MicroRNA-21 (miR-21) Affects Migration of Human Lung Cancer Cells in vitro via Regulation of MARCKS Expression

S Fang, Q Yin, J Park, AL Crews, KB Adler
College of Veterinary Medicine, North Carolina State University, Raleigh, NC, USA.
Correspondence: Kbadler@ncsu.edu

Abstract
MiR-21 is known to increase in different inflammatory and neoplastic diseases. We have shown previously that miR-21 regulation of expression of MARCKS protein in airway epithelial cells affects several cell functions, including secretion of mucin (Lampe et al, Open J Resp Dis, 3:99-96, 2013. doi:10.4236/ojrd.2013.32014). Here, we investigated the potential effect of miR-21 on migration of human lung cancer cells (PC-9 cell line derived from a human lung adenocarcinoma) and the potential role of MARCKS in these effects, as MARCKS has been shown to be critical protein in cancer cell migration in vitro and in metastasis in vivo in murine models of lung cancer (Chen et al, Oncogene. 2013 Aug 19 doi:10.1038/onc.2013.336). PC-9 cells, which have elevated levels of MARCKS protein compared to normal airway epithelial cells and are aggressively metastatic when orthotopically injected into mouse lungs, were grown to 70% confluence and treated with either a commercially available miR-21 inhibitor or a miR-21 mimic, both at 50 or 100 nM, for 72 hrs. Cell migration in chambers for a 12 hour period was then assessed via staining with Hoechst and cell counting. Treatment with the miR-21 inhibitor INCREASED migration of PC-9 cells about 3.5 fold, while treatment with the miR-21 mimic DECREASED migration of the cells by approximately 60%, compared to control medium or medium to which the vehicle for both the inhibitor and mimic, HiPerFect® (Qiagen), was added. The inhibitory effect of the miR-21 mimic correlated with a significant attenuation of expression of MARCKS in these cells at both the mRNA and protein levels. To demonstrate that inhibitory effects of the miR-21 mimic on MARCKS expression and migration were causally linked, additional studies in which MARCKS levels in PC-9 cells were knocked down via siRNA were performed. Cells were transfected with 100 nM of a MARCKS – specific siRNA for 72 hrs, then migration of the cells was assessed. siRNA treatment knocked down MARCKS expression in the PC-9 cells by ~ 60 % compared to a control siRNA (CTL). The decrease was with a highly significant decrease of ~ 90% in migration of the cells (p<0.001). Upregulation of miR-21 in lung and other cancers could affect metastasis via its targeting of MARCKS protein in the migrating cells.

Methods
MiR-21 Inhibitor and MiR-21 activator/mimic
A miR-21 inhibitor and a pre-miR-21 activator were both purchased from Ambion (Forster City, CA). The pre-miR-21 mimic siRNA contained the hsa-miR-21-5p sequence, and the miRNA mimic contained the stem loop sequence GUCGGCGAUCCAGUGAAUUCUCGUGCAAACACUGGGUGCGUGCGUGUUAA and 5’-GCAUACCGGAAGUUCUCCUCCGCAU-3’. Silencing of Gene Expression
Chemically synthesized double-stranded siRNA duplexes were purchased from Ambion for target gene MARCKS (siM) and the control siRNA (CTL siRNA). PC9 cells were transfected with HiPerFect transfection reagent from Qiagen according to manufacturers’ instructions. Silencing was quantified by immunoblotting.

In vitro migration assays
For the in vitro cancer cell migration assays, 1 x 10^6 cells were cultured in Transwell filters (24-well, 8-um pore size), with the top wells containing serum-free medium. 5 ml of complete medium was added to the lower wells, and after 12 hrs incubation, cells that migrated from the upper to lower surface of the chambers were stained with Diff-Quick Wright-Giemsa stain. Photomicrographs of invading cells were taken using a 20x objective and cells counted.

RT-PCR
RNA was extracted with an RNAeasy kit (Qiagen). For miRNA analysis, real-time qPCR was carried out using a qRTPCR System (Bio-Rad) using 5 ng RNA input, 2x SYBR Green Supermix (Bio-Rad ). For detection, 50 ng RNA input, 2x SYBR Green Supermix, and 5 pmol gene-specific primer pairs were used. Thermal cycling conditions were 95°C for 3 minutes, 40 cycles at 95°C for 10 seconds, and 55°C for 30 seconds, followed by melting curve analyses. RNA input was normalized to endogenous controls: beta-actin or 36B4. The 2^-ΔΔCt method was used to calculate the fold relationships in miRNA expression among the tested samples.

Results

Figure 1. miR-21 expression in various lung cancer cell lines (NCI-H226, CL-1, A549 and PC9) and normal epithelial cells (HBE1, NHBE). Cells were harvested and total RNA, including the miRNA fraction, was isolated. The level of miR-21 was determined by qRT-PCR using sense primer 5’-ccc gac tag att aca qtg-3’ and antisense primer 5’-ggc ggt gcc aat acx-3’. miR-21 expression is higher in the more aggressive tumor cells.

Fig. 2

Figure 2. Effects of the miR-21 inhibitor and mimic on MARCKS mRNA and protein expression in PC9 cells. The inhibitor increases expression of MARCKS mRNA, while the mimic decreases MARCKS expression in a concentration – dependent manner. Cells were transfected with the miR-21 negative control (miR-21 NC), miR-21 inhibitor, or miR-21 mimic. At 48h post-transfection, MARCKS levels (upper panel) were detected by Western blot. B-actin was used as a loading control (bottom panel).

Summary
Lung cancer cell lines show higher miR-21 expression than normal epithelial cells, such as HBE1 and NHBE.

PC9 cells were transfected with a miR-21 inhibitor that downregulates miR-21 expression, or a miR-21 mimic that increases expression. At 72 h post-transfection, MARCKS mRNA and protein levels were upregulated by the miR-21 inhibitor while the miR-21 mimic downregulated MARCKS mRNA and protein expression.

Treatment with the miR-21 inhibitor INCREASED migration of PC9 cells about 3.5 fold, while treatment with the miR-21 mimic DECREASED migration of the cells by approximately 60%, compared to control.

siRNA knockdown of MARCKS decreased migration of PC9 cells about 3.5 fold, while treatment with the miR-21 mimic DECREASED migration of the cells by approximately 60%, compared to control.

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