miR-218 suppresses epithelial-to-mesenchymal transition by targeting *Robo1* and *Ecad* in lung adenocarcinoma cells

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Aim: Although, miR-218 has been implicated in epithelial-to-mesenchymal transition process, the detailed mechanisms of miR-218 involvement in epithelial-to-mesenchymal transition in human lung adenocarcinoma cell are still unclear. **Materials & methods:** miR-218 function assays and its target gene analysis were performed. **Results:** miR-218 suppresses human lung adenocarcinoma cell migration and invasion and inhibits its target gene, *Ecad* and *Robo1* expression, which subsequently suppresses NF- κ B activity and its downstream targets. **Conclusion:** miR-218 inhibits human lung adenocarcinoma cell migration and invasion via the suppression of *Ecad* and *Robo1* expression, thus suggesting that miR-218 could serve as a potential therapeutic target.

First draft submitted: 8 August 2017; Accepted for publication: 1 September 2017; Published online: 22 September 2017

Keywords: *Ecad* • EMT • epithelial-to-mesenchymal transition • lung adenocarcinoma • miR-218 • *Robo1*

In tumor cells, the epithelial-to-mesenchymal transition (EMT) is an essential biological process that confers the capabilities of invasion and metastasis [1]. Once these capabilities are obtained, complete removal by surgical resection is difficult, thus leading to easy relapses and contributing to a great majority of deaths worldwide [2]. During the EMT process, cancer cells down-regulate gene expression relating to cell to cell contact, for example, E-cadherin, and upregulate the expression of mesenchymal markers, including vimentin, fibronectin and N-cadherin, thus enhancing cancer cell migration and invasion [3].

miRNAs are endogenous small noncoding RNAs that play a crucial role in gene expression regulation, with some target genes implicated in tumor cell invasion and metastasis and EMT suppression/promotion. One study found that miR-27 up-regulation increased EMT-associated gene expression to include ZEB1, ZEB2, slug and vimentin and decreased E-cadherin expression. This study also showed that miR-27 promoted human gastric cancer cell metastasis by inducing EMT [4]. Another study found that miR-214 downregulation promoted intrahepatic cholangiocarcinoma metastasis by targeting Twist, which is an important transcription factor regulating EMT, and by inhibiting E-cadherin expression and promoting N-cadherin, C-fos and MMP-9 expression, thus suggesting that miR-214 is an EMT-suppressive miRNA [5]. Furthermore, increasing evidence has shown that EMT-suppressive miRNAs have a reduced expression in human tumor tissue when compared with tumor-adjacent normal tissue, thus enabling EMT progression. Another EMT-suppressive miRNA, miR-655, when overexpressed has been shown to upregulate E-cadherin and suppress migration and invasion of mesenchymal-like cancer cells to include promoting a morphological shift toward an epithelial phenotype [6]. Furthermore, another recent study showed that the miR-200 family (miR-141, -200a, -200b, -200c and -429) and miR-205 act as EMT-suppressive miRNAs directly targeting ZEB1 and ZEB2 [7,8]. In colon cancer, miR-218 was found to suppress invasion and migration by inhibiting ZEB2 and N-Cadherin [9]. Moreover, miR-218 upregulation was found to inhibit gastric cancer invasion by targeting Gli2 and E-cadherin following Thermo-chemotherapy [10]. These findings suggest that miR-218 is involved in the EMT process by targeting N-Cadherin and E-cadherin, which are the key molecular makers associated with EMT. *Robo1*,

the axon guidance receptor gene, is conserved in animals ranging from fruit flies to mammals. In mammalian cells, the regulation of neural growth and development involves the interaction of Robo1 with Slit, with Robo1 also implicated in T-cell and monocyte chemotaxis [11]. Furthermore, Robo1 overexpression contributes to an enhanced migration capacity in human breast cancer cells [12]. Ecop, the EGFR-coamplified and -overexpressed protein, is overexpressed in human squamous cell carcinomas and prevents apoptosis while promoting cell migration [13]. Other downstream functional targets of miR-218 include *Robo1* and *Ecop*, which promote tumor cell invasion and metastasis [14,15]. Whether miR-218 is involved in the EMT process of human lung adenocarcinoma cells by targeting *Robo1* and *Ecop* is unclear.

Herein, we demonstrated that miR-218 suppressed EMT and inhibited human lung adenocarcinoma cells invasion and metastasis by targeting *Robo1* and *Ecop*, thereby resulting in a decreased NF- κ B transactivity. These findings suggest that the EMT-suppressive miR-218 can possibly serve as an important diagnostic marker and new therapeutic agent for human lung adenocarcinoma cells.

