miR-182 facilitates invasion and EMT by targeting Numb in Malignant Pleural Mesothelioma

Yanmeng Kang
The First Affiliated Hospital of Shandong first Medical University
Degan Lu
The First Affiliated Hospital of Shandong first Medical University
Lingxia Meng
The First Affiliated Hospital of Shandong first Medical University
Ruiping Ma
the First Affiliated Hospital of shandong first Medical University
Chuanjun Huang
the First Affiliated Hospital of shandong first Medical University
Mingxia Gao
the First Affiliated Hospital of shandong first Medical University
Caiqing Zhang (freecqz66@163.com)
the First Affiliated Hospital of shandong first Medical University

Research

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Abstract

Background

Malignant Pleural Mesothelioma (MPM) is a highly aggressive tumor which need effective therapeutic methods to improve the prognosis. We carried out this study to explore the role of miR-182 in MPM development, its correlation with Numb expression and EMT.

Methods

First, we investigated the level of miR-182 and Numb-mRNA by qRT-PCR. Furthermore, we introduced the putative miR-182 binding site into a luciferase reporter plasmid to illustrate the impact of miR-182 on Numb. Then, we down-regulated the expression of miR-182 with/without Numb knocked down in NCI-H2452 cells to investigated their effect. Data were presented as mean ± SD of three independent experiments. Student's test, correlation analysis and analysis of variance (ANOVA) were used.

Results

Our results revealed that miR-182 has a high level of expression in MPM, it has a negative correlation with Numb and targeted Numb in MPM cells. miR-182 facilitated the invasion of MPM cells while down-regulation of miR-182 restrained the progression of EMT and made MPM cells more susceptible to pemetrexed.

Conclusions

miR-182 and Numb can serve as potential therapeutic targets for MPM.

Introduction

Malignant Pleural Mesothelioma (MPM) is a highly aggressive tumor with a globally increasing incidence. MPM is resistant to conventional treatment which leads to dismal prognosis. The median overall survival of MPM was 4–14 months [1]. Therefore, it is urgent to develop novel diagnostic and therapeutic methods to improve the prognosis of MPM.

MicroRNAs (miRNAs) are highly conserved small non-coding RNA, 20–25 nucleotides in length, which post-transcriptionally regulate protein expression by binding to the 3'-untranslated region (UTR) of the target genes [2]. miRNA can be oncogenic factors or tumor suppressors in different tumors. Thereby, intensive efforts have been made to explore the miRNA-based therapeutic strategies for carcinoma. To this day, a number of miRNAs have been identified to participate in the development of MPM [3]. miR-182 is a member of miR-183-96-182 cluster encoded by a 5-kb DNA sequence that located on human

chromosome 7q32.2 [4]. miR-182 played an oncogenic role in many kinds of malignancies [5, 6], but the role of miR-182 in MPM is still unknown.

Many miRNAs including miR-182 have been confirmed to facilitate tumor metastasis by dysregulating epithelial-to-mesenchymal transition (EMT)-related genes and promoting EMT. *Numb* plays an important role in the transformation of cellular polarity and the plasticity of the stem cell (SC) compartment[7], both of which are closely related to tumor formation. In particular, the plasticity of the SC compartment is related to EMT, which contributes to the acquisition of invasive properties as well as resistance to cell apoptosis and chemotherapy [8, 9]. Our previous research indicated that the loss of *Numb* in epithelioid MPM correlated with tumor progression [10]. *Numb* is a versatile molecular adapter which can interact with multiple genes. We carried out this study to explore the role of miR-182 in MPM development, its correlation with *Numb* expression and EMT.

Materials And Methods

Patients and specimens

Tissue samples from 25 MPM patients who underwent surgery or thoracoscopic procedure in our hospital from 2007 to 2017 were collected. The diagnosis of MPM was based on the WHO criteria [11] and confirmed in all cases according to their clinical, morphologic, and immunohistochemical data. Besides, 25 normal pleura samples were collected as control from thoracoscopy-treated patients who were diagnosed with recurrent idiopathic spontaneous pneumothorax. This study was approved by the Ethical committee of Shandong Provincial Qianfoshan Hospital.

Cell culture

NCI-H2452 cells (stemming from ATCC) which belongs to human MPM cell lines were obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). Human pleural mesothelial cell line Met-5A cells were purchased from ATCC. NCI-H2452 cells were cultured in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), 1.5 g/l $NaHCO_3$, 2.5 g/l glucose, 0.11 g/l Sodium Pyruvate and maintained in a humidified atmosphere with 5% CO_2 at 37 °C. Met-5A cells were cultivated in medium 199, supplemented with 10% FBS, 20 mM HEPES, 24 mM sodium bicarbonate, 3.3 nM epidermal growth factor, 200 nM hydrocortisone, 4 mg/l insulin, 2 mM L-glutamine, and 100 mg/ml streptomycin in a humidified 5% CO_2 atmosphere at 37 °C.

