Title: *Bona fide* targets of deregulated microRNAs in non-small cell lung cancer as tool to identify novel therapeutic targets. A review.

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Abstract: *Background*: Non-small-cell lung cancer (NSCLC) is an aggressive neoplasm with a poor survival and novel therapies are urgently needed. The study of deregulated micro-RNAs (dereg-miRs) could constitute a strategy helping to detect specific genes playing a relevant role in the disease. Thus, the oncoproteins encoded by these genes could be exploited as novel therapeutic targets to be inhibited by small molecules, aptamers, or monoclonal antibodies. *Methods*: The present review is focused on candidate genes having convincing biological evidences to be both *bona fide* targets for dereg-miRs and playing a role in NSCLC progression. These genes were evaluated according to the molecular pathway they belong. Moreover, in the attempt to provide an even broader list of candidate therapeutic targets for NSCLC, the full list of genes was analyzed using the online tool Interactome DB. *Results*: Among the identified targets, some of them belong to p53 or MAP kinase signaling pathways, and others include caspases, MCL1, and BCL2L2 (playing a role in apoptosis), ZEB1, ZEB2, and USP25 (epithelial-to-mesenchymal transition), EZH2, SOX9, and HOXA5 (differentiation), Paxillin, LIMK1 and MTDH (cytoskeleton remodeling), and HDGF (angiogenesis). In addition, other targets, such as TIMP-2, PIM-1, and components of the IGF-signaling pathways were suggested following the interactome analysis. *Conclusion*: Studies on dereg-miRs helped to identify a set of genes whose encoded proteins could constitute candidates for future therapeutic approaches.

Keywords: NSCLC, deregulated micro-RNAs, target genes, therapeutic targets, targeted therapy.

1. INTRODUCTION

Non-small-cell lung cancer (NSCLC) accounts for about 80% of all lung cancer cases. The development of NSCLC, as for the other cancers, is considered a multi-step process [1]. Early studies identified somatic mutations in genes involved in the development of this disease and led to the discovery of crucial activated oncogenes and abnormally deregulated signaling pathways. Moreover, changes in the expression of epigenetic regulators, such as micro-RNAs (miRNAs), were found to play an important role [2]. MiRNAs are short noncoding RNAs of 22–27 nucleotides that regulate gene expression through binding to cognate sequences, preferentially the 3′-untranslated regions (UTR) of mRNAs. MiRNAs usually act as negative regulators inhibiting the translation of a mRNA targets through two proposed mechanisms: (i) miRNAs can stop the beginning of translation by repressing the m7G cap recognition by eIF4E (thus preventing the recruitment of 40S ribosomal subunit or interfering with the 60S subunit joining); (ii) miRNAs can interfere with translation after the very initial steps by repressing ribosome elongation or inducing ribosome drop-off and nascent protein chain degradation. Any of these mechanisms could lead to a decrease of protein but not mRNA levels. Recent papers also showed that miRNAs are involved in the decrease of target mRNAs levels, directing the transcripts towards the degradation machinery [3]. As mentioned, the main miRNA binding sites are located within the 3'-UTRs of target mRNAs. Despite this, binding sites were found also within coding sequences and 5'-UTRs [4-7]. The involvement of miRNAs in the carcinogenesis has been demonstrated for the first time in 2002, when the cluster of miR-15 and miR-16 (at 13q14.3) was identified as a frequently deleted region in chronic lymphocytic leukemia (CLL) [8]. Nowadays, the role of miRNAs in cancer is well acknowledged with some having a tumor-suppressor activity (mainly when they inhibit oncogenes) and others acting as oncogenes (also called onco-miRs, when they inhibit tumor-suppressor genes). Figure 1 resumes these mechanisms [9]. The first evidence of the involvement of a miRNA (let-7) in NSCLC is dated 2004 [10].

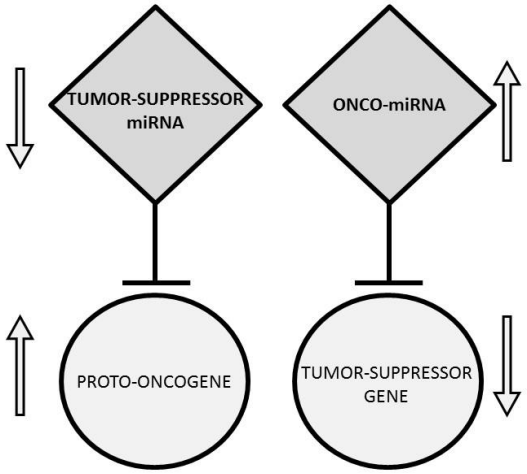

Figure 1. MicroRNAs: tumor suppressors and oncogenes.

NSCLC is often diagnosed at an advanced stage and shows a poor prognosis with only less than 15% of patients surviving beyond 5 years [11]. Current therapies have a limited success being NSCLC poorly sensitive to most of the available agents and to radiotherapy with response rates ranging from 10 to 25% [12]. Thus, understanding molecular mechanisms that control NSCLC growth and metastasis could help to develop novel therapeutic strategies improving the prognosis of these patients [13]. A way to reach this goal is to identify deregulated miRNAs (dereg-miRs; i.e. miRNAs up- or down-regulated) in NSCLC tissues as compared to their adjacent normal counterparts (ideal control) or to healthy tissues from different subjects. In fact, the deregulation of a specific miRNA affects many target genes and some of them could play a role in cancer progression. For this reason, the study of dereg-miRs could constitute a way for detecting novel cancer genes to be used for targeted therapy. For example, if the expression of a miRNA is lost causing an aberrant over-expression of specific oncogenes, one could hypothesize, as therapeutic approach, to suppress/inhibit with small molecules, aptamers, or monoclonal antibodies the oncoproteins encoded by these genes.

2. A BRIEF OVERVIEW OF PUBLISHED METHODS FOR IDENTIFYING CANCER GENES FROM DEREG-MIRS IN NSCLC

In this section we will briefly summarize the typical experiments reported in literature aimed to find interesting candidate therapeutic targets for NSCLC, as a consequence of the identification of dereg-miRs. Dereg-miRs can be identified with several updated methods, including microarrays or deep sequencing [14]. They, usually, are validated on an independent series of NSCLC tissues with standard qRT-PCR [15]. However, it is a wise strategy to further confirm these findings by extending the analyses on panels of NSCLC cell lines [16]. These approaches allow focusing on a smaller set of dereg-miRs truly relevant in sustaining the malignant phenotype, easing the successive work-flow. Once dereg-miRs are identified, typically the study continues with *in silico* analyses. Actually, the prediction algorithms are the most obvious, economic, and practical tools for target screening and they are an invaluable instrument for preliminary analyses and for "omics" investigations linking miRNAs and targets each other in complex networks. The list of tools is broad and it includes, among the others, miRanda (http://www.microrna.org), TargetScan (http://www.targetscan.org), PicTar (http://www.pictar.org), and miRWalk2.0 (http://www.zmf.umm.uni-heidelberg.de/apps/zmf/mirwalk2) [17].

However, there are several problems with the identification of miRNA-targets. Firstly, each dereg-miR has dozens or even hundreds of targets. Moreover, different prediction algorithms yield different results. Thus, the accumulation of biological evidences is needed in order to consider a candidate target as *bona fide* target for a given dereg-miR.

The most used test to verify the miRNA::mRNA interaction is the "dual luciferase assay". Cells grown *in vitro* are co-transfected with synthetic miRNAs and with vectors carrying reporter genes (e.g. Firefly luciferase) chimerized with the 3'-UTR of the candidate target. The drop of the expression of the reporter gene caused by the co-transfected miRNA suggests that the miRNA is inhibiting the chimeric target [18]. When a given 3'-UTR is found positive to this assay, a series of experiments is performed by down-regulating (with antago-miRs or miRNA inhibitors) [16] or by mimicking the over-expression of the endogenous miRNA (with an ectopic transfection of the pre-miR or short-hairpin RNA) [19] and measuring the mRNA/protein expression of the candidate target. Typically, an inverse relation between miRNA and target is expected to confirm their interaction. In addition, the same inverse correlation is also sought in tissues (both NSCLC and normal) [16]. When positive results are obtained in these experiments, the candidate gene could be considered a *bona fide* target for a specific dereg-miR. However, this fact does not imply necessarily that the target gene is also relevant for the disease.

