MicroRNA-mediated sensitization of lung cancer cells to chemotherapeutics: the roles of miR-21 and miR-155

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Background
MicroRNAs (miRNAs) are conserved short 22-nucleotide RNAs with important roles in regulating gene expression. Misregulation of genes that control cell-cycle and cell-fate determination often contributes to cancer. The aims of this work were to evaluate the expressions of two oncogenic miRNA (oncomiRs), miR-21 and miR-155, in lung cancer, and to see whether reintroduction or inhibition of these would affect progression or aid sensitivities of the lung cancer cells to major chemotherapeutics.

Methods
This work involved cell culture of lung adenocarcinoma cells H358 and A549 and normal lung cells. It compared the miRNA expression profiles of two miRNAs (miR-21 and miR-155) in cancer and normal lung cell lines using real-time PCR and then treated with three known chemotherapeutics, namely cisplatin, etoposide and paclitaxel, and concluded by inhibiting overexpressed miRNA by transfecting the cancer cells with miRNA inhibitors, and proliferation was measured with sulforhodamine-B assay. Results showed that miR-21 was overexpressed in the entire cell lines used, which is consistent with the role of miR-21 as an oncomir, whereas miR-155 was downregulated, suggesting that miR-155 could be acting as a tumor suppressor. Furthermore, inhibition of miR-21 function in H358 lines using 50 nM Ambion anti-miR led to a decreased proliferation of H358 cells compared with the 50 nM anti-miR-155-treated group. Downregulation of miR-21 seems to sensitize lung cancer cells to chemotherapeutics (etoposide).

Conclusion
This work demonstrated that miR-21 at 50 nM might sensitize lung cancer cells to chemotherapeutics (etoposide) and that miR-155, a known oncogenic miRNA, seems to be acting as a tumor suppressor in lung cancer, which promises to be of immense therapeutic importance.

Keywords:
cancer, chemotherapeutics, lung cancer, microRNAs, real-time PCR

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Introduction

MicroRNAs (miRNAs) are commonly believed to be a class of small, noncoding RNAs that posttranscriptionally control the translation and stability of mRNAs. miRNAs are known to hybridize to the 3' untranslated region of target mRNAs causing repression at a posttranscriptional level. Genes encoding miRNA molecules are found either inserted in introns as polycistronic clusters or in isolated regions of the genome (Jerome *et al.*, 2007).

MiR-21, in particular, is known to be highly expressed in various cancer types. miRNA functions can be cell context and tissue dependent. For example, inhibition of miR-21 using antisense oligonucleotides increased the growth of HeLa cells (derived from cervical cancer) but did not significantly affect the growth of A549 cells [nonsmall cell lung cancer (NSCLC); Lu *et al.*, 2005]. The inhibition of miR-21 resulted in increased apoptosis in human glioblastoma cells.

As noted by Chen *et al.* (2012), miRNA-21 promoter contains highly conserved regions with consensus binding sites for several transcriptional factors, including activator protein 1 and factors of the forkhead family (FOXO), such as FOXO3a.

MiR-21 is thought to play a significant role in the drug resistance to cisplatin in the neuroblastoma cells.

Liu *et al.* (2011) showed that miR-21 is involved in the later stages of tumorigenesis and not in tumor promotion, as it has no effect on tumorigenesis in the absence of oncogenic KRAS. These studies, combined with human tissue data and cell culture experiments, confirm that miR-21 is an oncogene and provide a rationale for the therapeutic inhibition of miR-21.

According to Liu *et al.* (2012), miR-21 has been reported to be overexpressed in many human malignancies including NSCLC. In previous studies, upregulation of miR-21 in NSCLC patients' serum is correlated with TNM stage and lymph node metastasis; Fu *et al.*, 2011; Wang *et al.*, 2011. Furthermore, the status of serum miR-21 expressions is found to be an independent prognostic factor for NSCLC patients. They further found that miR-21 could regulate adriamycin resistance of breast cancer cells, at least in part, by targeting the tumor suppressor gene PTEN. However, the associations of miR-21 expression with malignant phenotypes of NSCLC are still poorly understood (Stahlhut Espinosa and Slack, 2006).

