Review Article

MicroRNA and MET in lung cancer

Matteo Brighenti

Oncology Unit, Istituti Ospitalieri di Cremona, Cremona, Italy

Correspondence to: Matteo Brighenti. Oncology Unit, Istituti Ospitalieri di Cremona, Italy.
Email: m.brighenti@ospedale.cremona.it.

Abstract: MicroRNAs (miRNAs) are a class of small non-protein coding RNAs that modulate important cellular functions via their post-transcriptional regulation of messenger RNAs (mRNAs). Recent evidences from multiple tumor types and model systems implicate miRNA dysregulation as a common mechanism of tumorigenesis, cancer progression and resistance to therapy. Several miRNAs are dysregulated in cancers and a single miRNA can have multiple targets involved in different oncogenic pathways. MET, the tyrosine kinase receptor for hepatocyte growth factor (HGF), has a central role in lung cancer development and in acquired resistance to epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors; it has been predicted and shown to be the target gene of multiple miRNAs, which play a crucial role in controlling its activity in a stimulatory or inhibitory sense. In this review we will focus on the most important and recent studies about the role of miRNAs in the control of MET expression, reporting also the progress made using miRNAs for therapy of lung cancer.

Keywords: MET; microRNA; lung cancer

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Introduction

What are miRNAs ?

MicroRNAs (miRNAs) are 19 to 25 nucleotide-long non coding RNA molecules that regulate the gene expression at the level of messenger RNA degradation and translation. miRNAs are mainly transcribed by RNA polymerase II as long primary transcripts characterized by hairpin structures (pri-miRNA) and processed in the nucleus by RNAse III Drosha in a 70-nucleotide-long pre-miRNA. This precursor molecule is exported by the Exportin 5 to the cytoplasm, where RNAse III Dicer generates a dsRNA of approximately 22 nucleotides, named miR/miR*. The mature single stranded microRNA product is then incorporated in the complex, known as microRNA-containing RNA-induced silencing complex (RISC), whereas the other strand is likely subjected to degradation (1) .

miRNAs regulate the gene expression binding through partial complementarity for the most part to the 3'UTR of target mRNAs. The level of homology between guide and mRNA target determines which silencing mechanism will be employed: perfect matching of miRNAs to target sequences leads to transcriptional repression by cleaving and degrading the mRNA transcripts, while a limited base pairing inhibits the protein translation (*Figure 1*).

miRNA genes represent approximately 1% of the genome and it has been calculated that the human genome contains more than 1,800 miRNAs (2) with the potential of regulating around 30% of human genes (3).

By targeting multiple transcripts a single miRNA can regulate many fundamental cellular processes such as cell proliferation, apoptosis, differentiation, and migration; in the same way any gene can be regulated by multiple miRNAs.

Several databases are available to predict miRNA target genes, however the verification of these is essential, because the interaction between miRNA and the target is complex, with a poor overlap between databases (4). A reporter assay is one of the verification methods for miRNA target, because an alteration in the luciferase expression indicates whether a miRNA can bind to a target mRNA; using miRNA mimics and inhibitors and then measuring predicted targets of miRNAs is another method.

Although there is currently no gold standard for measuring miRNA expression (5) oligonucleotide microarray (microchip)