Transfection

Synthetic miR-182 mimics, miR-182 inhibitors, negative control (NC) miRNAs (miR-182 mimics-NC, miR-182 inhibitor-NC), small interference RNA (siRNA) targeting *Numb* were purchased from GenePharma Co. Ltd. (Shanghai, China). Plasmid pcDNA3.1- *Numb* was synthesized by GenePharma Co., Ltd. (Shanghai, China). Cells at a density of 2×10^5 per well were grown to 70% confluence. 1×10^6 cells were put in a 60-mm dish and transfected with either the empty vector（mock）or pcDNA3.1- *Numb* (*Numb*) using

Lipofectamin™ 2000 (Invitrogen) according to manufacturer's instructions. The detailed steps were the same as previously described [12]. After incubating for 48 h, the cell transfection and interference efficiency were assessed. The transfected cells were thereafter split into three sets: one was used to analyze protein expression by Western blot (WB), the other one used for cell viability assay, and the remaining one for cell apoptosis analysis.

The corresponding miRNA sequences were as follows:

miR-NC, 5'-AUAUGACGUACGUGUAACGUACUC-3';

miR182 mimics, 5'-UUUGGCAAUGGUAGAACUCACACU-3';

anti-miR NC, 5'-UCCGAGUGCUAUACGCUAGUAAAU-3';

and anti-miR182 oligonucleotides, 5'-AGUGUGAGUUCUACCAUUGCCAAA-3'.

Quantitative real-time reverse transcription polymerase chain reaction

The procedure of quantitative real-time polymerase chain reaction (qRT-PCR) for detection of miRNA or mRNA was as follows. First, total RNA was extracted using TRIzol reagent (Invitrogen). cDNA was then synthesized using the TaqMan microRNA Reverse Transcription Kit (Life Technologies) for analysis of miRNA. While for the analysis of mRNA, reverse transcription was firstly carried out using ReverTra Ace qPCR RT Kit (Toyobo, Japan), and qRT-PCR was subsequently performed using SYBR Green Realtime PCR Master Mix (Toyobo, Japan) and solutions containing specific primers of target genes on the ABI 7500 Real-time PCR System (Applied Biosystems, Foster City, CA, USA). The expression levels of U6 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were used as control.

Western blot

48 h after transfection, cells were harvested for protein analysis via WB. Total protein was extracted using RIPA lysis buffer. Proteins were then transferred to PVDF membrane and subjected to the primary antibodies including anti- *Numb* (1:1000, Abcam, UK), E-cadherin, Vimentin and ZEB1 (1:500, Santa Cruz Biotechnology, USA) antibodies. Peroxidase labeled anti-mouse or anti-rabbit secondary antibodies were thereafter applied (Zhongshan Goldenbridge Biotechnology, Beijing, China). Blots were developed using enhanced chemiluminescence detection reagent (Applygen Technologies Inc., Beijing, China).

Luciferase reporter assay

WT- *Numb* -3'UTR pmiR-ReportTM and MUT- *Numb* -3'UTR pmiR-ReportTM were purchased from ABI. HEK293T cells (density, 1×10^4 per well) were planted in 96-well plates, incubated overnight and then transfected with WT- *Numb* -3'UTR pmiR-ReportTM or MUT- *Numb* -3'UTR pmiR-ReportTM. 24 h after the transfection, the cells were treated with miR-182 (100 ng/ml) and incubated for 48 h additionally. The

luciferase activity was measured using a dual-luciferase reporter system (Promega) according to the manufacturer's instructions.

Cell viability assay

We used MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay to evaluate cell viability. Cells with a density of 1×10^4 /well were seeded in a 96-well plate and incubated for 24 h. Then, these cells were treated with different concentrations of pemetrexed for 24 h in triplicate. 20 μl of 5 mg/ml MTT was added into each well and incubated for 4 h at 37 °C in culture hood. Media were carefully removed and 150 μl dimethyl sulfoxide was added. The absorbance was measured at 490 nm from which the background was subtracted. The cell survival index was calculated as [A490 (pemetrexed +)/A490 (pemetrexed −)]×100%.

Cell apoptosis analysis

The apoptosis rate was examined by double staining assays (Annexin V/FITC binding and propidium iodide (PI) uptake) using flow cytometry. 1×10^5cells were seeded into 6-well plates and were tested 72 h after transfection; 1×10^5cells were seeded into 6-well plates and incubated for another 24h, then these cells were treated with 5μmol/L pemetrexed for 24 h. After that, cells were incubated with FITC-conjugated Annexin V for 20 min at room temperature in the dark. PI was then added and the samples were immediately analyzed by fluorescence-activated cell sorting.

Transwell assay

The transwell assay was performed to assess the invasive ability of MPM cells. 24-well Boyden chambers coated with Matrigel diluted in 1:4 by precooled serum-free medium were used. The parental and transfected cells were resuspended at a density of 1×10^6/ml after starvation for 24 h. Then, 2×10^5 cells were seeded into the upper chambers, while medium supplemented with 10% FBS was placed in the lower chamber. After incubated for 24 h at 37 °C, cells on the top side of the filter were removed. The remaining cells were fixed by 4% methanol for 15 min and stained with 0.5% crystal violet for 10 min. The number of migratory cells from at least five randomly selected microscopic fields were counted and the average value was calculated. The experiments were repeated three times.