Materials & methods

Cell lines & culture conditions

Human lung adenocarcinoma cell lines A549, NCI-H2228, NCI-H1975, HCC4006, NCI-H23, Calu-3, NCI-H1435 and NCI-H1793 were purchased from ATCC (VA, USA). The cell line A549 was grown in DMEM/F12 medium (Invitrogen, CA, USA) and other cell lines were grown 1640 medium (Invitrogen), with both mediums supplemented with 10% FBS (Invitrogen) and 1% Penicillin/Streptomycin (Wisent Inc., St-Bruno, Canada). All the cell lines were cultured at 37°C in 5% CO₂ and 95% air.

Real-time RT-PCR & western blot

Real-time RT-PCR and western blot are described in detail in the Supplementary Data.

Establishment of stable cell lines

A miR-218 overexpression vector was constructed as previously described with slight modifications [14]. The detailed procedure is described in the Supplementary Data.

miRNA inhibitor or miR-218 vector transfection, siRNA transfection

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Luciferase reporter assays

Luciferase reporter assays were performed as previously described with modification [15]. The detailed procedure is described in the Supplementary Data.

Cell invasion & migration assays

siRNAs against Robo1 or Ecop or miR-218 inhibitor were individually transfected into A549 cells grown in a 12-well plate. Scrambled siRNAs or mismatched sequences were used as negative controls. Cell invasion and migration assays were performed according to the procedure [16,17].

Mice xenografts

All animal procedures were approved under the guidelines of the Health Sciences Animal Policy and Welfare Committee of The Chinese PLA General Hospital. Xenografts were constructed to assess tumor proliferation as previously described with slight modification [18,19]. 7-week-old female BALB/c nude (nu/nu; n = 8) mice were purchased (Weitonglihua Company, Beijing, China), anesthetized with an isoflurane/propylene glycol mixture and A549 cell lines were subcutaneously injected into the right and left flanks (1.5×10^6 cells in 100 μ l RPMI 1640 medium per flank). When tumor volume reached 50 mm³, the tumor volume was measured every other day. When tumor volume reached 100 mm³, 7.5 μ g miR-218 inhibitor complexed with 1.6 μ l siPORTAmine (Life Technology, MD, USA) reagent in 50 μ l PBS was injected into the tumor tissue in the right flank and the mismatched sequence was injected in the left flank. Inhibitors were injected every other day using the same dosage as the miR-218. Tumor volumes were calculated according to the formula $V = ab^2/2$, where 'a' represents the length of tumor tissue and 'b' represents the width of tumor tissue, thus 'a' is greater than 'b'. All the mice were sacrificed with CO₂ inhalation after the last treatment and tumors were collected. Mice were handled according to the

guidelines of the Health Sciences Animal Policy and Welfare Committee of The Chinese PLA General Hospital. Our animal experiments were performed after the approval of the Health Sciences Animal Policy and Welfare Committee of The Chinese PLA General Hospital. The mice are raised in the environment with comfortable temperature, humidity and all the mice used in experiment are healthy.

Statistical analysis

Statistical significance was determined using SPSS 10.0. The data are presented as mean $\pm$ SE (standard error), and statistical comparisons between groups were performed using one-way ANOVA followed by Student's *t*-test. A *p*-value ≤ 0.05 was considered statistically significant.

Results

miR-218 levels negatively correlate with *Robo1* & *Ecop* expression in human lung adenocarcinoma cells

qPCR analysis showed that miR-218 was lowly expressed in various human lung adenocarcinoma tissues and HCC4006 cells. However, miR-218 expression was lower in A549 cells (Figure 1A & 1B). Previous studies have indicated that *Robo1* is a downstream target of miR-218 [14] and that *Ecop* is a key functional target of miR-218 [10]. Therefore, *Robo1* and *Ecop* mRNA expression was examined in the same human lung adenocarcinoma cell lines and showed that *Robo1* and *Ecop* was in lower expression in A549 cells and in higher expression in HCC4006 cells (Figure 1C). When examining any possible correlations between miR-218 and *Robo1* and *Ecop* expression levels, a negative correlation was found between miR-218 and *Robo1* or *Ecop* expression (Figure 1D & E).