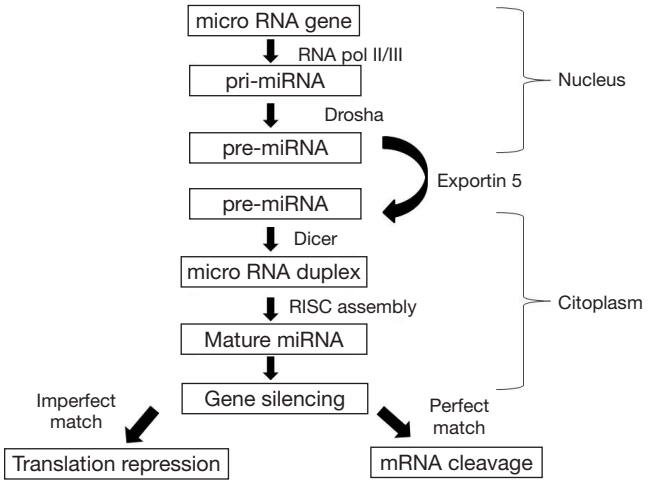

Figure 1 MicroRNA biogenesis: The generation of mature miRNAs is a multistage process. The miRNA genes are initially transcribed in the nucleus and are called primary miRNAs (pri-miRNAs). The enzyme Drosha then cleaves pri-miRNAs into precursors (pre-miRNAs) that are transported to cytoplasm by Exportin 5 and cleaved by Dicer to form a miRNA duplex. This duplex then unwinds into mature miRNA that is incorporated into an RNA-induced silencing complex (RISC) where it binds to a complementary sequence in the 3'UTR of target mRNA. The mechanism of inhibition of translation depends on the degree of miRNA-mRNA complementarity.

and quantitative real-time reverse transcription polymerase chain reaction (PCR) (qRT-PCR) are actually two of the most common methods to evaluate known miRNAs.

miRNA and lung cancer

miRNAs are involved in a variety of biological processes including cell cycle regulation, differentiation, development, metabolism, neuronal patterning and aging. Alterations in miRNA expression are not simply an effect of tumorigenesis but it has a causative role in cancer development: they are involved in the initiation, progression and metastasis of human tumors. Several miRNAs are dysregulated in cancers and a single miRNA can have multiple targets involved in different oncogenic pathways.

Given this powerful biology, numerous miRNAs have been implicated as either tumor suppressors or oncogenes ("oncomirs") in many different tumor types. Genomically they are frequently found to be at fragile sites in the human genome, but there are myriad additional mechanisms by which miRNAs can become dysregulated in cancer (6).

In 2002, a seminal study by Calin *et al.* (7) showed that miR 15 a/16-1 cluster is frequently deleted in chronic lymphocytic leukemia, implicating these miRNAs as tumor suppressor; after this discovery, several studies reported that miRNAs are aberrantly expressed in the majority of solid cancers and the diagnostic and prognostic value of miRNA expression in lung cancer has been intensely studied in recent years.

A first category of studies was conducted comparing miRNA expression of tumor samples to matched non-cancerous lung tissue: a report of Takamizawa *et al.* (8) showed that let-7 expression was reduced in lung cancer and this finding was confirmed later on by the group of Yanaihara *et al.* (9) where let-7 was part of a signature of miRNAs deregulated in tumor tissues compared to normal lung.

Recently a comprehensive meta-analysis of 20 miRNA expression studies in lung cancer, including a total of 598 tumors and 528 non-cancerous control samples, was published (10): using a robust rank aggregation method, the authors identified a statistically significant miRNA meta-signature of seven upregulated (miR-21, miR-210, miR-182, miR-31, miR-200b, miR-205 and miR-183) and eight downregulated (miR-126-3p, miR-30a, miR-30d, miR-486-5p, miR-451a, miR-126-5p, miR-143 and miR-145) miRNAs. Some of the upregulated miRNAs have a well-known role in cancer development: miR-21 is associated with worse prognosis of lung cancer patients and it was shown that directly target the tumor-suppressor PTEN, while miR-210 increases radioresistance of non-small cell lung cancer (NSCLC) cells by stabilizing HIF-1A. Oppositely, the downregulated miRNAs have a protective role: for example miR-126-3p and miR-126-5p work as regulators of angiogenesis and cell proliferation and miR-30a regulates the epithelial to mesenchymal transition by targeting the SNAI1 gene.

An alternative process to miRNA profiling of tumor tissues is measuring miRNA levels in relation to the outcome. This approach had principally been applied to address the question of whether miRNA signatures can be developed to stratify early-stage patients (those who have undergone surgical resection) into high or low risk for recurrence.

Landi *et al.* (11) reported a five-miRNA signature (miR-25, miR-34c-5p, miR-191, let-7e and miR-34a) that was first of all able to differentiate squamous from adenocarcinoma and then to correlate with poor overall survival among squamous patients; also Yu *et al.* (12) validated a 5-miRNA signature, including let-7a, miR-221, miR-137, miR-372 and miR-182, that was an independent predictor of cancer relapse and survival after surgery.