miRNA-155 is encoded in B-cell integration cluster, a conserved region of the noncoding gene (Medina and Slack, 2008). B-cell integration cluster was originally recognized as a common integration site for the avian leucosis virus, inducing B-cell lymphomas together with MYC (Zhang *et al.*, 2008). Overexpression of miR-155 has been observed in both hematological (Eis *et al.*, 2005; Kluiver *et al.*, 2005) and solid tumors (Zheng *et al.*, 2012). miR-155 is regarded as a 'master regulator' of many biological processes. It is a prominent miRNA that regulates genes involved in immunity and cancer-related pathways. MiR-155 is said to be overexpressed in lung cancer, which correlates with poor patient prognosis. It is unclear how miR-155 becomes increased in lung cancers and how this increase contributes to reduced patient survival (Mattiske *et al.*, 2012).

Overexpression of miR-155 specifically in the B-cell lineage results in preleukaemic pre-B-cell proliferation in the spleen and bone marrow, followed later in life by B-cell malignancy (Lawrie *et al.*, 2007).

Methodology

Cell lines

H358 and A549 cells were cultured in RPMI-1640 media supplemented with 10% Fetal bovine serum, FBS.

Normal lung-FC 3KT, bronchoalveolar cells were cultured with keratinocyte media, containing bovine pituitary extract, and epidermal growth factor. Cells were maintained in an incubator at 37° with 5% CO_2 according to standard procedures.

Cell transfection

All miRNA transfections and chemotherapeutic dose–response studies were performed in triplicate. Ambion mirVana anti-miR miRNA inhibitors (Carlsbad, California, USA), and Ambion mirVana anti-miR miRNA negative control were used. Anti-miR inhibitors are chemically modified, single-stranded nucleic acids designed to bind specifically to, and inhibit, endogenous miRNA molecules. The anti-miR-negative control no. 1 was used here, which is a random sequence anti-miR molecule that has been validated to produce no identifiable effects on miRNA function. To transfect cells with miRNA inhibitor, or control, 12.5 μl of 2 mmol/l oligomer was added to 100 μl with Opti-MEM (Invitrogen Corp., Carlsbad, California, USA); thereafter, 10 μl of Dharmafect 1 (Life Technologies, Carlsbad, California, USA) was diluted to 100 μl with Opti-MEM, mixed with the transfection components, and 2 μl of diluted cell suspension containing 20 000 cells was added, and the plate was incubated at 37°C. After 24 h, the medium was removed, and the cells were trypsinized. For the growth inhibition assay, the cells were treated with chemotherapeutics and then further processed for sulforhodamine-B (SRB) assay.

Sulforhodamine-B assay

Cell growth and drug potency were tested using the SRB proliferation assay. In each experiment, 3000–5000 cells per well were seeded in 96-well plates after transfection with miRNA inhibitors and incubated for 24 and 48 h. Chemotherapeutics (cisplatin, etoposide, and paclitaxel) were added in a seven-point dilution series, in three replicate plate columns per treatment. After 48 h, incubation was terminated by replacing the medium with 100 μl of 10% trichloroacetic acid (Sigma-Aldrich, Munich, Germany) in 1× PBS and incubating at 4°C for 1 h. The plates were then washed with water, air-dried overnight, and stained with 50 μl of 0.4% SRB (Sigma-Aldrich) in 1% acetic acid for 30 min at room temperature. Unbound SRB was washed off with 1% acetic acid. After air-drying, and resolubilization of the protein-bound dye in 10 mmol/l of Tris-HCl (pH 8.0), absorbance was read in a Wallac victor2 microplate reader at 570 nm. To determine IC_{50} values, the absorbance of control cells without the drug was set at 1, and dose–response curves were plotted using Prism software (GraphPad Software Inc., San Diego, CA, USA). Each experiment was carried out independently at least thrice.

Renilla luciferase reporter assay

The knockdown of 3' UTR-luciferase activity in the presence of miR-21 was measured by cotransfecting an miR-21 sensor reporter with a synthetic miRNA. DharmaFECT DUO was used to transfect H358 cells in triplicate in a 96-well format with 100 ng of miR-21 sensor reporter and serial dilutions (50–1.53 nmol/l) of

anti-miR-21 inhibitor or scrambled control miRNA. After 24 h of incubation, 100 μl of LightSwitch lysis reagent (SwitchGear Genomics, Carlsbad, California, USA) was added to each well, and luminescence was recorded; the plates were incubated at room temperature for 30 min and read on a Wallac Victor2 multilabel plate reader.