Statistical analysis

Statistical analyses were performed using SPSS 21.0 software package (SPSS Inc., Chicago, IL, USA). Data were presented as mean ± SD of three independent experiments. Student's test, correlation analysis and analysis of variance (ANOVA) were used. All statistical tests were two-sided with probability values <0.05 defined as statistically significant.

Results

1. The level of miR-182 and Numb-mRNA in MPM tissues and cell lines

The level of miR-182 in MPM tissues was much higher than normal pleura specimens ($P < 0.05$, Fig. 1A), while Numb has a reverse trend ($P < 0.05$, Fig. 1A). In cell lines, the levels of miR-182 were higher in NCI-H2452 than in Met-5A cells, whereas the expression level of *Numb* was quite the reverse (Fig. 1B). Therefore, a negative correlation was found between miR-182 and *Numb* expression in both MPM tissues and cell lines.

2. miR-182 targeted Numb in cell lines

Numb was predicted to be a potential target of miR-182 by silico algorithm and one research reported that the 3'UTR of *Numb* could be targeted by miR-182 using reporter assays [13](Fig. 2A). In the present research, we introduced the putative miR-182 binding site, i.e. the 3'UTR of *Numb*, into a luciferase reporter plasmid to illustrate the impact of miR-182 on *Numb* expression. The luciferase reporter assay showed that miR-182 markedly suppressed the expression of wildtype (WT) *Numb*, but not the mutant one (Fig. 2B). These results indicated that miR-182 could directly target the 3'UTR of *Numb*. WB analysis showed an increase of endogenous Numb in the NCI-H2452 cells after the introduction of miR-182 inhibitor (Fig. 2C). While in Met-5A, Numb was downregulated by miR-182 mimics (Fig. 2E). Next, NCI-H2452 cells with miR-182i were transfected with Numb-siRNA. WB showed that *Numb* knockdown could significantly inhibit the increase of Numb protein induced by miR-182 downregulation (Fig. 2C). But unlike proteins, the levels of corresponding mRNA in those groups were not significantly affected by *Numb* knockdown (Fig. 2D, E).

3. miR-182 facilitated invasion by targeting Numb in MPM cells

The invasive ability of miR-182i transfected NCI-H2452 cells was significantly decreased, compared with that of the mock and negative control groups($P < 0.05$, Fig. 3A,B). However, co-transfection of miR-182 inhibitor and *Numb*-siRNA had no significant effect on the capacity of cell invasion ($P > 0.05$ Fig. 3A,B).

4. Downregulation of miR-182 restrained the progression of EMT.

To further investigate the role of miR-182 on EMT in MPM cells, the expression levels of EMT-associated proteins (E-cadherin and Vimentin) were detected by WB and immunofluorescence. We found that the expression of E-cadherin which was an epithelial marker was up-regulated, while the mesenchymal markers Vimentin and ZEB1 were down-regulated in NCI-H2452 but not in control groups after being treated with miR-182i ($P < 0.05$, Fig. 4). That is, miR-182 inhibition could reverse EMT. Nonetheless, co-transfection of miR-182 inhibitor and Numb-siRNA had no significant effect on the progression of EMT ($P > 0.05$, Fig. 4).

5. Downregulation of miR-182 made MPM cells more susceptible to pemetrexed

MTT assay was used to determine the growth and proliferation of MPM cells after treated with pemetrexed at a range of clinically achievable concentration (0–5 μM) for 24 h. A significant decrease of cell viability was observed in miR-182i transfected MPM cells when pemetrexed was added compared to negative control groups (mock or i-NC) ($P < 0.05$; Fig. 5A). Nevertheless, as miR-182 inhibitor was

transfected along with Numb-siRNA, the chemosensitivity of MPM cells did not change significantly ($P > 0.05$; Fig. 5A). We next sought to determine whether miR-182i played a role in sensitizing MPM cell lines to pemetrexed-induced apoptosis. The apoptosis rate was assayed using flow cytometry 24 h after the application of 5 μM pemetrexed. The percentage of early and late apoptotic cells was significantly higher in miR-182i transfected cells than the control groups(mock or i-NC, $P < 0.05$) (Fig. 5B,C).

Discussion

It has been demonstrated that miRNAs take part in tumorigenesis in various types of cancers, of which MPM is no exception. miRNAs play a key role in the pathogenesis of MPM through targeting specific genes and pathways involved in cancer progression, invasion and treatment response [14, 15]. Our data demonstrated that the level of miR-182 was higher in MPM tissues and cell lines compared to normal pleural specimens and pleural mesothelial cells, respectively. Plenty of studies have investigated the profile of miRNAs in MPM, but the results were heterogeneous (sometimes even contradictory) because of 'technical factors' such as "diversity of the histological subtypes examined, different sample sources, diversity of the control groups, statistical approaches and different high-throughput platform used"[15]. Next, we plan to carry on research with different pathological types cell lines.

