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Restoration of miR-330 expression suppresses lung cancer cell viability, proliferation and migration

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Author contribution statement

Constructing an idea or hypothesis for research have been provided by Ali mohammadi, Behzad Mansoori.

Planning methodology to reach the conclusion have been provided by Behzad Baradaran and Pascal H.G. Duijf.

Organising and supervising the course of the project or the article and taking the responsibility have been provided by Behzad Baradaran, Ali Mohammadi.

Providing personnel, environmental and financial support and tools and instruments that are vital for the project have been provided by Behzad Baradaran

Biological materials, reagents and referred patients have been provided by Ali Mohammadi and Behzad Mansoori.

Taking responsibility in execution of the experiments, patient follow-up, data management and reporting have been provided by Ali Mohammadi and Behzad Mansoori

Taking responsibility in logical interpretation and presentation of the results have been provided by Ali Mohammadi and Behzad Mansoori.

Taking responsibility in this necessary function have been provided by Behzad Baradaran

Taking responsibility in the construction of the whole or body of the manuscript have been provided Ali Mohammadi, Behrooz Shokouhi, Leila Tebbi and Elham Safarzadeh.

Reviewing the article before submission not only for spelling and grammar but also for its intellectual content have been provided by Grith L. Sorensen**,** Uffe Holmskov.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

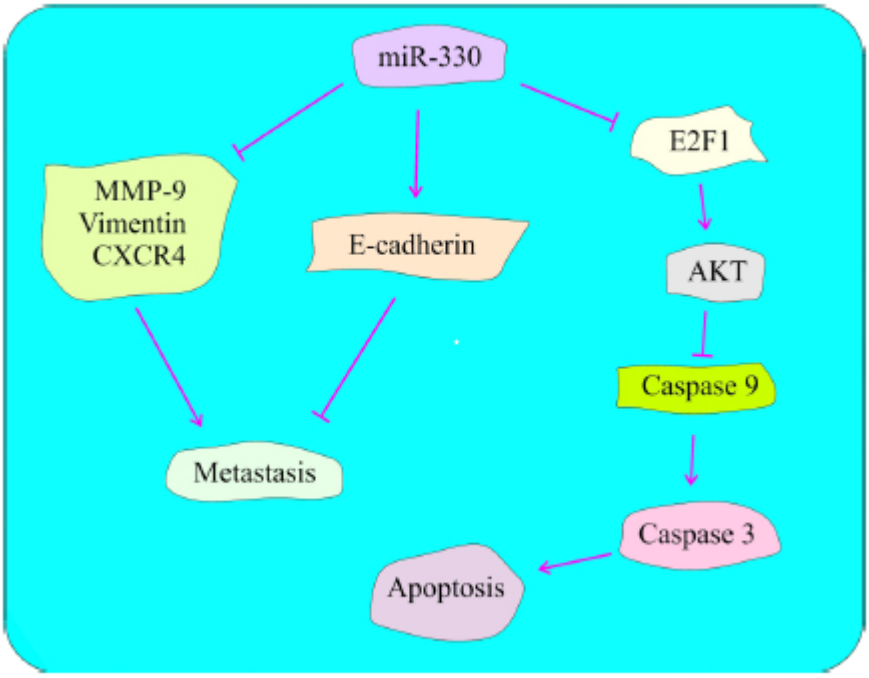
Abstract

Lung cancer is one of the most common cancers and its incidence is rising around the world. Various studies suggest that miR-330 acts as a tumor suppressor microRNA (miRNA) in different types of cancers, but precisely how has remained unclear. In this study, we investigate miR-330 expression in lung cancer patient samples, as well as *in vitro*, by studying how normalization of miR-330 expression affects lung cancer cellular phenotypes, such as viability, apoptosis, proliferation and migration. We establish that low miR-330 expression predicts poor lung cancer prognosis. Stable restoration of reduced miR-330 expression in lung cancer cells reduces cell viability, increases the fraction of apoptotic cells, causes G2/M cell cycle arrest and inhibits cell migration. These findings are substantiated by increased mRNA and protein expression of markers for apoptosis via the intrinsic pathway, such as caspase 9, and decreased mRNA and protein expression of markers for cell migration, such as vimentin, CXCR4 and MMP9. We showed that reduced miR-330 expression predicts poor lung cancer survival and that stable restoration of miR-330 expression in lung cancer cells has a broad range of tumor suppressive effects. This indicates that miR-

330 is a promising candidate for miRNA replacement therapy for lung cancer patients.

Graphical Abstract

We showed that reduced miR-330 expression predicts poor lung cancer survival and that stable restoration of miR-330 expression in lung cancer cells has a broad range of tumor suppressive effects. This indicates that miR-330 is a promising candidate for miRNA replacement therapy for lung cancer patients.