Isolation of total RNA

Cultured cancer cells were washed once with PBS and then lysed directly in the well by addition of 1 ml of TRIzol reagent; the lysed cells were removed from the well by scraping with a cell scraper. This mixture was transferred to a 1.5 ml Eppendorf tube; RNA was then extracted according to the manufacturer's instructions (Ambion).

Quantitative real-time reverse transcription PCR

The extracted total RNA was used to make cDNA, which was the template for real-time reverse transcription (RT)-PCR analysis, using the miScript Primer Assay kit (Qiagen, Valencia, California, USA) in combination with the miScript SYBR Green PCR Kit (Qiagen). In this assay, miRNAs are amplified using the miScript Universal Primer together with the miRNA-specific primer (the miScript Primer Assay). Briefly, qPCR was carried out using the QuantiTect or miScript SYBR green PCR kit (Qiagen) using the Roche Light cycler 480 RT-PCR system. RNUB6 was used as a control for miR-21-5p and miR-155-5p (miScript Primer Assay; Qiagen). Quantification was carried out using the $2^{-\Delta\Delta Ct}$ equation and then expressed as fold changes. PCR was performed in 384-well optical plates.

cDNA synthesis using miScript II RT Ki

Briefly, total RNA was reverse transcribed using the miScript II RT Kit (Qiagen), as per the manufacturer's protocol.

Inhibition of microRNA function

Transfection was carried out using Dharmafect 1 (Life Technologies) according to the manufacturer's manual as follows: H358 cells were trypsinized and counted. Cells were diluted in antibiotic-free complete medium to achieve the appropriate plating density in 100 μl of media solution. Hundred microliters of cells were then plated into each well of a 96-well plate and incubated at 37°C with 5% CO_2 overnight.

Results

miRNA profiling of H358 and A549 using miScript quantitative RT-PCR Assay (Qiagen).

MiR-21 is upregulated in H358 and A549 lung cancer cell lines.

Fig. 1, demonstrates the relative miRNA expression profile of miR-21 in H358 and A549 (cancer) compared with normal lung cells (bronchioaveolar cells); miR-21 is upregulated in cancer cells (H358 and A549) using QiagenMiScriptmiRNA PCR system. Data are shown as fold changes of miRNA levels in H358 lung cancer cells. The level of miR-21 in H358 cells was significantly higher than the normal lung cells ($P < 0.05$).

MiR-155 is downregulated in H358 lung cancer line

Fig. 2 shows relative miRNA expression level of miR-155 in H358 and A549 (cancer) compared with normal lung cells; miR-155 is upregulated in cancer cells (H358 and A549). miR-155 is upregulated in H358 cancer cells using Qiagen MiScript miRNA PCR system. Data are shown as fold changes of miRNA levels in H358 lung cancer cells. The result showed downregulation of miR-155 in two biological replicates of H358 lines.

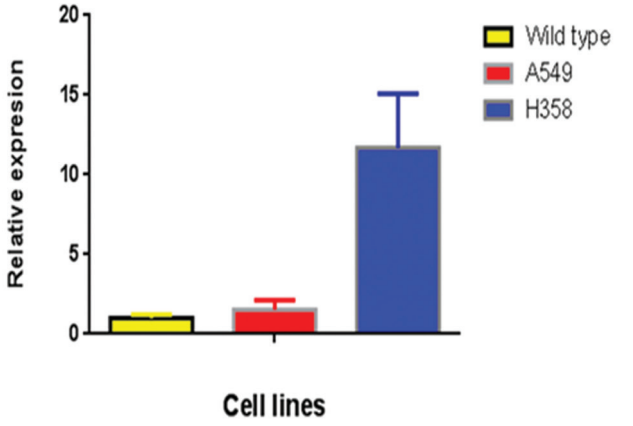
Determination of IC_{50} of the chemotherapeutics used

The dose–response curve of treating H358 cancer lines with three commonly used chemotherapeutics in the management of lung cancer were determined by SRB assay after 72 and 96 h of incubation with etoposide, cisplatin, and paclitaxel. The results are shown below.