Numb, originally identified in *Drosophila*, is a highly conserved protein and plays a critical role in cell fate determination [16]. We concentrate on *Numb* because it can modulate a wide range of pathological processes in solid tumors [17, 18]. Moreover, we have previously shown that *Numb* might be involved in the development of epithelioid MPM and up-regulation of this protein might help gain sensitivity to cisplatin [10]. In our present research, a negative correlation between miR-182 and *Numb* was observed in MPM tissues and cells, and *Numb* is identified as a target of miR-182 using the reporter assay. Meanwhile, inhibition of miR-182 upregulated the expression of *Numb* in vitro, which was in consistency with the results in Head and neck squamous cell carcinoma (HNSCC) [17]. This suggested that miR-182 might be an oncogenic factor for MPM and could serve as a potential biomarker to distinguish tumors from normal tissue. Interestingly, the mRNA level of *Numb* had not changed in miR-182-inhibited MPM cells, which means that the down-regulation of *Numb* expression by miR-182 occurred post-translationally. The exact mechanism needs to be further explored.

Recently, a new perspective on the function of miRNA has been put forward. Many miRNAs were reported to regulate tumor metastasis. In this study, we found miR-182 facilitated the invasion of MPM cells through targeting *Numb* which accelerated EMT progression. Cells undergo EMT lose cell–cell adhesion and gain the capacity of migration and invasion. EMT cannot be exclusively explained by irreversible genetic alterations [19]. Epigenetic modulation also plays an important role in EMT, which involves the post-transcriptional regulation via miRNAs [20]. *Numb* has been regarded as a crucial regulator of EMT in mammalian epithelial cells [21] as well as tumors [22]. As we know, MPM is histologically classified into three subtypes: epithelioid, biphasic, and sarcomatoid. And the sarcomatoid MPM has the worst prognosis. Intriguingly, EMT may contribute partly to it. Some researchers found that EMT is a key determinant of the aggressive features of Mesothelial/Mesothelioma-derived cell lines [23].

According to several recent studies, *Numb* overexpression led to a loss of mesenchymal markers and features and turned the cells into epithelial phenotype [24]. Meanwhile *Numb* knockdown has the opposite effect by promoting EMT. We reached similar conclusions in our research. It is implicated that *Numb* acts as a brake on the completion of EMT [18]. *Numb* has been proven to stabilize p53 through interaction with MDM2 [25], to inhibit Notch signaling by promoting ubiquitination and degradation of the Notch1 intracellular domain (NICD) [26], and to suppress Hh signal via Itch-dependent ubiquitination [27]. Consequently, we speculate that the loss of *Numb* in tumors may lead to EMT via the following mechanisms: activating p53 pathway or inhibiting Notch or Hh pathway [24]. For example, a recent study found that *Numb* suppressed EMT by antagonizing Notch signaling in triple-negative breast cancer and stabilizing wild-type p53 in normal mammary epithelial cells and breast cancer cells [24]. Our previous work indicated that *Numb* had an inverse correlation with nuclear Gli1 in MPM [28]. Meanwhile, our preliminary experiment found that Hedgehog signaling was activated in NCI-H2452 cell line. And downregulation of Gli1 via Gli-siRNA or GANT-61 could inhibit the proliferation and invasiveness of NCI-H2452 cells. Whether or not *Numb* suppresses EMT though Hedgehog signaling in MPM needs more research to clarify.

The recommended chemotherapy-cisplatin plus pemetrexed-for mesothelioma remains unchanged for more than two decades [29]. Although this schedule has improved the survival, the prognosis of MPM is still poor. We found downregulating miR-182 sensitized MPM cells to pemetrexed, identifying a novel approach to combat with chemoresistance. The role of miR-182 in chemoresistance has also been reported by other researchers [30]. Meanwhile, a growing number of studies have focused on improving the efficacy of pemetrexed. The latest research found that pemetrexed-loaded gold nanoparticles was more effective in inhibiting MPM cells' growth [31]. EMT induced by IL-6 promoted the growth and metastasis of lung adenocarcinoma in vivo, while blocking EMT could abrogate resistance to pemetrexed [32, 33]. In MPM, we speculated that miR-182 inhibition made NCI-H2452 cells more susceptible to pemetrexed by interfering with EMT progression.

In conclusion, our results revealed that miR-182 targeted *Numb* in MPM cells. miR-182 facilitated the invasion of MPM cells while down-regulation of miR-182 restrained the progression of EMT and made MPM cells more susceptible to pemetrexed. miR-182 and *Numb* can serve as potential therapeutic targets for MPM. However, more researches are needed to illustrate the function of miR-182- *Numb* - Hedgehog axis on EMT in MPM.

Declarations

Acknowledgements

Not applicable.

Authors' contributions:

YMK and CQZ conceived and designed the study. YMK, CJH, LXM, RPM and MXG performed the experiments, analyzed and interpreted the data. YMK and CJH drafted or revised the manuscript. All authors read and approved the final manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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Availability of data and materials

The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

This study was approved by the medical ethics committee of The First Affiliated Hospital of Shandong First Medical University and all participants gave informed consent.

Consent for publication

This manuscript is approved by all participants for publication.

Declaration of competing interest

All authors declare no financial competing interests.

