

## Original Article

# MiR-22-3p suppresses cell growth via MET/STAT3 signaling in lung cancer

Xia Yang<sup>1\*</sup>, Wenmei Su<sup>2\*</sup>, Yu Li<sup>3</sup>, Zhiqing Zhou<sup>3</sup>, Yi Zhou<sup>3</sup>, Hu Shan<sup>1</sup>, Xiaoling Han<sup>2</sup>, Ming Zhang<sup>1</sup>, Qihong Zhang<sup>1</sup>, Ying Bai<sup>1</sup>, Chunfang Guo<sup>4</sup>, Shuanying Yang<sup>1</sup>, David G Beer<sup>4</sup>, Guoan Chen<sup>3</sup>

<sup>1</sup>Department of Respiratory and Critical Care Medicine, The Second Affiliated Hospital of Xi'an Jiaotong University, Xi'an, China; <sup>2</sup>Department of Oncology, Affiliated Hospital of Guangdong Medical University, Zhanjiang, China; <sup>3</sup>School of Medicine, Southern University of Science and Technology, Shenzhen 518055, China; <sup>4</sup>Department of Surgery, University of Michigan, Ann Arbor, Michigan, USA. \*Equal contributors.

Received August 5, 2020; Accepted December 18, 2020; Epub March 15, 2021; Published March 30, 2021

**Abstract:** *MiR-22-3p* has been reported to be down-regulated in several cancers, but its expression pattern and roles in lung cancer is unclear. Given the crucial role of microRNAs in cancer progression, we examined the expression and function of *miR-22-3p* in lung adenocarcinoma. *MiR-22-3p* expression in lung cancer tissues and cell lines was measured by qRT-PCR. Cell proliferation was measured by WST-1 and colony formation assays were used to reveal the role of *miR-22-3p* in lung cancer *in vitro*. *MiR-22-3p* was notably down-regulated in lung cancer tissues as compared to normal lung tissues, but it was not associated with the clinical characteristics of tumor stage, differentiation and patient's smoking status. Colony formation ability and cell proliferation were suppressed by *miR-22-3p* mimics in lung cancer cell lines. Mechanistically, *miR-22-3p* mimics could reduce MET and STAT3 protein expression and induce apoptosis as measured by PARP protein. We conclude that *miR-22-3p* may play a tumor suppressor role via inhibiting MET-STAT3 signaling and have potential to be a therapeutic target and biomarker in lung adenocarcinoma.

**Keywords:** *miR-22-3p*, MET, STAT3, lung cancer

## Introduction

Lung cancer is the most common cause of cancer-related death for both men and women globally [1]. Non-small cell lung cancer (NSCLC) is the major type of lung cancer. Although treatment options are available, the mortality associated with NSCLC remains high [2]. The incidence of NSCLC is accompanied by a series of RNA and protein expression changes in lung cancer cells, and the mechanisms of cancer development and progression are not fully understood [3-5]. Elucidating the molecular basis of cancer progression is vital for the identifying potential new biomarkers and therapeutic targets [6].

MicroRNAs (miRNAs) are one type of non-coding small RNAs that can regulate gene expression and numerous cellular processes. Their expression can often be dysregulated in human

tumors and causing cancer progression [7]. MiRNAs regulate gene expression through several mechanisms [8]. Studies have shown that miRNAs can regulate more than 80% of the gene transcripts via their untranslated and protein coding regions [9]. MiRNAs play critical roles in multiple biological processes including cell cycle regulation, cell differentiation and cell death [10]. An exponentially growing number of publications have shown that miRNAs not only play important roles in tumor initiation and progression, but are also potential biomarkers for cancer diagnosis and prognosis [11, 12].

*MiR-22* was reported to play a tumor suppressor role in several cancers including breast, ovarian, liver, prostate and cervical cancers [13-17]. However, *miR-22* can also work as an oncogene to promote cancer progression in liver cancer, prostate cancer and chronic lymphocytic leukemia [17-19]. Several studies

## Role of *miR-22-3p* in lung adenocarcinoma

**Table 1.** The *miR-22-3p* expression and clinical variables in 91 lung adenocarcinomas

Variables	No. of cases	<i>miR-22-3p</i> (Mean ± SD)	t value	P
Age				
< 65	43	1.09±0.53		
≥ 65	48	0.98±0.32	1.182	0.240
Gender				
Male	40	1.05±0.34		
Female	51	1.02±0.49	0.281	0.779
Clinical stage				
1	50	1.07±0.48		
2	23	0.93±0.35	1.186	0.240
3	18	1.06±0.35	0.062	0.951
Smoking (pk/y)				
< 50	58	1.05±0.50		
≥ 50	33	1.00±0.27	0.542	0.590
Nodal status				
0	61	1.03±0.46		
1	12	1.03±0.38	0.012	0.991
2	18	1.06±0.35	0.293	0.771
Differentiation				
Well	10	1.09±0.27		
Moderate	53	1.01±0.4	0.500	0.619
Poor	28	1.06±0.35	0.267	0.791

**Table 2.** Primer sequences of miRNAs and genes utilized for qRT-PCR

Gene name	Primer ID	Primer sequence (5'→3')
<i>miR-22-3p</i>	miR-22-3p F	TCAGTGCATCACAGAACCTTTGT
<i>miR-22-3p</i>	miR-22-3p R	GCGAGCACAGAATTAATACGAC
<i>U6 snRNA</i>	U6 F	CTCGCTTCGGCAGCACA
<i>U6 snRNA</i>	U6 R	AACGCTTCACGAATTTGCGT

show *miR-22* levels are lower in lung cancer tissues and can function as tumor suppressors [20-22]. We and others have found that *miR-22* levels are increased in serum [23, 24] and decreased in pleural effusion [25]. In the present study, we sought to explore *miR-22-3p* expression patterns in patients using NSCLC tissues, to examine the functional role and underlying mechanism of *miR-22-3p* in lung cancer cells.

### Materials and methods

#### Lung cancer tissues and cell lines

All lung adenocarcinomas cancer tissue samples including 91 NSCLC tumors and 10

matched normal tissues were acquired from the Section of Thoracic Surgery, University of Michigan (USA), and approved by the Ethics Committee of the University of Michigan. The details of the patient information are listed in **Table 1**. This set of samples has been used in our previous study [11]. Two human NSCLC cell lines: H1299 (*NRAS* mutation) and H1975 (*EGFR* mutation) were purchased from ATCC (Manassas, VA, USA) and used for *in vitro* studies. Both cell lines were maintained in RPMI-1640 medium (Thermo Fisher Scientific, Waltham, MA) supplemented with 10% FBS (Thermo Fisher Scientific, Waltham, MA) and in a humidified atmosphere of 5% CO<sub>2</sub> at 37°C.

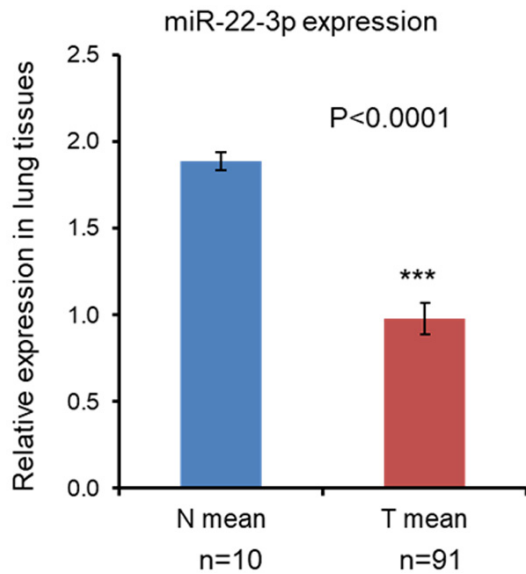
#### MiRNAs and siRNAs transfection

*MiR-22-3p* (mimic and inhibitor), miR-non-targeting control (miR-NC), siRNA for MET (siMET) and its non-targeting control (siCtrl) were purchased from Dharmacon (Lafayette, CO). Briefly, cells were transiently transfected at a final concentration of 30 nM for *miR-22-3p* mimic or *miR-22-3p* inhibitor, combined with or without 10 nM of siCtrl and siMET using Lipofectamine 2000 (Life Technologies, New York, USA) following the manufacturer's protocols. After 24 h to 72 h post-transfection, cells were used for function and mechanism study.