Thus, since a target can be regulated by different miRNAs, another important aspect to investigate is when several miRNAs, insisting on the same target, are deregulated. In fact, implicitly, this type of redundancy could reveal that the target is truly important for the disease. For this reason, once the candidate gene is detected, more experimental evidences (ranging form *in vitro* analyses to experiments on animal models) need to be obtained. Thus, series of *in vitro* phenotypic assays are performed to evaluate whether the up- or down-regulation of the miRNA or of its target can affect the proliferation, the cell cycle, the migration, the adhesion, the invasion, the apoptosis, the senescence (and so on) in various cell lines. Sometimes the study progresses in *in vivo*. For example, nude SCID mice are xenografted with NSCLC cell lines stably expressing specific miRNAs or their inhibitors and the effect on metastasizing capacity is measured together with the level of expression of the target (at mRNA and protein level). Other times, the high-/low-expression of dereg-miRs or targets (measured in tissues) is related also to prognostic factors (e.g. the overall survival).

3. METHODS AND REVIEWED STUDIES

The works reviewed here, reported in Table 1, were searched in Pubmed (http://www.ncbi.nlm.nih.gov/pubmed) using the following keywords: (micro-rna OR microrna OR mirna OR mi-rna) AND therapeutic target AND non-small cell lung [ti] AND (cancer OR carcinoma OR neoplasia). The same table also reports the full list of candidate targets of dereg-miRs. However, as consequence of the work-flow illustrated before, it appears obvious that only a fraction of predicted targets are *bona fide* targets and among them, only a minority will be relevant for NSCLC. Thus, the present review will not discuss all predicted targets of dereg-miRs described in NSCLC. Rather, it will be focused only on those having convincing biological evidences to be both *bona fide* targets for dereg-miRs and playing an active role in NSCLC progression.

These cancer genes, candidate for a targeted therapy, will be reviewed and discussed in the following sections according to the molecular pathway they belong.

Moreover, in the attempt to provide an even broader list of candidate therapeutic targets for NSCLC, the full list of genes of table 1 was used for an interactome analysis, using the online tool Interactome DB (http://www.mirob.interactome.ru; [20]). It should be stressed here that these methods allowed identifying also known cancer genes, giving a confirmation that the analysis of dereg-miRs could constitute an effective strategy for the detection of novel true targets relevant for NSCLC carcinogenesis.

Table1. List of dereg-miRs in NSCLC and their candidate targets. The first column reports the miRNA found deregulated in NSCLC tissues (irrespectively to their validation on NSCLC cell lines). The second shows the direction of deregulation (↑ up- or ↓ down-) as compared to non-malignant lung tissues. The fourth illustrates the target genes and the remaining columns reports what type of evidences support the identification of a given target (*in silico*, *in vitro*, *in vivo*-human, *in vivo*-mouse). The last column shows the bibliographic references of these studies.

Dereg-miRs discovered in NSCLC		**Was the miRNA studied in relation to diagnosis or prognosis?**	**Candidate Target Gene**	***In silico analysis***	***In vitro validation of the target***	**Human: *mRNA analysis in vivo***	***In vivo exp. on animal models***	**Was the target studied in relation to diagnosis or prognosis?**	**Reference**
miR-153	↓	no	*ADAM19*	yes	yes	yes	no	yes	Shan,2015 [229]
miR-185	↓	no	*AKT1*	yes	yes	yes	yes	no	Li,2015 [45]
miR-99a	↓	yes	*AKT1*	yes	yes	yes	yes	no	Yu,2015 [66]
miR-486-5p	↓	yes	*ARHGAP5*	yes	yes	yes	yes	no	Wang,2014 [230]
miR-143	↓	no	*ATG2B*	yes	yes	no	no	no	Wei,2015 [231]
miR-150	↑	no	*BAK1*	yes	yes	yes	no	no	Gu,2014 [232]
miR-21	↑	no	*BCL2*	yes	yes	no	no	no	Xu,2014 [55]
miR-15a	↓	yes	*BCL2L2*	yes	yes	yes	no	no	Yang,2015 [90]
miR-203	↓	no	*BMI1*	yes	yes	yes	no	no	Chen,2015 [233]
miR-137	↓	no	*BMP7*	yes	yes	yes	no	no	Yang,2015 [155]
miR-224	↑	no	*CASP3*	yes	yes	yes	no	yes	Cui,2015 [86]
miR-224	↑	no	*CASP7*	yes	yes	yes	no	yes	Cui,2015 [86]
miR-25	↑	no	*CDC42*	yes	yes	yes	yes	no	Yang,2015 [234]
miR-137	↓	no	*CDC42*	yes	yes	no	yes	no	Zhu,2013 [235]
miR-544a	↑	no	*CDH1*	yes	yes	no	no	no	Mo,2014 [236]
miR-223	↓	no	*CDK2*	yes	yes	no	yes	no	Nian,2013 [237]
miR-613	↓	no	*CDK4*	yes	yes	yes	yes	no	Li,2016 [238]
miR-137	↓	no	*CDK6*	yes	yes	no	yes	no	Zhu,2013 [235]
miR-512-5p	↓	no	*CDKN1A*	yes	yes	yes	no	no	Chu,2016 [239]
miR-148b	↓	no	*CEA*	yes	yes	yes	no	no	Liu,2014 [240]
miR-195	↑	yes	*CHEK1*	yes	yes	yes	no	yes	Liu,2015 [241]
miR-375	↑	yes	*CLDN1*	yes	yes	yes	no	yes	Yoda,2014 [242]
miR-522	↑	no	*DENND2D*	yes	yes	yes	no	no	Zhang,2016 [243]
miR-126	↓	no	*EGFL7*	no	yes	no	yes	no	Sun,2010 [244]
miR-330-3p	↑	no	*EGR2*	yes	yes	yes	no	no	Liu,2015 [245]
miR-7	↑	yes	*ERF*	yes	yes	no	yes	no	Chou,2010 [256]
miR-30a	↓	no	*EYA2*	yes	yes	yes	no	no	Yuan,2016, [247]
miR-101	↓	no	*EZH2*	yes	yes	yes	no	no	Zhang,2011 [123]
miR-138	↓	no	*EZH2*	yes	yes	yes	yes	no	Zhang,2013 [121]
miR-25	↑	no	*FBXW7*	yes	yes	yes	no	no	Xiang,2015 [248]
miR-99b	↓	no	*FGFR3*	yes	yes	yes	no	no	Kang,2012 [249]
miR-134	↓	no	*FOXM1*	yes	yes	no	no	no	Li,2012 [102]
miR-149	↓	no	*FOXM1*	yes	yes	no	no	no	Ke,2013 [103]
miR-183	↓	no	*FOXO1*	yes	yes	no	yes	no	Zhang,2015 [78]
miR-138	↓	no	*GIT1*	yes	yes	no	no	no	Li,2015 [250]
miR-675-5p	↓	yes	*GPR55*	yes	yes	yes	no	no	He,2015 [251]
miR-16	↓	no	*HDGF*	yes	yes	yes	no	no	Ke,2013 [168]
miR-195	↓	no	*HDGF*	yes	yes	yes	no	no	Guo,2014 [169]
miR-497	↓	yes	*HDGF*	yes	yes	yes	yes	no	Zhao,2014 [170]
miR-199a	↓	yes	*HIF1A*	no	yes	no	yes	no	Ding,2013 [252]
miR-142-3p	↓	no	*HMGB1*	yes	yes	yes	no	no	Xiao,2015 [253]
miR-1271	↑	no	*HOXA5*	yes	yes	yes	no	no	Wang,2015 [135]
miR-196a	↑	yes	*HOXA5*	yes	yes	yes	no	no	Liu,2012 [134]
miR-196b	↑	no	*HOXA9*	yes	yes	yes	no	no	Yu,2015 [254]
miR-140	↓	no	*IGF1R*	yes	yes	yes	yes	no	Yuan,2013 [203]
miR-195	↓	no	*IGF1R*	yes	yes	yes	no	no	Wang,2014 [204]
miR-30a	↓	no	*IGF1R*	yes	yes	yes	no	no	Wen,2015 [205]
miR-223	↓	no	*IGF1R*	yes	yes	no	yes	no	Nian,2013 [206]
miR-503	↓	yes	*IKBKB*	yes	yes	yes	yes	no	Yang,2014 [63]
miR-761	↑	yes	*ING4*	yes	yes	yes	no	no	Yan,2015 [255]
miR-23a	↓	no	*IRS1*	yes	yes	yes	no	yes	Cao,2014 [104]
miR-205	↓	no	*ITGA5*	yes	yes	no	yes	no	Larzabal,2014 [256]