Recently several authors (13,14) have studied miRNAs measurement also from patient serum or plasma: MiR-21, in combination with miR-210 and miR-486-5p, was shown to be expressed significantly higher in the plasma of patients with malignant solitary pulmonary nodules compared to those with benign nodules (15) and MiR-155 with miR-197 and miR-182 was able to distinguish between NSCLC patients and control samples by real time PCR of plasma (16). This technique has the advantage to repeatedly measure miRNAs during the course of treatment or in post-treatment surveillance and to overcome the problem of small amounts of tissue in lung cancer patients but it needs to be validated.

MET and miRNA in lung cancer

In the last two decades, it has been consistently proven that one of the players of the intricate scenario leading to tumorigenesis is MET, the tyrosine kinase receptor for hepatocyte growth factor (HGF). MET can be activated either by binding to its ligand HGF, overexpression/amplification, mutation or decreased degradation. Deregulated activation of the MET-driven "invasive-growth" program confers unrestricted proliferation and metastatic properties to cancer cells (17) and recently it was demonstrated that the expression of an activated MET is required also for the maintenance and sustaining of metastatic colonies in the lung (18). MET ability to control all these ways has an explanation in its signal transducing properties. The receptor, in fact, is known to concomitantly activate both the Ras-MAPK and the phosphatidylinositide 30-kinase-AKT pathways controlling together growth, resistance to apoptosis and cytoskeletal rearrangement.

MET has been predicted and shown to be the target gene of multiple miRNAs (*Tables 1,2*), which play a crucial role in controlling its activity in a stimulatory or inhibitory sense, creating an intricate system that we have started to know only from few years and that opens new therapeutic possibilities, too. In this review the studies on miRNAs that control MET, which have been mainly published in later years, will be reported.

MiR-34 family controls MET acting as an effector in the p53 tumor suppressor network

MiR34 and MET

MiR-34 family consists of three members: miR-34a, miR-34b and miR-34c and MET is negatively regulated by all of them. MiR-34a is encoded in the second exon of a gene located on chromosome 1p36.22, whereas miR-34b and miR-34c share a common host gene located on chromosome 11q23.1. All members of miR-34 family, targeting more than 77 target mRNAs, were shown to suppress tumor growth and metastasis by inhibiting the processes that stimulate the cancer development, including cell cycle, EMT, metastasis, stemness and by promoting the processes that inhibit carcinogenesis, such as apoptosis (38). MiR-34 family is frequently decreased in expression in solid tumors including NSCLC and this is a prognostically negative factor. CpG hypermethylation of miR-34a has been reported in multiple types of cancer including NSCLC and could decrease the miR-34a expression level (39); also miR-34b/c was found to undergo specific hypermethylation-associated silencing and this has a role in the metastasis formation in NSCLC (40).

Gallardo *et al*. (41) examined the role of miR-34 in 70 patients who underwent surgical resection for NSCLC: miR-34a and miR-34b were significantly repressed versus paired normal tissue and low levels of miR-34a in tumor samples correlated with a high rate of relapse; the authors also reported that the miR34b expression was lower in NSCLC tissue compared to that in pericarcinous tissue and confirmed that lower miR34b expression was correlated with higher lymph node metastasis. In 2008, Migliore *et al*. (42) showed that miR 34 b-c (and miR 199) contribute to control MET activity: they first confirmed that these miRNAs bind to the 3'UTR of MET and then transfected human carcinoma cells with chemically synthesized 34b-c miRNA precursors showing a significant reduction of MET protein even in cells displaying overexpression and MET gene amplification; furthermore the miRNA34 transfected cells were unable to migrate and to scatter (to break intercellular junctions) in response to HGF. Conversely, the inhibition of these endogenous miRNAs, by use of antagomiRs, resulted in increased expression of MET.

p53 and MET

The tumor-suppressor protein p53 is a master regulator of the stress response and provides a key barrier to cellular transformation and tumorigenesis; the p53 activation results in cell cycle arrest, apoptosis, or senescence, depending on the cellular context and the type of stimulus (43). p53 is mutated in more than 50% of NSCLC (44) and the loss of p53 signaling leads to uncontrolled cellular division and apoptotic avoidance.