#### RNA isolation and gene expression

Total tissue/cell RNAs were extracted with Trizol reagent (Invitrogen, Carlsbad, CA). The cDNA was synthesized using the RT System Kit (Applied Biosystems) followed the company's guidelines. qRT-PCR was conducted with inventoried TaqMan assays (Life Technologies) as described previously [26]. *GAPDH* and *U6 snRNA* were used as loading controls for mRNAs and miRNAs, respectively. The primer sequences for *miR-22-3p* and *U6 snRNA* are listed in **Table 2**. The primer sequences for *STAT3*, *MET* and *GAPDH* were described in our previous study [27]. The qRT-PCR reactions were completed with SYBR Green Master Mix (Life Technology Inc.) and the ABI StepOne RT-PCR System (Applied Biosystems, Foster City, CA). The qRT-PCR reactions were analyzed in triplicate. The  $\Delta Ct = Ct_{\text{gene}} - Ct_{\text{reference}}$  formula was used to calculate the relative levels of gene expression and the 2<sup>- $\Delta\Delta Ct$</sup>  method was used for the fold-change of gene expression.

## Role of *miR-22-3p* in lung adenocarcinoma



**Figure 1.** *MiR-22-3p* levels were lower in lung cancer tissues. The expression levels of *miR-22-3p* in 91 lung adenocarcinoma tissues (T) and 10 normal lung tissues (N) were measured by qRT-PCR. The bar plot (values represent the mean  $\pm$  SD) indicated that the levels of *miR-22-3p* were significantly lower in lung cancer tissues as compared with normal lung tissues. Student's t-test, T vs N, \*\*\* $P < 0.0001$ .

### Cell proliferation and colony formation analysis

For cell proliferation assays, 96-well plates were used for cell plating at a density of  $2 \times 10^3$  cells per well. WST-1 reagent was then added, and the plates returned to a humidified atmosphere for 0.5-1.5 hrs. A microplate reader was used to measure the light absorbance at 450 nm. All experiments were done in triplicate. For colony formation analysis, 200 cells per well were plated in 6-well plates and incubated for 7-10 days in a 5%  $\text{CO}_2$  humidified environment at 37°C. After fixation with 20% methanol, the cells were stained with crystal violet (0.5% w/v). The colony-forming units (CFUs) consisting of  $> 50$  cells were then counted and all experiments were done in duplicate.

### Immunoblotting analysis

Cells were harvested 72 h after either *miR-22-3p* mimic, inhibitor or *MET* siRNA treatment. The immunoblotting procedures (Western blot) including protein extraction, quantification, polyacrylamide gel electrophoresis and target protein visualization were completed as described previously [3, 28]. The primary antibodies

ies purchased from Cell Signaling Technology and used in this study were MET, STAT3, PARP, p21, p27, AKT, p53, CREB, S6K and GAPDH. After incubation of the primary antibodies at 4°C overnight, the membranes were washed three times with TBST (tris-buffered saline with 0.1% Tween20). After secondary antibody incubation, the membranes were treated with ECL reagent and the resulting protein bands exposed using the ChemiDoc MP Imaging System (BIO-RAD).

### *Mir-22-3p* target genes prediction and gene ontology analysis

The prediction of potential *miR-22-3p* target genes was performed using TargetScan [29]. To uncover the oncogenic pathways and biological process involved from the predicted *miR-22-3p* target genes, KEGG (Kyoto Encyclopedia of Genes and Genomes) and Gene Ontology (GO) database analyses were performed using an online analysis software, the Database for Annotation, Visualization and Integrated Discovery (DAVID) [30].

### Statistical analysis

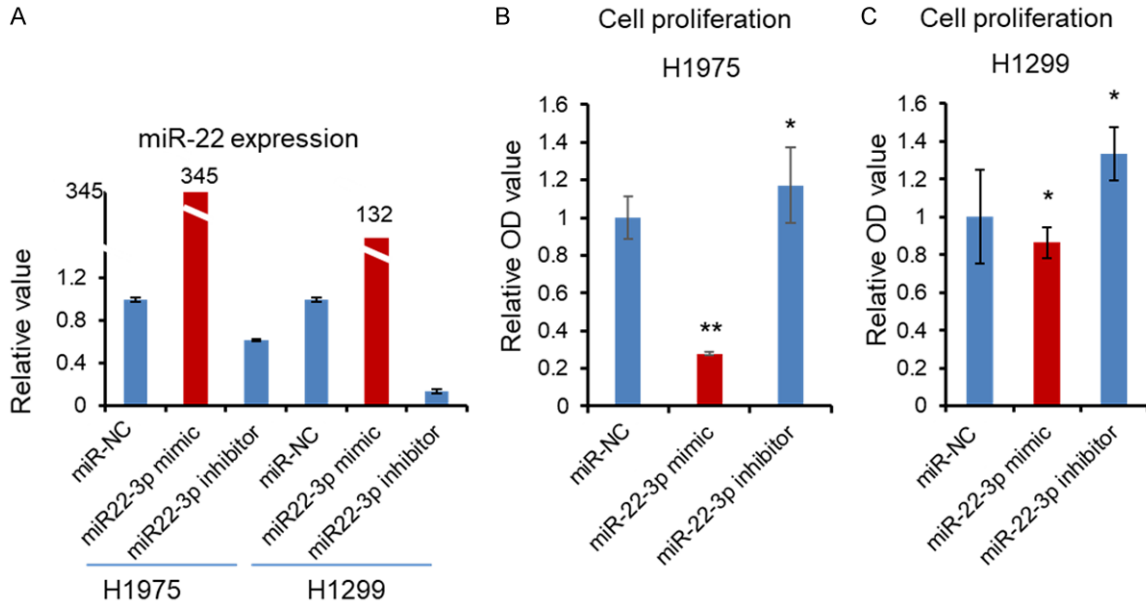
The difference between *miR-22-3p* level and clinical-pathological variables was analyzed using a Student's t-test. Results of experiments were shown as means  $\pm$  SD and a  $P < 0.05$  was judged as statistically significant.

## Results

### *MiR-22-3p* is down-regulated in lung adenocarcinoma tissues

To assess the levels of *miR-22* in patients with lung cancer, we first compared its expression in a cohort of 91 lung adenocarcinomas relative to those in 10 normal adjacent lung tissues using qRT-PCR. *MiR-22-3p* levels were significantly lower in NSCLC tissues as compared with the normal lung tissues (**Figure 1**). The patient's clinical information is shown in **Table 1**. In all variable groups, no statistically significant differences were found including age, gender, clinical stage, smoking status, TNM stage, cell differentiation or patient survival ( $P > 0.05$ ). These results suggested that *miR-22-3p* may be tentatively a tumor suppressor in NSCLC tumorigenesis and down-expression of *miR-22-*

## Role of *miR-22-3p* in lung adenocarcinoma



**Figure 2.** The effects on *miR-22-3p* expression and cell growth upon *miR-22-3p* mimic and inhibitor transfection in lung cancer cell lines. A. Expression level of *miR-22-3p* after *miR-22-3p* mimics and inhibitors transfection in lung cancer cell lines, H1975 and H1299, measured by qRT-PCR. B and C. Cell proliferation was determined by WST-1 assays following *miR-22-3p* mimics and inhibitors transfection in H1975 and H1299 cells. Values represent the mean  $\pm$  SD from three independent experiments. Student's t-test, compared to miR-NC control, \* $P < 0.05$ , \*\* $P < 0.001$ .

