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MicroRNAs as a drug resistance mechanism to targeted therapies in *EGFR*-mutated NSCLC: Current implications and future directions

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ABSTRACT

The introduction of EGFR-tyrosine kinase inhibitors (TKIs) has revolutionized the treatment and prognosis of non-small cell lung cancer (NSCLC) patients harboring epidermal growth factor receptor (*EGFR*) mutations. However, these patients display disease progression driven by the onset of acquired mechanisms of drug resistance that limit the efficacy of EGFR-TKI to no longer than one year. Moreover, a small fraction of *EGFR*-mutated NSCLC patients does not benefit from this targeted treatment due to primary (*i.e.* intrinsic) mechanisms of resistance that preexist prior to TKI drug treatment. Research efforts are focusing on deciphering the distinct molecular mechanisms underlying drug resistance, which should prompt the development of novel antitumor agents that surmount such chemoresistance modalities.

The capability of microRNAs (miRNAs) to regulate the expression of many oncogenic pathways and their central role in lung cancer progression, provided new directions for research on prognostic biomarkers, as well as innovative tools for predicting patients' response to systemic therapies. Recent evidence suggests that modulation of key miRNAs may also reverse oncogenic signaling pathways, and potentiate the cytotoxic effect of anti-cancer therapies.

In this review, we focus on the putative emerging role of miRNAs in modulating drug resistance to EGFR-TKI treatment in *EGFR*-mutated NSCLC. Moreover, we discuss the current implications of miRNAs analyses in the clinical setting, using both tissue and liquid biopsies, as well as the future potential use of miRNA-based therapies in overcoming resistance to targeted agents like TKIs.

1. Introduction

Lung cancer is the second most common malignancy constituting the leading cause of cancer-related deaths worldwide, accounting for > 25% of all cancer deaths (Siegel et al., 2018). Even though lung cancer incidence rates are decreasing in the last years, mainly because of changes in smoking habits, the outcome of lung cancer patients remains dismal (Siegel et al., 2018). Non-small cell lung cancer (NSCLC) accounts for approximately 85% of lung cancer cases, whereas small-cell lung cancers, which embody a distinct biological entity, constitute the remaining cases (Howlader et al., 2016). Concerning NSCLC, enormous improvements in diagnostic and therapeutic approaches have been made in the past decades. The deeper understanding of NSCLC biology and identification of specific driver mutations have led to the development of targeted treatments that supplanted standard platinum-based chemotherapy in the subset of so-called 'oncogene-addicted' NSCLC patients (Network et al., 2014; Ding et al., 2008). The mutations of the *Epidermal Growth Factor Receptor* (*EGFR*) gene represent the most common class of targetable genetic aberrations; they can be found in approximately 10–16% of NSCLC patients from Western countries, and this percentage is even higher (up to 50%) in Asian patients (Rosell et al., 2009; Shi et al., 2014). *EGFR* mutations usually cluster within *EGFR* exons 18–21, thus impairing the kinase domain of the receptor, that becomes constitutively active, leading to cell proliferation and survival regardless of the presence of the extracellular ligand (Sordella et al., 2004). In-frame deletions in exon 19 and exon 21, and the L858R point mutation are the most frequent mutations (> 90%), which confer increased sensitivity to tyrosine kinase inhibitors (TKIs) (Dearden et al.,

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2013). To date, three generations of TKIs have been developed and tested in the clinical setting: first-generation (gefitinib, erlotinib, and icotinib), second-generation irreversible inhibitors (afatinib and dacomitinib) and highly selective third-generation inhibitors (osimertinib and rociletinib) (Recondo et al., 2018). The introduction of first- and second-generation TKIs for the management of *EGFR*-driven NSCLC dramatically changed the natural course of the disease. Frontline treatment of patients with these targeted drugs experiences a gain of 3.4–6.9 months in terms of progression-free survival (PFS) over standard chemotherapy (Recondo et al., 2018). However, despite an initial high overall response rate (56–85%), most tumors acquire molecular mechanisms to escape this pathway blocked, resulting in an inexorable progression (Recondo et al., 2018). Over 50% cases of resistance to first- and second-generation TKIs are caused by the onset of the 'gatekeeper' mutation T790 M, which compromises the binding of the abovementioned compounds to EGFR and increases the receptor affinity for ATP (Sequist et al., 2011). Third-generation TKI osimertinib can overcome this drug resistance mechanism, and it has proven to be effective in *EGFR*-mutated NSCLC irrespective of T790 M status. However, the clinical benefit of this drug is limited by the further emergence of drug resistance (Soria et al., 2017). Besides the aforementioned *EGFR* T790 M mutation leading to detraction of drug binding due to target alteration, other intensively investigated mechanisms of drug resistance in cancer such as overexpression of efflux transporters, increased drug metabolism, epigenetic modifications, DNA damage response and epithelial-to-mesenchymal transition (EMT) were reported (Housman et al., 2014). Another more recently investigated resistance mechanism, lysosomal sequestration, has been described for hydrophobic weak base drugs such as sunitinib and nintedanib (Zhitomirsky and Assaraf, 2016; Gotink et al., 2011; Zhitomirsky and Assaraf, 2017, 2015; Englinger et al., 2017). Focusing on *EGFR*-mutated NSCLC, secondary *EGFR* mutations, activation of bypass signaling pathways and histological transformation to small-cell lung cancer constitute peculiar mechanisms that limit the efficacy of EGFR-TKI (Van Der Steen et al., 2018). Thus, an earlier identification of drug resistance mechanisms as well as the development of new strategies to overcome the limitations of EGFR blockade alone, are urgently needed.

2. An overview of miRNAs

MicroRNAs (miRNAs) are 18–25 nucleotides in length, single-stranded, noncoding RNAs that function as post-transcriptional regulators of gene expression (Krol et al., 2010; Cai et al., 2009). They are synthesized from large precursor RNAs (pri-miRNAs), which are then processed in the nucleus by the RNase III, Drosha, and the double-stranded RNA-binding protein, Pasha, into pre-miRNAs. Pre-miRNAs are then released into the cytoplasm by Exportin 5 and undergo further steps to form the mature miRNAs. Subsequently, a mature miRNA strand is incorporated in the RNA-induced silencing complex (RISC). In this manner, the protein complex can bind a complementary sequence in the 3' untranslated regions (3'-UTR) of the target mRNA and silence target genes by either degrading mRNA or preventing mRNA to be translated Cai et al., 2009; Lee et al., 2004). Noteworthy, each miRNA can target multiple mRNAs that in turn encode for hundreds of genes, and each gene can be targeted by different miRNAs (Jonas and Izaurralde, 2015). Due to their key role in central biological processes, miRNAs attracted much attention in the field of cancer research. Overall, gene silencing mediated by miRNAs reflects on regulation of different cellular processes, including cell differentiation, proliferation, apoptosis and stem cell self-renewal. Thus, aberrations in miRNAs' expression have been related to loss of cellular homeostasis that leads to a wide spectrum of diseases, including cancer (Croce, 2009). With respect to carcinogenesis, miRNAs can act as either oncogenes or tumor suppressors, depending on the cellular context and the multiple target genes affected by miRNA silencing (Shenouda and Alahari, 2009). However, cumulative evidence suggests that dysregulation of specific miRNAs may also influence cancer cell resistance to conventional chemotherapy and novel targeted agents.