miR-218 suppresses the markers of EMT in human lung adenocarcinoma cell

To explore the effect of miR-218 on EMT in human lung adenocarcinoma cells, miR-218 was upregulated via transfection with an miR-218 expression vector or downregulated with an miR-218 antagomir. To examine the impact of miR-218 up- or downregulation on EMT, expression levels of key EMT molecular biomarkers were examined in human lung adenocarcinoma cells. Following miR-218 knockdown, E-cadherin and miR-200b were found to be downregulated, while N-cadherin and ZEB1 were up-regulated, thus suggesting EMT promotion (Figure 2A & B). Conversely, miR-218 upregulation increased E-cadherin and miR-200b expression, while decreasing N-cadherin and ZEB1 expression (Figure 2C & D), thus indicating EMT suppression.

miR-218 represses cancer cell invasion & migration by inhibiting *Robo1* & *Ecop* expression

Malignant tumor cells are endowed with the capacity of invasion and metastasis by means of the EMT process. Herein, alterations of miR-218 expression were found to modulate EMT in human lung adenocarcinoma cells, with miR-218 downregulation promoting invasion and metastasis, while miR-218 upregulation had the opposite effect (Figure 3A & B). After determining that miR-218 suppresses EMT in human lung adenocarcinoma cells, possible targets were examined. Previous studies have indicated that miR-218 inhibits gastric cancer invasion and metastasis by targeting *Robo1* [14], while another study showed that miR-218 promoted glioma cells apoptosis and inhibited proliferation by modulating *Ecop* [10]. Therefore, these targets were examined in human lung adenocarcinoma cells. When miR-218 was overexpressed, *Robo1* and *Ecop* mRNA and protein expression levels were downregulated, while the opposite effect was seen following miR-218 knockdown (Figure 3C & D). Next, dual-luciferase reporter assays were performed to confirm that *Robo1* and *Ecop* are functional downstream targets of miR-218. These results showed that pcDNA3.1-miR-218 and pGL3-*Robo1*-3'UTR or pGL3-*Ecop*-3'UTR cotransfection significantly suppressed luciferase activity, by 30 and 35% respectively, relative to the control (Figure 3E). However, pcDNA3.1-*cel-miR-67*, as a control for miR-218, with pGL3-*Robo1*-3'UTR or pGL3-*Ecop*-3'UTR cotransfection, did not suppress luciferase activity. Also, since *Robo1* and *Ecop* are key regulators of the NF- κ B signal pathway [20], we examined whether miR-218 up- or down-regulation altered I κ B α expression. When miR-218 was overexpressed, both I κ B α and several NF- κ B target genes, including Bcl-xL, c-MYC and CCND1, were found to have an increased expression, while miR-218 knockdown had the converse effect (Figure 3F). The above results proved that miR-218 regulated the expression of *Robo1* and *ECOP* and thereby mediated I κ B α expression and resulted in the alteration of NF- κ B activity.

Figure 1. miR-218 negatively correlated with *Ecop* or *Robo1* expression. Total RNA was extracted from several human lung adenocarcinoma tissues and corresponding nontumor tissues ($n = 30$) and cell lines and reverse transcription was performed and miR-218 (A & B), *Ecop* and *Robo1* (C) levels were measured using qPCR, with U6 used as an internal control for miR-218 and β -actin used for *Ecop* or *Robo1*. The values displayed represent a mean $\pm$ standard deviation; $*p \leq 0.05$. (D & E) Correlation analyses comparing miR-218 with *Ecop* or *Robo1* using the SPSS 10.0 software.

Figure 2. miR-218 suppresses the markers of epithelial-to-mesenchymal transition in human lung adenocarcinoma cells. (A & B) A549 cells were cultured in 12-well plates and transfected with anti-miR-218 or mismatched sequence (negative control), while (C & D) HCC4006 cells were transfected with pcDNA3.1-miR-218 or pcDNA3.1-cel-miR-67 (negative control) vectors. Total RNA or protein was extracted at 48-h post-transfection and the levels of miR-218, E-Cad, miR-200b, N-Cad and ZEB1 were detected with qPCR or western blot. For qPCR, U6 was used as internal control for miRNAs and β -actin was used for E-Cad, N-Cad and ZEB1. The values presented represent a mean $\pm$ standard deviation; * $p \leq 0.05$. NC: pcDNA3.1-cel-miR-67.