Keywords: Lung cancer, miR-330, Apoptosis, Cell cycle, Migration

1-Introduction

Lung cancer development is a multistage process that includes the accumulation of genetic and epigenetic changes, which cause damage to DNA and finally turns lung epithelial cells into cancerous cells (Asghariazar, Sakhinia, Mansoori, Mohammadi, &

Baradaran, 2019). Lung cancer is the most fatal cancer among Americans (Larsen & Minna, 2011). It is estimated that there are going to be 200,221 new cases of lung cancer with 162,470 deaths until 2015 (R. L. Siegel, Miller, & Jemal, 2015). According to statistics, in America, 16.8% of lung cancer patients only live for 5 years after diagnosis (R. Siegel, Ma, Zou, & Jemal, 2014). Due to changes in lifestyle and increased risk factors, lung cancer is among the fifth most common tumors with recently a rapid growth in prevalence in Iran (Hosseini et al., 2009). In 8% of the cases, the risk factors are inherited, which show the importance of genetics in such cancer (Yang, Holloway, & Fong, 2013).

Different therapies are considered for lung cancer, including surgery, chemotherapy, immunotherapy and targeted therapy (Boolell, Alamgeer, Watkins, & Ganju, 2015; You et al., 2015). Today, the goal of the therapies is to increase the specificity of the target and decrease the side effects. In this regard, gene therapy can be an effective therapeutic method for genetic diseases (Brown, Venneri, Zingale, Sergi, & Naldini, 2006; Morgan et al., 2006).

Various studies have shown clinically relevant correlations between the expression of different miRNAs and various kinds of cancer. miRNAs can be either oncogenic or tumor suppressive. Expression of tumor suppressor miRNAs is often reduced in different cancers (Caldas & Brenton, 2005; Ghazanchaei, Mansoori, Mohammadi, Biglari, & Baradaran, 2019). It has been shown that changes of miRNA expression in lung cancer affect cancer progression. For instance, the miRNAs let-7, let-7b, let-7c, let-7d, let-7f, let-7g and miR-330 are down-regulated in lung cancer (Gao, Yin, & Fei, 2013). Therapeutic methods with miRNA are based on either suppression of miRNA expression or miRNA replacement (N. Hosseinahli, M. Aghapour, P. H. Duijf, & B.

Baradaran, 2018a; Behzad Mansoori, Mohammadi, Shirjang, & Baradaran, 2017). Tumor suppressor miRNAs are down-regulated in the specific cell lines, therefore, miRNA replacement can increase miRNA expression and reduce cancer progression (Hosseinahli et al., 2018a).

Lee and colleagues studied miR-330 in prostate cancer and discovered that it acts as a tumor suppressor (Lee et al., 2009). Mayo et al showed that miR-330 plays its tumor suppressor role in prostate cancer by targeting the SP1 gene (Mao et al., 2013). Paradoxically, another research study by Qu S et al found that the tumor suppressor gene *SH3GL2* is down-regulated by miR-330 in glioblastoma, suggesting that miR-330 acts as an oncogene in this tumor type (Qu et al., 2012). Simiolarly, Hui Meng et al. confirmed the oncogenic role of miR-330 in esophageal cancer (Meng et al., 2015). There are a lot of study that shoes miR-330 has a tumor suppressor role in cancers (Behzad Mansoori et al., 2020; Sehati et al., 2020; Shirjang et al.).One of the target genes of miR-330 is E2F1, a transcription factor belonging to the E2F family, which has a vital role in cell cycle regulation. Reduced expression of miR-330 is responsible for the overexpression of E2F1 and activation of the E2F1/AKT signaling pathway, which induces cell division (Lee et al., 2009). Despite several studies on miRNAs in lung cancer, the role of miR-330 has not been clearly defined yet. In the present study, we replaced miR-330 in the A549 lung cancer cell line using miRNA replacement and evaluated the effect of replacement on apoptosis and migration of these lung cancer cells.

2. Materials and methods

2.1. Patient survival analysis

For miR-330 expression and survival analysis, miRNA data (Illumina miRNASeq) and clinical data were downloaded from The Cancer Genome Atlas (TCGA)(Nature, 2012). Data were processed and analyzed essentially as previously described (Vaidyanathan, Cato, et al., 2016; Vaidyanathan, Thangavelu, & Duijf, 2016), except that the ratio between the low and high expression groups was 1:3. In addition, for statistical analyses, aside from Mantel-Cox (log-rank) tests and Cox proportional hazards regression analyses, Gehan-Breslow-Wilcoxon (GBW) tests were performed on all patients, as well as separately for patients with stage 1, 2, 3 or 4 tumors (Thangavelu, Krenács, Dray, & Duijf, 2016; Thangavelu et al., 2017).

2.2. Cell culture

The A549 lung cancer cell line was obtained from Pastor Institute (Tehran, Iran) and cultured in RPMI 1640 (Gibco، Lot No. 1703986X) containing 10% Fetal Bovine Serum (FBS) (Gibco ، Lot NO. 42F8160K ،USA), 1% antibiotic (penicillin 100 IU/ml, streptomycin 100µg/ml), 2 mM glutamine, 1% sodium pyruvate and incubated at 37˚C, 95% moisture and 5% carbon dioxide. After passaging and incubation time, 70% to 80% of the cells' confluency was used for each experiment.