3p could be a marker of NSCLC and particularly lung adenocarcinomas.

### *MiR-22-3p* inhibits NSCLC cell proliferation

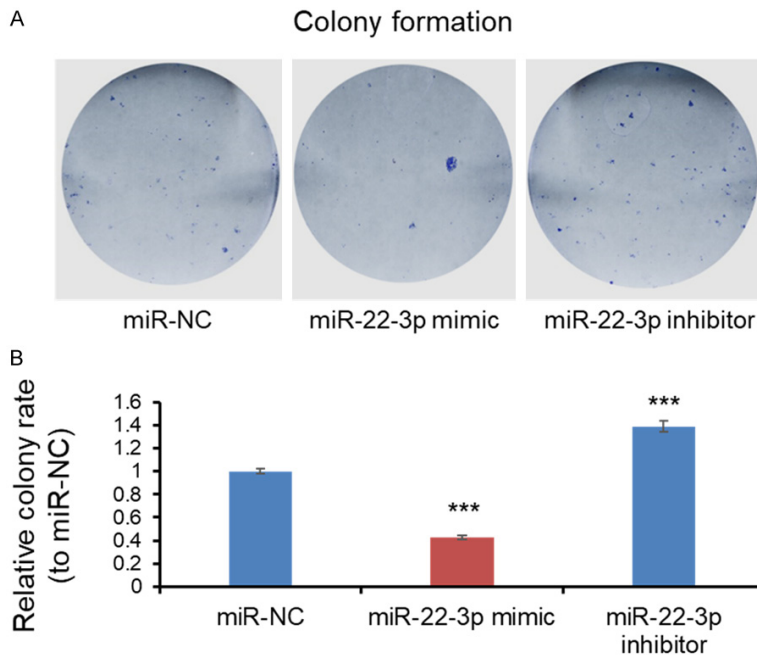
To further investigate whether *miR-22* might interfere with the oncogenic properties of NSCLC cells *in vitro*, we applied a synthetic *miR-22* mimic as well as a *miR-22* inhibitor by transfection for 72 hours of H1975 and H1299 NSCLC cell lines having different genetic backgrounds. The expression of *miR-22* before and after transfection was evaluated by qRT-PCR. A significant increase in *miR-22* expression was observed after transfection of the *miR-22* synthetic mimic, while a significant decrease in *miR-22* was seen in the *miR-22* inhibitor group as compared to the miR-non-targeting control (miR-NC) (**Figure 2A**). Subsequently, cell proliferation was measured using the WST-1 assay, and further compared among the three different miRNA-transfected groups (*miR-22-3p* mimics, *miR-22-3p* inhibitors and the control miR-NC) in both H1975 and H1299 cells (**Figure 2B** and **2C**). We found that cell proliferation was reduced after *miR-22* mimic treatment and increased after *miR-22* inhibitor treatment with statistical significance observed, especially in the H1975 cell line. The H1975 cell line was

then used as our ideal *in vitro* model for the following studies.

### *MiR-22* mimic abrogates the clonogenic potential of lung cancer cells

In this study, we asked whether the exogenous *miR-22* would affect the tumorigenic ability of NSCLC cells. To this end, a clonogenic assay was performed using H1975 cells with treatment of *miR-22* mimic, *miR-22* inhibitor, or miR-NC. After 10 to 14 days of culture, there was about a 60% reduction in the colony-forming capacity of *miR-22*-mimic transfected cells as compared to the miR-NC control cells. In contrast, H1975 cells transfected with the *miR-22* inhibitor showed a 1.5-fold increase in the number of cell colonies as compared to miR-NC cells (**Figure 3A**). Both treatments have statistical significance compared to the control group (**Figure 3B**). Additionally, the relatively smaller size of colonies was also observed in *miR-22* mimic group relative to those in miR-NC and *miR-22* inhibitor groups, yet no size difference was found between the latter two groups (**Figure 3A**). These data further supported a tumor suppressive role of *miR-22* in the H1975 lung cancer cell line.

## Role of *miR-22-3p* in lung adenocarcinoma



**Figure 3.** *MiR-22-3p* mimic inhibits colony formation in H1975 cells. (A) Image of the cell colony formation after *miR-22-3p* mimic and inhibitor treatment for 7-10 days in H1975 cell line. (B) The bar chart represents the relative number of clones from (A). Values represent the mean  $\pm$  SD from three independent experiments. Student's t-test, compared to miR-NC control, \*\*\* $P < 0.0001$ .

### Function and pathway analysis of *miR-22-3p* target genes

To obtain further define the biological processes influenced by *miR-22-3p*, database analyses of GO and KEGG were performed utilizing the DAVID online software. There were 620 *miR-22-3p* target genes predicted by Targetscan. The top 20 GO Biological Processes with significant  $P$  values are shown in **Figure 4**. The most significant biological processes for *miR-22-3p* were the positive regulation of nucleobase-containing compound metabolic processes, positive regulation of RNA metabolic processes, cell communication and signaling. *MiR-22* has been reported to regulate cellular metabolism [31, 32] which was further confirmed our analysis.

### *MiR-22* negatively regulates NSCLC cells proliferation through the inhibiting of MET-STAT3 axis

Current literature regarding *miR-22*-target genes in NSCLC is limited. MET is an important cell surface receptor tyrosine kinase where its dysregulation can lead to cancer progression through uncontrolled cell proliferation, apopto-

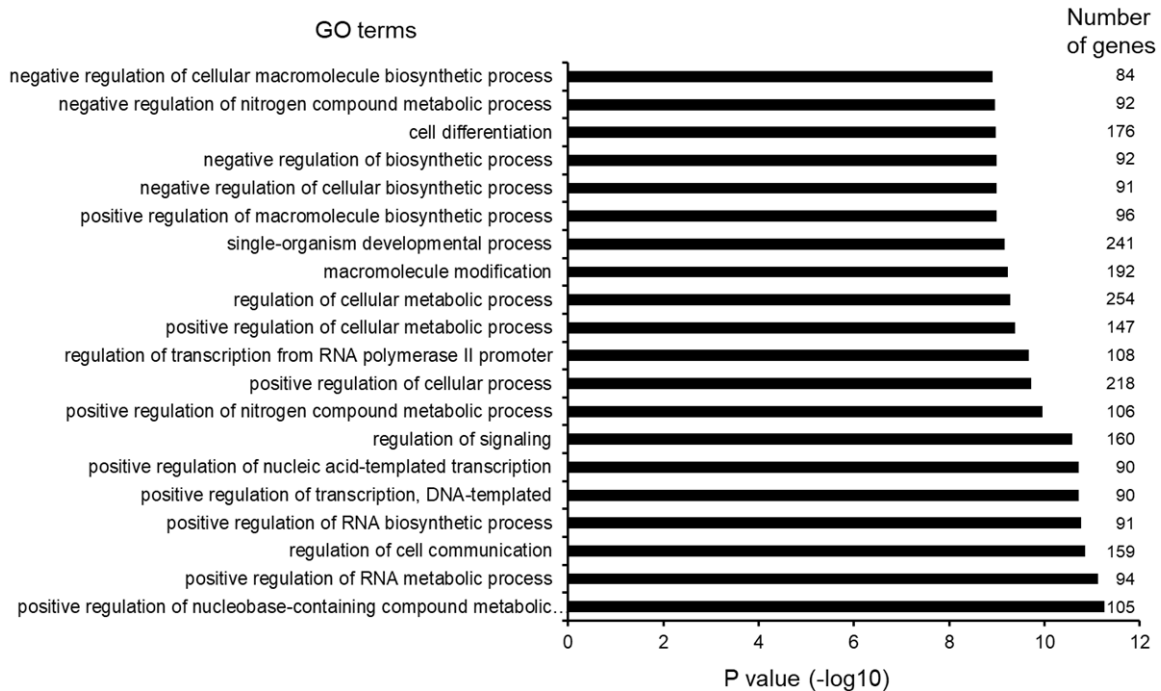
sis resistance and angiogenesis [33]. Our previous paper indicated that MET is involved in long non-coding RNA *MIR22HG*-mediated inhibition of lung cancer cell invasion and proliferation [28]. We observed that phosphorylated MET (p-MET) was the only one of 49 different phosphorylated proteins that were decreased after siRNA-mediated *MIR22HG* knockdown and assessment of receptor tyrosine kinases (RTK) by protein array in PC-9 lung cells [28]. This data caused us to then ask whether MET is also involved in *miR-22*-mediated cell growth inhibition. We compared the expression of total MET (t-MET), and phosphorylated MET (Tyr1234/1235) (p-MET) 72 hours after transfection of H1975 cells with *miR-22-3p* mimic, *miR-22-3p* inhibitor, or miR-NC (control).