miR-125b	↓	no	*KLC2*	yes	yes	yes	no	yes	Wang,2015 [257]
miR-135a	↓	yes	*KLF8*	yes	yes	yes	no	no	Shi,2015 [101]
miR-181a-5p	↓	no	*KRAS*	yes	yes	yes	yes	no	Ma,2015 [53]
Let-7	↓	yes	*KRAS*	yes	yes	yes	no	yes	Xia,2010, [54]
miR-31	↑	yes	*LATS2*	yes	yes	yes	yes	no	Liu,2010 [289]
miR-1238	↓	no	*LHX2*	yes	yes	yes	no	no	Shi,2015 [258]
miR-143	↓	yes	*LIMK1*	yes	yes	yes	no	no	Xia,2014 [152]
miR-27b	↓	no	*LIMK1*	yes	yes	yes	no	no	Wan,2014 [153]
miR-630	↓	no	*LMO3*	yes	yes	yes	no	no	Song,2015 [259]
miR-101	↓	yes	*MCL1*	yes	yes	yes	no	yes	Luo,2012 [87]
miR29b	↓	no	*MDM2*	yes	yes	no	no	no	Avasarala,2013 [31]
miR-660	↓	yes	*MDM2*	yes	yes	yes	yes	no	Fortunato,2014 [30]
miR-140-5p	↓	no	*MMD*	yes	yes	yes	yes	no	Li,2014 [260]
miR‑133a	↓	no	*MMP14*	yes	yes	no	no	no	Xu,2013 [261]
miR-25	↑	no	*MOAP1*	yes	yes	yes	no	no	Wu,2015 [262]
miR-125a-3p	↓	no	*MTA1*	yes	yes	yes	no	no	Zhang,2015 [177]
miR-30c	↓	no	*MTA1*	yes	yes	yes	no	yes	Xia,2013 [178]
miR-495	↓	no	*MTA3*	yes	yes	yes	no	no	Chu,2014 [263]
miR-193a-3p	↓	yes	*MTDH*	yes	yes	yes	no	yes	Ren,2015 [160]
MiR-145	↓	yes	*MTDH*	yes	yes	yes	no	yes	Wang,2015 [161]
miR-184	↓	no	*MYC*	yes	yes	no	no	no	Liu,2014 [74]
MiR-449c	↓	no	*MYC*	yes	yes	no	yes	no	Miao,2013 [70]
miR-124	↓	no	*MYO10*	yes	yes	yes	no	no	Su,2015 [264]
miR-24	↑	yes	*NAIF1*	yes	yes	yes	yes	yes	Zhao,2015 [265]
mir-365	↓	yes	*NKX2-1*	no	no	yes	no	no	Sun,2015 [266]
miR-152	↓	no	*NRP1*	yes	yes	yes	no	no	Zhang,2015 [267]
miR‑326	↓	no	*NSBP1*	yes	yes	yes	no	no	Li,2016 [268]
miR-138	↓	no	*PDK1*	yes	yes	no	no	no	Ye,2015 [269]
miR-212	↓	no	*PED*	yes	yes	yes	no	no	Incoronato,2010 [270]
miR-205	↑	no	*PHLPP2*	yes	yes	yes	yes	no	Cai,,2013 [57]
miR-503	↓	yes	*PIK3*	yes	yes	yes	yes	no	Yang,2014 [63]
miR-486-5p	↓	no	*PIM1*	yes	yes	yes	no	yes	Pang,2014 [221]
miR-296-5p	↓	no	*PLK1*	no	yes	yes	no	no	Xu,2016 [271]
miR-136	↑	yes	*PPP2R2A*	yes	yes	yes	no	no	Shen,2014 [272]
miR-143	↓	no	*PRKCE*	yes	yes	yes	no	no	Zhang,2013 [273]
miR-7	↓	yes	*PSME3*	yes	yes	yes	yes	yes	Xiong,2014 [274]
miR-106a	↑	yes	*PTEN*	yes	yes	yes	no	no	Xie,2015 [56]
miR-205	↑	no	*PTEN*	yes	yes	yes	yes	no	Cai,2013 [57]
miR-21	↑	no	*PTEN*	yes	yes	no	no	no	Xu,2014 [55]
miR-26b	↓	no	*PTGS2*	yes	yes	yes	no	no	Xia,2015 [275]
miR-340	↓	no	*PUM1*	yes	yes	yes	no	no	Fernandez,2015 [276]
miR-340	↓	yes	*PUM2*	yes	yes	yes	no	no	Fernandez,2015 [282]
miR-137	↓	no	*PXN*	yes	yes	no	no	no	Bi,2014 [144]
miR-218	↓	yes	*PXN*	yes	yes	yes	no	no	Wu,2010 [145]
miR-342-3p	↓	no	*RAP2B*	yes	yes	yes	yes	no	Xie,.2015 [277]
miR-92b	↑	no	*RECK*	yes	yes	yes	no	no	Lei,2014 [191]
miR-21	↑	no	*RECK*	yes	yes	no	no	no	Xu,2014 [192]
miR-186	↓	no	*ROCK1*	yes	yes	yes	no	no	Cui,2014 [278]
miR-138	↓	no	*SEMA4C*	yes	yes	no	no	no	Li,2015 [256]
miR-340	↓	no	*SKP2*	yes	yes	yes	no	no	Fernandez,2015 [276]
miR-137	↓	yes	*SLC22A18*	yes	yes	yes	no	yes	Zhang,2015 [279]
miR-224	↑	yes	*SMAD4*	yes	yes	yes	yes	no	Cui,2015 [80]
miR-19a	↑	no	*SOCS1*	yes	yes	yes	yes	no	Wang,2015 [281]
miR-132	↓	no	*SOX4*	yes	yes	no	yes	no	Li,2015 [287]
miR-206	↓	no	*SOX9*	yes	yes	yes	no	no	Zhang,2015 [131]
miR-32	↓	yes	*SOX9*	yes	yes	yes	no	no	Zhu,2015 [132]
miR-211	↑	no	*SRCIN1*	yes	yes	yes	no	no	Ye,2015 [282]
miR-124	↓	yes	*STAT3*	yes	yes	yes	no	yes	Li,2015 [283]
miR-489	↓	yes	*SUZ12*	yes	yes	yes	no	yes	Xie,2015 [284]
miR-200c	↑	no	*THBS1*	yes	yes	yes	yes	no	Pacurari,2013 [50]
miR-761	↑	yes	*TIMP2*	yes	yes	yes	no	no	Yan,2015 [261]
miR-429	↑	no	*TIMP2*	yes	yes	yes	no	no	Lang,2014 [285]
miR-224	↑	yes	*TNFAIPI*	yes	yes	yes	yes	no	Cui,2015 [80]
miR-150	↑	no	*TP53*	yes	yes	yes	no	no	Zhang,2013 [23]
miR-125b	↑	no	*TP53INP1*	yes	yes	yes	yes	yes	Li,2015 [33]
miR-19	↑	no	*TP53INP1*	yes	yes	no	no	no	Yamamoto,2015 [36]
miR-33a	↓	yes	*TWIST1*	yes	yes	yes	yes	no	Yang,2015 [100]
miR-200c	↓	yes	*USP25*	yes	yes	yes	yes	no	Schliekelman,2011 [51]
miR-4295	↓	yes	*USP28*	yes	yes	yes	yes	no	Zhang,2015 [286]
miR-200c	↓	yes	*ZEB1*	yes	yes	yes	yes	no	Schliekelman,2011 [51]

miR-132	↓	yes	*ZEB2*	yes	yes	yes	yes	no	You,2014 [287]
miR-215	↓	yes	*ZEB2*	yes	yes	yes	yes	no	Hou,2015 [288]

4. TP53 PATHWAY

TP53 pathway components, in particularly p53 itself, its negative regulatory MDM2 and the protein kinase TP53INP have been shown to play an important role in NSCLC. More than twenty years ago p53 was acknowledged as the "guardian of the genome", referring to its capabilities to block cell cycle progression in the presence of DNA damages [21]. About half of all neoplasia have mutations within *TP53* gene, while the other half with wild-type *TP53* yet develops a number of mechanisms to circumvent its function. Among them, also a role of dereg-miRs, such as miR-150, was found. In fact, *in vivo*, the expression of miR-150 in T2 stage tissue samples was higher than that in T1 stage tissue samples and *TP53* activity was inversely correlated with miR-150 expression [22]. MiR-150 specifically targets the 3'-UTR of *TP53* and regulates its expression. This was showed *in vitro* in co-transfection experiments with the use of specific vectors carrying *TP53* with or without the 3'-UTR and with vectors where a reporter gene was chimerized with the wild-type or a mutant *TP53* 3'-UTR. Thus, the over-expression of miR-150 leads to a reduced activity of wild-type p53 in a tumorigenic mechanism affecting cell cycle progression, proliferation, and apoptosis [23].