Robo1 & Ecop promote invasion & metastasis in human lung adenocarcinoma cells

Since, Robo1 and Ecop are direct functional downstream targets of miR-218, it seemed likely that their suppression would contribute to a suppression of metastasis and invasion in human adenocarcinoma cells. To further examine this, transwell assays were performed following Robo1 and Ecop knockdown. These results showed that miR-218 knockdown promoted the migration and invasion capacity, whereas Ecop or Robo1 knockdown inhibited them. However, the migration and invasion capacities were unaltered after the knockdown of both miR-218 and Ecop or Robo1 (Figure 4A & D), thus suggesting that miR-218 suppresses EMT and subsequent invasion and metastasis in human adenocarcinoma cells by inhibiting Robo1 and Ecop expression.

miR-218 suppresses the growth of human lung adenocarcinoma cells *in vivo*

To examine the effects of miR-218 on human adenocarcinoma cell growth *in vivo*, BALB/c (nu/nu) mice xenografts were constructed. A549 cells were subcutaneously injected into the right and left flanks, followed by the injection of miR-218 inhibitor into the tumor tissue in right flank and mismatched sequences into the left flank. Visually,

Figure 3. miR-218 suppresses the invasion and migration of lung adenocarcinoma cells via the inhibition of Ecop and Robo1 expression (see facing page for parts A-D). (A & B) HCC4006 cell lines with miR-218 overexpression or A549 cells with miR-218 inhibitor knockdown were seeded in 12-well plates and subsequently subjected to starvation for 12 h. Transwell assays for migration or invasion were performed and the capacity of migration and invasion of cells were analyzed. The values represent a mean $\pm$ standard deviation; $*p \leq 0.05$. HCC4006 cells with cel-mir-67 overexpression or A549 cells with mismatched sequences were used as negative controls. (C & D) Total RNA and protein were extracted from miR-218 overexpressing HCC4006 cells or A549 cells treated with miR-218 inhibitor and miR-218. Ecop and Robo1 levels were quantified with qPCR and Western blot. For qPCR, U6 was used as internal control for miR-218 and β -actin was used as internal control for Ecop and Robo1. For western blot, GAPDH was used as internal control. The values presented represent a mean $\pm$ standard deviation; $*p \leq 0.05$. (E) The comparative analysis of Robo1 and Ecop 3'UTR and miR-218 via TargetScan (left). 293T cells were cultured in 96-well plates and cotransfected with pcDNA3.1-miR-218 and pGL3-Robo1-3'UTR or pGL3-Ecop-3'UTR. Transfection efficiencies were normalized by cotransfection with a renilla construct, with individual luciferase activities shown relative to the activity of the empty reporter vector pGL3-basic (right). Values are displayed as a mean $\pm$ standard deviation ($n = 3$); $*p \leq 0.05$. (F) Total protein was extracted from miR-218 overexpressing HCC4006 cells or A549 cells treated with miR-218 inhibitor and NF- κ B, Bcl-xL, c-MYC and CCND1 expression was examined via Western blot. Rabbit anti-NF- κ B, rabbit anti-Bcl-xL, rabbit anti-MYC and rabbit anti-CCND1 were used as primary antibodies, goat antirabbit IgG coupled with HRP was used as the second antibody and β -actin was selected as internal control (cont.).

HCC: Hepatocellular carcinoma; HRP: Horseradish peroxidase; MYC: c-MYC; NC: pcDNA3.1-cel-miR-67.

the mean tumor volumes from miR-218 inhibitor transfected cells were bigger than the mismatched sequence transfected cells (Figure 5A & B). To further evaluate this observed difference, the mice were sacrificed and the whole tumor tissues were weighed. The tumor tissues treated with miR-218 inhibitor were in fact heavier than the control group samples (Figure 5C). These increases in growth rate with anti-miR-218 could be due to enhanced proliferation but reduced apoptosis could also be a factor.

Discussion

EMT plays a crucial role in invasion and metastasis of human tumor cells, with an understanding of the molecular mechanisms relating to the EMT process important for intervention. Increasing evidence has implicated microRNAs as key EMT modulators through their targeting of EMT-related target genes. In the present study, miR-218 was found to down-regulate Robo1 and Ecop expression by targeting their 3'-UTRs and subsequently inhibiting NF- κ B activity (Figure 3E & F). While EMT-suppressive miRNAs have been previously identified, the previously described mechanisms are distinct from those of miR-218 in human lung adenocarcinoma cells. Thus, the findings presented herein are beneficial to enabling a further understanding of the EMT process and subsequent invasion and metastasis of tumor cells.

While many potential miR-218 target genes were identified, Robo1 and Ecop are considered key functional downstream targets and key regulators of NF- κ B activity [14], thus they were examined. While Ras and TGF- β

Figure 4. Robo1 and Ecop promote migration and invasion in human lung adenocarcinoma cells. A549 cells were cultured in 12-well plates and treated with miR-218 inhibitor or siRNA against Ecop (A & B) or Robo1 (C & D), with cells individually transfected or cotransfected with miR-218 inhibitor and siRNA against Ecop or Robo1. After 24 h, the cells were starved for 12 h without serum and transwell assays were performed to assess the migration and invasion capacities. * $p \leq 0.05$. NC: Scrambled siRNA.