3. Techniques for miRNAs analysis

Some of the most established molecular biology-based methods, such as Reverse Transcription-Polymerase Chain Reaction (RT-PCR) and microarrays, remain powerful tools in the quantitative analysis of miRNA. However, these techniques are constantly evolving due to the continuous incorporation of new technologies. RT-PCR methodology to determine miRNAs is based on the role of reverse transcriptase, which converts RNA into their complementary DNA (cDNA) sequences and proceeds to the amplification of the newly synthesized cDNA according to the classic PCR procedure. The recent development of innovative primers has promoted template specificity, thermodynamic stability and superior detection from challenging clinical samples such as biofluids and formalin-fixed paraffin-embedded (FFPE) tissue. In particular, LNA™ oligonucleotides have substantially increased affinity for its complementary strand, resulting in unprecedented sensitivity and specificity. In addition, these primers enable robust detection of all miRNA sequences, regardless of GC content, as well as of single nucleotide mismatches. Another novel approach for RT-PCR-based miRNA expression profiling is based upon the hybridization of stem-loop RT primers which can offer specificity and sensitivity for miRNAs as compared to linear RT primers (Farrell, 2010; Qavi et al., 2010). The expression levels of miRNAs are frequently monitored by microarray technology. In particular, microarrays are powerful tools for the simultaneous screening of hundreds of target sequences within a single sample volume. Moreover, microarrays can be used to identify both precursor and mature miRNAs. Probe-target hybridization is the central concept of this technology to determine the relative abundance of nucleic acid sequences through fluorescence-based detection. However, the diversity of miRNA microarray platforms and lack of reliable analytical and statistical methods to normalize microarray data make cross-platform comparison, reproducibility and integration challenging (Wang and Xi, 2013). New methods for miRNA analysis are therefore warranted. Some emerging methods are based on the electrical and optical detection of miRNAs whether due to changes in circuit properties that occur upon target miRNA hybridization, or with the involvement of biosensors and innovative examples of fluorescence, bioluminescence, spectroscopic, and refractive index-based detection platforms[Qavi et al., 2010]. In particular, the analysis of the different localization in tissue compartments and cell populations of specific miRNAs might be extremely relevant for dissecting their roles during pathophysiological processes and after treatment or acquisition of drug resistance. In this sense, *in situ* hybridization (ISH) represents a powerful technology that allows for the analysis of subcellular miRNA localization (Nielsen, 2012). However, ISH is still considered a challenging technology in terms of detection and specificity analysis (Urbanek et al., 2015). Moreover, this technique is unable to distinguish between functional and nonfunctional RNA states, such as for RNA stored and destined for degradation. The co-localization of a specific miRNA with its target mRNAs or Ago proteins can at least in part solve this problem, but another important challenge is the development of an ISH method for single molecule detection.

The recent implementation of Next Generation Sequencing (NGS) technologies has provided new tools to perform a more detailed research of miRNAs expression profiles. This high-throughput and high-resolution method also offers the opportunity to quickly and accurately discover new miRNAs. NGS allows indeed the simultaneous analysis of sequences and expression levels of all microRNAs present in the analyzed samples, and has the advantage of profiling more miRNAs at the same time, thus representing a unique technique for novel miRNA discovery (Hu et al., 2017). However, it should be noted that NGS-based miRNA profiling studies typically identify a plethora of small RNAs with novel sequences (*i.e.*, putative miRNAs), yet not all of these small

Table 1
Summary of miRNAs involved in drug resistance to EGFR-TKI.

miRNAs	TKI	miRNA's effect on TKI resistance	Targets	Biological effect	Reference
miR-7	gefitinib	Reverse	EGFR IGF1R PIK3CD	- Downregulation of EGFR expression	(Ge et al., 2015)
miR-21	gefitinib	Promote	PTEN PI3K/AKT	- PTEN loss - Activation of PI3K/AKT pathway - Inhibition of apoptosis	(Garofalo et al., 2012; B. Li et al., 2014; Shen et al., 2014)
miR-23a	gefitinib erlotinib	Promote	CDH1 PTEN	- TGF-β1-induced EMT - ↓ E-Cadherin expression - PTEN loss - Activation of PI3K/AKT pathway	(Cao et al., 2012; Han et al., 2017)
miR-30a	gefitinib	Reverse	PIK3R2	- Inhibition of PI3K/AKT pathway	(Meng et al., 2016)
miR-30b/c	gefitinib	Promote	BIM	- Inhibition of BIM-induced apoptosis	(Garofalo et al., 2012)
miR-34a	gefitinib erlotinib	Reverse	EGFR PI3K/AKT c-MET	- Inhibition of PI3K/AKT pathway - Induction of apoptosis	(Li et al., 2017; Zhao et al., 2017, 2014; Zhou et al., 2014)
miR-103, miR-203	gefitinib	Reverse	SRC PKC-ε Dicer GSK3β AKT ERK	- Inhibition of PI3K/AKT pathway - ERK pathway inactivation - Induction of MET - Induction of apoptosis	(Garofalo et al., 2012)
miR-124	gefitinib	Reverse	SNAI2 STAT3	- Inhibition of cell migration - Induction of MET	(Hu et al., 2016)
miR-126, miR-145	gefitinib	Reverse	PI3K VEGF	- Inhibition of PI3K/AKT pathway - ERK pathway inactivation	(Zhong et al., 2010)
miR-128	gefitinib	Reverse	c-MET	- Downregulation of c-MET expression in CSCs - Inhibition of PI3K/AKT pathway in CSCs	(Jiang et al., 2016)
miR-134, miR-487b, miR-655 (cluster)	gefitinib	Promote	MAGI2	- TGF-β1-induced EMT - PTEN loss	(Kitamura et al., 2014)
miR-138-5p	gefitinib	Reverse	GPR124	unknown	(Gao et al., 2014)
miR-147	gefitinib	Reverse	CDH1 ZEB1 SNAI2	- Reverse TGF-β1-induced EMT - ↑ E-Cadherin expression - AKT inhibition	(Lee et al., 2014)
miR-181a	gefitinib	Promote	GAS7	- Activation of PI3K/AKT pathway - ERK pathway activation - Induction of EMT	(Ping et al., 2018)
miR-200a	gefitinib	Reverse	EGFR c-MET	- Downregulation of EGFR and c-MET expression - Inhibition of cell migration	(Zhen et al., 2015)
miR-200c	gefitinib	Reverse	AKT ZEB1	- Inhibition of PI3K/AKT pathway - Induction of apoptosis - Inhibition of cell migration	(J. Li et al., 2014; Zhou et al., 2017)
miR-214	gefitinib erlotinib	Promote	PTEN LHX6	- PTEN loss - loss of LHX6 function	(Liao et al., 2017; Wang et al., 2012)
miR-221/222	gefitinib	Promote	APAF-1 PTEN TRAIL	- Inhibition of APAF-1 induced apoptosis - PTEN loss - Inhibition of TRAIL-induced apoptosis	(Garofalo et al., 2012, 2009)
miR-223	erlotinib	Reverse	IGF1R	- Inhibition of PI3K/AKT pathway	(Han et al., 2016)
miR-223	erlotinib	Promote	FBXW7	- Downregulation of FBXW7	(Zhang et al., 2017)
miR-483-3p	gefitinib	Reverse	Integrin ß3	- Repression of FAK/ERK signaling pathway - Induction of MET - Induction of apoptosis	(Yue et al., 2018)