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Figures

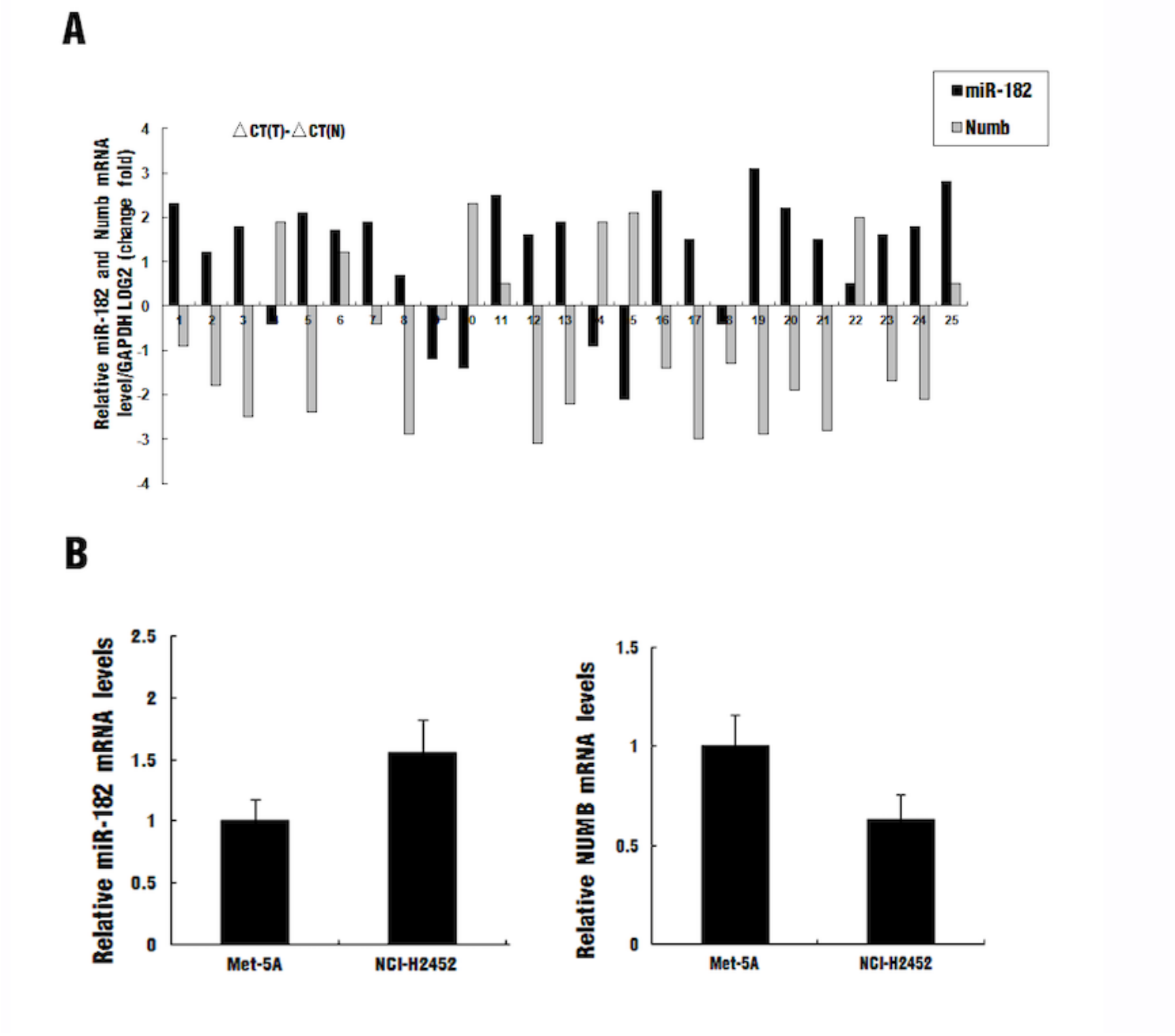

Figure 1

The level of miR-182 and Numb-mRNA in MPM tissues and cell lines. Panel A showed the levels of miR-182 and Numb-mRNA in MPM (T)(n=25) and normal pleural tissues (N)(n=25). Panel B displayed the levels of miR-182 and Numb-mRNA in NCI-H2452 and Met-5A.

