regulated and eight down-regulated miRNAs in lung cancer (again miRNA-21, miRNA-143, and miRNA-145). In a retrospective analysis, a benefit from chemotherapy in addition to current clinical guidelines[149] could be identified by high levels of miRNA-21 for high-risk patients after TNM stage I resection.[19] Furthermore, by using two different normalization strategies, Charkiewicz et al.[150] determined that miRNA-21 and miRNA-205 expression allow NSCLC subtype classification and patient selection for targeted therapy. Nevertheless, monitoring miRNA-21 expression to detect early lung cancer compared to healthy controls may be challenging.[151] Further miRNAs which are currently under investigation as biomarkers and diagnosis tools include miRNA-486 and miRNA-150 as blood markers,[152] miRNA-486-5p and miRNA-30a-5p as tissue markers,[153] and extra cellular miRNA-198 as biomarkers warning against AC-associated malignant pleural effusion.[154]

However, clinical studies have identified diagnostic miRNA markers either in lung tumor tissue or blood samples. It should be noted that tumor tissue analysis results vary depending on tissue preparation: studies conducting analysis from frozen tissues reveal that high miRNA-21 expression correlates with low patient survival,[110,155] whereas in FFPE (formalin-fixed paraffin embedded) samples, this correlation could not be confirmed.[113,156] This points out the challenge of technical accuracy necessary for clinically reliable miRNA profiling. For this reason, it was recommended to evaluate multiple slices for standardization and normalization. Furthermore, oncomiRs should be used as controls.[157,158] Importantly, sample preparation should be carried out in a lab where standard operating procedures (SOPs) are well established.

MiRNA detection from blood or biological fluids is a new technique and monitors patients non-invasively. For example, Sozzi and colleagues demonstrated in 2014 in their MILD trial (Multicenter Italian Lung Detection) that a miRNA signature classifier (*MSC*) from patients' blood samples has a predictive, diagnostic as well as prognostic value that reduces false-positive rates of low-dose computer tomography (LDCT) when used as an additional diagnostic tool.[16] Screening with false-double positive results could reduce false-positive rates to an impressive 3.7% compared to 19.7% for LDCT alone.[16] It has been speculated that the *MSC* derives from the tumor environment which is related to the tumor aggressiveness and hence distinguishes between lung cancers and benign nodules that are both detected by LDCT.[16] Low *MSC* of 24 circulating miRNAs predicted the absence of lung cancer mortality in the first 3 years correctly.[159] Similarly, a signature of 34 different regulated miRNAs from the COSMOS trial (Continuous Observation of Smoking Subjects) detected with 80% accuracy high-risk asymptomatic participants, representing a useful blood test for early lung cancer detection as it was shown in a large-scale clinical validation study.[146,160]

From the technical point of view, it is critical to avoid hemolysis of red blood cells or platelets as they also contain miRNAs such as miRNA-451, miRNA-486-5p, miRNA-16, and miRNA-92a and here, the levels and changes are completely different from serum. Moreover, different miRNA ratios may also reflect different blood cell counts.[161] Regarding further technical challenges for miRNA detection in blood samples, it is important to choose the correct housekeeping genes for real-time quantitative polymerase chain reaction (qRT-PCR) evaluation: they should always be present with robust and good expression levels in all samples.[162]

Therapeutic implications

MiRNA can beneficially affect multiple targets and pathways in cancer tumorigenesis and resistance development.[25,27,94] Several studies illustrate that miRNAs have promise in treatment of lung cancer, for instance, breaking resistance of TKIs erlotinib or gefitinib by modulating miRNA let-7 and miRNA-34 regulation of EGFR and p53 pathways in NSCLC cells.[20,25,94,97] Both miRNAs are down-regulated by c-Myc oncogene which explains their low expression and reduced anti-proliferative and pro-apoptotic function in cancer cells.[163] Excitingly, chemopreventive agents can modulate smoke-induced miRNAs and connected proteome.[164] Thus, Delta-tocotrienol down-regulates the Notch-1 pathway as it induces miRNA-34a in NSCLC cells.[17] Several studies confirmed that miRNA-21 overexpression is involved in EGFR-TKI resistance in NSCLC. Experimental modulation of the miRNA-21 expression level in NSCLC tissues and cells show first promising therapeutic results through regulation of PTEN, PDCD4, and the PI3K/AKT signaling pathways.[18,165]

Moreover, targeting the miRNA-dependent interaction network in NSCLC patients which do not respond any more to gefitinib/erlotinib represents a promising alternative therapeutic strategy. Thus, c-MET receptors often involved in EGFR-TKI therapy resistance are currently under investigation for their application in the clinic.[27,56] However, most miRNA studies focus on cell biology and are still far away from direct therapeutic application. As mentioned before, expression levels of miRNA-21, miRNA-221/222, miRNA-34a, and miRNA-30b/c are altered in gefitinib-resistant lung cancer cells due to EGFR and c-MET alterations.[27,97] Consequently, experiments considering these miRNAs have been carried out: modulation of miRNA-221/222 and miRNA-30b/c expression levels in vitro and in vivo reverses gefitinib resistance in NSCLC,[27] whereas modulation of miRNA-34a rescues

gefitinib and miRNA-130a modulation reduces the migratory capacity and overcomes gefitinib treatment failure in NSCLC cells.[82,97]

Conclusion

Diagnosis and treatment of lung cancer is challenging. However, observing miRNA-21 and miRNA-34 family levels and connected networks provide important new handles. Combined bioinformatics and experimental approaches exploit high-throughput RNA sequencing and omics data for a detailed understanding of the complex interactions of these and other miRNAs with their cognate mRNA and protein networks in lung cancer. miRNAs can regulate several targets and pathways. Thus, the modulation of specific miRNAs such as miRNA-21 and miRNA-34 promises new diagnostic options and novel therapeutic interventions. However, miRNA signatures and miRNA-inspired therapies are currently explored in first clinical trials. They have a high potential to improve significantly the diagnosis and treatment of lung cancer, but still hampered by various specificity considerations. In this context, we hope our overview stimulates future research as a basis for an improved clinical management of lung cancer.

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