Table 1 Tumor suppressor miRNAs that target MET

Tumor suppressor miRNAs (MET inhibition)	Molecular mechanism	Some of other targets
Mir 34 family, (19)	Induces upregulation of p53	CDK4 - CCNE2 - CCND1- CDK6 - BCL2 B-SIRT1-YY1-BIRC5-JAG1-WNT1- NOTCH1, LEF1-WNT3-CTNNB1-LRP6- MTA2- TPD52- AXL- CD44 – NANOG- SOX2- MAP2K1-RRAS- PDGFRA- E2F3-MYB- ACSL1- LDHA IMPDH
Mir 130, (20-32)	Increases TRAIL sensitivity	EGFR-PPARc -GAX-HOXA5
Mir 103/203, (21-33)	Promote the mesenchimal to epithelial transition; downregulate expression of PKC-ε, SRC and Dicer	EGFR-MEF2D -CASK
MiR 27a, (22,34,35)	Down-regulates EGFR and Sprouty2	EGFR-PLK2-MAP2K4-SGPP1-Smad2
Mir 1, (23)	Promotes the mesenchimal-epithelial transition	FoxP1- HDAC4-Slug- PIK3CA- LASP1- TAGLN2-SRSF9- PTMA-PNP1-CCND2-CXCR4-CXCL12-ET-1
Mir 409-3p, (24)	Inactivates of AKT signaling by targeting c-MET	PHF10-ANG
Mir 449a, (25)	Cell cycle regulation; regulates migration and invasion	HDAC1-CDK6-CDC25A-pRb-E2F1
Mir 199a, (26,27,36)	Down-regulates MET proto-oncogene and also down-regulates ERK2	FZD7 -MYC; Tnfsf10- CD 44
Mir 7515, (28)	Reduces Akt and ERK1/2	p-Rb-CDK2-cyclin E
Mir 340, (29)	Inhibits MET and stimulates p27 accumulation	ROCK1-RHOA-CDH1

Table 2 Oncogenic miRNAs that target MET

Oncogenic miRNAs (MET stimulation)	Molecular mechanism	Some of other targets
Mir 222/221 cluster, (30)	Promotes cancer cell proliferation; impair TRAIL-dependent response. Repress PTEN and TIMP3 expression	EGFR-c-KIT-P27-CDKN1B-P57-CDKN1C-ESR1-TRSP1
Mir 30b/c, (31-37)	Implicated in EGFR TKI resistance	Smad1-Runx2 -p53
Mir 21, (30)	Represses negative regulators of the Ras/MEK/ERK, Ras/PI3K/Akt patways	EGFR-BCL2-MASPIN-PDCD4-PTEN-TPM1-RECK-RASA1- TIMP3

MET is a critical player in p53-mediated control of motility and invasion and antagonizes p53 through Mdm2 upregulation via PI3K-AKT and mTOR (45) (*Figure 2*); similarly the wild type of p53 negatively regulates MET expression by two mechanisms: suppression at the transcriptional level with inhibition of SP1 binding to MET promoter and transactivation of miR-34 (46). In fact the two genomic loci encoding the three miR-34 family members have each one a p53 binding site in their promoter and their expression is induced by oncogenic stress or DNA damage (47). They directly act repressing HDM4, a potent negative regulator of p53, which positively regulates Mdm2 to promote p53 degradation through polyubiquitination. So, p53 and miR-34 form a positive feedback loop to strengthen the downstream effects of p53 and these miRNAs seem to act in parallel with the other p53 effectors, creating a sort of signal strengthening (48).