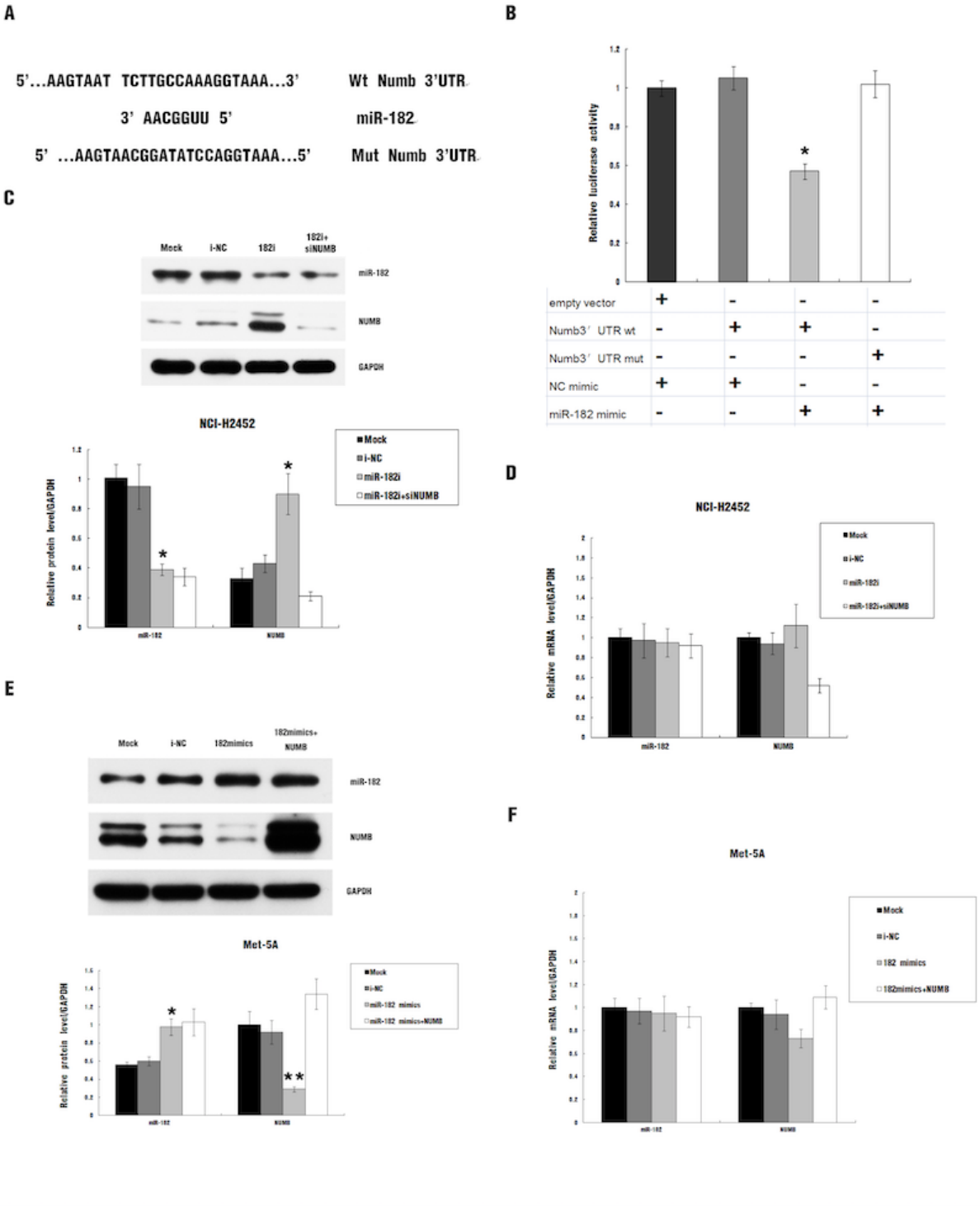

Figure 2

Numb is a direct target of miR-182. A, a sketch of the predicted miR-182 binding site within 3'UTR of Numb, as well as the wildtype (WT) and mutant (Mut) type sequences of the miR-182 binding site. B, luciferase reporter assay of HEK293T cells co-transfected with miR-182 mimics and WT or Mut luciferase plasmids. *P < 0.05. C-D, the protein or mRNA levels of Numb in NCI-H2452 cells transfected with mock, negative control (NC), miR-182 inhibitor and miR-182 inhibitor + Numb-siRNA, respectively. E-F, the protein

or mRNA levels of Numb and miR-182 in Met-5A transfected with mock, NC, miR-182mimics and miR-182 mimics + Numb, respectively.