We found that *miR-22-3p* mimic transfection reduced both mRNA and protein levels of MET (t-MET and p-MET) when compared to those in the control or *miR-22* inhibitor treated cells (**Figure 5A** and **5B**). Notably, phosphorylation at Tyr1234/1235 in the MET kinase domain is critical for triggering its downstream signaling components [34]. The phosphorylation ratio of MET (p-MET: t-MET) was also calculated by Image J with the resultant ratio of miR-NC:*miR-22* mimic:*miR-22* inhibitor being 1:0.5:1.2. This indicates that *miR-22* negatively regulates the kinase activity of MET at both the transcriptional and translational levels.

Our GO analysis of *MiR-22* target genes indicated that cellular metabolism was the top biology process involved, and which was confirmed by others [31, 32]. MET and STAT3 were also reported to regulate cancer metabolism [35-37]. This suggested this *miR-22*-MET axis may be involved in cancer metabolism in lung cancer.

We next asked whether a similar impact of the *miR-22* mimic might occur on the downstream pathway molecules of the MET kinase. As STAT3 was reported to be persistently activat-

## Role of *miR-22-3p* in lung adenocarcinoma



**Figure 4.** GO biological process enrichment analysis for predicted target genes of *miR-22-3p*. GO biological process enrichments were accomplished by the predicted target genes using DAVID online software. The top 20 biological processes sorted by *P* value were shown in this figure.

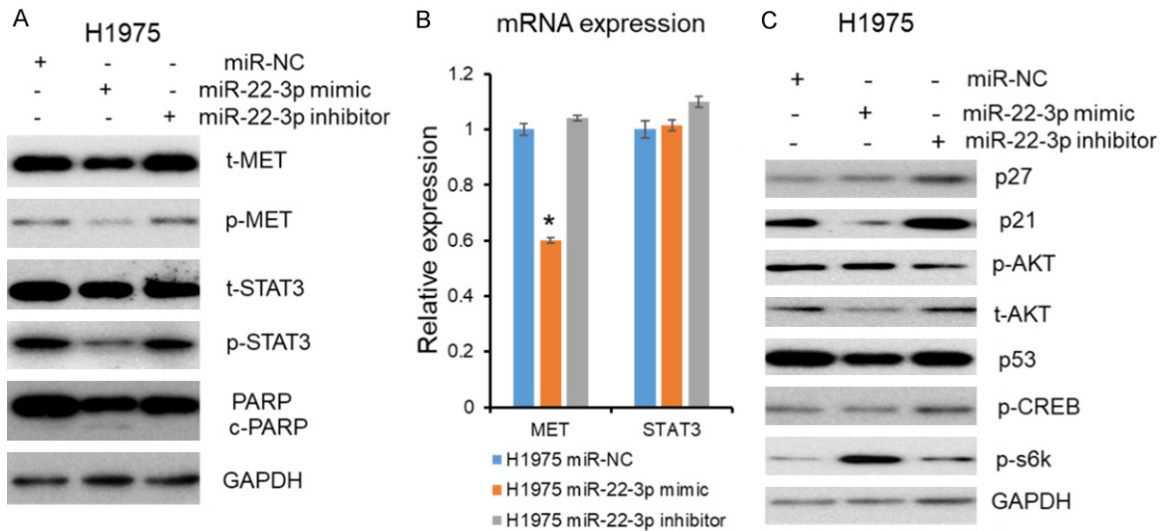
ed in 22%~65% of NSCLC [38], we examined total STAT3 (t-STAT3) and phospho-STAT3 (p-STAT3) levels after transfection of the *miR-22-3p* mimic or inhibitor using Western blot analysis. We found that both t-STAT3 and p-STAT3 were decreased in *miR-22* mimic treated cells as compared to the control group. No obvious difference was observed between the *miR-22* inhibitor and control (**Figure 5A**). The levels of *STAT3* mRNA were not changed upon *miR-22-3p* treatment (**Figure 5B**). These results suggest that *miR-22-3p* can down-regulate STAT3 at the translational level.

We also performed Western blot analysis for other proteins including p21, p27, AKT, p53, CREB, and S6K (Ribosomal Protein S6 Kinase B1) after *miR-22-3p* mimic treatment. We found that p21, t-AKT, p53, and CREB were decreased, while p-S6K levels were increased (**Figure 5C**). The basis and potential interpretation for these changes are currently unknown and the subject of future studies.

To verify the effect of MET/STAT3 on *miR-22*-mediated inhibition of cell growth in NSCLC cells, a series of transfections were performed

in H1975 cells: *miR-22-3p* inhibitor, *miR-22-3p* inhibitor plus siMET, and siCtrl plus *miR-NC* as control. Cell proliferation was then evaluated by WST-1 assay 72 h post-transfection. As shown in **Figure 6A**, the cell growth rate after co-transfection of the *miR-22* inhibitor plus siMET was about half of that seen in the *miR-NC* control. Since the *miR-22* inhibitor can increase cell proliferation, we suspected that decreased cell proliferation was caused by MET knockdown, which was also consistent with our previous report [39]. Western blot analysis indicated that siMET specifically decreased total and phosphor MET levels in *miR-22* inhibitor-treated cells. Consequently, total STAT3 was reduced in the *miR-22* inhibitor plus siMET group (**Figure 6B**), while *STAT3* mRNA was not changed upon siMET treatment (**Figure 6C**). Knockdown of MET without *miR-22* inhibitor or mimic can decrease STAT3 protein expression was confirmed in an independent Western blot analysis in PC-9 cells (**Figure 6D**). This suggested that STAT3 may be one of the downstream targets of MET as reported by others [40]. Taken together, *miR-22* inhibits cell growth at least in part through deactivating the MET-STAT3 pathway in lung cancer.

## Role of *miR-22-3p* in lung adenocarcinoma



**Figure 5.** Proteins and mRNAs affected by *miR-22-3p* mimic and inhibitor. A. Western blots indicating that protein levels of t-MET, p-MET, t-STAT3, and p-STAT3 were decreased after *miR-22-3p* mimic treatment in H1975 cells. Cleaved-PARP (c-PARP) was induced by *miR-22-3p* mimic transfection in H1975 cells. The protein loading control was GAPDH. B. qRT-PCR showing MET mRNA expression was decreased by *miR-22-3p* mimic transfection, while STAT3 mRNA not changed in H1975 cells. The loading control was GAPDH. Student's t-test, compared to miR-NC control, \* $P < 0.05$ . C. Western blots showing that proteins of p21, t-AKT, p53, and CREB were decreased, while p-S6K increased after *miR-22-3p* mimic treatment. GAPDH was used as loading control.