Another important mechanism to regulate *p53* is the over-expression of MDM2 (mouse double minute 2), an E3 ubiquitin ligase which leads p53 to degradation [24]. As a matter of fact, *MDM2* gene is found overexpressed in a variety of human cancers, such as sarcoma [25], lymphoma [26], breast cancer (BC) [27], lung cancer (LC) [28], and testicular germ cell tumor [29]. MDM2 is over-expressed, among other mechanisms, when its negative regulators, such as miR-660 are lacking. This dereg-miR was showed to bind *MDM2* 3'-UTR [30] and the results of preliminary *in silico* analyses were confirmed by *in vitro* and *in vivo* experiments. The luciferase reporter assay showed that following miR-660 transfection, mRNA and protein levels of *MDM2* were decreased. Moreover, subcutaneous injections of miR-660 in nude mice xenografted with NCI-H460 or A549 cells (both wild-type for p53) caused a delay in tumor growth of 10-15 days and 30-35 days, respectively [30]. The functional role of miR-660 in lung tumorigenesis was evaluated in a series of experiments using miRNA mimics in four different lung cancer cell lines (NCI-H460, LT73, A549, and H1299). The transfection with miR-660 mimic caused an arrest of cell cycle in G0/G1 phase, and a reduction of migration and invasive capacities in all three cell lines with p53 wild-type. No effects were noticed in H1299 cells, lacking an active p53 [30]. Also miR-29b was showed to act on *MDM2* mRNA with mechanisms similar to miR-660, but it was found to affect also the Wnt7a/Frizzled9 pathway affecting the regulation of NSCLC cell proliferation [31].

Another player involved in the regulation of p53 is *TP53INP1*. TP53INP1 protein promotes the phosphorylation at serine 46 of p53. Post-translational modifications of p53 mediated by TP53INP1 were shown to be involved in the regulation of apoptosis and it could explain the role of this protein in the carcinogenesis [32]. TP53INP1 is now widely recognized as a tumor suppressor gene with anti-proliferative and pro-apoptotic functions. In 2015, Li and co-workers showed that the expression of miR-125b in 37 patients affected by NSCLC was statistically significant higher in cancer tissues compared to the adjacent normal ones. Moreover, within cancer tissues the expression was higher in poorly differentiated than in well and moderately differentiated tissues [33, 34]. To understand the putative target genes of miR-125b, TargetScan and PicTar programs were used and the best candidate was *TP53INP1*. *TP53INP1* exhibited a relevant increase in mRNA and protein levels when primary NSCLC cells from patients were transfected with a miR-125b inhibitor. The transfection caused also an inhibition of invasive capacities of NSCLC cells in a matrigel-matrix. On the contrary, an increase of invasion was observed when cells were transfected with a silencing RNA (siRNA) against *TP53INP1*. These results were confirmed also in BALB/c nude mice and, in agreement with these findings, the expression of *TP53INP1* was found significantly lower in 20 tumor tissues compared with their adjacent counterparts [35]. Moreover, the post-transcriptional regulation of *TP53INP1* is affected also by miR-19a [36] and high level of miR-19a expression was significantly correlated with NSCLC TNM stage and lymph node metastasis [37]. The expression of *TP53INP1* was found reduced not only in NSCLC but also in BC, pancreatic carcinoma (PC), and gastric cancers (GC) [38-40]. In PC this gene is involved in a feedback loop involving p53 and miR-19a, modulating proliferation and apoptosis [41].

In the last decade, small molecule compounds targeting mutant p53 to reactivate its normal functions were developed, and they are employed in pre-clinical phases or currently being tested in clinical trials. Gendicine, a recombinant adenovirus encoding human wild-type p53 was the first p53-based therapy approved in China in 2003 and it has been used locally for treating several types of solid tumors [42]. Another approach to restoring p53 is to directly suppress MDM2 ubiquitin ligase activity. Yang and co-workers [43] discovered a family of small molecules closely related to the 7-nitro-5-deazaflavin compounds (named HLI98s), whereas Sasiela and collaborators have identify inhibitors, such as sempervirine, following a high-throughput screening of natural product extracts. Like 5-deazaflavin analogs, sempervirine suppresses MDM2-mediated p53 ubiquitination, stabilizes p53, and induces apoptosis in wild-type p53 cancer cells44]. In summary, reactivating p53 to its normal activity, empowering TP53INP1, or regulating MDM2 or miR-150 and miR-19a could be a promising strategy for developing novel therapies for NSCLC.

5. MAP KINASE SIGNALING PATHWAY

MAP-kinase signaling pathway is acknowledged as one of the most important pathway altered in human cancers. Changes in this pathway can promote uncontrolled cell proliferation, epithelial-mesenchymal transition (EMT), invasion, and metastasis [45,46].

Growth factors receptors, e.g. EGFR, activates (by phosphorylation) KRAS that in its turn can trigger the activation of PI3K and, in cascade, of AKT1. PTEN (phosphatase and tensin homolog deleted on chromosome 10) is an inhibitor of these processes and can de-phosphorilate specific intermediates antagonizing KRAS activation. KRAS is a member of the small GTPase superfamily and activating mutations correspond in almost all cases to single aminoacid substitutions at codons 12 or 13. These are responsible for a constitutively activated protein with transforming properties that is found in human malignancies, including lung adenocarcinoma (LA) [47], mucinous adenoma, ductal carcinoma of the pancreas [48], and colorectal carcinoma (CRC) [49]. In addition, as alternative to gene mutation, increased activity of *KRAS* can be reached also through dereg-miRs. For example, the down-regulation of miR-200c, that directly targets KRAS, could lead to *KRAS* over-expression. As a matter of fact, miR-200c is an important player in NSCLC progression, it is often down-regulated in human cancer tissues and cell lines and its expression inversely correlates with the expression and activity of KRAS. Biological evidences suggested also that the ectopic up-regulation of miR-200c could inhibit KRAS mRNA and other oncogenic pathways leading to a potent anti-proliferative activity [50, 51, 52]. In analogy, KRAS activity could be increased also with a reduced expression of miR-181a-5p [53] or of let-7, whose low expression was associated with a short survival of NSCLC patients [54].

In NSCLC tissues and cell lines [55-57], in LA [58-60], and in SCLC [61] the pathway was found activated also following the inhibition of *PTEN*, through the up-regulation of miR-21, miR-106a, and miR-205. The central role of miR-21 in NSCLC is well-documented in literature. A high expression of miR-21 is associated with disease recurrence and chemo-resistance in LC patients [55, 62]. The over-expression of miR-106a negatively correlated with protein expression level of PTEN, and with an increased pAKT protein expression [56]. The inhibitory effect of miR-106a on migration and invasion capabilities was showed in *in vitro* experiments on A549 cells transfected with anti-miR-106a [56]. High levels of miR-106a were also associated with a short overall survival of NSCLC patients [56]. Concerning miR-205, it was showed that its up-regulation directly represses not only *PTEN* but also *PHLPP2* leading to the activation of AKT/FOXO3a signaling pathway [57]. Also miR-503, miR-185, and miR-99a are relevant for NSCLC. MiR-503 targets PI3K and it was found down-regulated in NSCLC and its expression was associated positively with

