miRNA-26a blocks interleukin-2-mediated migration and proliferation of non-small cell lung cancer cells via vascular cell adhesion molecule-1

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Background: Literatures have confirmed role of inflammatory mediators like interleukin-2 (IL-2) in non-small cell lung cancer (NSCLC). microRNA-26a (miR-26a) is involved in variety of signaling pathways and functions as tumor suppressor and regulating the responsible genes at posttranscriptional level. The aim of study was to study the correlation of IL-2 and miR-26a in lung cancer (LC) cells.

Methods: For the study we selected 32 subjects reported for NSCLC and 20 with chronic obstructive pulmonary disease (COPD) as control in the present study. For *in vitro* analysis, H-460 and H-226 cell lines were selected and received treatment of varying dose of IL-2 to study the effect of IL-2 on expression of miR-26a and vascular cell adhesion molecule-1 (VCAM-1). miR-26a mimic and miR-26a inhibitor were transfected to study the effect on progression and migration of tumor cell as well as on levels of VCAM-1.

Results: We evidenced that, expression of miR-26a was suppressed, whereas levels of IL-2 and VCAM-1 were increased in NSCLC tissues. IL-2 down-regulated the levels of miR-26a but upregulated the levels of VCAM-1 in H-460 and H-226 cells. miR-26a modulated the expression level of VCAM-1 via binding the 3'-UTR region. We found that miR-26a attenuated the migration and proliferation of H-460 and H-226 cells. IL-2 modulated the levels of miR-26a via activating p65 but not STAT-3.

Conclusions: All together, the finding of our study show that miR-26a blocks IL-2 mediated proliferation and migration of NSCLC via targeting VCAM-1.

Keywords: microRNA-26a (miR-26a); lung cancer (LC); vascular cell adhesion molecule-1 (VCAM-1); non-small cell lung cancer (NSCLC)

Introduction

Due to increasing rate of air pollution and cigarette smoker's worldwide it is anticipated that the lung cancer (LC) cases will be to their highest level in coming years (1,2). Studies have suggested that LC is one of the major causes accounting for cancer associated deaths globally (3). LC is divided into non-small cell lung cancers (NSCLC) and small cell lung cancers (SCLC). The NSCLC is further divided into 3 different types such as adenocarcinoma, squamous cell carcinoma and large cell carcinoma (4). It is reported that NSCLC's account for about 90% cases of

all LCs (5). Platinum derivatives-based chemotherapy has attained a dead end on account of efficacy, research's now are being targeted in search of new treatment approaches for improving the survival rate in LC (6).

Researchers have confirmed that inflammation is critically linked to tumorigenesis (7). Interleukin-2 (IL-2) is one of the important members of cytokines; it exerts beneficial effects on immune system. IL-2 also described to be T-cell growth factor (TCGF) is reported to play important role in immunotherapy for human cancer (8). In a study earlier IL-2 was reported as an important candidate for cancer immunotherapy in treating metastatic renal cell carcinoma and metastatic melanoma (9). Several studies have come up describing role of IL-2 in immunity development and in structural biology of cytokines (10-12). In addition to this, expression of IL-2 is induced by IL-1β (13). It was found that high levels of IL-2 resulted in elimination of large local burdens of prostate cancer in rats (14). Furthermore, in a meta-analysis study, it was found that IL-2 enhanced the efficacy and overall survival when combined with chemotherapy in treatment of NSCLC patients (15). Also, a study evaluating the behavior of IL-2 in advanced NSCLC patients suggested that all the involved subjects showed higher serum levels of IL-2 compared to control (16). Nevertheless, the pathway of IL-2 mediated progression and migration of NSCLC and not been explored.

microRNA-26a (miR-26a) is a subtype belonging to has-miR-26 family, it 21–22 nucleotide in length. The sequence of miR-26a is an important region having potential binding sites to target mRNA. Studies involving microarray analysis of various human tumors have suggested variable expression of miR-26a (17,18). miR-26 has been found to be down-regulated in various tumors such as bladder cancer, irrespective of the cancer stage (19), breast cancer via the caspase-8 and 9 dependent pathway (20), oral squamous cell carcinoma (21) and anaplastic carcinomas (22). Contrary to this, the expression of miR-26a was upregulated in malignancies such as high-grade glioma via PTEN pathway (23) and glioblastoma multiforme (24). Recently, some of the researches have decoded the role of IL-2 and the possible interconnection between various diseases and miRNA (25,26). This important connection between IL-2 and miRNAs can hold key in therapy and diagnosis of diseases. Here in the present study, we evaluated whether miR-26a is involved in IL-2 induced progression and migration of NSCLC.