2.3. MTT assay for determining of the selection capacity of antibiotic Geneticin (G-418)

Tetrazolium salt is used in this method. First, 15×10^3 of A549 cells were collected along with RPMI1640 medium and 10% of fetal bovine serum in each well of a 96

plate and incubated in 37°C, 95% humidity and 5% carbon dioxide for 24 hours. After 24 hours, 50 µl of MTT solution (2mg/mL, Bio Basic‹Lot No.DU21373R2, Canada) was added into each well along with 100 µl of medium (RPMI and 10% Fetal Bovine Serum) and incubated in 37°C, 95% humidity and 5% carbon dioxide for 4 hours. After that, the surface layer was discarded. Then, 200 µl dimethylsulfoxide (DMSO) (AppliChem Lot No.8S011641, Germany) was added to each well along with 25 µl of Sorenson buffer and incubated at 37°C for 30 min. Following incubation, the cells were analyzed by ELISA reader at 570/630 nm.

2.4. A549 cell transfection with miR-330

A549 cells were counted and 3×10^5 cells per well were seeded in a 6-well plate. One well was cultured in order to be transfected by PCMVmiR-330 (Origene, USA) referred to as Vec+, and the other wells were cultured with empty PCMV cells as a negative control. The PCMV vector expressed miRNA percourser from CMV promoter. It have GFP reporter for evaluating percentage of stable cells. The neomycin antibiotic use for selection of miRNA postive cells. The map of miRNA vector displayed in figure 1.

The plate was incubated at 37°C and 5% of carbon dioxide. After 24 hours, the cells were controlled for numbers and also ensured that the cells were attached to the bottom of the flask. As the first step in the transfection, 2 sterilized microtubes were prepared for Vec+ (miR-330 carrier). As per the manufacturer's protocol, 6µl of JetPEI (Polyplus, lot No. 1502C1ZF, Frence), a DNA transfection solution, and 94µl of NaCl (Polyplus‹ lot No. N130925, Frence) were added to a microtube. Since the suggested density for miR-330 carrier vector for transfection was 6000 ng, in the other microtube we used NaCl to increase the volume to 100µl. The contents of these

microtubes were mixed within the first microtube (containing JetPEI+ NaCl), then the mixture was added to the cell culture. After 24 hours, the optimum media was replaced by RPMI containing 10% FBS and antibiotics. Since the vectors contain a neomycin resistant gene, 444.3 µg/ml of genticin (Gibco‹Lot No. 1743630 ‹USA) was added to each well to select the cells that contained the vectors. Medium replacement was continued by addition of antibiotic until completion of cell selection.

2.5. Quantitative real-time PCR

After finishing the 14-day stage of transfecting the A549 cells with a vector that contained miR-330 and selection of these cells using genticin, total RNA was extracted from the cells. This was done by using RiboEx reagent (Gene All ‹ Lot NO. REX15J12014, Korea). Then, cDNA synthesis was done according to the EXICON kit protocol. The concentration and quality of the extracted RNA were determined by NanoDrop 2000c (Thermo, USA). According to the manufacturer's protocol, miRCURY LNA™ Universal RT microRNA PCR (Exiqon, Denmark) was used for cDNA synthesis. After cDNA synthesis, miR-330 expression was measured using qRT-PCR (Roche REF:05815916001 J SN:11769 Germany). The device was programmed using the following protocol. First step: 95°C for 10min, second step: 95° for 10sec, 60°c for 60sec in 45 cycles over 1.6 sec ramp, and last step was melting. Using this method, miR-330 expression was measured by recording the intensity of fluorescent emission form cDNA amplification. SNORD48 (EXIQON Lot No. 177600, Denmark) was also used as an internal control for miRNA. Finally, we analyzed the data using the $2^{-\Delta\Delta CT}$ formula.

2.6. Western blotting

First, total proteins of the samples were separated using SDS polyacrylamide gel electrophoresis (PAGE), then the proteins were transferred to a nitrocellulose membrane. Next, we studied MMP9, CXCR4, AKT, G) Caspase-9 and Caspase-3 expression in the A549 cell line after transfection by PCMVmiR-330 and empty PCMV as a negative control. RIPA buffer(Santa Cruz Biotechnology, Santa Cruz, CA) was used for protein extraction. SDS-PAGE method was also used for electrophoresis of extracted proteins. Following electrophoresis, proteins were transferred to PVDF membrane and finally probed with specific antibodies against MMP-9, CXCR4, AKT, Caspase-3 and Caspase-9.

2.7. Flow cytometry

A549 cells were seeded into six-well culture plates (1.0×10^6 cells/well) with RPMI 1640 media and incubated under growth conditions. The cells were dislodged and separated using Trypsin-EDTA and supernatants were discarded after centrifugation at 1300 rpm for 10 min at room temperature (RT). According to the AnnexinV/PI apoptosis kit (EXBIO, Czech) instructions, cell pellets were washed once in PBS and supernatants were disposed at each step. After that, the cells were resuspended in 190 µl of 1X binding buffer. Then, 5 µl of FITC-conjugated Annexin V was added to 100 µL of the cell suspension and incubated for 15 min in the dark at RT. After centrifugation, the cells were resuspended in 190 µL of 1X binding buffer. At the final stage, 5 µl of propidium iodide (PI) staining solution was added to the cells and analyzed by flow cytometry. Data analyses were performed using FlowJo (Treestar, Inc., San Carlos, CA).