The IC_{50} of cisplatin following incubation of H358 cells in cisplatin

Fig. 3 is the dose–response curve IC_{50} of cisplatin following 96 h incubation of H358 in serial concentrations of cisplatin. The cells were fixed with 1% Trichloroacetic acid (TCA) and analyzed using SRB assay. The IC_{50} of cisplatin (72 h incubation) is

Figure 1



Expression level of miR-21 in H358 and A549 (cancer) and normal lung cells; miR-21 is upregulated in cancer cells, H358 and A549, using Qiagen MiScript miRNA PCR system. Data are shown as fold changes of miRNA levels in H358 lung cancer cells ($P < 0.05$).

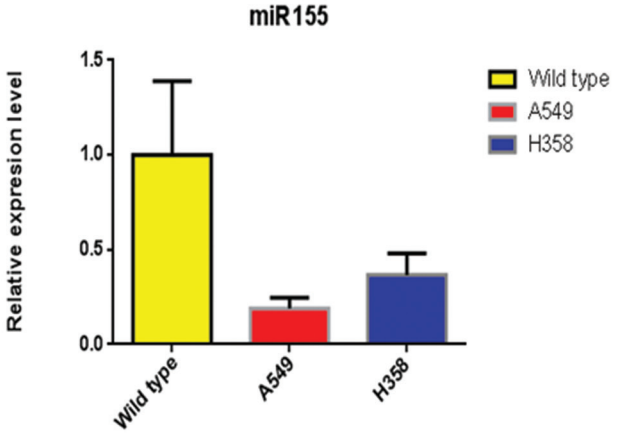
10 and 5.47 μmol/l for 96 h incubation. The data were generated using GraphPad Prism, version 6.

The IC_{50} of etoposide following incubation of H358 cells in etoposide

Fig. 4 is the dose–response curve IC_{50} of etoposide following 72 and 96 h incubation of H358 in serial concentrations of paclitaxel. The cells were fixed with 1% TCA and analyzed using the SRB assay. Data were generated using GraphPad Prism, version 6.

The IC_{50} of etoposide (72 h incubation) is 15.8 and 0.1 μmol/l for 96 h incubation. The data were generated using GraphPad Prism, version 6.

Figure 2



Expression level of miR-155 in H358 and A549 (cancer cells) and normal lung cells; miR-155 is downregulated in H358 and A549 cells using Qiagen MiScript miRNA PCR system. Data are shown as fold changes of miRNA levels in H358 and a549 compared with FC 7333 3KT (normal lung) cells.

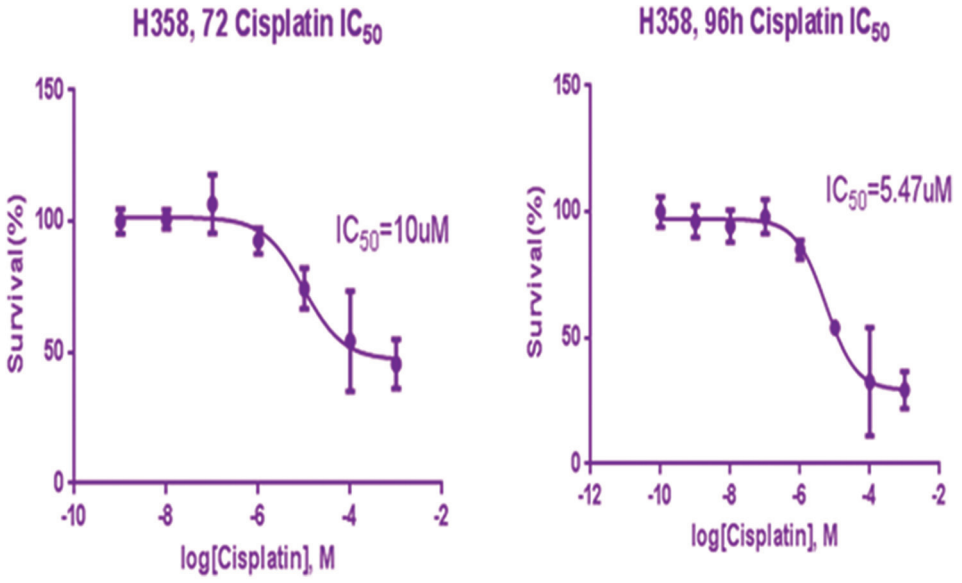
Downregulation of miR-21 inhibited survival capacity of H358 cells after chemotherapeutic treatment