Methods

Collection of samples

For the study, a total of 36 patients having diagnosed for NSCLC were selected, among them 21 were male and 15 females aging between 33–78 years, the mean age was 47.12±11.24. All the patients underwent surgery at the department of surgery, Affiliated Hospital of Inner Mongolian University for The Nationalities, Tongliao, Inner Mongolia, China. The tumors were confirmed by histology; the grading was done by hematoxylin and eosin (H&E) staining in accordance to WHO guidelines for classification. All the 36 tumor specimens were re-investigated in accordance to their histological subtypes, the progression stage and differentiation status. The specimens were found to have 14 cases of squamous carcinoma and remaining 22 were reported to be adenocarcinoma. The p-TNM grading system was used for classifying tumor specimens. Among the 36 subjects about 24, i.e., 66.67% had a previous record of smoking. In addition to this 24 subjects reported for chronic obstructive pulmonary disease (COPD) were included in the study; the lung tissues of such subjects were isolated for the study. The tumor tissues were freezed at –80 ℃ in liquid nitrogen. All the subjects were educated about the study and written consents were obtained from them. The protocol was approved by the ethical committee of Affiliated Hospital of Inner Mongolian University for The Nationalities, Tongliao, Inner Mongolia, China (No. MU40012LC).

Cell lines and culture conditions

For the study, human derived NSCLC cell lines along with A-549 and H-226 were included, the cell lines were obtained from the American Type Cell Collection (USA) and were maintained by incubating in RPMI media added with fetal bovine serum (FBS) (10%), penicillin (100 U/mL) (Merck, USA) and streptomycin (100 µg/mL) (Merck, USA) at 37 ℃ under 5% CO_2. For conditioning the cells with IL-2, the A-549 and H-226 cell lines were treated with varied concentrations of recombinant IL-2 (20, 40, 60 and 80 ng/mL) for 12 hours. The 293T human embryonic kidney cells maintained in DMEM medium added with FBS (10%), penicillin (100 U/mL) and streptomycin (100 µg/mL).

Transfection of miR-26a mimic and miR-26a inhibitor

The cancerous NSCLC cell lines received transfection of

miR-26a mimic and inhibitor as well as the negative control (miR-NC). The mimic and inhibitor (50 nM/L) were transfected using Lipofectamine-2000 transfection reagent following the supplied instructions.

Luciferase assay

The 3'-UTR region in the sequence of VCAM-1 having target sites for miR-26a were cloned into the luciferase vector of pGL3. After cloning the constructs were named as pGL3-Mut and pGL3-VCAM-1. Sequence analysis was done for confirming the constructs. The 293T cells were transferred to 96 well plates followed by transfection with pGL3-Mut or pGL3-VCAM-1 (100 ng) along with miR-26a mimic, inhibitor or NC (50 nM) with the aid of Lipofectamine-2000 reagent. The cells were collected after 24 post transfection and were subjected to lysis for performing the luciferase assay. Dual luciferase reporter assay was done for measuring firefly luciferase. Every analysis was performed in triplicate.

Cell proliferation studies

The A-549 and H-226 cell lines received transfection of miR-26a mimic, inhibitor or VCAM siRNA followed by exposure to IL-2. The cells after receiving the treatment were rinsed by phosphate buffer saline and incubated in RPMI-1640 media supplemented with FBS (10%). The cells were monitored after 24, 48, 72 and 96 hours post incubation, the extent of cell proliferation was determined by incubating the cells with Thiazolyl blue tetrazolium bromide (MTT, 5 mg/mL) for 2 hours. The samples were evaluated for optical activity at 570 nm using a plate reader.

Cell migration studies

The cell migration assay was performed by transwell chambers (Corning, USA) following the supplied instructions. Briefly, 3×10^5 A-549 and H-226 cells were maintained in RPMI buffer (100 mL) and transferred to the chamber in the upper portion. The lower portion of chamber was loaded with RPMI media (100 mL) supplemented with FBS (10%). The cells were incubated for 6 hours at room temperature with CO_2 (5%) and the upper surface was scratched, the membranes were fixed and the migrated cells received staining of crystal violet. For the assay, the cells from 5 random fields were counted.

Transfection of siRNA

For the study, VCAM-1 siRNA, STAT3 siRNA, NF-κB (p65) siRNA or STAT3 siRNA along with respective scramble control were procured from Santa Cruz biotech, USA. The transfecting the cells with siRNA, the A-549 cells were trypsinized in suspension and 3×10^5 cells were transferred in 6 well plates. The cells received transfection of STAT3 siRNA and NF-κB siRNA following the manufacturers protocol, the cells were collected after defined time intervals for further study. Western blot analysis was done for confirming the transfection.

Quantitative real-time PCR (qRT-PCR)

For qRT-PCR analysis, the total RNA was isolated from the cancerous tissues or the selected cancer cell lines with the help of Trizol reagent. After extraction of RNA, 5 μg was submitted to reverse transcription in the 1st strand of cDNA. qRT-PCR analysis was done using AccuPower® Dualstar™ qPCR PreMix (Bioneer, South Korea). qRT-PCR premix (5 μL), cDNA (1 μL) and primer (2 μL) (both forward and reverse) were used. The primers for the work are described in *Table 1*.