Furlan *et al*. (49) identified for the first time in HCC cells the c-Abl as a signaling node interconnecting MET and p53 core pathways and showed that its inhibition impairs MET-dependent tumorigenesis. MET ensures cell survival through a new pathway in which c-Abl leads to wt

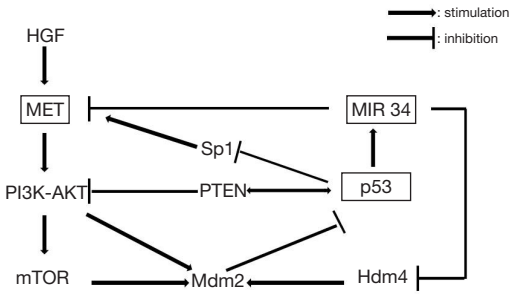

Figure 2 MET triggers signaling pathways that stimulate survival via PI3K-AKT and mTOR. Mdm2 promotes p53 poliubiquination. P53 and miR-34 family form a positive feedback to inhibit MET. PTEN is one of the targets of p53 and antagonizes PI3K activity.

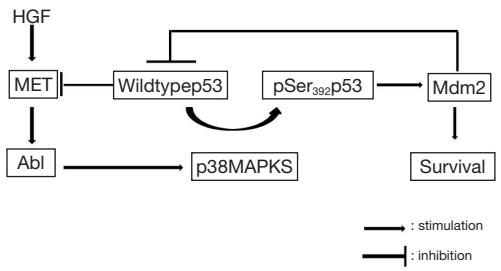

Figure 3 MET acts on c-Abl that by p38MAPKs leads to p53 phosporylation on Ser 392 and Mdm2 up regulation.

p53 phosphorylation on Ser392 by p38-MAPK, leading to Mdm2 upregulation and finally stimulating survival and tumorigenesis (*Figure 3*).

Wang *et al*. (50) confirmed these results in lung cancer cells, showing that there is an association between lower miR34b and overexpression of Phospho-MET, Phospho p53 (on S392) and Mdm2.

The authors noticed also that there was no increased expression levels of miR-34b corresponding to the overexpression of $p53^{S392}$ observed in the samples with NSCLC, probably because the phosphorylation on S392 decrease the transcriptional activities of p53 in lung cancer tissues and its capacity to activate miR-34 family.

Okada *et al*. (51) discovered another mechanism that human lung adenocarcinoma acts escaping miR-34 control: the cancer cells are able to generate an elevated level of a short HDM4 isoform that lacks miR-34-binding sites evading thereby miR-34 regulation to disable the p53-miR-34 positive feedback.

This is one of the mechanisms known as p53 mutations, deregulation of miR-34 through either genomic deletion or promoter hypermethylation to disrupt the p53-miR-34 positive feedback and ultimately contribute to tumor development.

MiR 222/221 and MiR 130a

MiR-222/221 cluster is among the most dysregulated miRNA implicated in cancer.

The expression of miR-222/221 is highly upregulated in a variety of solid tumors and their oncogenic role is known in NSCLC and many others cancers. In 2008, Garofalo *et al*. (52) reported that NSCLC cells overexpressing miR-221&222 are TRAIL-resistant and show an increase in migration and invasion capabilities. The TNF-related apoptosis inducing ligand (TRAIL) is a cytokine of the TNFα family that induces apoptosis by binding to death receptors (DR) 4 and 5 on the cell surface, which then leads to a cascade of death-inducing signaling complex (DISC) formation, caspase activation and ultimately the execution of the apoptotic program.

The same authors founded that MET, through the upregulation of miR 221&222 expression, confer resistance to TRAIL-induced-cell death and enhance tumorigenicity of lung and cancer cells (53). Indeed, upon HGF stimulation, MET increased phosphorylation of ERK1/2 and JNK; phosphorylated JNKs activate the oncoprotein c-Jun, which forms the activator protein-1(AP-1) transcription factor as a homodimer; they show that c-Jun has one binding site in the miR 221/222 promoter region and that miR 221&222, after c-Jun stimulation, represses *PTEN* and *TIMP3* expression (*Figure 4*).