To assess whether miR-21 downregulation could sensitize NSCLC H358 cells to chemotherapeutics, H358 cells were transfected with either anti-miR-scrambled control (Ambion) or anti-miR-21 (Ambion). The following serial concentrations were used for both the control and the miRNA inhibitor (5, 10, 20, 40 nmol/l). After 48 h incubation, the cells were in turn treated with various concentrations of etoposide, which was the selected chemotherapeutic due to its robust activity from the previous IC_{50} experiments. Their response was analyzed using SRB assay. In SRB survival assay, there was decreased survival capacity of H358 cells transfected with 40 nmol/l anti-miR-21 7 days post-transfection and 7 days postetoposide treatment (Fig. 5). Forty-eight hours after transfection, H358 cells were treated with various doses of etoposide (1.25, 2.5, 5, 10, 20 μmol/l), and the survival percentage upon incubation was detected. As shown in Fig. 5, the survival fraction of H358 cells in 40 nmol/l anti-miR-21-transfected group was significantly lower than that in the Dharmafect-transfected control group. There was a clear decrease in survival of all anti-miR-21-transfected cells (5, 10, 20, 40 nmol/l, respectively) at the lower doses of etoposide, indicating that downregulation of miR-21 could significantly enhance the sensitivity of H358 cells to chemotherapeutics (Fig. 5).

Does inhibition of miR-21 and miR-155 functions in H358 cells lead to reduced survival of cancer cells?

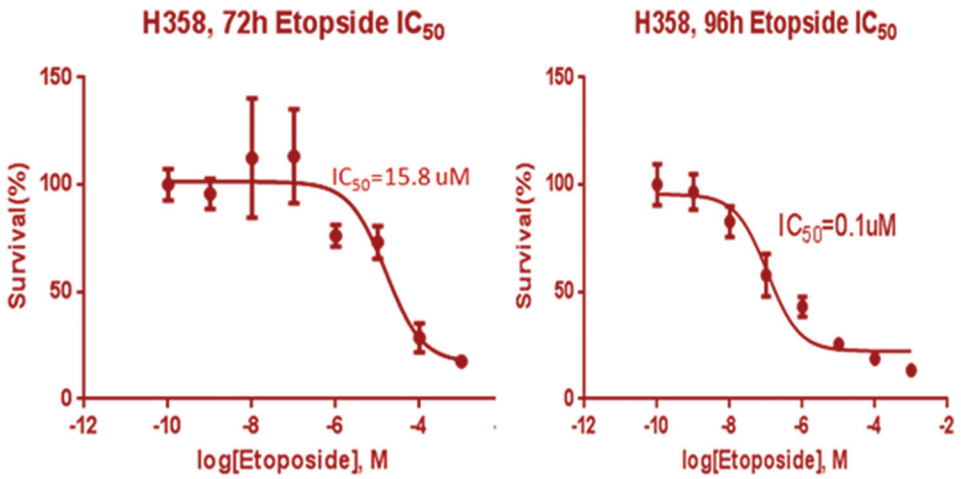
H358 cells were transfected with 25 and 50 nmol/l of anti-miR-21 and 155 using Dharmafect 1 transfection solution and incubated for 3, 5, and 6 days

Figure 3



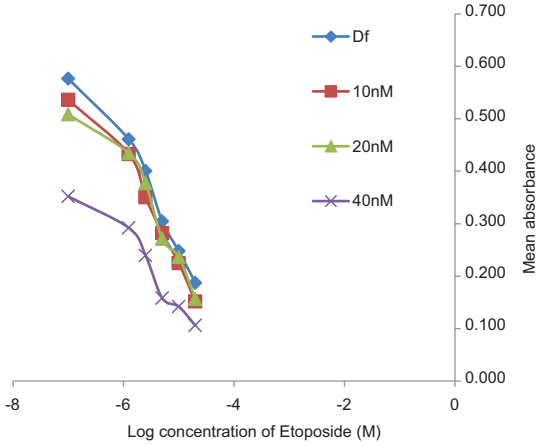
The dose–response curve IC_{50} of cisplatin following 72 and 96 h incubation of H358 in various concentrations of cisplatin and analyzed using SRB assay. The data generated were plotted with GraphPad Prism, version 6. SRB, sulforhodamine-B.