Western blot study

For expression levels of proteins western blot study was done. The cancerous tissues or cell lines were homogenized followed by lysis using a lysis buffer (Sigma Aldrich, USA). The proteins were separated using SDS-PAGE (12%) followed by blotting on a PVDF membrane. The non-specific proteins were blocked by nonfat milk (5%) at 37 ℃ for 60 minutes. The proteins were incubated for 12 hours at controlled temperature conditions (4 ℃) along with I^{ry} antibodies (anti-VCAM-1, 1:1,000) (anti-IL-2, 1:1,000), anti- NF-κB (1:1,000), anti-STAT3 (1:1,000), anti-phosphorylated p65 (1:1,000) and anti-p-STAT3 (1:1,000). The membranes were then incubated with horseradish peroxidase-conjugated II^{ry} antibodies for 60 minutes. All the antibodies were obtained from Santa Cruz Biotech, USA. The membranes were washed with Tris-buffered saline, the blots were visualized using enhanced chemiluminescence. Actin was used as loading control and internal standard.

Statistical analysis

All the results presented are mean ± SD. All the statistics

Table 1 Primers used for the study

Name of gene	Sequence	
	Forward	Reverse
miR-26a	CCGCCGTTCAAGTAATCCAG	CGCGGGGGCUGUUCAUAACUUACAUUGG
U6	CGCTTCGGCAGCACATATAC	CAGGGGCCATGCTAATCTT
IL-2	CCTGAGCAGGATGGAGAATTACA	TCCAGAACATGCCGCAGAG
β-Actin	CATCCTCACCCTGAAGTACCC	AGCCTGGATAGCAACGTACATG
STAT3	CCGCTCGAGATGGCCCAATGGAATCAGCTAC	ATCGTTAACTCACATGGGGGAGGTAGCGC
VCAM-1	AGAGGCAAGACUUCCCUGAAUGUA	GACTGTGATCGGCTTCCCAG

was done using GraphPad prism software. Student's *t*-test or one-way ANOVA was done for comparing the results. Pearson's correlation was measured for establishing correlation between mRNA levels of miR-26a or between miR-26a and mRNA levels of VCAM-1. Value of $P<0.05$ was regarded significant.

Results

Levels of miR-26a are negatively linked to VCAM-1 and IL-2 in NSCLC

miR-26a has been reported to behave as tumor suppressor in number of cancers such as colorectal cancer (27), gastric cancer (28), hepatic carcinoma (29), nasopharyngeal cancer (30) and breast cancer (31). However, role of miR-26a under the IL-2 mediated inflammation which is the common feature in LC showing metastasis haven't been explored. Here the study evaluated the levels of IL-2 and miR-26a in NSCLC (36 cases) and normal lung tissues (24 cases). The outcomes of qRT-PCR analysis suggested decreased levels of miR-26a (*Figure 1A*), whereas the levels of IL-2 were over-expressed in NSCLC (*Figure 1B*).

VCAM-1 is important contributing for adhesion of cancer cells. Studies involving abnormal expression of VCAM-1 associated with cancer have been previously studied (32,33). We observed that levels of VCAM-1 were upregulated significantly in NSCLC cells (*Figure 1B*). The findings also suggested that the levels of miR-26a were linked negatively to IL-2 and VCAM-1 (*Figure 1C,D*). The analysis by western blot analysis of cancer tissues for expression of VCAM-1 and IL-2 also suggested a significant elevation in NSCLC tissues compared to normal (*Figure 1E*).

Treatment of IL-2 decreases the expression of miR-26a but increases the levels of VCAM-1 in NSCLC cells

In direction to evaluate the effects of IL-2 on expression of miR-26a in A-549 and H-226 cells, the cells were treated overnight to varied concentrations of IL-2 (20–80 ng/mL). The outcomes suggested that treatment of IL-2 decreased the levels of miR-26a significantly when the concentrations exceeded 40 ng/mL compared to control cells (*Figure 1F*). Consequently, exposure of IL-2 resulted in a significant elevation in protein levels of VCAM-1 in A-549 and H-226 cells (*Figure 1G*), however when both the NSCLC cell lines received exposure of other members of IL family such as IL-6, IL-1β and IL-8 at concentration of 80 ng/mL. did not cause any significant change in levels of VCAM-1 (*Figure 1H*). These findings indicate that IL-2 suppressed the expression levels of miR-26a and upregulated the levels of VCAM-1 in the NSCLC cells.