PTEN is an oncosuppressor that acts directly antagonizing the activity of PI3 kinase (PI3K) (54). Its inactivation results in constitutive activation of the PI3K/AKT pathway and in a subsequent increase in protein synthesis, cell cycle progression, migration and survival; TIMP3 (55) is a member of the tissue inhibitors of metalloproteinases (MMPs) that inhibits the activity of MMPs by binding with a 1:1 stoichiometry to the active site. Previous studies (56,57) have shown that the overexpression of TIMP3 in vascular smooth muscle cells and melanoma cell lines inhibits invasion and promotes apoptotic cell death.

In 2012, Acunzo *et al*. (58) showed that miR-130, an oncosuppressor expressed at low level in lung cancer cell lines, was able to increase TRAIL sensitivity in NSCLC TRAIL resistant cell lines; miR 130a, decreasing MET expression, is able to reduce the binding of c-Jun to the

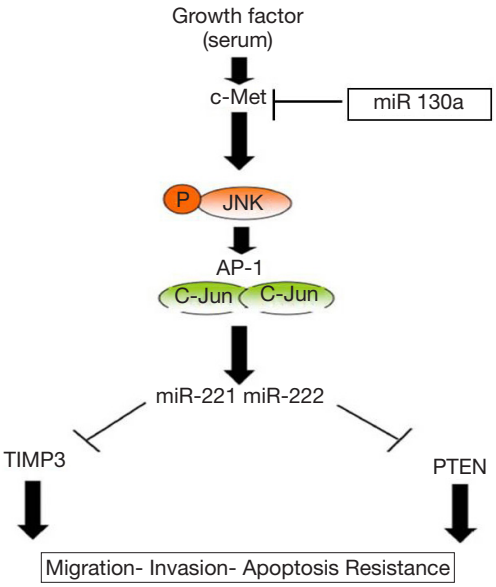

Figure 4 MiR 130a, reducing MET expression, is able to stop the binding of c-Jun to the miRNA-221 and miRNA-222 promoter region increasing TRAIL sensitivity and inhibiting migration and invasion. Adapted from MiR-221&222 regulate TRAIL-resistance and enhance tumorigenicity through PTEN and TIMP3 down-regulation (53).

miRNA-221 and miRNA-222 promoter region inducing TRAIL sensitivity in NSCLC cells.

miRNAs targeting MET implicated in EGFR resistance

The epidermal growth factor receptor, EGFR (ErbB1/Her-1), is a member of the ErbB family of tyrosine kinase growth factor receptors that is frequently found to be activated by mutation or amplification in epithelial malignancies. In lung cancer it is mutated in approximately 10% of Caucasian patients and 30% of Asian patients. The EGFR tyrosine kinase inhibitors (TKIs) are effective clinical therapies for advanced NSCLC patients with EGFR activating mutations; however, despite initial dramatic benefits from EGFR TKIs, all of these patients ultimately develop resistance (referred to as acquired resistance) to these agents. In EGFR mutant lung cancers more than 50% of resistance cases are due to the occurrence of a secondary T790M mutation in EGFR. Amplification of the MET receptor has also been shown to maintain ERBB3/PI3K/AKT signaling in presence of gefitinib and cause resistance to EGFR targeted therapies in approximately 20% of NSCLC patients (59). In fact, clinical trials using combined EGFR and MET inhibitors in NSCLC patients with acquired resistance to gefitinib/erlotinib are currently underway. Garofalo *et al*. (60) studied the miRNAs implicated in EGFR resistence: to identify EGFR- and MET-regulated-miRNAs, they first silenced EGFR and MET in Calu-1 cells (a NSCLC cell line) using shRNA lentiviral particles and found 8 miRNAs that are regulated by both EGFR and MET (miRNA 221-222-30b/c-21-29a/c-100). Then they focused on miR-30b-c and miR 221-222 showing the highest downregulation after both MET and EGFR silencing: they demonstrated that in sensitive cells the gefitinib treatment triggers programmed cell death through downregulation of miR 30b-c and miR 221-222 and the consequent upregulation of APAF-1 (apoptotic peptidase activating factor 1) and BIM (BCL2-like 11); MET overexpression is able to induce resistance to gefitinib through the upregulation of miR 30b-c and miR 221-222 making uneffective the EGFR inhibition alone. They also showed that this resistance could be overcome using MET inhibitors or anti-miRNA 221-222 and anti-30c which strongly increase gefitinib-sensitivity in xenograft mouse models *in vivo*.