2.8. DAPI staining

DAPI or (4′, 6-diamidino-2-phenylindole) is a fluorescent color which binds to adenine-thymine enriched regions of DNA. Firstly, the cells were seeded in a 96-well plate in triplicates with 1×10^5 cells in each well. 24 hours later, the surface layer was discarded, and the cells were washed with PBS. Then, 200µl of paraformaldehyde 4% was added to each well and incubated for 1 hour. Next, the cells were washed with 200µl of PBS and 200µl of Triton X-100 0.1% was added to the cells to reduce the surface tension and it was incubated at the room temperature for 10 min. The cells were subsequently washed with 200µl of PBS, stained with DAPI for 10 min and again washed with 200µl of PBS. Finally, cells were imaged.

2.9. Cell cycle assay

The cells were harvested, fixed with ethanol (75 %), treated with RNase A (5 mg/ml) (Pishgham, Iran), stained with 1 mg/ml of DAPI and analyzed by flow cytometry instrument (MACS quant 10. miltiny biotech, Germany) for DNA synthesis. Also, the cell cycle status was analyzed using FlowJo software (Treestar, Inc., San Carlos, CA).

2.10. Wound healing assay

For wound healing assay, we trypsinized the A549 cells, including control cells and Vec+ cells separately and then cultured these in a six-well plate at 5×10^5 cells in each well. When the cells were growing in a monolayer, a scratch was applied using the sterile yellow micropipette tip and the cells were washed with PBS. Images of cell migration towards the scratch line were taken 0, 24 and 48 hours later. We used an inverted microscope (Optika XDS_3, Italy) to capture these images.

3. Results

3.1 Low miR-330 expression predicts poor lung cancer prognosis

We studied whether miR-330 expression is associated with lung cancer patient survival. Patients with low miR-330 expression show significantly poorer overall survival than patients with high miR-330 expression, as determined by three independent statistical tests: Cox proportional hazards model (n=471; Hazard Ratio (HR)=0.71 with 95% confidence interval (CI)=0.53-0.97 and p=0.0261; log-rank test p=0.0311 and Gehan-Breslow-Wilcoxon (GBW) test p=0.0374) (Fig 2A). In addition, analysis of patient survival at different stages revealed that reduced miR-330 expression is a particularly strong predictor of poor overall survival for patients with stage 1 tumors (n=228, p=0.0187, p=0.0181 and p=0.0358, respectively) (Fig 2B). Low miR-330 expression was not significantly associated with poor survival for stage 2, stage 3 or stage 4 patients (all p>0.05; data not shown). Thus, low miR-330 expression predicts poor lung cancer patient survival, in particular for patients with early-stage tumors.

3.2. Increasing miR-330 expression in A549 lung cancer cells

Our clinical data above suggest that there may well be a therapeutic benefit to elevating miR-330 expression in lung tumors. To test this hypothesis *in vitro*, we used the A549 lung cancer cell line – because of its confirmed low miR-330 expression level (Ruike et al., 2008) – and increased miR-330 expression in these cells by transfection and selection. First, we perfromed an MTT test to calculate the IC_{50} value of genticin (G418) for untransfected cells. A549 cells were treated with 100 to 1200 µg/ml of the antibiotic. This showed that the IC_{50} for A549 cells was 444.3 µg/ml (Fig 3A), thus enabling us to distinguish between transfected and non-transfected cells.

Next, we transfected the pCMV-miR-330 vector, which also contained cDNAs encoding Green Fluorescent Protein (GFP) and G418 resistence, into the A549 cells. To confirm transfection, images were taken from the plates using an immunofluorecent microcope. This showed GFP-positive cells, indicating successful transfection (Fig 3B). After genticin selection, we extracted RNA, performed cDNA synthesis and detected miR-330 expression in control cells and transfected cells (vec+) using qRT-PCR (Fig 3C).

3.3. Elevated miR-330 expression in A549 cells reduces cell viability and induces apoptosis in A549 cells

We used MTT assays to study the cytotoxic effect of increased miR-330 expression in A549 cells. We found that cell survival in transfected cells was decreased in comparison to control cells (Fig 4A). This indicates that increasing miR-330 expression reduces lung cancer cell survival. Next, we used DAPI staining to determine the type of cell death that A549 cells undergo after stably increasing miR-330 expression. As shown in Fig. 4B and C, in contrast to control cells, miR-330-transfected cells suffer from DNA fragmentation, typical of cells undergoing apoptosis. AnnexinV/PI assays independently confirmed induction of apoptosis in the stably transfected miR-330 cells compared to the control group (Fig 4D, E and F). Thus, increased miR-330 expression in A549 lung cancer cells induces apoptosis.

3.4. miR-330 replacement causes G2-M cell cycle arrest in A549 cells

Then we performed FACS analysis to determine the effect of miR-330 gene silencing on the cell cycle. When miR-330 was transfected into A549 cells, this resulted in an increased fraction of cells in G2-M phase of the cell cycle compared to cells transfected with empty vector, which were used as controls. Quantification and

statistical analysis indicated that this difference was significant (Fig 5). Thus, miR-330 replacement causes a G2/M cell cycle arrest.