the overall survival of 97 NSCLC patients [63]. The expression of miR-185 was found low in NSCLC tissues, pleural fluids and in H460, A549 and H1299 NSCLC cell lines, when compared to their respective controls [64,65]. MiR-185 binds the 3'-UTR of *AKT1*. This was showed with *in silico* and *in vitro* experiments. When H1299 and A549 cells were transfected with miR-185 mimic, the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide assay) assay showed a reduction of cell proliferation (compared to a miRNA of control) and migration. Interestingly, these effects were lost if miR-185 was co-transfected with a vector over-expressing *AKT1* lacking of the 3'-UTR (i.e. unable to be regulated by miRs) [64]. *AKT1* was found targeted also by miR-99a [66]. MiR-99a was down-regulated in 105 NSCLC tissues (when compared to the adjacent non tumor ones), and this deregulation was associated with a poor prognosis. Moreover, in BALB/c-nu/nu nude male mice xenografted with NCI-H1299 cells stably over-expressing miR-99a in a lentiviral construct (NCI-H1299-miR-99a group) showed a decreased number of lung metastases as compared to the controls (mice xenografted with cells stably over-expressing an empty lentiviral construct) [67]. AKT1/protein kinase B α is the most extensively investigated member of the serine/threonine protein kinase subfamily [68] and its activity is elevated in PC, BC, and ovarian carcinomas [69]. An important effector of the MAP kinase signaling pathway is C-MYC (V-Myc Avian Myelocytomatosis Viral Oncogene Homolog), a multifunctional nuclear phosphor-protein that acts as transcription factor and it promotes cell growth, proliferation, and transformation in numerous cell types [70,71]. MYC plays an important role in metastasis and its activation is associated with aggressive cancer phenotypes [72]. Its activity is regulated by the stabilization of Mad1 under the control of Ras/MAPK and PI3K/mTOR pathways [73], and in its turn its activity can regulate PI3K, AKT, and C-Jun expression. Two miRNA studies identified miR-184 and miR-449c as down-regulated in NSCLC and further experiments showed that both can directly target *MYC* [74,70]. Liu and co-workers showed also that once MYC mRNA has been targeted and inhibited by miR-184 a cascade of cell growth suppression, mediated by CCDC19, is triggered, finally affecting the PI3K/AKT/C-Jun pathway [74]. Among other important effectors of MAP kinase signaling pathway there are other pleiotropic molecules such as tuberous sclerosis complex 2 (*TSC2*), forkhead box O1 (*FOXO1*), eIF4E [75], IκB kinase (*IKK*), NF-κB [76], and *MDM2* [77]. These downstream molecules were found deregulated in LC not only by an aberrant activation of the PI3K/PTEN>AKT1 signaling pathway but also by dereg-miRs, such as for example miR-183 [78] that targets *FOXO1* mRNA.

Nowadays, there is the critical need to develop the appropriate drugs against the above mentioned partners, including mutant KRAS. However, we propose here that the use of inhibitors against the endogenous miR-106a, miR-205, miR-21 (such as AC1MMYR2, [79]), *AKT1* (such as the novel

agent NSC156529, [80]) or *FOXO3a*, or the restoration of the expression of miR-99a, miR-185, miR-183, *PHLPP2*, or *PTEN* could be at the basis for developing novel therapies. Concerning MYC, several MYC inhibitors, namely the small molecules 10058-F4, KSI-3716, 10074-G5, "Omomyc" [81-84], have been developed. However, no clinical trials are ongoing with this class of molecules, yet.

6. APOPTOSIS

The apoptotic pathway includes a family of cysteine proteases called Caspases with pro-apoptotic activities and Bcl-2 family members, with pro-survival functions, that counteract caspases' signals. Dereg-miRs can affect their function leading to cancer progression by suppressing the pro-apoptotic or enhancing the pro-survival signaling. MiR-224 was found up-regulated in NSCLC [85, 86] and CASP3 and CASP7 were identified as their targets [86]. *In vitro* experiments showed that the up-regulation of miR-224, leading to the down-regulation of CASP7, significantly increased the resistance to apoptosis and the migratory and proliferative abilities of H1299 and H460 malignant cells, thus suggesting a role of miR-224 and Caspase-7 in apoptosis and lung metastasis [86].

In another miRNA study, Luo and co-workers (2012) reported a down-regulation of miR-101 which was associated with an over-expression of *MCL1* (mRNA and protein) in 45 NSCLC tissues [87]. MCL1 is a potent multi-domain anti-apoptotic protein that hetero-dimerizes with other BCL2 family members to protect against apoptotic cell death [88]. It was found that the co-expression of MCL1 with MYC could constitute a useful biomarker for identifying aggressive forms of NSCLC and for predicting patient outcomes [89].

Finally, miR-15a was found down-regulated in 18 paired NSCLC and adjacent non-tumor lung tissues [90]. MiR-15a binds the 3'-UTR of *BCL2L2*, another pro-survival member of the BCL2 protein family which acts as an inhibitor of apoptosis [91]. The over-expression of miR-15a caused a significant decreased of the protein levels of BCL2L2 and an increase of the downstream effectors of BCL2L2, i.e. Caspase-9, Caspase-3, and BAX [90].

In summary, studies on dereg-miRs could help in selecting, among a multitude of molecules, specific players (i.e. *CASP3*, *CASP7*, *MCL1* and *BCL2L2*) of the apoptotic and anti-apoptotic pathways involved in the progression of NSCLC. Theoretically, the rescue of the normal expression levels of these genes could help in developing novel therapeutic strategies. As an example, recently, it has been discovered that ABL, a natural chemical component obtained from *Inula britannica*, a plant used in the Chinese traditional medicine, alone or in combination with gemcitabine induced apoptosis in NSCLC cells through the down-regulation of Bcl-2 and the up-regulation of Bax [92].

7. EPITHELIAL-MESENCHYMAL TRANSITION (EMT)

EMT is an evolutionarily conserved process in which cells undergo transformation from a more differentiated status (e.g. epithelial-like) to a less differentiated one (i.e. mesenchymal). In this modification, epithelial cells lose cell-cell adhesion and cell polarity, decrease the expression of epithelial cells' markers such as E-cadherin, increase the expression of mesenchymal cell markers such as Vimentin, fibronectin, N-cadherin, alpha-smooth muscle actin (α-SMA), as well as increase the activity of matrix MMPs, associated with an invasive phenotype [93,94]. Thanks to miRNAs studies, changes in the expression of a number of transcriptional factors have been identified, including ZEB1/2, Twist1, KFL8, IRS1, and FOXM1, all potential candidate target genes for NSCLC [95].

ZEB1 and ZEB2, major transcriptional repressors of E-cadherin, are zinc finger E-box binding homeobox transcription factors playing an important role in the carcinogenesis of NSCLC [95]. *In vitro* and *in vivo* studies showed they are *bona fide* targets of miR-200c a potent anti-tumor oncomiR (already mentioned as regulator of KRAS) and of miR-215 [96], both miRNAs often down-regulated in NSCLC [97-99].

In NSCLC tissues and cell lines, EMT was also related to a low expression of miR-33a, miR-135a miR-134, miR-149, and miR-23a [100-104]. Concerning miR-33a, various experiments *in vitro* and *in vivo* showed that Twist1 is one of its preferred targets [100]. Notably, in NCI-H1299 cells the inhibition of Twist1 by siRNA induced the expression of CDH1 and suppressed the expression of Vimentin suggesting a regulatory network between miR-33a, CDH1, and Vimentin. Twist1 belongs to the basic helix-loop-helix (bHLH) family of transcription factors involved in cell lineage determination and differentiation, and it is expressed preferentially by mesodermal-derived tissues. Moreover, also miR-135a was down-regulated in NSCLC cells and in clinical tissue samples. It was showed to target KLF8, and *in vitro* experiments proved that silencing *KLF8* was able to inhibit migration and invasion of lung cancer cells [101]. MiR-134 and miR-149, both targeting *FOXM1* mRNA, were found down-regulated in NSCLC tissues [102, 103]. FOXM1 belongs to the forkhead box transcription factor family and it is involved in EMT induced by TGF-β1 (at least in A549 cells) [105]. *FOXM1* was found highly expressed in various tumors, including NSCLC, prostate (PaC), head and neck squamous cell carcinoma (HNSCC), GC and acute lymphoblastic leukaemia [106-111]. In NSCLC, it was found associated with poor prognosis [112]. Finally, miR-23a promotes TGF-β-induced EMT in NSCLC in a Smad-dependent manner [113] by targeting IRS1, a critical EMT suppressor in NSCLC cells [114].

Another candidate gene player of the EMT is *USP25*, encoding for a poorly studied member of the

ubiquitinating-specific proteases family. Interestingly, the 3'-UTR of this gene is targeted directly by miR-200c (the same miRNA that regulates KRAS) that causes a decreased expression of USP25 both at mRNA and protein level. The silencing of *USP25* gene mimicked the effects of miR-200c over-expression. In NSCLC tissues miR-200c and USP25 expression was found inversely correlated. Interestingly, the mRNA and protein expression of USP25 was higher in NSCLC patients, compared to healthy controls, and its expression also correlated with the clinical stage and lymphatic node metastases [97]. The over-expression of miR-200c or the down-regulation of *USP25* gene was found to inhibit NSCLC cells migration, invasion, and EMT *in vitro* and lung metastasis formation *in vivo* [50, 51].