miR-26a modulated the levels of VCAM-1 in NSCLC cells

To evaluate the involvement of miR-26a in the expression of VCAM-1, both the NSCLC cell lines i.e., A-549 and H-226 cells were transfected with miR-26a mimic and inhibitor prior to exposing them to IL-2 (80 ng/mL). The outcomes of experiment demonstrated that transfection of miR-26a mimic upregulated the expression of miR-26a whereas inhibitor down-regulated it (*Figure 2A*). It was also found that the upregulated miR-26a inhibited the expression of VCAM-1 in both the NSCLC cell lines (*Figure 2B,C*). These outcomes demonstrate involvement of miR-26a in regulation of VCAM-1 via IL-2. Simultaneously, we performed bio-informatics analysis by searching the database of TragetScan, we found that the seed sequence of VCAM-1
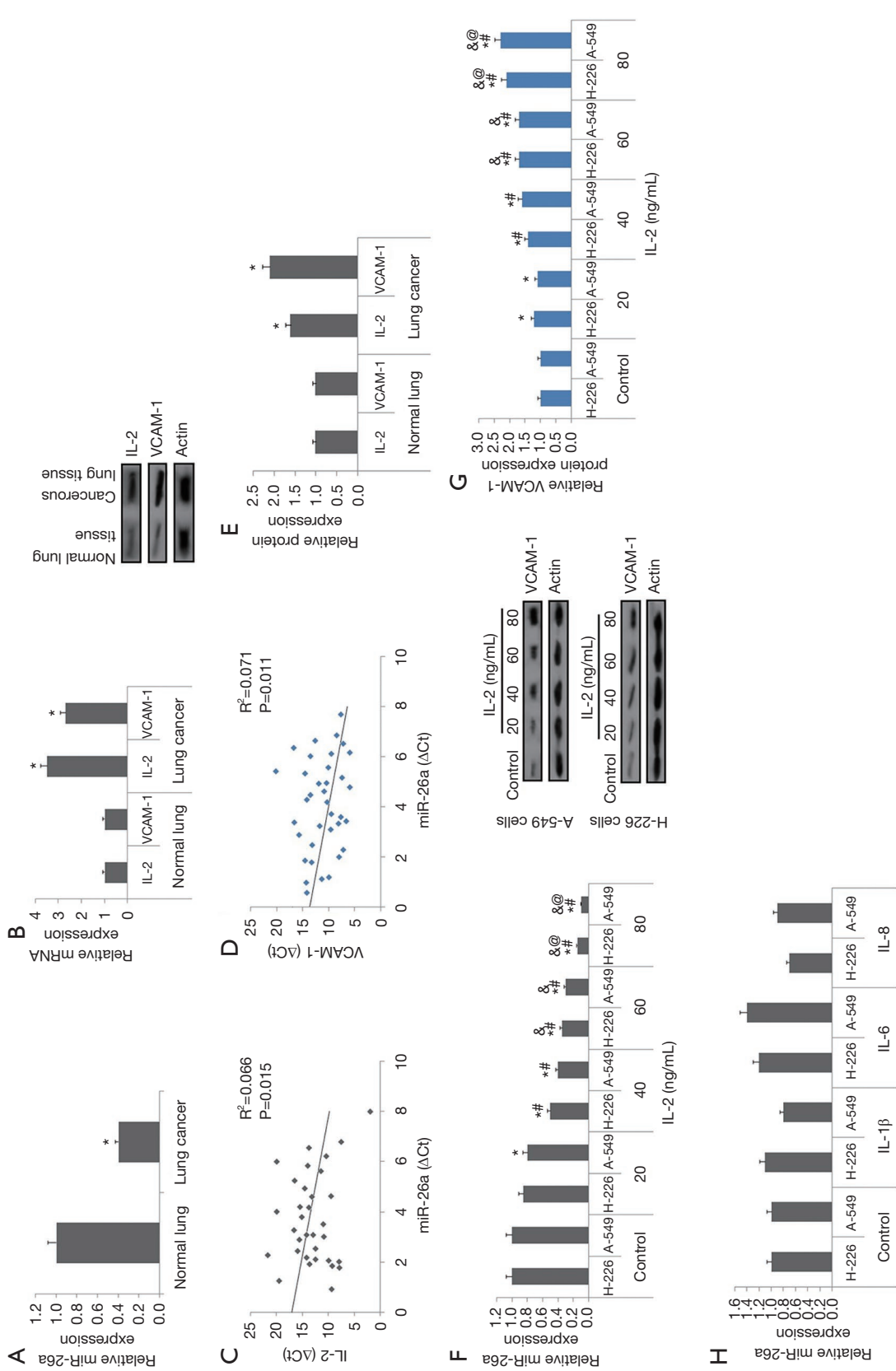
Figure 1 Expression levels of miR-26a, VCAM-1 and IL-2 in NSCLC cells and tissue. (A) Levels of miR-26a compared to cancerous and normal lung tissues; (B) mRNA expression levels of IL-2 and VCAM-1 compared to cancerous and normal lung tissues; (C) correlation between expression of miR-26a and levels of IL-2 with Pearson correlation test; (D) correlation between miR-26a and levels of VCAM-1; (E) immunoblotting analysis