3.5. Elevated miR-330 expression inhibits A549 cell migration

Next, we performed a wound healing assay or scratch test on the A549 cell line in order to determine the effects of miR-330 replacment on cell migration. We gathered images that were taken at 0, 24 or 48 hours after applying the scratch. We found that miR-330 replacement caused a significant reduction in cell migration, which was apparent at both 24 and 48 hours after the start of the experiment (Fig. 6). Thus, elevated miR-330 expression reduces the migration capacity of lung cancer cells.

3.6. miR-330 replacement induces apoptosis and suppresses metastasis of A549 cells by altering the expression of genes involved in these processes

We evaluated mRNA expression of the MMP9, Ecadherin, CXCR4, Vimentin, E2F1, AKT, Caspase-9 and Caspase-3 genes in the A549 lung cancer cell line by qRT-PCR. This revealed a significant reduction in the expression of MMP9, CXCR4, E2F1 and AKT after miR-330 replacement. However, we detected a significant increase in Ecadherin, Caspase-9 and Caspase-3 expression in the transfected cells (Fig. 7). Importantly, using Western blot analysis, we found that these observations were substantiated at the protein level (Fig. 8). This indicates that apoptosis is induced via the intrinsic pathway. Thus, consistent with our previous phenotypic observations, increasing miR-330 expression leads to an increase in pro-apoptotic and anti-migration genes and proteins.

Discussion

miRNA expression is highly regulated in cells. Studies have shown that miRNA expression changes in different diseases, such as cancer, and this leads to new phenotypes. Thus, these changes could be used as biomarkers in cancer diagnosis (Lu et al., 2005). miRNAs are also important, since they can target multiple genes (B Mansoori, Mohammadi, Shirjang, & Baradaran, 2015). If a class of miRNAs is reduced in cancerous cells, then their target genes are often oncogenes. Such miRNAs are known as a tumor suppressors)TsmiRs). TsmiRs can be used in new therapeutic strategies in cancer treatment, such as in miRNA replacement therapy (N. Hosseinahli, M. Aghapour, P. H. Duijf, & B. J. J. o. c. p. Baradaran, 2018b; Sassen, Miska, & Caldas, 2008).

In the last few years, miR-330 has been studied as miRNA with a low expression in different cancers. Ruike Y et al. studied 16 cancerous cell lines and confirmed the low expression of miR-330 in some of these cell lines, including A549 cells (Ruike et al., 2008). Here, we show that reduced miR-330 expression predicts poor lung cancer patient prognosis. Thus, we used A549 cells, in which miR-330 expression was shown to be low (Ruike et al., 2008), as a model to test *in vitro* how miR-330 replacement would affect lung cancer cell behavior. We found that induction of miR-330 leads to a reduction in the proliferation and migration of A549 cancerous cells. miR-330 was stably transfected into A549 cells using the GFP-pCMV-miRNA-330 vector. miR-330 induction was confirmed by qRT-PCR. We showed that miR-330 was increased in the transfected cells in comparison to the control cells. Study on E2F1 expression showed a low expression of this gene in transfected cells with miR-

330 in comparison to control cells. miR-330 reduces E2F1 expression by targeting its mRNA.

Mao Y et al showed that the transcription E2F1 and is involved in cell cycle regulation through AKT phosphorylation. E2F1 mRNA contains a target sequence for miR-330 in the 3'UTR region. The E2F1 expression is regulated post-transcriptionally by miR-330 (Mao et al., 2013). These results are in accordance with results from our study, as we find an inverse correlation between E2F1 and miR-330 expression. Other studies have also confirmed that E2F1 overexpression is related to autogenesis and promotes neoplastic behaviour (Pierce et al., 1999). Previous studies have indicated that silencing of E2F1 by siRNA could inhibit proliferation and migration in different types of cancer (Pierce et al., 1999). E2F1 causes proliferation and migration by activating downstream target genes (Kumari et al., 2015). This gene also causes tumor cell migration by regulating expression of Ecadherin, Ncadherin and Vimentin (Alla et al., 2010). These studies are in accordance with the results of our study. For example, our MTT assays proved that miR-330 could lead to a significant reduction in cell division and proliferation in lung cancer cells. Seventy percent reduction in proliferation of lung cancer shows the crucial role of miR-330 in cell division.

DAPI staining and annexinV/PI assays also comfirmed that miR-330 induces apoptosis in A549 cells. In another research study, it was reported that miR-330 inhibits Cell Division Cycle 42 (CDC42) in colorectal cancer, reduces cell division and induces apoptosis (Li, Zhu, Xu, Wang, & Yan, 2013). Our results from real-time PCR also showed a reduction in AKT and E2F1 expression and higher expression of

Caspase-3 and Caspase-9 (Fig 8). Therefore, miR-330 induces apoptosis through the intrinsic pathway.