Abbreviations: AKT, Protein kinase B; APAF-1, Apoptotic protease activating factor-1; BIM, Bcl-2-like protein 11; CDH1, Cadherin 1; c-MET, Hepatocyte growth factor receptor; CSCs, cancer stem cells; EGFR, Epidermal growth factor receptor; EMT, epithelial-to-mesenchimal transition; ERK, Extracellular signal-regulated kinase; FAK, Focal adhesion kinase; FBXW7, F-box/WD repeat-containing protein 7; GAS7, Growth Arrest Specific 7; GPR124, G-protein coupled receptor 124; GSK3β, Glycogen synthase kinase 3 beta; IGF1R, Insulin-like growth factor 1 receptor; LHX6, LIM Homeobox 6; MAGI2, Membrane-associated guanylate kinase inverted 2; MET, mesenchymal-to-epithelial transition; miRNA, micro RNA; PI3K, Phosphatidylinositol-3-kinase; PIK3CD, Phosphatidylinositol-4,5-Bisphosphate 3-Kinase Catalytic Subunit Delta; PIK3R2, Phosphoinositide-3-Kinase Regulatory Subunit 2; PKC-ε, Protein kinase C epsilon type; PTEN, Phosphatase and tensin omolog; SNAI2, Snail Family Transcriptional Repressor 2; SRC, Proto-oncogene tyrosine-protein kinase Src; STAT3, Signal transducer and activator of transcription 3; TGF- β1, Transforming growth factor beta 1; TKI, tyrosine kinase inhibitor; TRAIL, TNF-related apoptosis-inducing ligand; VEGF, Vascular endothelial growth factor; ZEB1, Zinc finger E-box-binding homeobox 1.

RNAs may not be *bona fide* miRNAs. Additional drawbacks to NGS include time-consumption, high cost, the bioinformatics infrastructure and staff required for analysis and interpretation of the output data. Moreover, NGS analysis is less accurate in quantifying the levels of a specific miRNA than RT-PCR. In this scenario, RT-PCR still remains the most suitable technology for clinicians and, to date, it represents the gold standard methodology for miRNA diagnostics.

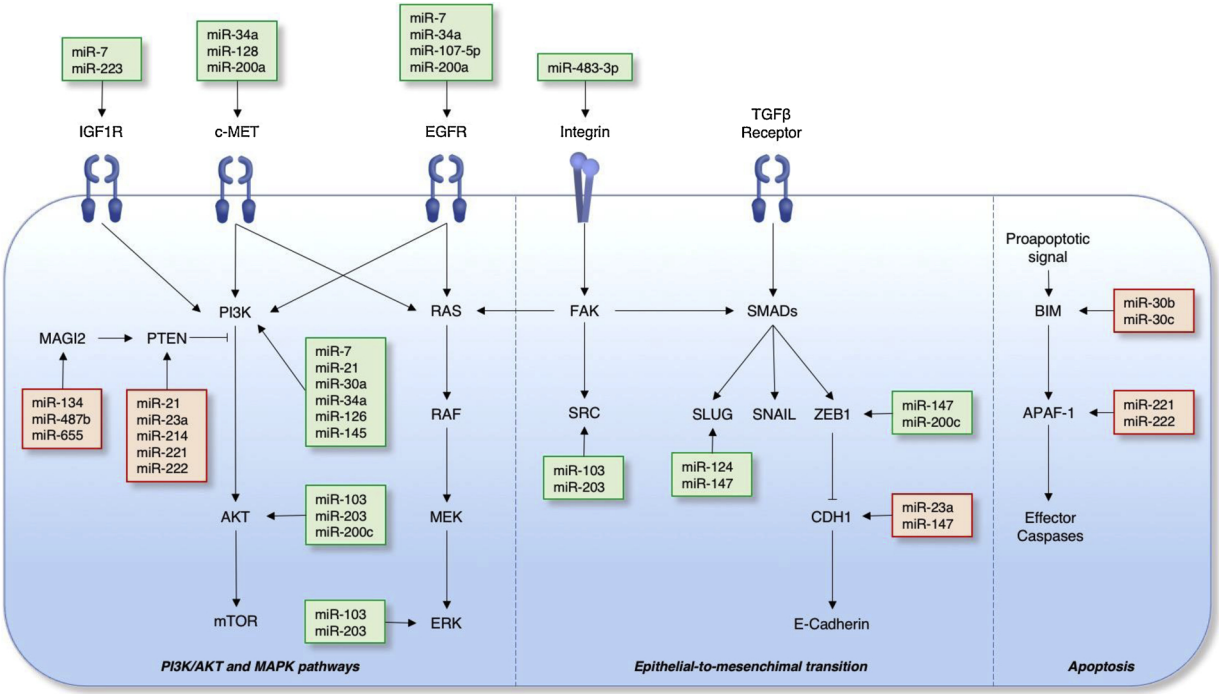

Fig. 1. Mechanisms of acquired resistance to EGFR-TKI mediated by miRNAs. Several key miRNAs can deregulate pivotal pathway involved in cell survival, metabolism, EMT and apoptosis in *EGFR*-mutated NSCLC. This figure provides a schematic representation of known miRNAs' targets involved in modulation of resistance to EGFR-TKI. miRNAs depicted in red boxes enhance EGFR-TKI resistance. As opposite, miRNAs depicted in green boxes reverse resistance to EGFR-TKI (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).
Abbreviations: AKT Protein kinase B; APAF-1 Apoptotic protease activating factor-1; BIM Bcl-2-like protein 11; CDH1 Cadherin 1; c-MET Hepatocyte growth factor receptor; EGFR Epidermal growth factor receptor; ERK Extracellular signal-regulated kinase; FAK Focal adhesion kinase; IGF1R Insulin-like growth factor 1 receptor; MAGI2 Membrane-associated guanylate kinase inverted 2; MAPK Mitogen-activated protein kinase; MEK Mitogen-activated protein kinase kinase; mTOR Mammalian target of rapamycin; PI3K Phosphatidylinositol-3-kinase; PTEN Phosphatase and tensin omolog; RAF v-raf murine sarcoma viral oncogene homolog B1; RAS Rat sarcoma; SLUG Snail Family Transcriptional Repressor 2; SMAD Small mother against decapentaplegic; SNAIL Protein snail homolog 1; SRC Proto-oncogene tyrosine-protein kinase Src; ZEB1 Zinc finger E-box-binding homeobox 1.

4. The link between miRNAs and *EGFR*-mutated NSCLC

The link between miRNAs and lung cancer has been demonstrated by numerous studies over years, as reviewed by Iqbal and collaborators (Iqbal et al., 2018). Cumulative evidence suggests that miRNAs are involved in lung cancer initiation as well as progression. Moreover, miRNAs may be used as biomarkers for the early diagnosis and as predictors of patients' prognosis (Bjaanæs et al., 2014; Yanaihara et al., 2006; He et al., 2015). For instance, miR-21 has been shown to have a significantly high expression in lung cancer compared to normal lung tissues. Similar results were reported in studies on plasma samples from NSCLC patients, compared to healthy individuals. Therefore, this miRNA could serve as a minimally invasive biomarker for NSCLC diagnosis (Yanaihara et al., 2006; Seike et al., 2009; Bica-Pop et al., 2018).