for levels of IL-2 and VCAM-1; (F) levels of miR-26a in NSCLC cells (A-549 and H-226 cells) in presence of IL-2; (G) levels of VCAM-1 in A-549 and H-226 cells in presence of IL-2; (H) levels of miR-26a against exposure to IL-8, IL-8 and IL-1β in NSCLC cells. *, P<0.05 against normal lung tissues or control; #, P<0.05 against IL-2 treatment; @, P<0.05 against IL-2 (60 ng/mL) and &, P<0.05 against IL-2 (40 ng/mL). miR-26a, microRNA-26a; VCAM-1, vascular cell adhesion molecule-1; IL-2, interleukin-2; NSCLC, non-small cell lung cancer.
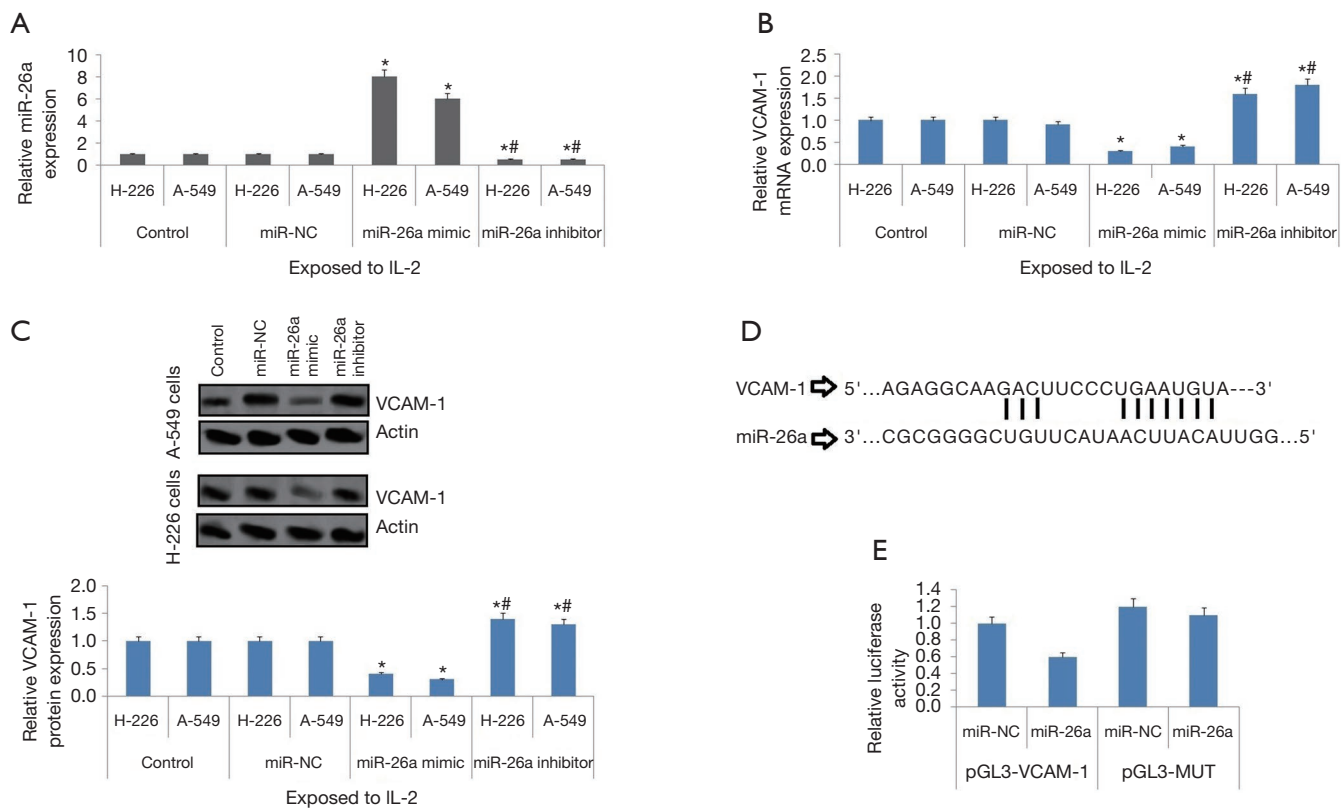
Figure 2 miR-26a regulates the expression of VCAM-1. (A) The levels of miR-26a post transfection of miR-26a mimic and inhibitor in A-549 and H-226 cells; (B) mRNA expression levels of VCAM-1 in NSCLC cells post transfection of miR-26a mimic and inhibitor, these cells were treated to IL-2 (80 ng/mL) for 12 hours before subjected to quantification; (C) protein levels of VCAM-1; (D) seed sequence of VCAM-1 showing potential binding site for miR-26a; (E) luciferase activity in 293T cells. *, P<0.05 *vs.* control; #, P<0.05 *vs.* miR-26a mimic. miR-26a, microRNA-26a; VCAM-1, vascular cell adhesion molecule-1; IL-2, interleukin-2; NSCLC, non-small cell lung cancer.