Lee KH et al. also reported the anti-proliferative role of miR-330 in prostate cancer cells. We show that miR-330 suppresses E2F1 expression in AKT phosphorylation, which induces apoptosis and inhibits cell growth in prostate cancer (Lee et al., 2009). Our cell cycle analyses indicated that the replacement of miR-330 significantly arrests A549 cells in G2-M phase of the cell cycle. These results suggest that cells do not start mitosis before they have a chance to repair damaged DNA after replication, leading to death after cell division (Esmailzadeh, Mansoori, Mohammadi, Shanehbandi, & Baradaran, 2017).

Our wound healing assays showed that miR-330 can inhibit cell migration up to 56% in the first 24 hours, but in the next 48 hours this number was reduced to 47%. Mao Y et al. reported that miR-330 reduces SP1 protein levels in prostate cancer, which leads to the suppression of cell migration. They also reported a low expression of miR-330 in cancerous cells. These results are consistent with our results and show the reduction in miR-330 expression in a number of cancerous cell lines (Mao et al., 2013). A small number of miR-330 targets have been identified, but it was also proven that miR-330 is able to down regulate KRAS (Wang, Gu, & Gao, 2016). CXCR4, Vimentin, Ecadherin and MMP9 gene expression correlate with KRAS expression. Several studies have indicated that overexpression of CXCR4, Vimentin and MMP9, as well as downregulation of Ecadherin promote tumor growth, angiogenesis, metastasis and the resistance of cancer cells to various treatments (Hunt, Green, & Buchholz, 2012; J. Kim et al., 2006; Nikkola et al., 2005; Ye et al., 2016). In this study, we showed that miR-330 downregulates CXCR4, Vimentin and MMP9 expression and upregulate

Ecadherin expression (Fig 9). Thus, these regulations strongly correlate with miR-330 expression. OTaken together, our results suggest that miR-330 simultaneously acts as a tumor suppressor in various ways. First, it inhibits metastasis by suppressing pro-metastatic and promoting anrti-metastatic gene expression. Our results are also similar to results of Kim BK et al., who showed that miR-330-5p can inhibit the proliferation and migration of keratinocytes in mice. They also showed that this could be as a result of a miR-330-5p regulatory effect on mRNA PDIA3 (B. K. Kim, Yoo, Choi, & Yoon, 2015).

In conclusion, microRNA replacement therapy is an emerging method for cancer treatment (Hosseinahli et al., 2018b). Our studies have shown that miR-330 is a strong candidate for miRNA replacement in lung cancer. We demonstrate that reduced miR-330 expression predicts poor lung cancer patient prognosis and restoration of its expression in lung cancer cells inhibits cell viability, proliferation and migration, whereas it induces apoptosis. While further studies are needed to clarify the precise tumor-suppressive mechanisms of action of miR-330 in lung cancer, our findings suggest that miR-330 is a strong candidate for restoration of its expression via miRNA replacement therapy for the treatment of lung cancer.

Confilict of interest

All the authors declare no confilict of interest.

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Figures

Fig 1. Schematic drawing of hsa-miR-330 plasmid vector.

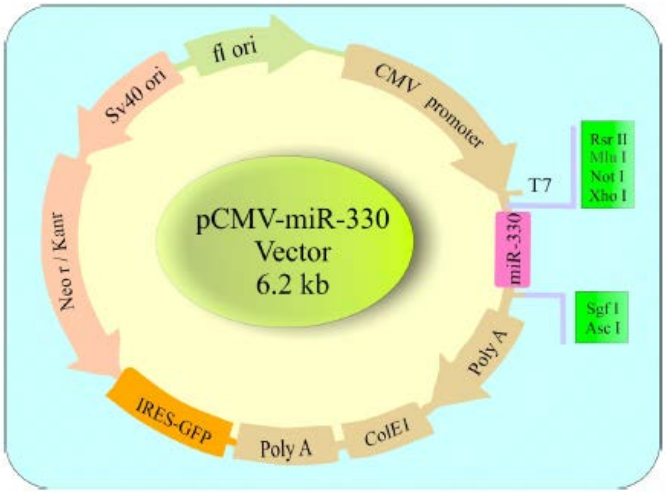

Fig 2. Low miR-330 expression predicts poor lung cancer patient survival. (A) Overall survival curve for lung cancer patients with low or high tumor-specific mir-330 expression (n=471). HR, Hazard Ratio; CI, Confidence Interval; GBW, Gehan-Breslow-Wilcoxon. (B) Overall survival curve as in (A), but showing stage 1 lung cancer patients only (n=228).