Another oncoMir regulated by both EGFR and MET is miR-21; data from transgenic tumor models show that it drives tumorigenesis by repressing negative regulators of the Ras/MEK/ERK, Ras/PI3K/Akt and Ras/RalGDS/JNK pathways (61); when it is overexpressed it represses *PTEN* inducing gefitinib resistance (62), while its blockade is able to reverse the EMT phenotype associated with EGFR-TKI resistance (63).

Recently Shen *et al*. (64) showed that the upregulation of miR-21 and the downregulation of *PTEN* in tumor tissues from human NSCLC patients negatively correlate with shorter disease free survival; furthermore, by analyzing miR-21/*PTEN* expression levels and clinical response to TKI treatment in 46 NSCLC patients, they found that high miR-21 expression levels and low PTEN protein levels were associated with poor clinical response to TKIs and shorter overall survival.

When miR-21 was knockdown, the TKI sensitivity was restored both *in vitro* and *in vivo* (mouse models), by inactivation of ERK and AKT pathways and PTEN upregulation. MiR 103 and miR 203 work as tumor suppressors promoting the mesenchymal to epithelial transition (65). They are upregulated after MET silencing, inducing apoptosis in gefitinib-resistant NSCLC. They reduce mesenchymal markers and increase epithelial cell junction proteins, by downregulating the expression of

PKC-ε, SRC and Dicer that exert pro-survival effects and contribute to gefitinib resistance by activating AKT and ERK signaling pathways (66,67).

MiR 27a is an oncosuppressor that is tipically downregulated in NSCLC patients (68); it belongs to the miR-23a-27a-24-2 cluster, whose expression is induced through ELK1 after HGF binding to MET.

Recently, Acunzo *et al*. (69) have showed that MiR-27a negatively regulates MET and EGFR in NSCLC with direct and indirect mechanisms: it directly targets MET and EGFR 3UTRs downregulating their expression; miR-27a also downregulates Sprouty2, that normally increases MET and EGFR levels by attenuating their degradation through ubiquitination.

The lack of miR 27 regulatory action on Met-EGFR axis can then lead to an uncontrolled proliferation.

Mir130a, as we have seen in the previous section, is an oncosuppressor that increases TRAIL sensitivity directly targeting MET and it is also implicated in the EGFR resistance.

Zhou *et al*. (70) demonstrated that miR-130a expression was negatively correlated with that of MET and that is overexpressed in gefitinib-sensitive NSCLC cell lines, but is low in gefitinib-resistant NSCLC cell lines.

This group also showed that overexpression of miR-130a increased cell apoptosis and inhibited proliferation of NSCLC cells treated with gefitinib, whereas lowering the expression of miR-130a decreased cell apoptosis and promoted cell proliferation after treatment with gefitinib in both gefitinib-sensitive and -resistant NSCLC cell lines; they also demonstrated that miR-130 overcomes gefitinib resistance downregulating MET protein levels.

Zhou *et al*. (71) assessed the anti-tumour effect of miR-34a in a gefitinib- resistant lung cancer mouse model and showed that the combination of miR-34a and gefitinib caused dramatic regression of tumours compared with either gefitinib or miR-34a monotherapy. In the mouses treated with the combination therapy they found that the expression of MET and p-ERK were decreased, suggesting that miR-34 replacement therapies might sensitize resistant tumors to EGFR-TKIs by suppressing MET expression and its activation of oncogenic signaling pathways.

Therapeutic applications of miRNAs targeting MET

The increasing evidence of the important role of miRNAs in cancer development and the capacity of a single miRNA to control many known oncogenes have strengthen the interest in using miRNAs in cancer treatment.

miRNA therapeutic approaches can be divided into two different categories: miRNA inhibition therapy when the target miRNA is overexpressed and miRNA replacement therapy when the miRNA is repressed.

miRNA antagonists are generated to inhibit endogenous miRNAs that show a gain-of-function in diseased tissues. This technique involves the introduction of a highly chemically-modified miRNA passenger strand (anti-miR) that binds with high affinity to the active miRNA strand. Since binding is frequently irreversible, the new miRNA duplex is unable to be processed by RISC and/or degraded.