*MiR-22* inhibits the expression of the apoptotic cell marker, cleaved-PARP

The lower cell survival rate is echoed by reduced colony formation, suggesting that there are higher fractions of cells dying in the population. Apoptosis is one of the important forms of cell death. The MET/STAT3 pathway has been reported to be associated with apoptosis-inducing ability by regulating multiple pro- and anti-apoptotic genes, such as cleaved-PARP (poly(ADP-ribose) polymerase) [41]. Cleaved-PARP also has been used extensively as a marker of apoptosis [42]. In the current study, we asked whether there was a link between cleavage-PARP and *miR-22*-mediated inhibition of colony survival in NSCLC cells. Western blot indicated that *miR-22* mimic could induce cleaved-PARP expression in H1975 cells, but not in *miR-22* inhibitor treated cells (Figure 5A). Reducing MET expression using siMET strongly increased cleaved-PARP levels in *miR-22* inhibitor treated cells as compared to the levels in either cells treated solely with *miR-22* inhibitor, or the miR-NC treated cells (Figure 6B). Collectively, these results suggest up-regulation of cleaved-PARP is one of the important biological consequences associated with the reduced clonogenic potential seen in NSCLC

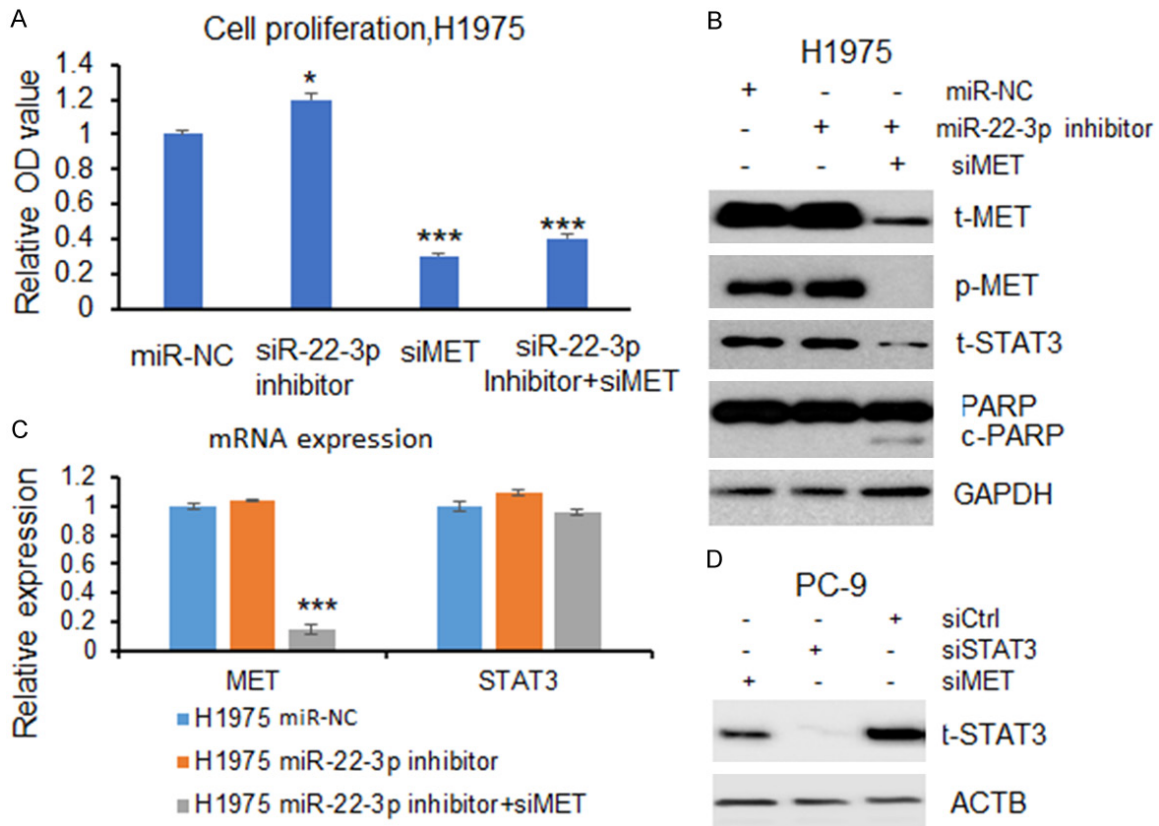
cells following *miR-22*-mediated inactivation of the MET/STAT3 pathway.

### Discussion

In this study, we demonstrate that *miR-22-3p* plays a tumor suppressive role in NSCLC, which is supported by the reduced *miR-22-3p* expression in human lung adenocarcinoma tissues as compared to normal lung tissues, and decreased cell proliferation and clonogenic ability of lung cancer cell lines upon *miR-22-3p* over-expression. Supporting a molecular mechanism, we found that *miR-22* down-regulates MET at both transcriptional and translational levels and thereby induces apoptosis of lung cancer cells which may be via a negative regulation of the MET-STAT3 signaling pathway (Figure 7).

*MiR-22-3p* was reported to be abnormally expressed in many types of malignancies with functions as tumor suppressor or oncogene [17]. For example, You et al showed that *miR-22* can inhibit tumor progression in hepatocellular carcinoma (HCC) by inhibiting its direct target oncogene galectin-1 expressed in the HCC microenvironment by hepatic stellate cells [43]. *MiR-22* reduced cell cycle-related genes *cyclin*

## Role of *miR-22-3p* in lung adenocarcinoma



**Figure 6.** Proteins and mRNAs affected by *miR-22-3p* inhibitor and/or MET siRNAs. A. Cell proliferation was decreased after combining *miR-22-3p* inhibitor and MET siRNAs (siMET) or siMET only treatment as measured by WST-1 assays. Values represented the mean  $\pm$  SD. Student's t-test, compared to miR-NC control, \* $P < 0.05$ , \*\*\* $P < 0.0001$ . B. Western blots showing the proteins of MET and STAT3 were decreased after combining of *miR-22-3p* inhibitor and MET siRNA treatment in H1975 cells. The cleaved-PARP (c-PARP) was also induced by *miR-22-3p* inhibitor plus siMET treatment. GAPDH was used as protein loading control. C. qRT-PCR showing MET mRNA was decreased by the combining of *miR-22-3p* inhibitor and MET siRNA treatment in H1975 cells, while STAT3 not changed. The protein loading control was GAPDH. D. Western blots showing STAT3 protein was decreased upon MET knockdown by siRNAs. ACTB was used as protein loading control.

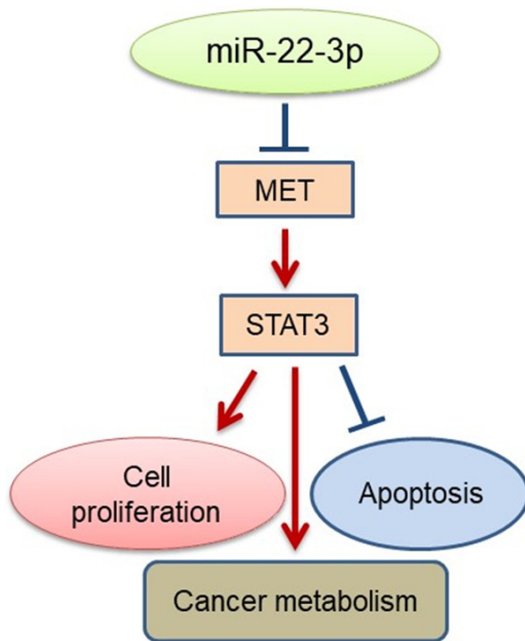
A2 and *CDKN1A* and hindered tumor malignant processes in colorectal carcinoma and liver cancer [44, 45]. Xu found that *miR-22* can promote anticancer effects by reducing cell proliferation and invasion in cervical cancer and breast cancer via stimulating pRb signaling and inducing p53 expression [46]. Conversely, *miR-22* can drive tumor progression in some cancers [17-19, 47]. Palacios et al reported that *miR-22* promoted tumor aggressiveness through active PI3K/AKT signaling via directly inhibition of the *PTEN* gene in chronic lymphocytic leukemia [18]. Regarding in lung cancer, *miR-22* was down regulated in lung cancer tissues and can inhibit tumor progression [21, 22, 48]. Consistent with these published studies, our current investigation supports the tumor suppressive role of *miR-22* based on its

reduced expression in a large cohort of human lung cancer tissues, and our *in vitro* studies confirming a tumor suppressive effect of *miR-22* using both cell proliferation and colony formation. Interestingly, we and others have found that *miR-22* levels were higher in serum in patients with lung cancer as compared to healthy controls [23, 24] but decreased in pleural effusions [25]. The connection between *miR-22* expression between tissues and serum is not clear.