Focusing on *EGFR*-mutated NSCLC, previous studies showed that miRNAs are distinctly expressed according to *EGFR* status (Han et al., 2015). *EGFR* mutation or overexpression can indeed regulate miRNAs' levels. On the other hand, miRNAs can target and thus inhibit *EGFR* transcription (Han et al., 2015). When analyzing miRNAs' expression profiles of 154 surgical specimens of both *EGFR*-mutated and wild-type (wt) NSCLC by Agilent microarrays, 17 miRNAs were differentially expressed in the two groups (Bjaanæs et al., 2014). Similarly, Gasparini and colleagues developed a diagnostic classifier based on three miRNAs (miR-1253, miR-504, and miR-26a-5p) which could discern between FFPE NSCLC samples based on Anaplastic Lymphoma Kinase-translocation, mutant *EGFR*, or mutant Kirsten Rat Sarcoma (*KRAS*) *versus* wt status. In particular, tissue expression of miR-504 was associated with the presence of *EGFR* mutation (odds ratio 2.86; 95% Confidence Interval [CI] 1.07–7.71, $p = 0.04$) (Gasparini et al., 2015). In another study, miR-183 levels appeared significantly higher in FFPE of *EGFR*-mutated NSCLC than in the wt group ($p = 0.028$) (Pak et al., 2015). However, miR-25 was also upregulated more than twofold only in *EGFR*-mutated lung cancer specimens compared to *KRAS*-positive and *EGFR/KRAS*-negative samples ($p = 0.0016$) (Dacic et al., 2010).

Moving to diagnostic miRNA testing on plasma samples, miR-122 was found to be differently expressed in a cohort of 105 female never-smoker NSCLC patients when comparing *EGFR* mutation carriers and *EGFR*-wt patients, regardless of disease stage ($p = 0.033$ to 0.018) (Zhang et al., 2013). In addition, circulating miR-122 appeared to be a specific biomarker for the L858R mutation (Qu et al., 2017). Similarly, Zhao and collaborators identified five selected miRNAs (miR-25, miR-122, miR-195, miR-21 and miR-125b), which were upregulated in both tumor tissues and plasma of 150 NSCLC patients (all $p < 0.001$), and significantly associated with *EGFR* mutation, with a discriminatory power demonstrated by the area under the curve (AUC) of 0.869 ($p < 0.001$, 95% CI 0.808-0.930) (Zhao et al., 2015). A similar attempt to identify a panel of circulating plasma miRNAs that can predict *EGFR* status was made by Qu and colleagues: a combination of miR-107 (upregulated) and miR-195 (downregulated) had a sensitivity and specificity of 71.8% and 78.9%, respectively, in discriminating in the presence of *EGFR* mutation (Qu et al., 2017). These studies clearly provide hints for the potential use of miRNAs as a complementary tool for molecular characterization of NSCLC, especially when considering the feasibility and minimal invasiveness of miRNA analysis of plasma.

By targeting *EGFR*, miRNAs can silence EGFR protein translation, thus exerting a tumor suppressive function. Yamaguchi and collaborators found that miR-542-5p had a tumor suppressive role in NSCLC by targeting *EGFR* mRNA in H3255, A549 and HCC827 lung cancer cell lines. When analyzing lung cancer specimens, an inverse correlation

between miR-542-5p and EGFR protein levels was observed (Yamaguchi et al., 2012). *EGFR* is negatively regulated by miR-7 as well. Indeed, gefitinib-resistant A549 lung cancer cells transfected with a miR-7 mimic, showed a marked decrease in EGFR protein levels, suggesting that miR-7 may improve the sensitivity of cancer cells to gefitinib (Ge et al., 2015). An analogous inverse correlation between miRNA levels and EGFR expression was demonstrated for miR-34a (Li et al., 2017), miR-133 (Wang et al., 2014) and miR-107-5p (Wang et al., 2017). Moreover, *EGFR* is a direct target of miR-200a, in addition to MET (Zhen et al., 2015). A summary of the miRNAs involved in EGFR-TKI resistance in NSCLC is provided in Table 1, while the following paragraphs include a brief description of the known mechanisms by which miRNAs can cause primary/acquired resistance to targeted drugs in *EGFR*-mutated NSCLC (Fig. 1).

5. Mechanisms of EGFR-TKI resistance mediated by miRNAs

5.1. Modulation of PI3K/AKT/mTOR signaling pathway

Beyond secondary *EGFR* mutations, an important mechanism that NSCLC cells can adopt in order to escape EGFR-TKI blockade, relies on the activation of parallel downstream signaling pathways, among which PI3K/AKT/mTOR constitutes a key transduction cascade responsible for cell survival, proliferation and invasion (Vara et al., 2004; Fumarola et al., 2014). PI3K/AKT/mTOR can be activated through the interaction of c-MET, also known as hepatocyte growth factor receptor (HGFR), with its ligand HGF. Among the inhibitory proteins of this pathway, PTEN is known to downregulate AKT activity, and loss of *PTEN* contributes to erlotinib and gefitinib resistance in *EGFR*-mutated NSCLC due to the lack of the inhibitory input (Van Der Steen et al., 2018). Considering this scenario, several miRNAs could interfere with the PI3K/AKT/mTOR pathway. By activating the PI3K/AKT/mTOR signaling pathway, miRNAs could thus mediate resistance to TKIs. In contrast, miRNAs could reverse resistance to these compounds through inhibition of the PI3K/AKT/mTOR pathway.

Shen and collaborators demonstrated that miR-21 induced gefitinib resistance in NSCLC PC9 cells through *PTEN* silencing and subsequent activation of AKT and ERK (Shen et al., 2014). Interestingly, miR-21 knockdown dramatically restored gefitinib sensitivity *in vitro* and *in vivo* in PC9 cells, accompanied by upregulation of PTEN (Shen et al., 2014). Parallel experiments identified miR-214 as an inhibitor of *PTEN* in the human lung adenocarcinoma HCC827 cell line as well as a potential miRNA to be targeted in order to reverse the gefitinib acquired resistance (Wang et al., 2012). *PTEN* is also targeted by miR-23a, as shown in a study conducted on cancer stem cells (CSCs) isolated from the PC9 NSCLC tumor cell line: upregulation of miR-23a induced erlotinib resistance in PC9 CSCs, and this effect was reversed by miR-23a knockdown (Han et al., 2017).

Besides *PTEN*, numerous miRNAs can interact with PI3K/AKT oncoproteins, and hence eliminate the trigger of the aberrant signaling in the case of TKI resistance, thereby re-sensitizing the cells. Indeed, in NSCLC, *PI3K/AKT* signaling determinants are silenced by miR-30a (Meng et al., 2016), miR-34a (Zhao et al., 2017, 2014), miR-103 (Garofalo et al., 2012), miR-203 (Garofalo et al., 2012), miR-126 (Zhong et al., 2010), miR-145 (Zhong et al., 2010) and miR-200c (Zhou et al., 2017). Among these miRNAs, a mimic of miR-34a showed a synergic effect with erlotinib, afatinib, rociletinib and osimertinib in multiple *EFGR*-mutant and *EGFR*-wt cell lines, and the most powerful anticancer effects were documented in erlotinib-resistant cell models (Zhao et al., 2017, 2014). The same synergy was observed with miR-126 and gefitinib *in vitro* (A549 and H460 cells) and *in vivo* (xenografts): when tested on tumor cell lines, forced expression of miR-126 increased the gefitinib sensitivity sixfold (Zhong et al., 2010).