had potential binding site for miR-26a (*Figure 2D*). For confirming the outcomes of bio-informatics analysis, we constructed plasmid having VCAM-1 3'-UTR or the mutant named pGL3-VCAM-1 or pGL3-Mutant respectively (*Figure 2D*). The 293T cells received transfection of miR-26a mimic and plasmids. The outcomes demonstrated that miR-26a halted the luciferase activity in the NSCLC cells transfected with pGL3-VCAM-1 and failed to do the same in cells transfected with pGL-3-mutant (*Figure 2E*). The outcomes confirmed that miR-26a modulated the expression VCAM-1 in NSCLC by binding to the 3'-UTR binding sequence.

miR-26a modulates the proliferation and migration of NSCLC cell lines induced by IL-2

To evaluate the effect of miR-26a on behavior of NSCLC

cells, the cells were transfected with miR-26a mimic and miR-26a inhibitor before exposing them to IL-2. The outcomes of proliferation studies suggested that miR-26a mimic halted *in vitro* proliferation of cells, whereas the inhibitor caused cell proliferation (*Figure 3A,B*). The results also suggested that miR-26a mimic caused a significant inhibition of cell migration in A-549 and H-226 cells induced by IL-2. However, it was also found that miR-26a inhibitor stimulated the cell migration (*Figure 3C*).

IL-2 regulates expression of miR-26a via NF-κB pathway

It was evidenced that IL-2 can lead to activation of STAT3 and NF-κB cascades (34,35). In the present work we evaluated the effects of IL-2 on STAT3 and NF-κB which are the inflammatory factors. We evidenced that IL-2 encouraged the activation of STAT3 and NF-κB in the
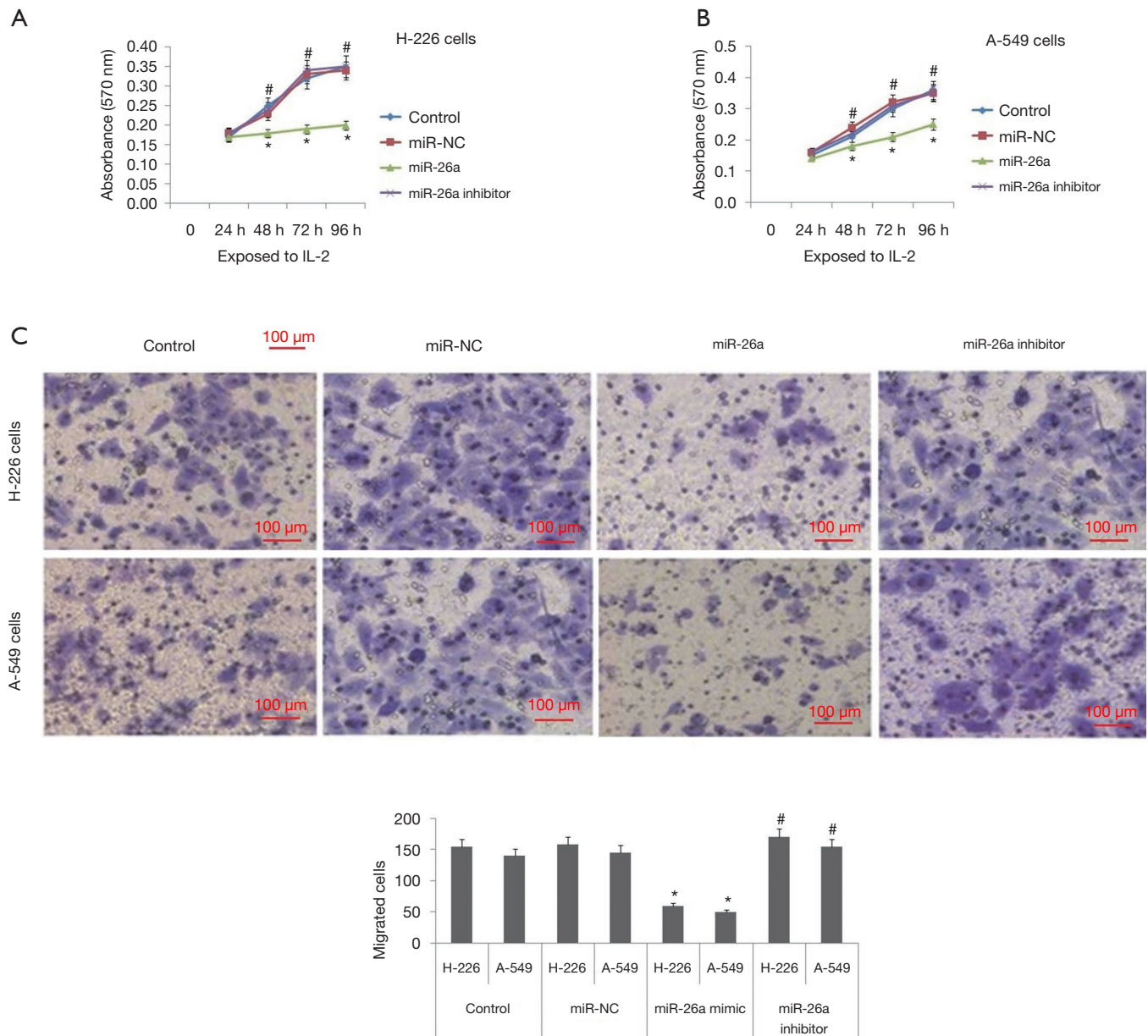
Figure 3 miR-26 a causes alteration in migration and proliferation of NSCLC cells. (A,B) Cell proliferation study post transfecting the A-549 and H-226 cells with miR-26a mimic or inhibitor, the cells were exposed to IL-2 (80 ng/mL) for 12 hours before evaluation; (C) cell migration study post, transfecting the A-549 and H-226 cells with miR-26a mimic or inhibitor, the cells were exposed to IL-2 (80 ng/mL) for 12 hours before evaluation. *, P<0.05 *vs.* control; #, P<0.05 *vs.* miR-26a mimic. miR-26a, microRNA-26a; IL-2, interleukin-2; NSCLC, non-small cell lung cancer; NC, negative control.