Silibinin, a natural flavonolignan, either alone or in combination with DNA methyl transferase inhibitors or 5'-Aza-deoxycytidine (Aza), was showed to modulate the expression of ZEB1 [115]. Inhibitors of FOXM1, including the thiazole antibiotic thiostrepton, the more recently developed small drug FDI-6, and the natural compound plumbagin [114-119], have been used in *in vitro* and *in vivo* experiments on xenografted mice showing the ability to reduced invasiveness and metastatic capacities of cancer cells. Until now, no small molecule inhibitors of USP25 were developed. In summary, molecules developed to inhibit the endogenous ZEB1, ZEB2, USP25, Twist1, Vimentin, miR-23a, or FOXM1 or to restore the expression of CDH1, IRS1, miR-33a, miR-215, or miR-200c could be of help in the therapy of NSCLC.

8. DIFFERENTIATION

Genes involved in differentiation are deregulated in cancer, as de-differentiation is part of the malignant phenotype. This process involves shifts between cell proliferation and differentiation, thus mutations or changes in these genes are observed in cancer and this mechanism is often related to a poor prognosis [120]. In the context of NSCLC, dereg-miRs helped to identify *EZH2* a member of the polycomb complex, *SOX9* and *HOXA5* as candidate target gene for therapeutic approaches. The expression level of miR-138 was investigated in 18 human NSCLC tissue samples and matched normal ones, in 4 NSCLC cell lines, and in the non-malignant 16HBE cell line. Overall, a down-regulation of this miRNA was observed [121]. The role of miR-138 in NSCLC was also reinforced by another independent study showing that miR-138 down-regulation was significantly associated with advanced stages, positive lymph node metastasis, and short overall survival of NSCLC patients [122]. *In silico* predictions [121] showed that *EZH2* (Enhancer of zeste homolog 2), a fundamental member of the polycomb repressive complex 2, is a target for miR-138 [122, 123]. In A549 and H460 cells stably over-expressing miR-138, increased levels of apoptosis and a slow cell cycle with

a high percentage of cells accumulating in the G1/S transition were reported when compared to cells infected with a lentivirus carrying the empty construct (controls) [121]. These results were replicated also in BALB/c nude mice (injected with A549 cell overexpressing miR-138 or control miRNA) [121].

Also miR-101 was found deregulated in NSCLC and it was showed as another miRNA targeting *EZH2* mRNA [124] providing a further evidence of the role of EZH2 in NSCLC. In fact, in a recent meta-analysis including 1,695 patients with LC the combined hazard ratios suggested that EZH2 protein overexpression was associated with poor prognosis and short overall survival [125]. EZH2 is frequently over-expressed in different types of human neoplasms including BC [126], PC [127], GC [128], and CRC [129] reinforcing the importance of the polycomb complex in cancer. This complex is known to have pleiotropic effects by regulating hundreds of genes by causing epigenetic changes in the chromatin conformation. EZH2, in particular, has a histone methyl-transferase activity (HMT) and mediates the down-regulation of gene transcription through post-translational histone modifications [130].

MiRNA studies showed that the expression of miR-206 and miR-32 was decreased in NSCLC tissues compared with adjacent non-tumor tissues [131,132]. Further research identified *SOX9* mRNA (Sry-related high-mobility group HMG box 9) as their main target. *SOX9* is a transcription factor involved in the development and differentiation [133-136] and its over-expression plays a role in the process of metastasis, by enhancing cell migration, invasion and EMT, at least in part, through the activation of Wnt/β-catenin signaling pathway [137]. SOX9 was found up-regulated in BC, CRC, and PC [138-140]. In particular, *SOX9* was found up-regulated in NSCLC tissues and its elevated levels were associated with poor prognosis [133].

Another gene involved in cell differentiation and deregulated in NSCLC is *HOXA5*. It belongs to the homeobox gene family that contains a common 183-nucleotide sequence (homeobox) and encodes for specific transcription factors involved in the morphogenesis of vertebrate embryonic cells, providing regional information along the main body axis. HOXA5 is found down-regulated in NSCLC likely depending on the up-regulation of miR-196a [134] or of miR-1271 [135], as showed in NSCLC tissues [134, 136], cell lines [134], and in an independent analysis on GEO datasets [137]. The down-regulation of *HOXA5* mRNA causes a reduced apoptosis mediated by retinoic acid (RA) and enhances the cellular growth acting directly downstream of RARβ [138]. HOXA5 has an important role in modulating cell-cell and cell-matrix interactions, as its activity was also shown to suppress cell migration and invasion [138]. HOXA5 is hypothesized to bind promoters of cytoskeleton-related genes and down-regulate their mRNA and protein expression levels. In fact, the ectopic expression of HOXA5 is involved in the down-regulation of the expression of

cytoskeleton proteins, in tissue-remodeling pathways, and it inhibited filopodia formation [139].
EZH2 inhibitors could be employed as potential agents for NSCLC therapy. The S-adenosyl-l-homocysteine hydrolase inhibitor, 3-Deazaneplanocin A (DZNep), has been shown to deplete and inhibit EZH2 [140]. More recently, the Ursolic acid (UA), a pentacyclic triterpenoid, was shown to inhibit the growth of NSCLC cells through SAPK/JNK-mediated inhibition of SP1. This, in turn, results in the inhibition of DNMT1 (DNA methyl-transferase 1), an interactor of EZH2, and thus it could be considered an indirect inhibitor of EZH2 [141]. At the present time, small drugs inhibitors of SOX9 are not reported in literature, however a natural endogenous inhibitor, i.e. the extracellular protein Epimorphin [142], has been identified and this could of help for future therapies.

9. CYTOSKELETON REMODELING

Cell migration is essential for tumor invasion and it is a highly integrated multistep process that is initiated by the protrusion of the cellular membrane spatially and temporally regulated by actin cytoskeleton polymerization. Genes of cytoskeleton remodeling altered in NSCLC are *PXN, LIMK1* (LIM kinase 1), and *MTDH*. Paxillin seems particularly important in NSCLC and it is one of the key components within the focal adhesions machinery, forming a structural link between the actin cytoskeleton and the extracellular matrix [143]. MiRNA studies found that PXN is deregulated also by the aberrant expression of PXN-targeting dereg-miRs, such as miR-137 and miR-218 [144, 145]. Paxillin is a protein of 68 kDa that was found over-expressed not only in NSCLC, but also in HNSCC [146], in CRC, [147], in BC, in PaC [148], and in GC [149].
LIMK1 protein is a downstream effector of PAK4 (P21-activated kinase 4) and it acts as a regulator of the actin cytoskeleton, cell motility, and invasion [150]. In NSCLC, the LIMK1 is over-expressed and the low levels of miR-143 and miR-27b (both found significantly decreased in tissues and cell lines [151, 152]) is part of the mechanism. LIMK1 positivity in NSCLC was associated also with high TNM stages and lymph node metastases [153]. Finally, MTDH protein is a component of the tight junction complexes and it is a marker of matured tight junctions [154]. MTDH over-expression was showed to play an important role in the carcinogenesis of NSCLC [155-158] by promoting NSCLC metastasis and invasion and by suppressing apoptosis. A variety of pathways, including PI3K/AKT1 MAP kinase, are aberrantly activated following MTDH over-expression. Also cancer proteins such as matrix metalloproteinase-9 (MMP-9) and the anti-apoptotic Bcl-2 are enhanced [159]. The over-expression of MTDH could be due, at least in part, to the down-regulation of two miRNAs, i.e. miR-193a-3p and miR-145 [160,161]. *MTDH* is up-

regulated in various human cancers, including BC [162, 163], hepatocellular carcinoma (HCC), and HNSCC [164, 165].

Molecules that can inhibit MTDH would have potential to be developed for cancer therapeutics. Recently, *in vitro*, it was showed that evodiamine suppresses the proliferation of LC cells through the inhibition of MTDH [166] holding promises as novel NSCLC therapeutic agent. In summary, empowering HOXA5 or inhibiting PXN, SOX9, LIMK1, or MTDH could provide benefits in NSCLC patients.