Last but not least, miRNAs can also target upstream effectors of the PI3K/AKT pathway. For instance, c-MET gene amplification is a well-known cause of acquired resistance to TKIs (Van Der Steen et al., 2018) and is found to be directly targeted by miR-128 (Jiang et al., 2016). Through c-MET silencing, miR-128, when combined with gefitinib, successfully suppressed the entire EGFR/ PI3K/AKT pathway in PC9-CSCs, and it reversed gefitinib resistance in this peculiar cell line (Jiang et al., 2016). Insulin-like growth factor 1 receptor (*IGF1R*) gene, which is an upstream effector of PI3K/AKT signaling responsible for TKIs' secondary resistance (Van Der Steen et al., 2018), is targeted by miR-223. Accordingly, overexpression of miR-223 partially restored drug sensitivity in PC9 cells resistant to erlotinib by suppressing *IGF1R* (Han et al., 2016). However, miR-223 was found to enhance drug resistance in erlotinib-resistant HCC827 clones by down-regulating *FBXW7* expression and activating the Notch pathway (Zhang et al., 2017). Thus, the role of miR-223 in mediating EGFR-TKI resistance remains controversial.

5.2. Modulation of epithelial-to-mesenchymal transition (EMT)

EMT constitutes a relevant resistance mechanism to EGFR-TKI treatment *per se*, beyond acquired *EGFR* mutations (Weng et al., 2018; Poh et al., 2018), and it has been historically related to the development of metastases in cancer (Bastid, 2012). It consists of a plastic switch of cancer cells from an epithelial to mesenchymal phenotype, which implies augmented cell motility, invasiveness, elevated resistance to apoptosis and increased production of extracellular matrix components (Kalluri and Weinberg, 2009). Transforming growth factor-β1 (TGF-β1) plays a dominant role in this phenotype transition, through different mechanisms across tumor types (Wendt et al., 2009). Schematically, TGF-β1 activates zinc finger E-box binding 1 (*ZEB1*), which encodes for a key transcriptional repressor of the cadherin 1 gene (*CDH1*) which is responsible for transcription of the cell-adhesion glycoprotein, E-cadherin.

Concerning the implications of miRNA in EMT, TGF-β1 has been shown to induce miR-23a expression in A549 cells, which in turn suppressed E-cadherin and stimulated EMT in a SMAD-dependent manner (Cao et al., 2012). E-cadherin levels also decreased after transfection of miR-181a mimics in PC9 cells. On the other hand, downregulation of miR-181a in gefitinib-resistant PC9 cells increased E-cadherin expression and decreased the levels of mesenchymal markers, resulting in enhanced gefitinib sensitivity (Ping et al., 2018). Moreover, miR-134 and miR-487b, triggered by TGF-β1, directly target membrane-associated guanylate kinase inverted 2 (MAGI2), that is a scaffold protein required for PTEN function. PTEN inactivation, induced by miR-134 and miR-487b, consequently led to a mesenchymal phenotype and resistance to gefitinib in A549 cells (Kitamura et al., 2014). Through this study, the authors identified miR-134 and miR-487b as key effectors of TGF-β1-induced EMT (Kitamura et al., 2014).

Since EMT is reversible, miRNAs can also modulate the opposite conversion, mesenchymal-to-epithelial transition (MET), in order to restore TKI efficacy in drug resistant cells. In keeping with this hypothesis, miR-147 (Lee et al., 2014) and miR-200c (Zhou et al., 2017) were found to inhibit *ZEB1*, as demonstrated by preclinical studies performed on A549 and PC9 cell lines, respectively. In both cases, the expression of the respective miRNA dramatically reversed the intrinsic/acquired drug resistance of these cells (Zhou et al., 2017; Lee et al., 2014). In a more complex manner, miR-103 and miR-203 induced MET by targeting parallel EMT-associated pathways, as observed by Garofalo and colleagues (Garofalo et al., 2012). This research demonstrated that miR-103 and miR-203 silenced protein kinase C-ε (PKC-ε) and proto-oncogene tyrosine-protein kinase Src (SRC), and in turn increased E-cadherin and decreased protein snail homolog 1 (*SNAIL*), *ZEB1,* zinc finger E-box binding 2 (*ZEB2*), vimentin and fibronectin mRNA levels. The result was an augmented Calu-1 sensitivity to gefitinib (Garofalo et al., 2012). The same study showed that c-MET activation reduced *in vitro* levels of miR-103 and miR-203, which normally function as onco-suppressor miRNAs. Therefore, miR-103 and miR-203 could drive acquired resistance to EGFR-TKI in case of *c-MET* gene amplification and

overexpression (Garofalo et al., 2012). More recently, miR-483-3p was found to target integrin ß3, which participates in cell adhesion, and miR-483-3p levels were dramatically decreased in PC9 and HCC827 gefitinib-resistant cell lines (Yue et al., 2018). Forced overexpression of miR-483-3p reversed EMT and inhibited migration, invasion, and metastasis of the abovementioned tumor cell lines, thus re-sensitizing them to the targeted drug (Yue et al., 2018). Finally, miR-124 has been proven to be a tumor suppressor that reversed EMT *via* Snail Family Transcriptional Repressor 2 (*SNAI2*) and Signal Transducer And Activator Of Transcription 3 (*STAT3*), and it was found to re-sensitize clones of PC9 cells which display resistance to gefitinib (Hu et al., 2016). Interestingly, miR-124 was also significantly downregulated in plasma samples of gefitinib-resistant NSCLC patients (Hu et al., 2016).

5.3. Interference with EGFR-TKIs-induced apoptosis

Several studies demonstrated that miRNAs can influence TKI response in *EGFR*-mutated NSCLC by modulating cancer cell apoptosis-associated genes. Such effect has been identified for miR-214, which targets Lim homeobox 6 (*LHX6*) (Liao et al., 2017), a tumor suppressor gene that regulates apoptosis-related genes *TP53* and *BCL-2* in lung cancer, as well as cell cycle-related gene *P21* and cell proliferation-associated genes *CYCLIN D1* and *C-MYC* (Liu et al., 2013). In addition, miR-221/222, whose expression is reduced following gefitinib treatment, target apoptotic protease activating factor-1 (*APAF-1*) (Garofalo et al., 2012). APAF-1 protein has a central role in the apoptosis regulatory network, since it forms the oligomeric apoptosome together with caspase-9 (Li et al., 1997). When APAF-1 levels were reduced by miR-221/222, gefitinib-sensitive HCC827 and PC9 cells became less responsive to EGFR-TKI. Conversely, overexpression of APAF-1 in A549 resistant cells, induced gefitinib-related PARP cleavage (Garofalo et al., 2012). Moreover, miR-221/222 target *TIMP3*, a gene which encodes for metalloproteinase inhibitor 3, essential for activation of apoptotic caspases (Garofalo et al., 2009). Similar to miR-221/222, miR-30b-c promotes gefitinib resistance through post-transcriptional regulation of *BIM-1*, which encodes for a Bcl-2 family anti-apoptotic protein (Garofalo et al., 2012). Importantly, both levels of miR-221/222 and miR-30b-c increased after EGFR and c-MET stimulation, respectively, suggesting a feedback regulatory mechanism underlying drug resistance (Garofalo et al., 2012). Modulation of TKI-induced apoptosis has been also demonstrated with apoptotic assays for the oncomir miR-21, that reduced apoptosis in PC-9 cells treated with gefitinib by both down-regulating *PTEN* and activating AKT and ERK pathways (Shen et al., 2014). Lastly, miR-483-3p promoted apoptosis of a subpopulation of gefitinib-resistant HCC827 lung cancer *in vitro* and *in vivo* (Yue et al., 2018).