Figure 4



The dose–response curve IC_{50} of etoposide following 72 and 96 h incubation of H358 in various concentrations of etoposide and analyzed using SRB assay. The data generated were plotted with GraphPad Prism, version 6. SRB, sulforhodamine-B.

Figure 5



Transfection of H358 with different concentrations of anti-miR-21 inhibitor (D5 post-transfection and D3 postchemotreatment), compared with Dharmafect alone treated control. A total volume of 40 nmol/l of anti-miR-21 significantly reduced the survival of H358 cells, increasing the sensitivity of the cells to the chemotherapeutic etoposide in a dose dependent manner.

post-transfection and analyzed using SRB survival assay. Fig. 7 shows the result obtained.

Transfection of H358 with 50 nmol/l of anti-miR-21 miRNA inhibitor (Ambion) significantly inhibited the growth of H358 cells in all the three times points (3, 5, and 6 days) compared with anti-miR-155, which seems to support qPCR data showing downregulation of miR-155 in H358 cells relative to normal lung cells. The effect of 50 nM concentration of the anti-miR seems to be very marked compared with the 25 nM concentration, showing that the effect could be dose dependent. This observation could also be a preliminary validation of the qPCR data, suggesting that miR-155 could be acting as a tumor suppressor in lung cancer cells (Figs. 6 and 7).

Luciferase assay

In order to verify transfection efficiency and to show that the anti-miR-21 specifically binds to the target, Renilla luciferase using miR-21 sensor (Switchgear Genomics) was cotransfected with anti-miR-21 miRNA inhibitor (Ambion) as well as with scrambled anti-miR control (Ambion) using Dharmafect Duo (Life Technologies). Serial dilution of anti-miR-21 and negative control (scramble) were made from 50 to 1.53 nmol/l. Fig. 8 shows the result obtained, which clearly demonstrates derepression of reporter activity in H358 cells indicating the functioning or transfection efficiency of the anti-miR-21 used. The scramble controls are seen not evoking similar responses like anti-miR-21. This shows that the killing seen in the scramble control could be due to the normal toxicity of nucleic acid.

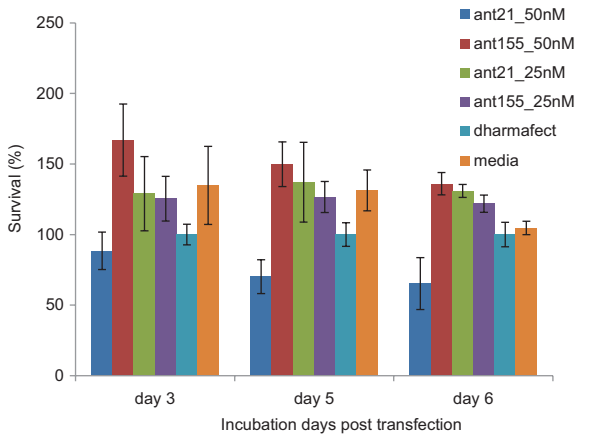
Discussion

MicroRNA-mediated sensitization of lung cancer cells to chemotherapeutics

Lung cancer continues to be the leading cause of cancer-related death globally. Using microarray data, Gao *et al.* (2011) compared miRNA expression profiles in primary squamous cell lung carcinoma with normal cells and highlighted the potential relevance of miRNA to clinical events and patient survival periods. They observed that miR-21 was upregulated in nearly 75% of cancer specimens and that this signature was significantly correlated with shortened survival.

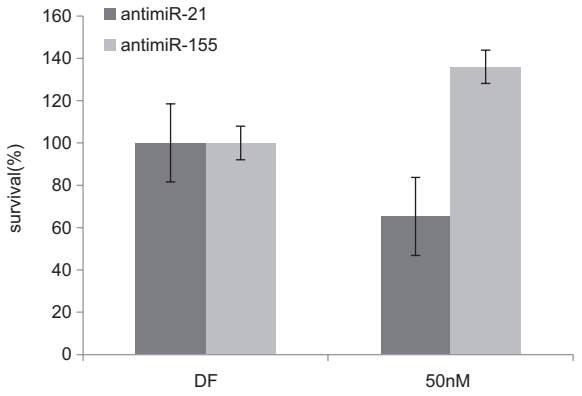
MiRNA expression signatures seem to hold great promise in cancer diagnosis and treatment, and strategies to interfere with miRNA function are considered to offer novel opportunities for cancer treatment (Adams *et al.*, 2014, 2017).