10. ANGIOGENESIS

Angiogenesis plays a key role in tumorigenesis, thus controlling players involved in this process could be an efficacious therapeutic strategy. Independent studies showed that miR-16, miR-195, and miR-497 are down-regulated in NSCLC tissues and cells lines [167-169] and that these miRNAs can bind to 3'-UTR of the hepatoma-derived growth factor (*HDGF*). HDGF is a secreted growth factor and could promote cellular processes like proliferation, differentiation, migration [170] and in particular angiogenesis through the stimulation of VEGF release [171, 173, 179]. Its overexpression has been detected in several cancers including HCC, cholangiocarcinoma, gastrointestinal stromal tumors, PC, and GC [174]. In NSCLC, the expression of HDGF is also a prognostic predictor for patients with early-stages [175]. In HCC, cells stably transfected with an anti-HDGF shRNA assayed in *in vitro* and in xenografted mice resulted in a decreased proliferative activity with suppressed VEGF expression and reduced angiogenesis of developing tumors [176]. Overall, these findings suggested that the targeted inhibition of HDGF could be a novel anti-cancer therapy.

11. METASTASIS

MiR-125a-3p and miR-30c were found down-regulated in NSCLC [177, 178]. Both miRNAs were showed to target *MTA1* mRNA (metastasis-associated protein 1). The encoded protein plays an important role in nucleosome remodeling and histone deacetylation complex, regulating many genes involved in promotion of malignant tumor metastasis [179-181]. MTA1 increased also the expression of VEGF vascular endothelial growth factor thus promoting tumor angiogenesis [182,183]. Overexpression of MTA1 was reported in BC, PaC, and LC [184-186] and it was suggested as prognostic biomarker of poor survival in BC, esophageal, and urinary cancers [187,188]. MTA1 can activate the Wnt/β-catenin signaling pathway [189], another

important pathway playing a critical role in lung tumorigenesis [190]. Another metastasis-associated gene, *RECK* (reversion-inducing-cysteine-rich protein with kazal motifs), was shown to be involved in NSCLC by studies on miR-92b and miR-21 [191, 192]. Both were showed to target *RECK* mRNA and their expression was found increased in NSCLC tissues and cell lines. It was showed that the miRNA-dependent down-regulation of *RECK* gene expression could constitute an important step in the tumorigenesis process [192]. RECK is a membrane-anchored glycoprotein and it could act as a negative regulator for matrix metalloproteinase-9, a key enzyme involved in tumor invasion and metastasis. The appropriate levels of RECK could modulate cell growth and motility in lung and bladder cancers [191].

Interestingly, researchers showed that curcumin could inhibit the proliferation and invasion of NSCLC cells through the inhibition of MTA1 and the Wnt/β-catenin signaling pathway. Thus, these investigators provided novel insights into the mechanisms of curcumin on inhibition of NSCLC cell growth and invasion, suggesting potential therapeutic strategies for NSCLC [190].

However, therapeutic approaches aimed to restore a lost expression of cancer-inhibiting molecules like RECK are intuitively less feasible than others based on inhibiting up-regulated targets. The inhibition of miR-92b or miR-21 could be a possibility.

12. NETWORK OF INTERACTIONS AMONG MIRNAS AND THEIR *BONA FIDE* TARGETS

In order to get the maximal information from the collected studies, in this review the miRNAs and their targets reported in table 1 were also analyzed *in silico* with the use of the tool Interactome DB. The output (strictly related to LC) was implemented with more information from the manuscripts listed in table 1 (related to NSCLC only) and the results are showed as regulatory network in figure 2.

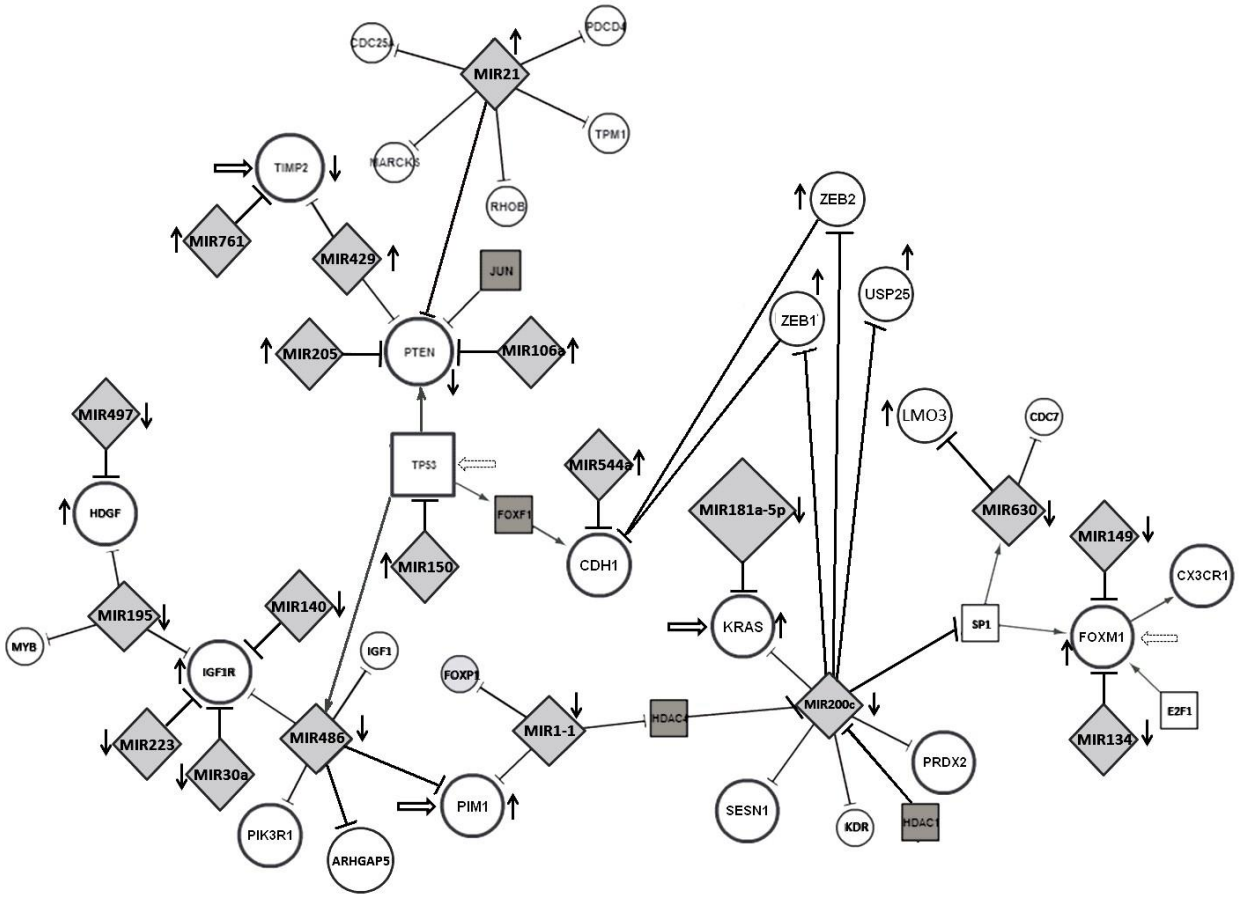

Figure 2. The regulatory network found in NSCLC by Interactome DB, implemented with data of literature.

The displayed network should be considered as a simplified graph showing only a subset of miRNAs together with their *bona fide* targets found (experimentally) deregulated in NSCLC. Although the picture shows a small part of the actual interactions, the observation of the arrows (an alias for the up- or down- expression, all experimentally confirmed in NSCLC) tells that cancer is a very complex phenomenon where each node is affected by multiple signals. Thus, in practice, a given player can be deregulated by several different mechanisms and this suggests also that, likely, one therapeutic drug alone could not counteract the overwhelming changes happened once the disease is triggered. As a summary view of the network, it is interesting to note that studies are basically all in agreement to show how miRNAs and targets have a coherent and unique flux of deregulation. In fact, in general, there is an inverse correlation between miRNAs and targets

detected by Interactome DB confirming what observed in NSCLC tissues. Moreover, the analysis links most of the players each other into an unique regulatory network where each node is found up- or down-regulated coherently to the status of its neighbors. For example, the figure shows that miR-21 is up-regulated in NSCLC and this is consistent with the down-regulation of its target, *PTEN*. In turn, *PTEN* was showed to be down-regulated also by the up-regulation of miR-205, miR-106a, and miR-429. This latter, in turn, inhibits *TIMP2*, in concert with the up-regulation of miR-761. Thus, this analysis could allow identifying more nodes (such as TIMP-2) involved in NSCLC. In this last section of the review, we will give a particular focus on *TIMP2*, IGF-signaling pathway, and *PIM1* as additional putative therapeutic targets.