Several proposed molecular targets of *miR-22* affecting the downregulation of ErbB3 [22], ATP citrate lyase (ACLY) [21] or snail [48] by *miR-22* in lung cancer cell lines have been reported previously. Because we observed that MET was involved in long non-coding RNA *MIR22HG*



## Role of *miR-22-3p* in lung adenocarcinoma



**Figure 7.** The schematic showing the possible signaling of *miR-22-3p* in lung cancer. *MiR-22-3p* inhibits cell proliferation, induces apoptosis and regulates cancer metabolism through MET-STAT3 signaling.

mediated inhibition of lung cell invasion and proliferation [28] we asked whether MET is also associated with the tumor suppressive functions of *miR-22*. MET is a cell surface tyrosine kinase receptor which regulates the expression of cell-cycle regulators, cytoskeletal proteins, and anti-apoptotic effectors. The amplification, mutation or overexpression of MET and the subsequent activation of its downstream genes, such as *AKT*, *MARK*, and *STAT3*, play important roles in tumorigenesis of many types of malignancies, including NSCLC [49, 50]. *MET* gene amplification, exon 14 skipping mutation and/or protein overexpression were reported to be associated with a worse prognosis in NSCLC patients [51, 52]. In our *in vitro* studies, we found that the overexpression of *miR-22* in the NSCLC cell line H1975 using a synthetic *miR-22* mimic, reduced the levels of both MET mRNA and protein. Most importantly, this was accompanied by suppression of the tyrosine kinase activity of MET as demonstrated by a significant reduction of phosphorylated MET. However, we did not observe a significant increase in t-MET and p-MET levels by the inhibition of *miR-22*, which might be related with the very low level of *miR-22* in H1975 cells at baseline. *STAT3* is an important downstream

signaling component of MET [40] and reported to be constitutively activated in primary NSCLC and NSCLC cell lines either dependent or independent of activating *KRAS*, *EGFR* and *PDGFR* or *MET* pathways [38, 40, 53]. *STAT3* was related to poor survival in NSCLC patients and associates with anti-apoptosis and resistance to chemotherapy or targeted therapy [38, 40]. In our Western blot analysis, we found that p-*STAT3* showed the same trend of downregulation as did p-MET in *miR-22* mimic-treated H1975 cells. Decreasing MET expression by siMET could significantly inhibit p-*STAT3* expression as well as reduce cell proliferation of *miR-22* inhibitor treated H1975 cells. Moreover, cleaved-PARP, an apoptosis marker and effector of the MET/*STAT3* axis, was also found to be upregulated either by *miR-22* mimic treatment or by siMET treatment. Collectively, these data indicate that *miR-22-3p* plays tumor suppressive effects at least in part through targeting the MET-*STAT3* signaling axis in lung cancer cells.

Bioinformatic analyses of *miR-22* regulated genes may suggest other roles for this miRNA. Several reports suggested that *miR-22-3p*, MET and *STAT3* were involved in cancer metabolism [35-37]. In this study, DAVID with GO and KEGG analysis suggested functions of *miR-22-3p* target genes that were significantly enriched in positive regulation of nucleobase-containing compound metabolic processes and positive regulation of RNA metabolic processes (Figure 4). This indicated that the *miR-22*-MET/*STAT3* axis was not only involved in cell growth/death pathways, but also regulated cellular metabolism in lung cancer (Figure 7).

One intriguing finding is that *miR-22* mimic treatment reduced MET at both the RNA and protein level, but *miR-22-3p* inhibitor had no effect on MET expression (Figure 5A and 5B). However, we did find that the *miR-22* inhibitor can promote cell colony formation (Figure 2B) and cell proliferation (Figures 2B, 6A) in H975 cells. We don't know the exact reason, but the possible explanations may be: (1) The concentration of *miR-22-3p* inhibitors is insufficient. In this study, we used 30 nM of *miR-22-3p* for both mimic and inhibitor. There is only 2-fold decrease of *miR-22-3p* expression by *miR-22-3p* inhibitor, while 345-fold increase of *miR-22-3p* expression by *miR-22-3p* mimic (Figure 2A).

## Role of *miR-22-3p* in lung adenocarcinoma

Thus, the knockdown efficiency of the inhibitor is lower than the mimic. Higher concentration of an inhibitor may be used as compared to miRNA mimic in the future. (2) The basal level of *miR-22-3p* in tumor are already low (**Figure 1**), making it difficult to see the effect of *miR-22-3p* inhibitor on the target gene (e.g. MET). Since *miR-22-3p* inhibitor may have less of an effect on MET expression, it is unclear why it affects cell proliferation, although there was only 10-20% increase (*miR-22-3p* mimic decreased cell proliferation by 70%, **Figure 2B**). The reason may be that the *miR-22-3p* inhibitor targets other genes. We know one miRNA can have many target genes and one gene could be targeted by many miRNAs. Whether *miR-22-3p* inhibitors increase colony formation and proliferation by targeting other genes is unclear, and we plan to design and perform additional experiments to define the exact reasons in the future.

In summary, *miR-22-3p* was down-regulated in NSCLC tissues and may act as an anti-oncogene via regulating the MET-STAT3 axis. The detailed molecular mechanisms require further analysis, however, this study provides new interpretations into the role of *miR-22-3p* in NSCLC tumorigenesis and may present a new biomarker as well as a therapeutic target for lung cancer.

### Acknowledgements

This project was supported in part by National Natural Science Foundation of China (NSFC) (32070625 to G.C.; 81702270 to W.S.); Affiliated Hospital of Guangdong Medical University Doctoral Foundation (Grant No. 2018052638) to W.S.; General Project of Key Research and Development Program of Shaanxi Province (Grant No. 2017SF-173) to Q.Z.; Southern University of Science and Technology Starting Found (G.C.). This study was supported by Center for Computational Science and Engineering of Southern University of Science and Technology.

### Disclosure of conflict of interest

None.

### Abbreviations

NSCLC, Non-small cell lung cancer; MET, MET Proto-Oncogene, Receptor Tyrosine Kinase;

STAT3, Signal Transducer And Activator Of Transcription 3; PARP, poly (ADP-ribose) polymerase; qRT-PCR, Quantitative real-time PCR; MiR-22-3p, MicroRNA 22-3p.