Figure 6



Inhibition of H358 with anti-miR-21 and 155. H358 cells were transfected with 50 and 25 nmol/l of Ambion microRNA inhibitors (anti-miR-21 and miR-155). 50 nmol/l of anti-miR-21 significantly inhibited the growth of H358 cells in all the three time points.

Figure 7



H358 was transfected with 50 nmol/l of Ambion microRNA inhibitors (anti-miR-21 and miR-155) using Dharmafect 1 transfection reagent (Thermoscientific). A total volume of 50 nmol/l of anti-miR-21 significantly inhibited the growth of H358 cells 6 days posttransfection.

This work was designed to look at the expression profile of two known oncomirs, miR-21 and miR-155, in NSCLC, and to see whether reintroduction or inhibition of these would affect progression or aid sensitivities of the lung cancer cells to major chemotherapeutics used in the management of lung cancer.

The finding of this study showed that miR-21 in lung adenocarcinoma cells lines H358, H23, H440, and A549, were highly expressed than that in the normal human bronchial epithelial cell line. However, expression of miR-155 was down-regulated in the lung cancer lines compared with the normal cells. This result seems to suggest that miR-155 could be acting as a tumor suppressor in some lung cancer lines. An earlier report by Slack and colleagues showed that hypoxia leads to the upregulation of miR-155 in lung cancer cells. They showed that the HIF-1α gene drives the induction of miR-155. They believed their data were supported by the observation that miR-155 was also induced in renal cancer cells lacking functional Von Hippel-Lindau and that this induction depended in part on HIF-1α (Babar *et al.*, 2011).

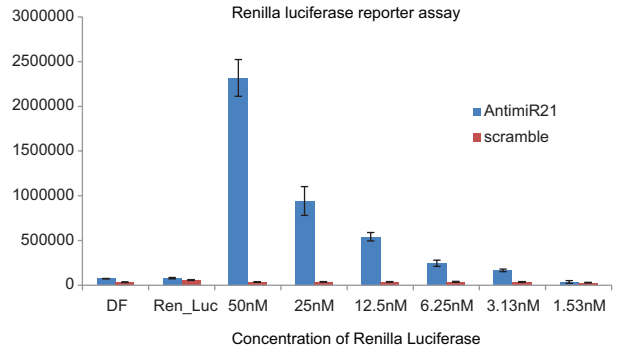
Furthermore, the Slack group (Babar *et al.*, 2012a) equally showed that 'overexpression of miR-155 in lymphoid tissues resulted in disseminated lymphoma characterized by a clonal, transplantable pre-B-cell population of neoplastic lymphocytes. Again, withdrawal of miR-155 in mice with established disease resulted in rapid regression of lymphadenopathy, in part due to apoptosis of the malignant lymphocytes, demonstrating that these tumors are dependent on miR-155 expression. They equally showed that systemic delivery of antisense peptide nucleic acids encapsulated in unique polymer nanoparticles inhibits miR-155 and slowed the growth of 'addicted' pre-B-cell tumors *in vivo*, suggesting a promising therapeutic option for lymphoma/leukemia' (Babar *et al.*, 2012b). From these reports, downregulation of miR-155 in H358, an equally NSCLC cell line, seems to support two reports suggesting that miR-155, the so called 'master regulator' of numerous biological processes, most notably those involved in immune function and cancer development, could also be acting as a tumor suppressor.

This finding that miR-155 could be acting as a potential tumor suppressor in H358 lung cancer line is supported by the work of Gasparini *et al.* (2014a) in triple-negative breast cancer. They showed that 'overexpression of miR-155 in human breast cancer cells reduced the levels of RAD51 and affects the cellular response to IR. MiR-155 directly targets the 3'-untranslated region of RAD51. Overexpression of miR-155 decreased the efficiency of homologous recombination repair and enhanced sensitivity to IR *in vitro* and *in vivo*' (Gasparini *et al.*, 2014b).