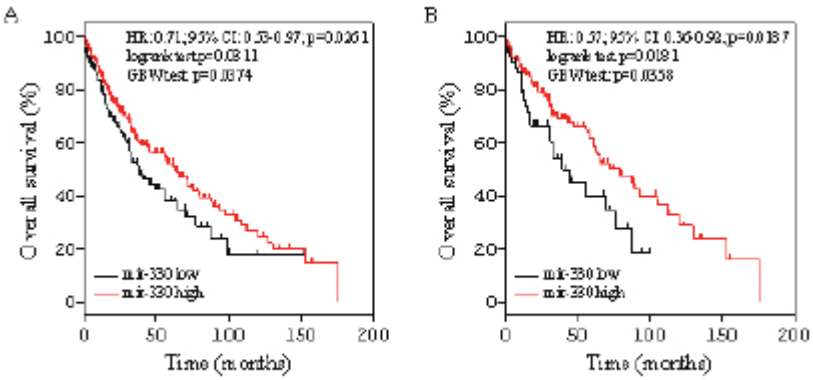

Fig 3. Increasing miR-330 expression in A549 lung cancer cells. (A) Untransfected A549 cell survival in different dosages of genticin (G418). P<0.5 shows the reduction in cell survival. (B) Fluorescence microscopy image of A549 cells transfected with a vector encoding miR-330, GFP and genticin resistance. (C) Quantification comparing miR330 expression levels in transfected and control A549 cells (* p<0.05 *** P <0.001).

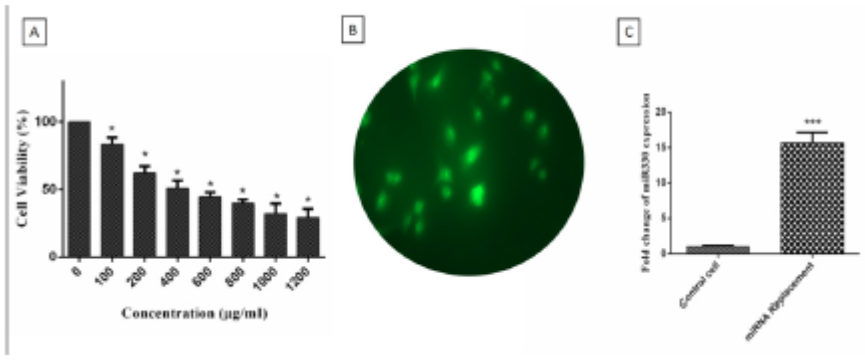

Fig 4. Increasing miR-330 expression in A549 cells reduces cell viability and induces apoptosis. (A) Cell viability was determined by MTT assays. (B) DAPI-stained control A549 cells. (C) DAPI-stained A549 cells transfected with pCMV-miR-330 vector. (D, E) AnnexinV/PI assay for control and stable miR-330-expressing A549 cells, respectively. (F) Quantification of the number of apoptotic cells in control and stable miR-330-expressing cells, (*** P <0.001).

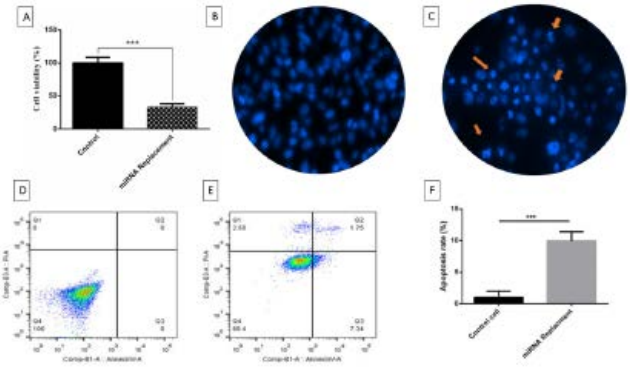

Fig 5. miR-330 replacement arrests cells in G2-M phase of the cell cycle. The FACS analysis graphs of PI-stained control and miR-330 replaced cells (top left and right, respectively). Percentages of cells in each cell cycle phase are represented. A bar graph comparing the percentages is shown on the bottom. (* p<0.05 compared to the control group).

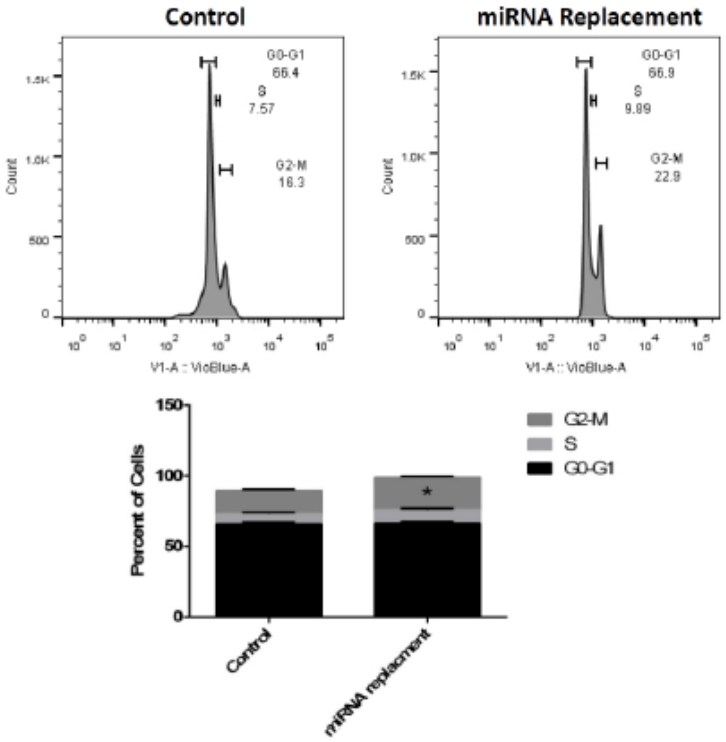

Fig 6. miR-330 replacement reduces cell migration of A549 cells. Images were taken 0, 24 and 48 hours after applying a scratch and the migration capacity of transfected cells in comparison with the control cells was determined by quantification of the numbers of cells in the scratch areas. (** p<0.01, *** p<0.001).