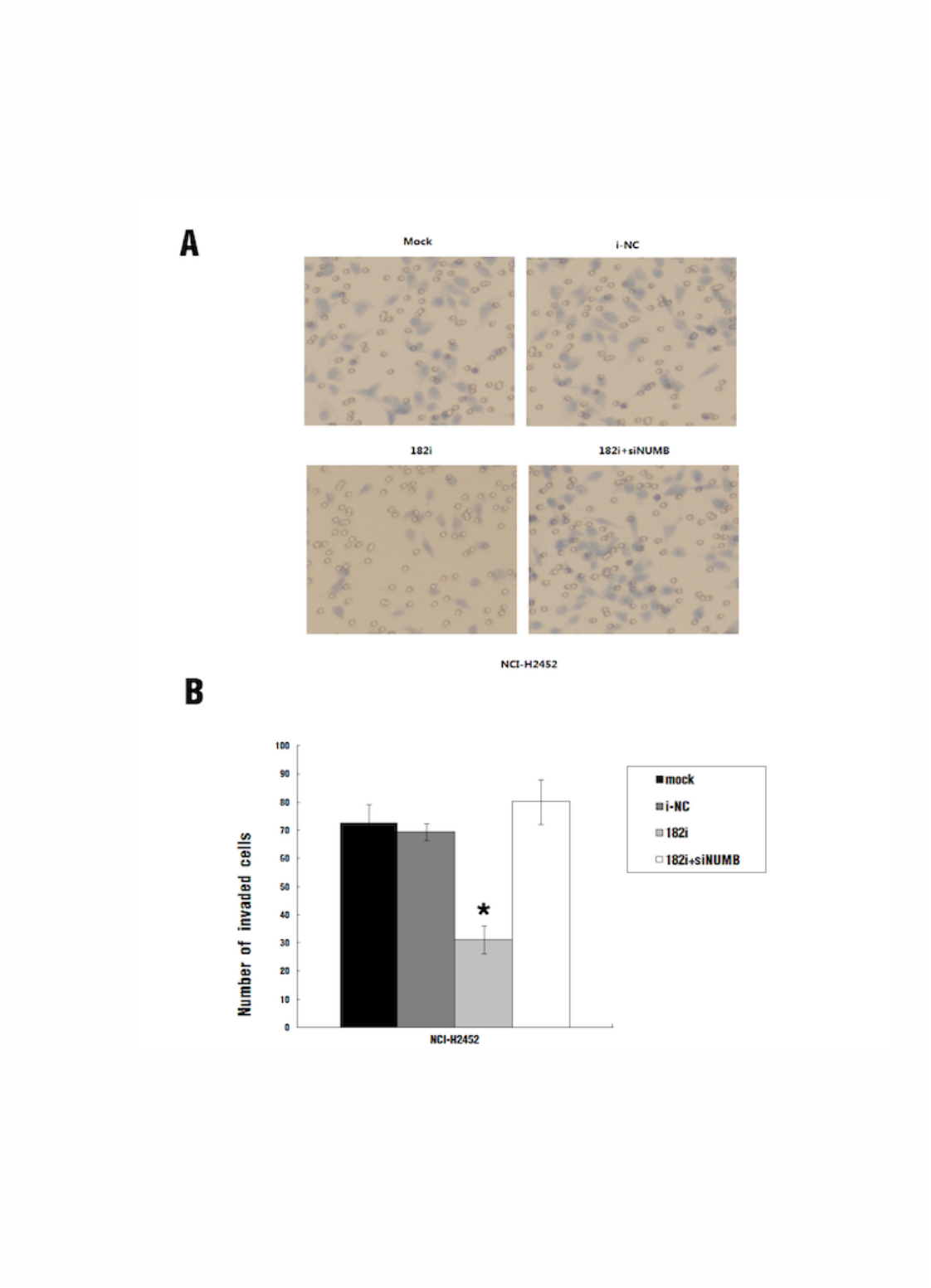

Figure 3

Illustrations of the invasive ability of NCI-H2452 cells transfected with mock, i-NC and miR-182i plasmids with/without Numb knocked down.