**Address correspondence to:** Guoan Chen, School of Medicine, Southern University of Science and Technology, No. 1088, Xueyuan Road, Nanshan District, Shenzhen 518055, Guangdong Province, China. Tel: +86-755-88018042; E-mail: cheng@sustech.edu.cn; Shuangying Yang, Department of Respiratory and Critical Care Medicine, The Second Affiliated Hospital of Xi'an Jiaotong University, Xi'an, Shaanxi Province, China. E-mail: yangshuangying66@163.com

### References

- [1] Siegel RL, Miller KD and Jemal A. Cancer statistics, 2020. *CA Cancer J Clin* 2020; 70: 7-30.
- [2] Mattiuzzi C and Lippi G. Current cancer epidemiology. *J Epidemiol Glob Health* 2019; 9: 217-222.
- [3] Chen X, Mao R, Su W, Yang X, Geng Q, Guo C, Wang Z, Wang J, Kresty LA, Beer DG, Chang AC and Chen G. Circular RNA circHIPK3 modulates autophagy via MIR124-3p-STAT3-PRKAA/AMPKalpha signaling in STK11 mutant lung cancer. *Autophagy* 2020; 16: 659-671.
- [4] Chen G, Gharib TG, Thomas DG, Huang CC, Misk DE, Kuick RD, Giordano TJ, Iannettoni MD, Orringer MB, Hanash SM and Beer DG. Proteomic analysis of eIF-5A in lung adenocarcinomas. *Proteomics* 2003; 3: 496-504.
- [5] Kim N, Kim HK, Lee K, Hong Y, Cho JH, Choi JW, Lee JI, Suh YL, Ku BM, Eum HH, Choi S, Choi YL, Joung JG, Park WY, Jung HA, Sun JM, Lee SH, Ahn JS, Park K, Ahn MJ and Lee HO. Single-cell RNA sequencing demonstrates the molecular and cellular reprogramming of metastatic lung adenocarcinoma. *Nat Commun* 2020; 11: 2285.
- [6] Xu JY, Zhang C, Wang X, Zhai L, Ma Y, Mao Y, Qian K, Sun C, Liu Z, Jiang S, Wang M, Feng L, Zhao L, Liu P, Wang B, Zhao X, Xie H, Yang X, Zhao L, Chang Y, Jia J, Wang X, Zhang Y, Wang Y, Yang Y, Wu Z, Yang L, Liu B, Zhao T, Ren S, Sun A, Zhao Y, Ying W, Wang F, Wang G, Zhang Y, Cheng S, Qin J, Qian X, Wang Y, Li J, He F, Xiao T and Tan M. Integrative proteomic characterization of human lung adenocarcinoma. *Cell* 2020; 182: 245-261, e217.
- [7] Di Leva G, Garofalo M and Croce CM. MicroRNAs in cancer. *Annu Rev Pathol* 2014; 9: 287-314.
- [8] Filipowicz W, Bhattacharyya SN and Sonenberg N. Mechanisms of post-transcriptional regulation by microRNAs: are the answers in sight? *Nat Rev Genet* 2008; 9: 102-114.

## Role of *miR-22-3p* in lung adenocarcinoma

- [9] Miranda KC, Huynh T, Tay Y, Ang YS, Tam WL, Thomson AM, Lim B and Rigoutsos I. A pattern-based method for the identification of MicroRNA binding sites and their corresponding heteroduplexes. *Cell* 2006; 126: 1203-1217.
- [10] Ortiz-Quintero B. Cell-free microRNAs in blood and other body fluids, as cancer biomarkers. *Cell Prolif* 2016; 49: 281-303.
- [11] Nadal E, Zhong J, Lin J, Reddy RM, Ramnath N, Orringer MB, Chang AC, Beer DG and Chen G. A MicroRNA cluster at 14q32 drives aggressive lung adenocarcinoma. *Clin Cancer Res* 2014; 20: 3107-3117.
- [12] Nadal E, Truini A, Nakata A, Lin J, Reddy RM, Chang AC, Ramnath N, Gotoh N, Beer DG and Chen G. A novel serum 4-microRNA signature for lung cancer detection. *Sci Rep* 2015; 5: 12464.
- [13] Xin M, Qiao Z, Li J, Liu J, Song S, Zhao X, Miao P, Tang T, Wang L, Liu W, Yang X, Dai K and Huang G. miR-22 inhibits tumor growth and metastasis by targeting ATP citrate lyase: evidence in osteosarcoma, prostate cancer, cervical cancer and lung cancer. *Oncotarget* 2016; 7: 44252-44265.
- [14] Zhang J, Yang Y, Yang T, Liu Y, Li A, Fu S, Wu M, Pan Z and Zhou W. microRNA-22, downregulated in hepatocellular carcinoma and correlated with prognosis, suppresses cell proliferation and tumorigenicity. *Br J Cancer* 2010; 103: 1215-20.
- [15] Li J, Liang S, Yu H, Zhang J, Ma D and Lu X. An inhibitory effect of miR-22 on cell migration and invasion in ovarian cancer. *Gynecol Oncol* 2010; 119: 543-548.
- [16] Chen B, Tang H, Liu X, Liu P, Yang L, Xie X, Ye F, Song C, Xie X and Wei W. miR-22 as a prognostic factor targets glucose transporter protein type 1 in breast cancer. *Cancer Lett* 2015; 356: 410-417.
- [17] Wang J, Li Y, Ding M, Zhang H, Xu X and Tang J. Molecular mechanisms and clinical applications of miR-22 in regulating malignant progression in human cancer (Review). *Int J Oncol* 2017; 50: 345-355.
- [18] Palacios F, Abreu C, Prieto D, Morande P, Ruiz S, Fernandez-Calero T, Naya H, Libisch G, Robello C, Landoni AI, Gabus R, Dighiero G and Oppezzo P. Activation of the PI3K/AKT pathway by microRNA-22 results in CLL B-cell proliferation. *Leukemia* 2015; 29: 115-125.
- [19] Budd WT, Seashols-Williams SJ, Clark GC, Weaver D, Calvert V, Petricoin E, Dragoescu EA, O'Hanlon K and Zehner ZE. Dual action of miR-125b As a tumor suppressor and oncomiR-22 promotes prostate cancer tumorigenesis. *PLoS One* 2015; 10: e0142373.
- [20] Zhang K, Li X, Wang Z, Han Z and Zhao YH. MiR-22 inhibits lung cancer cell EMT and invasion through targeting Snail. *Eur Rev Med Pharmacol Sci* 2017; 21: 3598-3604.
- [21] Xin M, Qiao Z, Li J, Liu J, Song S, Zhao X, Miao P, Tang T, Wang L, Liu W, Yang X, Dai K and Huang G. miR-22 inhibits tumor growth and metastasis by targeting ATP citrate lyase: evidence in osteosarcoma, prostate cancer, cervical cancer and lung cancer. *Oncotarget* 2016; 7: 44252-44265.
- [22] Ling B, Wang GX, Long G, Qiu JH and Hu ZL. Tumor suppressor miR-22 suppresses lung cancer cell progression through post-transcriptional regulation of ErbB3. *J Cancer Res Clin Oncol* 2012; 138: 1355-1361.
- [23] Franchina T, Amodeo V, Bronte G, Savio G, Ricciardi GR, Picciotto M, Russo A, Giordano A and Adamo V. Circulating miR-22, miR-24 and miR-34a as novel predictive biomarkers to pemetrexed-based chemotherapy in advanced non-small cell lung cancer. *J Cell Physiol* 2014; 229: 97-99.
- [24] Yang XZ, Q, Su W, Zhang M, Shan H, Zhang J, Chen G, Guo C and Li Y. Expression level of serum miRNA-22 and its clinical significance in patients with lung cancer. *Int J Respir* 2019; 39: 1373-1378.
- [25] Shin YM, Yun J, Lee OJ, Han HS, Lim SN, An JY, Lee KH, Lee KM and Choe KH. Diagnostic value of circulating extracellular miR-134, miR-185, and miR-22 levels in lung adenocarcinoma-associated malignant pleural effusion. *Cancer Res Treat* 2014; 46: 178-185.
- [26] Yang X, Su W, Chen X, Geng Q, Zhai J, Shan H, Guo C, Wang Z, Fu H, Jiang H, Lin J, Lagisetty KH, Zhang J, Li Y, Yang S, Massion PP, Beer DG, Chang AC, Ramnath N and Chen G. Validation of a serum 4-microRNA signature for the detection of lung cancer. *Transl Lung Cancer Res* 2019; 8: 636-648.
- [27] Su W, Wang L, Niu F, Zou L, Guo C, Wang Z, Yang X, Wu J, Lu Y, Zhang J, Beer DG, Yang Z and Chen G. LINC00857 knockdown inhibits cell proliferation and induces apoptosis via involving STAT3 and MET oncogenic proteins in esophageal adenocarcinoma. *Aging (Albany NY)* 2019; 11: 2812-2821.
- [28] Su W, Feng S, Chen X, Yang X, Mao R, Guo C, Wang Z, Thomas DG, Lin J, Reddy RM, Orringer MB, Chang AC, Yang Z, Beer DG and Chen G. Silencing of long noncoding RNA MIR22HG triggers cell survival/death signaling via oncogenes YBX1, MET, and p21 in lung cancer. *Cancer Res* 2018; 78: 3207-3219.
- [29] Garcia DM, Baek D, Shin C, Bell GW, Grimson A and Bartel DP. Weak seed-pairing stability and high target-site abundance decrease the proficiency of Isy-6 and other microRNAs. *Nat Struct Mol Biol* 2011; 18: 1139-1146.
- [30] Huang da W, Sherman BT and Lempicki RA. Systematic and integrative analysis of large