By reintroducing a tumor suppressor miRNA, miRNA replacement therapy seeks to restore a loss-of-function in cancer and to reactivate cellular pathways driving a therapeutic response.

Although successful delivery is an obstacle to effective miRNA-based therapeutics, new findings from recent trials and the rapid advances in systemic drug delivery systems led to a rapid progress in this field (72).

In 2010, Wiggins *et al*. (73) described the development of a therapeutic formulation using chemically synthesized miR-34a and a lipid-based delivery vehicle. They first demonstrated that intratumoral delivery of formulated miR-34a block lung tumor growth in mice inducing a specific inhibitory effect in tumor cells with an accumulation of miR-34a and concurrent repression of its direct target genes (MET, CDK4 and Bcl-2); then they evidenced that, similarly to the intratumoral injection of miR-34a, also the intravenous delivery of formulated miR-34a specifically blocked the tumor growth and did not induce an elevation of liver and kidney enzymes and an immune response. They also noticed that miR-34a inhibited tumor cells that showed normal levels of endogenous miR-34a, indicating that the therapeutic application of tumor suppressor miRNAs is not limited to replacement.

Also Xue *et al*. (74) recently investigated the effects of Mir-34a delivery on lung cancer development in a genetically engineered mouse model (called "KP") of lung cancer based on loss of p53 and Kras activation.

Tumor bearing KP mice were treated with intravenous Mir-34a in a lipid/polimer nanoparticle at a dose of 1.5 mg/kg twice each week for 4 weeks and 10 weeks after the first injection were scanned with microCT. They noticed that the therapeutic delivery of Mir-34a delayed tumor progression compared with control animals and tumors showed reduced levels of ki67 without increasing toxicity. They also demonstrated that the combination of

Mir-34 and siRNA targeting Kras is possible and improved the therapeutic response compared to miR-34a or siKras alones. Furthermore, they analized the combination of chemotherapy with nanoparticles: they treated tumor bearing Kp mice with either cisplatin, nanoparticles carrying both Mir-34a mimics and siKras or a combination of the two. The KP mice treated with the combination therapy survived significantly longer than the others, suggesting that restoring p53 functions with Mir-34a could be important to obtain an improved response to chemotherapy.

Xu *et al*. (75) used synthesized locked nucleic acid (LNA) antimiR-21 (Oncomir regulated by both c-MET and EGFR) showing the increased sensitivity of lung cancer cells to Cisplatin *in vitro* and *in vivo* and confirming that the combining chemotherapy with anti-Mir (or suppressor MiR) might be a potential strategy for the treatment of human NSCLC.

A combinatorial therapeutic approach was also studied by Kasinski *et al*. (76) who used for the first time a combination of two tumor suppressive miRNAs: the well-known miR 34a and the let7 (that targets Kras, cMYC, HMGA2 and the LIN 28 isoforms). They treated the aggressive "KP" lung cancer mouse models with systemic nanodelivery of both miRNAs showing that it is feasible and that they act reducing tumor proliferation and invasiveness in a synergistic manner: the average tumor volumes from the dual treated animals were significantly smaller than the average volumes from each of the single miRNA treated group. They show also that the combinatorial approach was able to downregulate more target genes than the use of each miRNA alone, suggesting that the combinatorial treatment, by simultaneously targeting multiple key factors in the cancer growth, could overcome the development of secondary resistance.

In summary, miRNA-based therapy has a great potential to be a more powerful tool in tumor treatment by the simultaneous modulation of multiple genes involved in distinct tumor-related signaling networks. Actually the first clinical trial with microRNA replacement therapy in cancer is ongoing: this is a phase I study (77) with miR34 (MRX34) in patients with liver cancer or solid cancer with liver involvement. It is delivered using a liposomal formulation and the first safety data from 26 patients show that it has a manageable safety profile.

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