12.1. TIMP2

The interactome analysis predicted that TIMP-2 is down-regulated in NSCLC because under the control of miR-761 and miR-429, that are often up-regulated in NSCLC, the latter controlling also the expression of PTEN. TIMP-2 has been poorly studied in relation to NSCLC and the expression status of TIMP-2 in this disease is unknown, however a reduced level of TIMP-2 was associated with a poor prognosis [193]. Thus, further studies on this specific target are warranted. TIMP-2, together with TIMP-1, -3, -4, belongs to the TIMP (Tissue Inhibitor of Metalloproteinase) family playing a role in remodeling the extracellular matrix (ECM). Each of their N- and C-terminal domains contains 6 conserved cysteine residues that form three disulfide loops. The N-terminal region binds to the (matrix-metalloproteinases) MMPs' catalytic domain and inhibits MMP activity, whereas, the C-terminal region interacts with the pro-forms of MMP-2 and MMP-9 C-terminal hemopexin domain to stabilize the pro-enzyme inhibitor complex [194]. TIMP-2 is the only TIMP member that specifically interacts on the cell surface with both MT1-MMP and pro-MMP-2 in order to facilitate the activation of pro-MMP-2. Thus, TIMP-2 could function both as a MMP- -inhibitor and -activator [195]. It was showed that TIMP-2 has a role also in inhibiting the neo-angiogenesis through the binding to endothelial cell receptors [202] such as the α3β1-integrin receptor [196] or acting as a Vascular Endothelial Growth Factor A (VEGF-A) antagonist and blocking endothelial cell proliferation [195]. The anti-angiogenic activity of TIMP-2 could be elicited also through the increase of the SHP-1 activity associated with FGFR1 (Fibroblast Growth Factor Receptor 1) and VEGFR2 (Vascular Endothelial Growth Factor Receptor 2). TIMP-2 could also affect the vascular permeability by increasing the vascular E-cadherin distribution in cell-cell contacts through increased association with the actin cytoskeleton TIMP-2 suppresses also endothelial cell migration through RECK expression that is considered a suppressor of angiogenesis [196]. Furthermore, TIMP-2 treatment was also able to inhibit endothelial cell growth, by mediating

the G1-growth arrest through the activation of the cyclin-dependent kinase inhibitor p27/Kip1 synthesis [197]. Studies with knock-out mice showed also that TIMP-2 modulates the recruitment of inflammatory cells within xenografted NSCLC cells, suggesting that it could modulate the cytokine release affecting the tumor micro-environment, tumor-immune-stealth, and the aforementioned intra-tumor neo-angiogenesis [196]. Finally, TIMP-2 was found to inhibit the signaling of Insulin-like Growth Factor Receptor 1 (IGFR1) [198]. Thus, in spite the fact that TIMP-2 could be not targeted directly in NSCLC because of its down-regulation, a therapeutic strategy could consist in rescuing or mimicking its (inhibitory) activity towards VEGFR2, FGFR1, α3β1-integrin receptor, SHP-1, p27, IGFR1, endothelial, or immune cells. For example, delphinidin, an anthocyanidin present in pigmented fruits and vegetables, was shown to be a potent inhibitor of both VEGFR2 (and EGFR) in NSCLC cells and it could be further developed for clinical use [199]. Inhibitory small molecules against FGFR1 such as NVP-BGJ398 or Ponatinib were developed and at least in H1581 NSCLC cells they showed to induce cell growth inhibition and death [200, 2011]. *In vivo*, mice xenografted with FGFR1-mutant NSCLC cells exhibited attenuated tumor growth and prolonged survival when the FGFR-specific tyrosine-kinase inhibitor AZ4547 was combined with an mTOR inhibitor, but the response was weak when AZ4547 was administered as monotherapy [202].

12.2. miR-486 and IGF signaling pathway

The interactome analysis implemented with miRNA studies showed that *IGF1R* could be up-regulated by several mechanisms that involve the down-regulation of various miRNAs such as miR-140, miR-195, miR-30a, miR-223, and miR-486 [203-207]. In a cohort of 81 NSCLC patients miR-486 was showed as the most down-regulated miRNA within tumor tissues compared with adjacent healthy lung tissues [207] and this finding was confirmed also in other studies [208,209]. Interestingly, miR-486 was proved to be regulated by p53 by using anti-p53 antibody in chromatin immunoprecipitation experiments [207] and it is an important regulator of the IGF1-signaling pathway targeting not only *IGF1R* but also *IGF1* mRNAs [207].

IGF1R is a membrane receptor-type tyrosine kinase that plays a crucial role in cancer cell proliferation, inhibition of apoptosis, angiogenesis, and anchorage-independent growth via the PI3K-AKT (phosphatidylinositol 3-kinase-AKT) and RAS/RAF/mitogen activated protein kinase signaling pathways [210, 211]. The axis p53>miR-486>IGF plays an important role in NSCLC. Indeed, the IGF1R was discovered as playing an important role in the pathogenesis of NSCLC already about 15 years ago [212, 213] and it was already suggested as therapeutic target in 2010 [214]. IGF1R is a potential therapeutic target also for patients affected by BC and sarcoma and

therapeutic agents include both monoclonal antibodies to IGF1R (dalotuzumab, figitumumab, cixutumumab, ganitumab, R1507, AVE1642) or IGF1R pathway targeting strategies such as the use of monoclonal antibodies to IGF1 and IGF2 (MEDI-573, BI 836845) or linsitinib, a small-molecule tyrosine kinase inhibitor of IGF1R [215].

The human IgG2 monoclonal antibody against IGFIR, figitumumab, was developed for the treatment of NSCLC [216] but the subsequent clinical trials (ended in 2013, see clinicaltrials.gov for CP-751,871) did not provide evidence of benefits in combination with the standard chemotherapy (paclitaxel plus carboplatin) [217]. Negative trials obtained on patients with BC and NSCLC contrasted with the sustained success of IGF1R inhibitor monotherapy in a subset of patients with sarcoma [217, 218]. This underlines, once more, the importance of combing inhibitors for a more effective approach to improve overall survival in NSCLC. Likely, in NSCLC the down-stream effectors of IGF1R receptor (i.e. the KRAS pathway) are activated by multiple mechanisms and this could explain the relatively poor effectiveness of these IGF1R-directed therapies. It is thought that specific biomarkers should be developed for predicting patients who could benefit from these therapies [219, 220].

12.3. PIM1

MiR-486 down-regulation could play a role also in the up-regulation of *PIM1* gene expression that could be up-regulated also by a low expression of miR-1 [221]. PIM1 was originally identified as a proviral integration site in Moloney murine leukemia virus-induced murine T-cell lymphomas. It is a serine/threonine kinase oncogene that plays a role during differentiation and it is a potent mediator of cell survival by inhibiting apoptosis, by promoting cell proliferation and genomic instability [222]. It is also a pivotal mediator for radio-resistance of NSCLC cells [222]. The overexpression of PIM1 protein was observed closely associated with transformation of malignant cells and acceleration of tumorigenesis in a significant fraction of human myeloid and lymphoid leukemia, as well as in lymphomas [223]. In HNSCC, immune-histochemical analyses revealed that PIM1 protein is expressed in tumors of different grades and stages, but not in normal tissues [224]. In GC it was found that immunoreactivity of PIM1 increased as the grade of malignancy increased, further emphasizing that PIM1 levels might serve as a tumor marker [225]. PIM1 up-regulation correlates with a poor prognosis [226] and it could be a critical survival signaling factor in NSCLC (213). Small molecules, such as SGI-1776, ETP-45299, tryptanthrin [222] and the imidazo-[1,2-b]-pyridazine-based Pim1 kinase inhibitors, were developed and are at the pre-clinical research stages with nearly 40 patents emerged in the last years [227, 228]. There is hope some of them will be of benefit for NSCLC patients.

CONCLUSIONS

Various omics approaches are ongoing in order to unravel the secrets of cancer. The more data are cumulated more knowledge is gained, with the perception that cancer is a moving target with hundreds of genes subject to plastic and adaptive deregulation. In the present review, although we did not approach the complexity of NSCLC with omics tools, we reported the collection of experimental studies where *bona fide* targets for specific miRNAs (found altered in NSCLC) were validated. We showed that these studies helped to identify a plethora of genes whose encoded proteins could constitute known/novel candidates to be studied in view of future therapeutic approaches. Thus, the study of deregulated miRNAs could be of help in this process and further experimentation is warranted with the aim to find novel targets to develop drugs, or for improving those already synthesized that are currently in pre-clinical stages or under clinical trials.

CONFLICT OF INTEREST

No potential conflicts of interest were disclosed.

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