## Role of *miR-22-3p* in lung adenocarcinoma

- gene lists using DAVID bioinformatics resources. *Nat Protoc* 2009; 4: 44-57.
- [31] Schweisgut J, Schutt C, Wust S, Wietelmann A, Ghesquiere B, Carmeliet P, Drose S, Korach KS, Braun T and Boettger T. Sex-specific, reciprocal regulation of ERalpha and miR-22 controls muscle lipid metabolism in male mice. *EMBO J* 2017; 36: 1199-1214.
- [32] Koufaris C, Valbuena GN, Pomyen Y, Tredwell GD, Nevedomskaya E, Lau CH, Yang T, Benito A, Ellis JK and Keun HC. Systematic integration of molecular profiles identifies miR-22 as a regulator of lipid and folate metabolism in breast cancer cells. *Oncogene* 2016; 35: 2766-2776.
- [33] Organ SL and Tsao MS. An overview of the c-MET signaling pathway. *Ther Adv Med Oncol* 2011; 3: S7-S19.
- [34] Bian C, Liu Z, Li D and Zhen L. PI3K/AKT inhibition induces compensatory activation of the MET/STAT3 pathway in non-small cell lung cancer. *Oncol Lett* 2018; 15: 9655-9662.
- [35] Huang X, Gan G, Wang X, Xu T and Xie W. The HGF-MET axis coordinates liver cancer metabolism and autophagy for chemotherapeutic resistance. *Autophagy* 2019; 15: 1258-1279.
- [36] Fafalios A, Ma J, Tan X, Stoops J, Luo J, Defranco MC and Zarnegar R. A hepatocyte growth factor receptor (Met)-insulin receptor hybrid governs hepatic glucose metabolism. *Nat Med* 2011; 17: 1577-1584.
- [37] Demaria M, Camporeale A and Poli V. STAT3 and metabolism: how many ways to use a single molecule? *Int J Cancer* 2014; 135: 1997-2003.
- [38] Harada D, Takigawa N and Kiura K. The role of STAT3 in non-small cell lung cancer. *Cancers (Basel)* 2014; 6: 708-722.
- [39] Su W, Feng S, Chen X, Yang X, Mao R, Guo C, Wang Z, Thomas DG, Lin J, Reddy RM, Orringer MB, Chang AC, Yang Z, Beer DG and Chen G. Silencing of long noncoding RNA MIR22HG triggers cell survival/death signaling via oncogenes YBX1, MET, and p21 in lung cancer. *Cancer Res* 2018; 78: 3207-3219.
- [40] Van Schaeybroeck S, Kalimutho M, Dunne PD, Carson R, Allen W, Jithesh PV, Redmond KL, Sasazuki T, Shirasawa S, Blayney J, Michieli P, Fenning C, Lenz HJ, Lawler M, Longley DB and Johnston PG. ADAM17-dependent c-MET-STAT3 signaling mediates resistance to MEK inhibitors in KRAS mutant colorectal cancer. *Cell Rep* 2014; 7: 1940-1955.
- [41] Ndolo KM, An SJ, Park KR, Lee HJ, Yoon KB, Kim YC and Han SY. Discovery of an indirubin derivative as a novel c-met kinase inhibitor with in vitro anti-tumor effects. *Biomol Ther (Seoul)* 2019; 27: 216-221.
- [42] Ferrer I and Planas AM. Signaling of cell death and cell survival following focal cerebral ischemia: life and death struggle in the penumbra. *J Neuropathol Exp Neurol* 2003; 62: 329-339.
- [43] You Y, Tan JX, Dai HS, Chen HW, Xu XJ, Yang AG, Zhang YJ, Bai LH and Bie P. MiRNA-22 inhibits oncogene galectin-1 in hepatocellular carcinoma. *Oncotarget* 2016; 7: 57099-57116.
- [44] Yang F, Hu Y, Liu HX and Wan YJ. MiR-22-silenced cyclin A expression in colon and liver cancer cells is regulated by bile acid receptor. *J Biol Chem* 2015; 290: 6507-6515.
- [45] Shi C and Xu X. MicroRNA-22 is down-regulated in hepatitis B virus-related hepatocellular carcinoma. *Biomed Pharmacother* 2013; 67: 375-380.
- [46] Xu D, Takeshita F, Hino Y, Fukunaga S, Kudo Y, Tamaki A, Matsunaga J, Takahashi RU, Takata T, Shimamoto A, Ochiya T and Tahara H. miR-22 represses cancer progression by inducing cellular senescence. *J Cell Biol* 2011; 193: 409-424.
- [47] Jiang R, Deng L, Zhao L, Li X, Zhang F, Xia Y, Gao Y, Wang X and Sun B. miR-22 promotes HBV-related hepatocellular carcinoma development in males. *Clin Cancer Res* 2011; 17: 5593-5603.
- [48] Zhang K, Li XY, Wang ZM, Han ZF and Zhao YH. MiR-22 inhibits lung cancer cell EMT and invasion through targeting Snail. *Eur Rev Med Pharmacol Sci* 2017; 21: 3598-3604.
- [49] Birchmeier C, Birchmeier W, Gherardi E and Vande Woude GF. Met, metastasis, motility and more. *Nat Rev Mol Cell Biol* 2003; 4: 915-925.
- [50] Mahtouk K, Tjin EP, Spaargaren M and Pals ST. The HGF/MET pathway as target for the treatment of multiple myeloma and B-cell lymphomas. *Biochim Biophys Acta* 2010; 1806: 208-219.
- [51] Pyo JS, Kang G and Cho H. Clinicopathological significance and diagnostic accuracy of c-MET expression by immunohistochemistry in gastric cancer: a meta-analysis. *J Gastric Cancer* 2016; 16: 141-151.
- [52] Moosavi F, Giovannetti E, Saso L and Firuzi O. HGF/MET pathway aberrations as diagnostic, prognostic, and predictive biomarkers in human cancers. *Crit Rev Clin Lab Sci* 2019; 56: 533-566.
- [53] Belalcazar A, Azana D, Perez CA, Raez LE and Santos ES. Targeting the Met pathway in lung cancer. *Expert Rev Anticancer Ther* 2012; 12: 519-528.