A-549 cells (*Figure 4A*). In addition to this, STAT3 siRNA and p65 siRNA were transfected in the NSCLC cells for inhibiting their expression (*Figure 4B,C*). It was evidenced that inhibition of NF-κB blocked the expression of miR-26a (*Figure 4D*), whereas the inhibition of STAT3 did not

influenced the expression of miR-26a (*Figure 4E*). It was also found that, inhibition of NF-κB decreased the levels of VCAM-1 (*Figure 4B*), which remained unaffected by inhibition of STAT3 in A-549 cells (*Figure 4C*). Also, expression of VCAM-1 in A-549 cells was blocked when
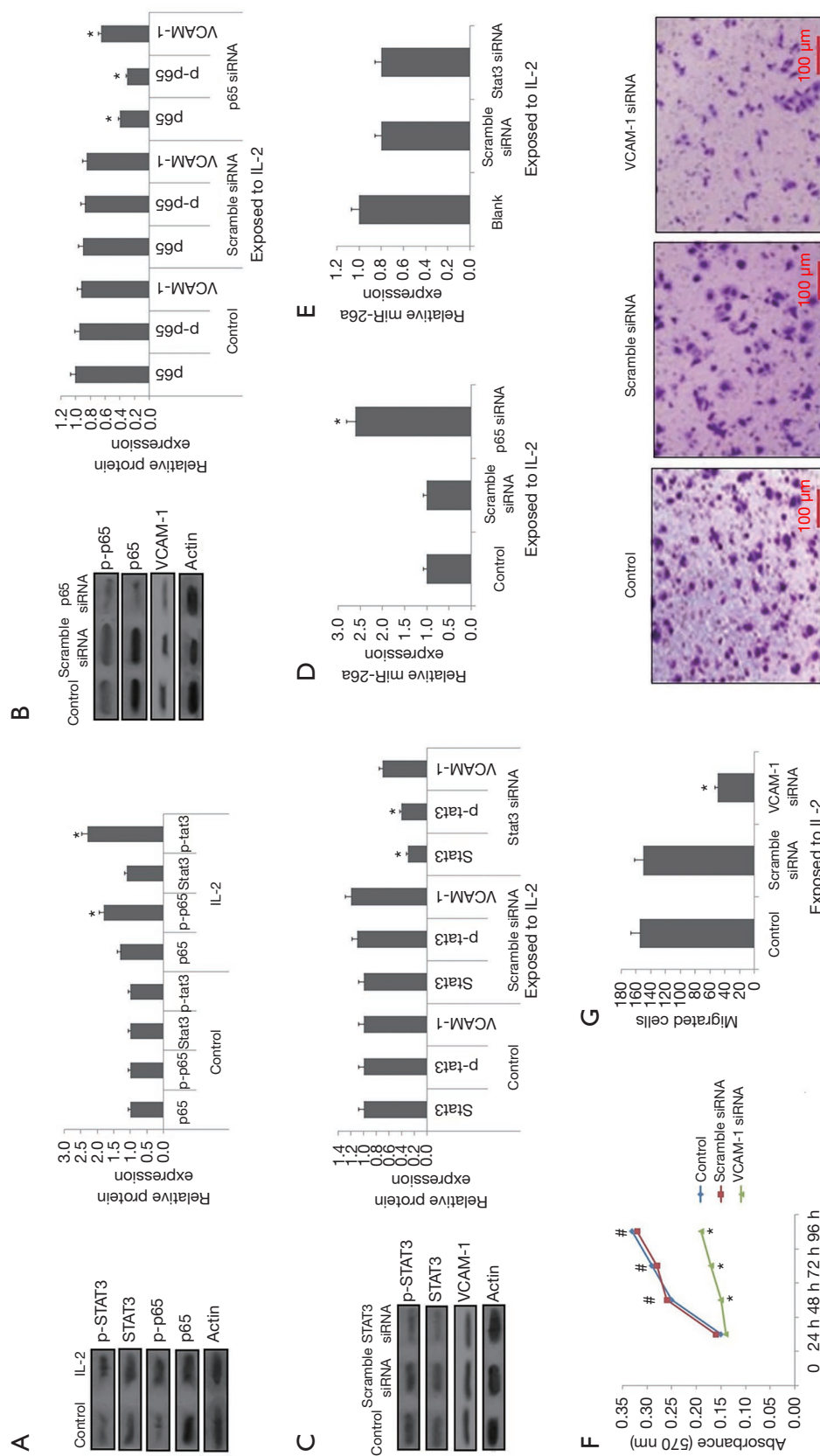
Figure 4 IL-2 regulates the expression of miR-26a. (A) Levels of p-STAT3, STAT3, p-p65 and p65 in A-549 cells treated with IL-2; (B,C) the NSCLC cells transfected with STAT3 siRNA and p65 siRNA and expression of p65, p-p65, STAT3 and p-STAT3 proteins was done by western blot analysis; (D,E) relative expression levels of miR-26a in after inhibiting the expression of p65 and STAT3 in A-549 cells; (F) cell viability of A-549 cells which received transfection of VCAM-1 siRNA or Scramble siRNA; (G) cell migration studies of A-549 cells transfected using VCAM-1 or scramble siRNA. *, P<0.05 compared to control. miR-26a, microRNA-26a; VCAM-1, vascular cell adhesion molecule-1; IL-2, interleukin-2; NSCLC, non-small cell lung cancer.