6. Clinical implications of miRNAs' analysis in EGFR-mutated NSCLC

6.1. Prognostic value

Besides the diagnostic potential of miRNAs in *EGFR*-mutated NSCLC, the expression of key miRNAs could have prognostic value in the clinical setting. High plasma expression of miR-122, miR-19a, miR-19b, miR-195 and miR-590-5p was associated with better overall survival (OS) among advanced non-smoking female NSCLC patients harboring an *EGFR* mutation compared to *EGFR*-wt patients, even after adjusting the results for the treatment regimen in order to reduce the confounding effect of EGFR-TKIs administration (Zhang et al., 2013). In contrast, tissue miR-197 and miR-184 overexpression was associated with the occurrence of brain metastases, a known negative prognostic indicator, in *EGFR*-driven NSCLC ($p = 0.017$ and $p = 0.01$, for miR-197 and miR-184, respectively) (Remon et al., 2016). Furthermore, high miR-21 plasma levels were associated with poor OS ($p = 0.0045$) in a cohort of 201 NSCLC patients with *EGRF* mutation who underwent surgery and were treated with gefitinib-based adjuvant therapy (Shen et al., 2013). MiR-500a, which is overexpressed in *EGFR*-mutant NSCLC, was also associated with shorter time to progression in the microarray analyses of 154 surgically resected lung adenocarcinomas ($p < 0.001$) (Bjaanæs et al., 2014). The impact of miRNAs on patients' prognosis can be partially explained by the notion that miRNAs are key regulators of the EGFR pathway, which represents the driving force of *EGFR*-mutated lung cancer. Furthermore, by interfering with EGFR signaling, miRNAs can influence and modulate the response to EGFR-targeted drugs, as further discussed in the following chapter.

6.2. Predictive value

Taking into account the strong preclinical evidence of miRNAs' modulation of EGFR-TKIs efficacy in distinct tumor cell lines, the evaluation of the expression levels of miRNA in patients' tumor specimens has been performed in multiple studies in order to predict the treatment's response (Qu et al., 2017; Ping et al., 2018; Hu et al., 2016; Liao et al., 2017; Shen et al., 2013; Weiss et al., 2008; Li et al., 2014a, b; Wang et al., 2015; Bisagni et al., 2018). Clinical investigations of a number of miRNAs on FFPE/plasma samples and their correlation with response to TKI therapy are listed in Table 2. In their original study, Weiss and collaborators described loss of heterozygosity of miR-128b as a positive predictive factor correlated with clinical response and survival to gefitinib treatment (Weiss et al., 2008). Other researchers explored a putative role of miRNAs in influencing inherent (primary) drug resistance to EGFR-TKIs, evaluating both tissue and blood of 54 NSCLC patients, divided in three cohorts (Wang et al., 2015). Among 153 miRNAs found to be differentially expressed between the sensitive and resistant groups, miR-21, miR-27a and miR-218 were significantly overexpressed in plasma of primary resistant patients (PFS < 3 months), compared to the sensitive group (PFS ≥ 3 months) ($p = 0.004$, $p = 0.009$ and $p = 0.041$ respectively) (Wang et al., 2015). A miRNA microarray analysis conducted by Ma and collaborators revealed 16 miRNAs, 15 of which were down-regulated (hsv2-miR-H19, miR-744-5p, miR-3196, miR-3153, miR-4791, miR-4803, miR-4796-3p, miR-372-5p, miR-138-2-3p, miR-16-1-3p, miR-1469, miR-585-3p, ebv-miR-BART14-5p, miR-769-3p, miR-548aq-5p), whereas only miR-503-3p was up-regulated in plasma samples from patients displaying inherent drug resistance to EGFR-TKIs (all $p < 0.05$) (Ma et al., 2017). In a more recent study performed on FFPE tumor specimens from 32 NSCLC patients treated with erlotinib in second or third line, high levels of miR-133b and miR-146 were associated with longer PFS ($p = 0.006$ and $p = 0.018$, respectively), with miR-133b showing the highest differential expression between responders and non-responders [Bisagni et al., 2018]. On the other hand, miR-7 levels were higher in non-responders ($p = 0.037$), defined as patients with disease stabilization on erlotinib for less than 6 months [Bisagni et al., 2018].

Several studies were also aimed at demonstrating a putative association between dynamic changes in circulating miRNAs during TKI treatment and tumor response. Among these, miR-21 expression was evaluated in plasma samples collected prior to EGFR-TKI treatment and at the time of progression of 25 *EGFR*-mutated NSCLC patients treated with gefitinib/erlotinib for advanced disease. The authors demonstrated that the mean plasma miR-21 level was significantly higher at the time of disease progression than at baseline ($p < 0.01$), thus supporting the role of miR-21 as a potential biomarker and a determinant of acquired drug resistance to EGFR-TKIs (Li et al., 2014a). This is concordance with *in vitro* findings, considering that miR-21 was more aberrantly expressed in the EGFR-TKI-resistant lung cancer cell line PC9R relative to its parent cell line, PC9 (Li et al., 2014a).

Other studies showed increased levels of miR-214 (Liao et al., 2017) and miR-181a (Ping et al., 2018) in patients' plasma after establishing resistance to TKIs when compared to baseline values. In contrast, miR-124 levels were decreased in serum of drug resistant patients when compared to pre-treatment samples (Hu et al., 2016). Qu and colleagues

Table 2
miRNA clinical investigations and correlations with response to EGFR-TKIs.