Figure 4. *Robo1* and *Ecop* promote migration and invasion in human lung adenocarcinoma cells. A549 cells were cultured in 12-well plates and treated with miR-218 inhibitor or siRNA against *Ecop* (A & B) or *Robo1* (C & D), with cells individually transfected or cotransfected with miR-218 inhibitor and siRNA against *Ecop* or *Robo1*. After 24 h, the cells were starved for 12 h without serum and transwell assays were performed to assess the migration and invasion capacities. * $p \leq 0.05$ (cont.). NC: Scrambled siRNA.

signal pathways exert important roles during tumor cell EMT, the roles of these two signal pathways in EMT are dependent on NF- κ B activation. In a previous study, NF- κ B was found to intersect with Ras and TGF- β signal pathways, with the three pathways found to cooperate in the induction and maintenance of EMT [21]. Since, *Robo1* and *Ecop* are key regulators of NF- κ B activation and were found to be involved in tumor cell invasion and metastasis, it seemed probable that the two genes function in the EMT process in human lung adenocarcinoma cells. Therefore, the present study focused on how miR-218 effects *Robo1* and *Ecop* expression and subsequent NF- κ B activity changes during its suppression of EMT in human lung adenocarcinoma cells.

The activation of NF- κ B is modulated by IKK- β . When miR-218 is upregulated, NF- κ B becomes inactivated via the downregulation of IKK- β , with its 3'-UTR directly targeted by miR-218 [22]. In the present study, miR-218 overexpression resulted in the downregulation of *Robo1* and *Ecop* by targeting their 3'-UTRs, thus resulting in a

Figure 5. miR-218 suppresses human lung adenocarcinoma cell growth. (A & B) A549 cells were subcutaneous injected into the right and left flanks (1.5×10^6 cells) of 7-week-old female BALB/c(nu/nu) mice ($n = 8$). When tumor volume reached 50 mm^3 , tumor volumes were measured every other day. When tumor volume reached 100 mm^3 , $7.5 \mu\text{g}$ miR-218 inhibitor was injected into tumor tissues in the right flanks, with the equivalent concentration of mismatched sequence injected into the left flanks, every other day. Tumor volumes were calculated according to the formula $V = ab^2/2$, where 'a' represents the length of tumor tissue and 'b' represents the width. **(C)** After 30 days, mice were sacrificed and the tumor tissues were isolated and weighed. The values displayed represents a mean $\pm$ standard deviation; * $p \leq 0.05$.

subsequent decrease in NF- κ B activity. However, this inhibition of NF- κ B activity may also be attributed to an inhibited NF- κ B transactivity through the direct targeting of the IKK- β via its 3'-UTR.

In conclusion, miR-218 can negatively regulate Robo1 and Ecop expression and inhibit the EMT, thereby subsequently inhibiting human lung adenocarcinoma cell invasion and metastasis. These findings suggest that enhancing miR-218 expression may benefit patients with human lung adenocarcinoma.

Summary points

- miR-218 is relatively low in human lung adenocarcinoma cells.
- miR-218 and Robo1 or Ecop expression is negatively correlated.
- miR-218 suppresses the markers of epithelial-to-mesenchymal transition in human lung adenocarcinoma cell.
- miR-218 inhibits human lung adenocarcinoma cell migration and invasion.
- Ecop and Robo1 are miR-218 target genes and miR-218 inhibits Ecop and Robo1 expression.
- Ecop and Robo1 promote human lung adenocarcinoma cell migration and invasion.
- miR-218 represses cancer cell invasion and migration by inhibiting Robo1 and Ecop expression.
- miR-218 suppresses the growth of human lung adenocarcinoma cells *in vivo*.

Conclusion

miR-218 inhibits human lung adenocarcinoma cell migration and invasion via the suppression of *Ecop* and *Robo1* expression, thus suggesting that miR-218 could serve as a potential therapeutic target.

Financial & competing interests disclosure

This research was funded by grants from the National Natural Science Foundation of China (81401910). The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

No writing assistance was utilized in the production of this manuscript.

Ethical conduct of research

All animal procedures were approved under the guidelines of the Health Sciences Animal Policy and Welfare Committee of The Chinese PLA General Hospital. This article did not contain any studies with human participants performed by any of the authors.

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Supplementary data

To view the supplementary data that accompany this paper please visit the journal website at: www.futuremedicine.com/doi/full/10.2217/fon-2017-0398.

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