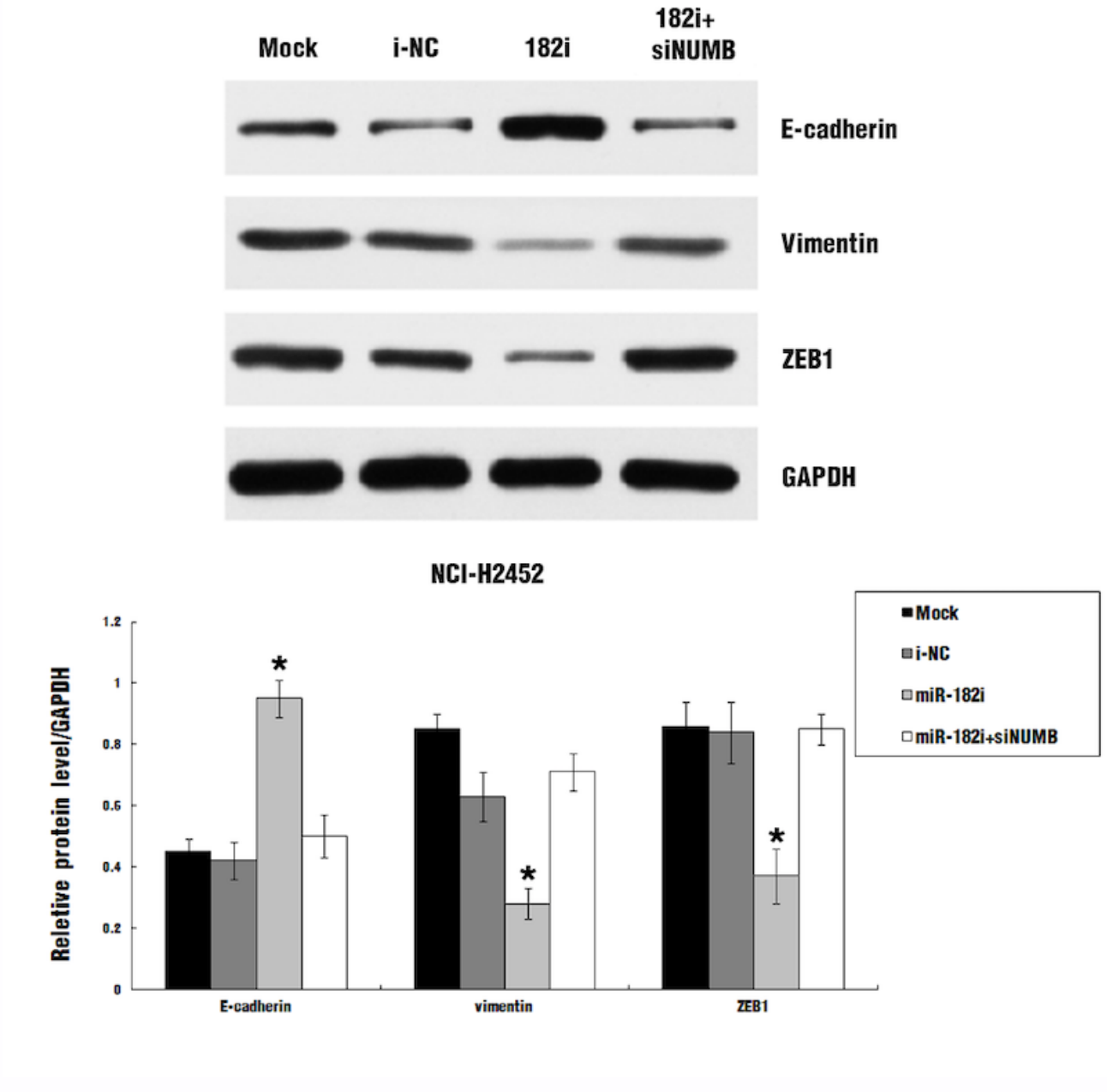

Figure 4

The influence of miR-182 down-regulation on the levels of E-cadherin, vimentin and ZEB1 in NCI-H2452 cells.

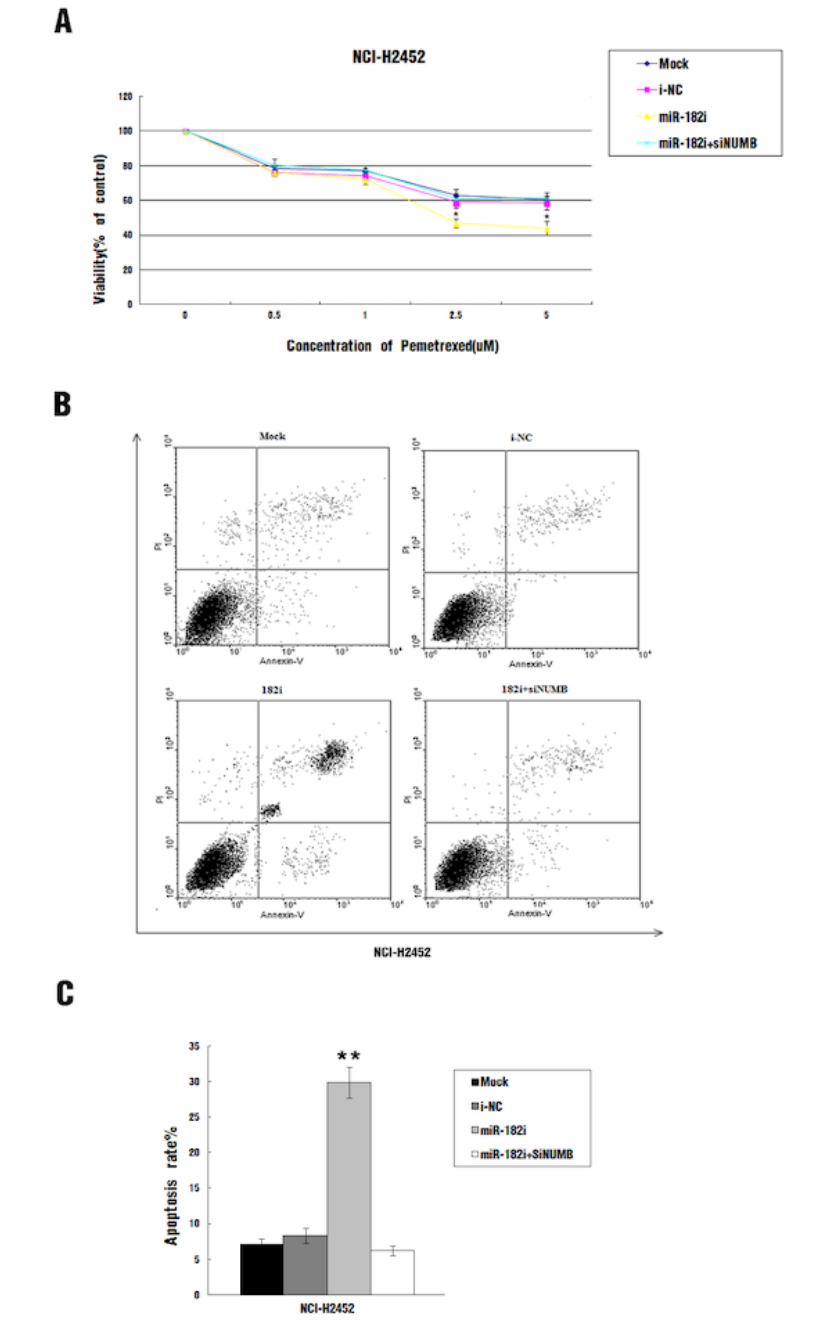

Figure 5

Effects of miR-182i on the susceptibility of MPM cells to pemetrexed. A: the cell viability of miR-182i±Numb-siRNA transfected MPM cells after pemetrexed was added compared to negative control groups (mock or i-NC). B-C: the apoptosis rate (the percentage of Annexin V/PI double-positive cells) of miR-182i±Numb-siRNA transfected cells compared to that of negative control groups（mock or i-NC）24 h after the application of 5 μmol/l pemetrexed.