miRNAs	Study population	Samples for miRNA analysis	TKI treatment	Clinical findings	Preclinical findings	Reference
miR-128b	58 *EGFR*-mut NSCLC patients	FFPE lung tissue	Gefitinib	• miR-128 LOH was correlated with clinical response and survival	• miR-128b regulates EGFR expression	Weiss et al, *Ann Oncol* 2008 (Weiss et al., 2008)
miR-21 miR-10b	261 NSCLC patiens: - cohort 1: 128 radically resected NSCLC (*EGFR*-mut and *EGFR*-wt) - cohort 2: 201 *EGFR*-mut NSCLC patients treated with adjuvant gefitinib	Plasma samples (at baseline, every follow-up visit and at PD)	Gefitinib (adjuvant)	• reduced miR-21 levels were correlated with better OS ($p = 0.0045$) • no correlation between miR-10b levels and OS was documented ($p = 0.634$)	Not assessed	Shen et al, *Med Oncol* 2013 (Shen et al., 2013)
miR-21	25 NSCLC patients (20 *EGFR*-mut, 5 *EGFR* status unknown)	Plasma samples (at baseline and PD)	Gefitinib/erlotinib	• miR-21 expression was higher at the time of acquired resistance to TKIs than at baseline ($p < 0.01$)	• miR-21 was overexpressed in TKI resistant cell lines (PC9R)	Li et al, *Lung Cancer* 2014 (Li et al., 2014aB. Li et al., 2014)
miR-200c	150 NSCLC patients with acquired resistance to EGFR-TKIs (73 *EGFR*-mut, 66 *EGFR*-wt, 11 *EGFR* status unknown)	FFPE lung tissue	Gefitinib/erlotinib as $2^{nd}/3^{rd}$ line therapy	• miR-200c was lower in *EGFR*-wt compared to *EGFR*-mut patients ($p = 0.049$) • High level of miR-200c were correlated with longer PFS, longer OS and higher DCR in *EGFR*-wt patients	• miR-200c expression decreased in gefitinib-resistant cell lines (A549, H23, H1299, H460, H1975 and PC9R) • Upregulation of miR-200c re-sensitized gefitinib-resistant cells (A549 and H1299)	Li et al, *Oncotarget* 2014 (Li et al., 2014bJ. Li et al., 2014)
miR-21 miR-27a miR-218	- Training group: 20 NSCLC patients with *EGFR* ex19del - Validation group: 34 NSCLC patients with *EGFR* ex19del + 48 NSCLC patients *EGFR*wt	Plasma samples and FFPE lung tissue	Gefitinib/erlotinib as 1^{st} line therapy	• miR-21, miR-27a and miR-218 levels were significantly higher in plasma of primary resistant patients (PFS < 3months) compared to the sensitive group ($p = 0.004$, $p = 0.009$ and $p = 0.041$ respectively)	Not assessed	Wang et al, *J Hematol Oncol* 2015 (Wang et al., 2015)
miR-124	15 *EGFR*-mut NSCLC patients (ex19, ex21)	Plasma samples (at baseline and PD)	Gefitinib	• miR-124 decreased in serum of resistant patients after treatment compared to pre-treatment samples	• miR-124 decreased in gefitinib-resistant cell lines (PC9 GR) • downregulation of miR-124 induced resistance to gefitinib and EMT (PC9) • overexpression of miR-124 re-sensitized gefitinib-resistant cells (PC9 GR) and inhibit EMT (A549)	Hu et al, *J Huazhong Univ Sci Technol* 2016 (Hu et al., 2016)
miR-214	7 *EGFR*-mut NSCLC patients (L858R)	Plasma samples (at baseline and PD)	Gefitinib/erlotinib as $1^{st}/2^{nd}$ line therapy	• miR-214 levels increased after acquiring of resistance to TKIs ($p = 0.398$)	• downregulation of miR-214 reversed resistance to erlotinib (HCC827)	Liao et al, *Sci Rep* 2017 (Liao et al., 2017)
hsv2-miR-H19 miR-744-5p miR-3196 miR-3153 miR-4791 miR-4803 miR-4796- 3p miR-372-5p	8 *EGFR*-mut NSCLC patients (ex19del, L858R)	Plasma samples	Gefitinib/erlotinib	• all the investigated miRNAs were downregulated, when only miR-503-3p was upregulated in primary resistant patients' plasma (PFS < 3months) compared to sensitive patients	Not assessed	Ma et al, *Oncotarget* 2017 (Ma et al., 2017)

(*continued on next page*)

Table 2 (*continued*)

miRNAs	Study population	Samples for miRNA analysis	TKI treatment	Clinical findings	Preclinical findings	Reference
miR-138-2-3p miR-16-1-3p miR-1469 miR-585-3p ebv-miR-BART14-5p miR-769-3p miR-548aq-5p miR-503-3p						
miR-107 miR-195	- Training group: 9 *EGFR*-mut NSCLC patients - Validation group: 153 NSCLC patients for validation (both *EGFR*-mut and *EGFR*-wt); 36 of 64 patients with ex19del had dynamic samples collected at 1,3 and 5 months during TKI treatment; 12 patients underwent plasma sample collection at PD	Plasma samples	Gefitinib/erlotinib/ icotinib	• miR-107 was upregulated in *EGFR* ex19del vs *EGFR*-wt • miR-195 was downregulated in *EGFR* ex19del vs *EGFR*-wt • miR-107 levels increased more sharply in responders than in patients with stable disease ($p < 0.05$), but no difference was detected between patients with a PFS $>$ or $<$ 8 months • miR-195 was downregulated in responders compared to patients with a SD • At the time of PD, miR-107 and miR-195 levels had a tendency to return at baseline levels	Not assessed	Qu et al, *Oncotarget* 2017 (Qu et al., 2017)
miR-181a	8 *EGFR*-mut NSCLC patients (ex19del, L858R)	Plasma samples	Gefitinib	• miR-181a levels increased in acquired gefitinib-resistant NSCLC patients compared with baseline values	• Authors screened miRNA expression through a miRNA microarray in PC9 cells and PC9GR cells (11 miRNAs upregulated, 3 miRNAs downregulated) • miR-181a is upregulated in gefitinib-resistant cells compared with gefitinib-sensitive cells, and it showed the great change in expression levels compared to other miRNAs (PC9GR/A549/H1975) • downregulation of miR-181a sensitized NSCLC cells to gefitinib (A549)	Ping et al, *Biochem Biophys Res Commun* 2018 (Ping et al., 2018)
miR-7 miR-21 miR-133b miR-146	32 NSCLC patients (*EGFR*-mut and *EGFR*-wt)	FFPE lung tissue	Erlotinib as $2^{nd}/3^{rd}$ line therapy	• miR-7 levels were higher in non-responders ($p = 0.037$) • miR-133b and miR-146 levels were higher in responders ($p = 0.006$ and $p = 0.018$, respectively) • miR-133b showed the highest differential expression and was correlated with longer PFS	• transfection of H1299 and A549 with mir-133b did not influence sensitivity to erlotinib	Bisagni et al, *Plos One* 2018

Abbreviations: DCR, disease control rate; EGFR, Epidermal growth factor receptor; EMT, Epithelial-to-mesenchimal transition; FFPE, Formalin-fixed paraffin-embedded; LOH, Loss of heterozygosity; NSCLC, Non-small cell lung cancer; OS, Overall survival; PD, Progression of disease; PFS, Progression-free survival; SD, stable disease; TKI, Tyrosine kinase inhibitor.

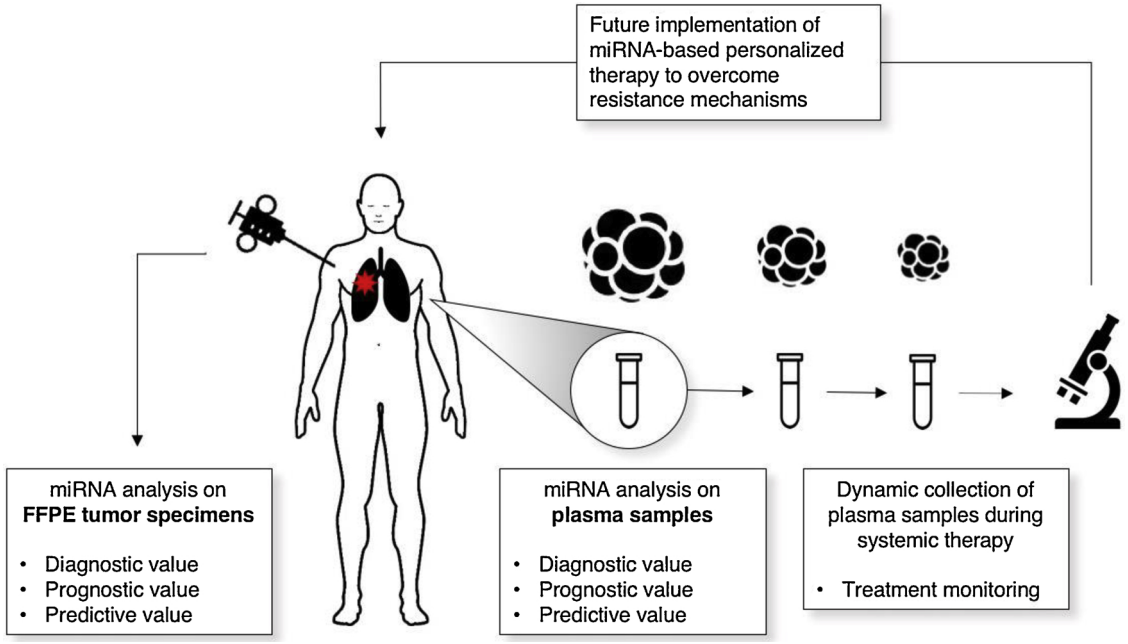

Fig. 2. Clinical implications of miRNA analysis.
MiRNA analysis can be performed on both FFPE tumor tissue and plasma samples of NSCLC patients. At the time of lung cancer diagnosis, miRNA levels could add relevant diagnostic and prognostic information. Moreover, in case of *EGFR*-mutated NSCLC, miRNAs could serve as a predictive biomarker of response to EGFR-TKIs. A dynamic collection of plasma samples taken under EGFR-TKI treatment can further help clinicians in monitoring EGFR-TKI response and early identifying acquired mechanisms of resistance. In the next future, miRNA levels modulation at the time of progression will allow researchers to develop a personalized miRNA-based therapy, which would be administered to the patient in order to overcome resistance mechanisms.
Abbreviations: EGFR, epidermal growth factor receptor; FFPE, formalin-fixed paraffin-embedded; miRNA, microRNA; NSCLC, non-small cell lung cancer; TKI, tyrosine kinase inhibitor.