This observation, therefore, supports the 'double-edged role of miR-155: from oncomir in most of the cancer models to protective in triple-negative breast cancer' and in H358 lung cancer line that this current work reported. Their proposed rationale for this completely unexplored role of miR-155 is believed to be based on triple-negative breast cancer's high dependency on the homologus recombination repair pathway (Gasparini *et al.*, 2014a).

In another study that seems to support miR-155 as a potential tumor suppressor, miR-155 was downregulated in melanoma cells, as compared with normal melanocytes, and its ectopic expression impaired proliferation and induced apoptosis (Levati *et al.*, 2011).

Figure 8



Cotransfection of H358 cells with 100 nM of Renilla miR-21 sensor and various concentrations of anti-miR-21 and scramble control serially diluted from 50 to 1.53 nmol/l solutions using Dharmafect duo transfection reagent. Result shows increased reporter activity indicating the functioning or transfection efficiency of the anti-miR-21 used. The scramble controls are seen not evoking similar responses like anti-miR-21, which shows that the killing seen in the scramble control could be due to the normal toxicity of nucleic acid.

Levati *et al.* (2011) further investigated whether miR-155 could mediate melanoma growth inhibition via SKI gene silencing; using luciferase reporter assays, they demonstrated that miR-155 interacted with SKI 3' UTR and impaired gene expression. This work seemed to highlight the role of miR-155 as a tumor suppressor despite being known to act as an oncomiR in many cell types. In this work, miR-155 was observed acting as a tumor suppressor in the context of the lung cancer cells used. The dual role of miR-155 as both oncogenic and tumor suppressor miRNA was equally noted by Slack's group (Svoronos *et al.*, 2016).

Development of resistance to chemotherapy or radiotherapy is a serious problem in the management of cancer, greatly increasing patient morbidity and mortality (Port *et al.*, 2011). Data from this work showed that inhibition of miR-21 significantly enhanced the sensitivity of lung cancer cells to chemotherapeutic agents (etoposide) (Fig. 5).

From IC_{50} data of various chemotherapeutics used, such as etoposide and cisplatin, effective dosage of the drugs and anti-miR were used to set up experimental matrix to assess the effect of the chemotherapeutics and miRNA-inhibition on cell death or cell survival. The result showed that in H358 lines, IC_{50} of etoposide after 72 h incubation was 15.8 and 0.1 μmol/l for 96 h incubation. The IC_{50} of cisplatin (72 h incubation) is 10 and 5.47 μmol/l for 96 h incubation.

Inhibition of miRNAs using anti-miRs in this study demonstrated that the knock-down of miR-21 could significantly inhibit the growth of lung cancer cells by inducing apoptosis enhancement. This observation is supported by various reports in other human cancers, showing that miR-21 upregulation promotes the growth of tumor cells. Furthermore, other laboratories have shown that miR-21 could affect the metastatic behavior of tumor cells. Yang *et al.* (2013) reported that miR-21 regulates the metastatic behavior of B16 melanoma cells by promoting cell proliferation, survival, and migration/invasion as well as by suppressing IFN action.

Moreover, Zhu *et al.* (2012) showed that miR-21 functions in both invasion and tumor metastasis by targeting multiple tumor/metastasis suppressor genes, suggesting that suppression of miR-21 may provide a novel approach for the treatment of advanced cancers. Therefore, inhibition of miR-21 in H358 in this current study would provide this novel tumor targeting approach in being discussed.

It is important, however, to keep in mind that miRNA functions can be cell context and tissue dependent. For example, inhibition of miR-21 using antisense oligonucleotides increased the growth of HeLa cells (derived from cervical cancer) but did not significantly affect the growth of A549 cells (NSCLC; Tang *et al.*, 2013). The later finding was also observed in this study in relation to A549 (data not shown).

Conclusion

In conclusion, the present study demonstrated the biological functions of miR-21, with the ability to promote growth, migration, invasion, and chemoresistance of NSCLC cells. This result indicates that inhibition of miR-21 might be a rational therapeutic strategy for the treatment of NSCLC in the future. More importantly, this work has shown that miR-155 could be acting as a tumor suppressor, which will change the therapeutic approach of miRNA-mediated targeting of lung cancers.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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