transfected with VCAM-1 siRNA. Being a potential target of miR-26a, the study was directed to evaluate whether blockade of VCAM-1 showed the similar effects on A-549 cells mediated via miR-26a mimic (*Figure 3*). The findings demonstrated that viability of cells decreased along with their migration when VCAM-1 was blocked (*Figure 4F,G*). The findings suggested that NF-κB may play a potential role in expression of miR-26a via IL-2.

Discussion

It is established that the extracellular matrix compartment and the stromal and cellular compartment of tumor is involved in metastasis of NSCLC (36). The tumor micro-environment majorly constitutes of inflammatory cells, immune cells and macrophages (37). In a study earlier involving animal model of LC showed presence of Th17 and Treg cells in the tumor tissues and also suggested there potential role in tumorigenesis (38). In the current study, expression of miR-26a was negatively associated with expression of VCAM-1 and IL-2 in the LC tumor tissues, suggesting involvement of miR-26a in the progression of NSCLC cells. We used the LC cell lines A-549 and H-226 and evidenced that IL-2 acted as modulator for the expression of miR-26a in VCAM-1. Outcomes of the study suggested about the micro environment of tumor is full of inflammatory cells may affect the expression levels of VCAM-1 and may also lead to progression and migration of NSCLC cells.

VCAM-1 has been found to be implicated in number of pathological conditions such as infections (39), auto-immune disorders (40) and cardiac diseases (41). Recently, role of VCAM-1 in development, progression and metastasis in cancer has made the researchers to focus on it. In a study reported earlier for subjects with LC demonstrated VCAM-1 can be an indicator for diagnosis after chemotherapy (42). Also it was reported that the expression of VCAM-1 in the respiration condensates of LC subjects was high compared to subjects reported for COPD or the controls (43). In addition to this, levels of VCAM-1 in tumor tissues may lead to inhibition of T-cell function, promotion of T-cell migration and confab the capacity of tumor cells expressing VCAM-1 for preventing the attack from immunity (44).

In the present research, transfection of miR-26a mimic inhibited the levels of VCAM-1 mediated by IL-2, whereas miR-26a inhibitor encouraged the expression of VCAM-1 in presence of IL-2. The outcomes of luciferase studies demonstrated that miR-26a may bind VCAM-1 on its

3'-UTR region and also suppressed the expression protein levels of VCAM-1. Our experiments also demonstrated that miR-26a mimic showed adverse effect on proliferation and migration of NSCLC. Contrary to this, miR-26a inhibitor caused proliferation and migration of NSCLC under IL-2 treatment.

We also demonstrated that IL-2 activated the expression of STAT3 and NF-κB in A-549 cells. Knockdown of expression levels of NF-κB and not of STAT3 faded the inhibition of IL-2 mediated expression of miR-26a. Our findings thus established the important role of NF-κB in regulating the expression of miR-26a via IL-2, the findings were in agreement to earlier a study which have identifiedmiR-26a mediated the regulation of IL-2 expression in avian lymphocytelines (45). We also found the link between IL-2 induced cell adhesion and inflammation shown by NSCLC. As reported earlier, NF-kB has been found to mediate modulation of miR-26a in cardiac fibrosis (46).

Conclusions

Altogether, the present study demonstrated that IL-2 modulated the levels of VCAM-1 in LC cells through miR-26a and found VCAM-1 as potential target for binding of miR-26a. miR-26a could halt the migration and proliferation of NSCLC in presence of IL-2, hence suggesting that increasing the expression of miR-26a in NSCLC may be an potential way in dealing the progression of NSCLC. More studies using *in vivo* models involving miR-26a can support our findings.

Acknowledgments

Footnote

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at http://dx.doi.org/10.21037/tcr.2020.02.36). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all

aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). The protocols were sanctioned by ethical committee of Affiliated Hospital of Inner Mongolian University for The Nationalities, Tongliao, Inner Mongolia, China. The present work complies with the guidelines for human studies and animal welfare. Informed consent was obtained from the subjects.

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