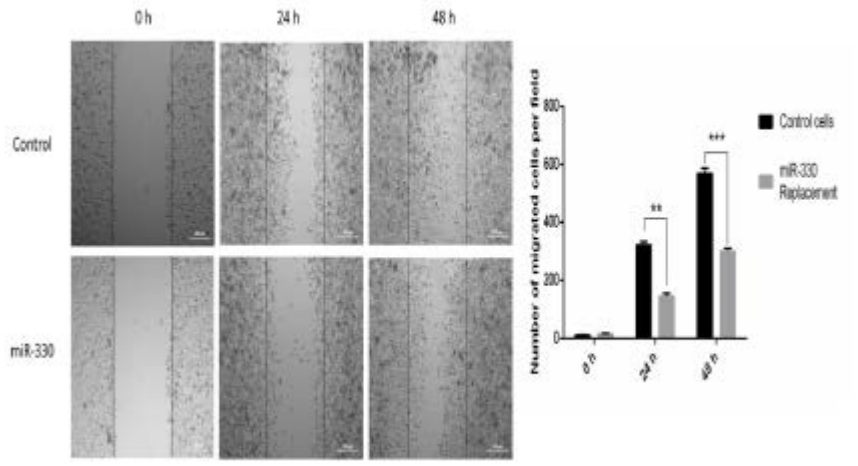

Fig 7. Replacement of miR-330 alters expression of key genes involved in apoptosis and metastasis. Using qRT-PCR, mRNA expression in A549 cells was measured for: (A) E2F1, (B) MMP9, (C) Vimentin, (D) CXCR4, (E) Ecadherin, (F) AKT, (G) Caspase-9 and (H) Caspase-3. (** p<0.01, *** p<0.001 and **** p<0.0001).

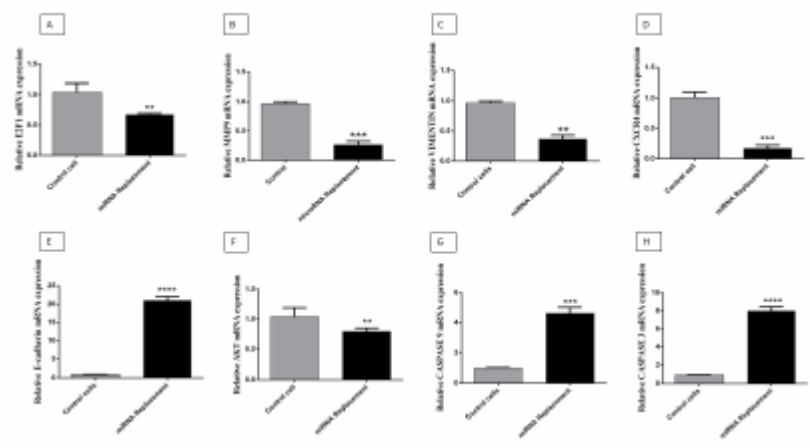

Fig 8. Replacement of miR-330 alters expression of key genes involved in apoptosis and metastasis. Protein level expression in A549 cells was performed by Western blotting (A) and normalized protein expression of these blots, relative to beta-actin expression, was quantified for: (B) MMP9, (C) CXCR4, (D) AKT, (E) Caspase-9, (F) Caspase-3, (G) E2F1, (H) Vimentin, and (I) E-cadherin. (** $p<0.01$, and *** $p<0.001$).

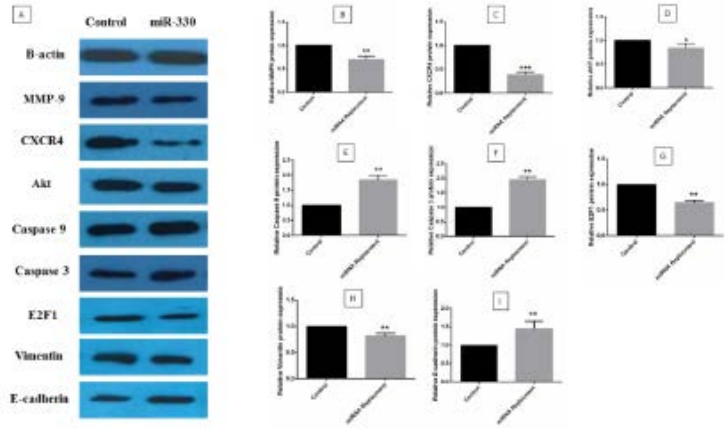

Fig 9. The scheme of effect of miR-330 in apoptosis induction and metastasis supressio. miR-330 by targeting E2F1 mRNA could decrease the AKT expression, caspase-9 and caspase-3 activation and apoptosis induction. miR-330 also could act as negative regulator of MMP-9, Vimentin and CXCR4 and positive regulator of E-cadherin. So these chenges cause metastasis inhibition.