studied the fluctuations in circulating miRNAs within the course of EGFR-TKI treatment (Qu et al., 2017). Prospective dynamic changes in two miRNAs (miR-107 and miR-195) were found to correlate with responses to the targeted therapy, although an association with differential PFS was not demonstrated. In particular, plasma miR-107 levels increased more sharply in responders than in patients with stable disease (SD) ($p < 0.05$). On the contrary, miR-195 levels were downregulated in responders compared to SD patients (Qu et al., 2017). These findings support the role of a specific miRNA signature as a powerful and intriguing surrogate for predicting EGFR-TKI performance in *EGFR*-mutated NSCLC patients and facilitating clinical decision-making process. Dynamic evaluation of circulating miRNAs surely deserves prospective large-scale validations, especially when considering the minimal invasiveness of the blood sampling procedure, before routine clinical implementation can be feasible.

7. miRNAs as therapeutic targets – Future Challenges

In addition to the promising diagnostic, prognostic and predictive value, miRNAs have the potential to become a target themselves for drug development, in order to overcome the acquired resistance to the currently used agents in *EGFR*-driven lung cancer. Preclinical findings on tumor cell lines clearly demonstrated that both restoring the tumor-suppressor miRNA function (by miRNA mimics) and inhibiting the oncogenic properties of onco-miRs (by antagomiRs) are effective strategies to re-sensitize resistant clones to EGFR-TKIs. However, implementing miRNA-based therapeutic strategies *in vivo* and subsequently translating the results in patients, constitutes a significant hindrance for clinicians. Indeed, to date, miRNA therapy has not yet been implemented in the clinical setting. One of the main concerns relies on the relative instability of miRNAs in body fluids, being that miRNAs are easily degraded by cellular and serum nucleases, phagocytosed by host immune cells, and rapidly cleared by kidneys (Christopher et al., 2016). These features can both impair reproducibility of miRNA levels determination in the plasma of cancer patients and limit the development of miRNA-based therapeutics. The synthesis of double-stranded miRNAs, protected by the addition of fluorine to cytosine and uracil nucleotides, represents a strategy to overcome this limitation (Chiu and Rana, 2003).

In addition, delivering miRNAs to the specific tumor sites is challenging, and it is going to be the focus of future studies in order to develop an effective therapeutic strategy (Barger and Nana-Sinkam, 2015). Indeed, the uptake of miRNAs by lung cancer cells remains to be determined (Fortunato et al., 2014). MiRNA delivery can be addressed by either systemic infusions or local intratumoral injections, the latter which is unlikely to be applied in lung cancer clinical setting. To date, one of the most compelling methods of introducing miRNAs into tumor tissues consists of transfection by a modified viral vector, including lentivirus, adenovirus, and adeno-associated virus. These vectors can easily provide a long-term and robust stable expression of miRNA-based therapeutics in transduced host cells. On the other hand, this approach is still considered controversial due to the potential risk of viral DNA integration into undesirable locations of the host genome and subsequent transformation of healthy somatic and germline cells (Chira et al., 2015). Of interest, a cationic lipoplexes-based carrier system represents an alternative method for delivering miRNA-based therapeutics in lung cancer cells, as demonstrated by Wu and collaborators (Wu et al., 2013). The cationic lipid is indeed able to confer a positive surface charge to the carrier in order to improve cellular uptake (Wu et al., 2013). Aptamer-conjugates have been also demonstrated to be an efficient selective tool for delivery of miRNAs into NSCLC cells (Esposito et al., 2016; Engelberg et al., 2018).

Recently, miRNAs have been identified in exosomes, which are nanovesicles released from many cell types into the extracellular milieu, with an intrinsic remarkable ability to cross biological barriers (Zhang et al., 2015). Such vesicles, which contain an increased amount

of genetic information compared to micro-vesicles, are widely distributed in various body fluids and may constitute a useful source for the detection of miRNAs as well as an efficient vehicle for miRNAs' delivery (Mathiyalagan and Sahoo, 2017). Despite numerous potential advantages, research concerning exosomal miRNAs in NSCLC is still at its infancy.

The safety profile of potential miRNAs-targeted therapies should be further investigated, considering the off-target effects that miRNAs' gene regulation can elicit, for the purpose of bringing miRNA therapeutics closer to the clinic. As an example, systemic administration of miRNA mimics, can result in the undesirable uptake by non-target tissues that do not express the miRNA of interest in physiological conditions, causing potential side effects (van Rooij and Kauppinen, 2014). In addition, miRNA-based therapies may induce a potentially deleterious adaptive immune response by triggering endosomal toll-like receptors signaling, thus consequently stimulating interferon and pro-inflammatory cytokines synthesis (Barber, 2011). Additionally, one cannot underestimate the cumulative toxic effect that therapeutic miRNAs could inflict when used in combination with current EGFR-directed therapeutic regimens. For instance, miRNAs can regulate the expression of EGFR-TKI metabolizing enzymes, such as cytochrome P450 s, at a post-transcriptional level, and enhance drug-mediated toxicity, but also cause the occurrence of several drug interactions that can result in reduced pharmacological effect, and adverse drug interactions (Yokoi and Nakajima, 2013; Zanger and Schwab, 2013).

Nevertheless, a miRNA-based therapy seems appealing when considering, at least theoretically, that a small number of miRNAs could be used to broadly silence multiple tumorigenic pathways. Furthermore, miRNAs' mutations are extremely rare due to the small size of the sequence, and thus resistance to miRNA therapy would require multiple mutations in several genes (Fortunato et al., 2014). In a futuristic view, the circulating miRNA signature, acquired at the starting time of EGFR-TKI treatment, could be used to develop a personalized miRNA-based therapy aimed at enhancing tumor sensitivity to the targeted agents. Upon disease progression, re-acquisition of the miRNA profile from a blood sample as well as a tumor biopsy could serve to adapt the personalized treatment for an optimal management of the disease (Fig. 2). Further research is warranted to assess all the above-mentioned challenges, including improvement of stability, delivery, and control of off-target effects of miRNAs. A better understanding of the complex biological network carried out by miRNAs in *EGFR*-mutated NSCLC and advancements in the field of genetic engineering will provide additional weapons to surmount well defined mechanisms of drug resistance.

Conflicts of interests

The authors have no conflicts of interest, including specific financial interests or relationship and affiliations relevant to the subject matter or materials discussed in the manuscript.

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