MIR-875-5P PROMOTES PROLIFERATION AND INVASION OF NON-SMALL CELL LUNG CANCER CELLS BY INHABITING SATB2

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ABSTRACT

Objective: *To explore the role of miR-875-5p in targeting non-small cell lung cancer to promote invasion and proliferation of non-small cell lung cancer cells by targeting SATB2.*

Methods: *The expression of SATB2, E-cadherin and other protein under the regulation of miR-875-5p detected by WB test. The effect of miR-875-5p on proliferation and invasion of non-small cell lung cancer cells was studied by miR-875-5p mimic, an inhibitor of miR-1209, scratch method and Tranwell assay, respectively. And the expression of miR-875-5p in non-small cell lung cancer cells was detected by Real-time quantitative PCR. The bioinformatics software was used to analyze its possible target and then adopt luciferase reporter gene to detect the regulation of SATB2. Finally, animal experiments were carried out to further characterize whether miR-875-5p affects cell proliferation and invasion of non-small cell lung cancer through SATB2 or not.*

Results: *The expression of miR-875-5p was increased in human non-small cell lung cancer cells compared with normal human lung cancer cells ($P<0.01$). Proliferation and invasion ability of non-small cell lung cancer cells was decreased through inhibition of miR-875-5p expression ($P<0.05$). The expression of SATB2 would be reduced when miR-875-5p binds to the SATB2 3'UTR region.*

Conclusion: *miR-875-5p acts as a cancer-promoting small RNA that promotes the proliferation and invasion of non-small cell lung cancer cells by inhibiting the expression of SATB2.*

Keywords: *non-small cell lung cancer, miR-875-5p, proliferation, invasion, SATB2.*

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Introduction

In this study, we found that miR-875-5p is up-regulated in NSCLC patients, by inhibiting SATB Homeobox 2 (SATB2) to promote proliferation and invasion of NSCLC cells[1,2]. From the Transwell experiment, it can be seen that Mir-875-5pmimics has contributed to the migration and invasion of NSCLC.

The cck-8 assay showed that the miR-875-5p mimetic promoted proliferation of NSCLC cells. Luciferase assay confirmed that miR-875-5p directly binds to the 3'untranslation region of SATB2, and western blot results showed that miR-875-5p inhibits the expression of SATB2 protein. In addition, miR-875-5p is inhibited from proliferating and invading NSCLC cell lines. Mir-875-5p15 is expected to be a potential target for future NSCLC treatment.

Materials and methods

Specimen collection

16 samples of tumor samples and paired non-tumor lung samples of non-small cell lung cancer patients were collected at Xuzhou Central Hospital from January 2018 to January 2019.

Real-time quantitative polymerase chain reaction (qRT-PCR)

The expression of mirna and genes was detected by qRTPCR kit (Life Technologies, Shanghai, China), QuantStudio 6 Flex Real-Time PCR system (Life Technologies, Shanghai, China) and SYBR Green kit (TaKaRa, Tokyo, Japan). The relative expression levels of mirna and genes were determined by quantitative assay.

Cell culture

H157 and A549 human NSCLC cell lines were obtained from American Type Culture Collection (USA) and RPMI 1640 medium (Life Technologies, Shanghai, China), respectively, and 10% fetal bovine serum (FBS) (Life Technologies, Shanghai, China) was added to culture. The cells were maintained in a humidified atmosphere of 37°C and 5% CO2.

Transfection

H157 and A549 NSCLC cells were seeded in 24-well plates at a cell density of 5105 cells/well and then hatched overnight. miR-875-5-p mimic, anti-miR-875-5-p, inactivated control group miR-67 (Living technique, Shanghai, China), or pMIR-Report vector transfected with Lipofectamine 2000 transfection reagent (Invitrogen, Shanghai, China). Total protein was isolated from H157 and A549 NSCLC cells after 48h.

Primers and sequences

The primers for miR-875-5p are 5-cgaatgggccataagatcccg-3 and 5-ggagcccagcactttgatct-; the primers for SATB2 are 5- AGGAGTTTGGGAGATGGTAT-3 and 5-ACTGAACCTGACCGTACACC CAGAACACAATAGTCTGAA-3, and the WT sequence of the SATB2 3'utr structure is 5-uaauggguaauuuuuugagguaug3. The MUT sequence of the SATB2 3'UTR structure is 5-UAAUGGGUGGUUUUUGACCU aug3.Results

Cell count Kit-8 (CCK-8) method

The proliferation of H157 and A549 NSCLC cells was measured by CCK-8 (Dojindo, Kumamoto, Japan). H157 and A549 NSCLC cells were seeded in 24-well plates at a cell density of 5105 cells/well. And then cultured in 10% CCK-8. After transfection, the proliferation rate of H157 and A549 NSCLC cells was measured at 24 h, 48 h and 72 h, respectively.

Transwell test

The transwell chamber (Corning, Shanghai, China) was used to measure the invasion of H157 and A549 NSCLC cells with matrix gel (Invitrogen, Shanghai, China).

The migration was determined by the same method without matrix gel (Invitrogen, Shanghai, China).Logistic Regression Analysis of Risk Factors of Coronary Heart Disease

Luciferase assay

H157 and A549 NSCLC cells were seeded in 24-well plates at a cell density of 5105 cells/well. Then hatched for 24 h. Luciferase reporter gene analysis, H157 and non-small cell lung cancer A549 cells cotransfected 0.8 μg pGL3-SATB2 3 'utr wild type or pGL3 - SATB2 3 'utr mutant plasmid, 0.08 ng phRL-SV40 control vector (Promega, Shanghai, China)), and 200 nautical miles mir-875-5- p imitate or control rna Lipofectamine 2000 (Invitrogen, Shanghai, China). After 24h of transfection, firefly and renal luciferase activities (Promega, Shanghai, China) were determined by luciferase double enzyme digestion method.

Western blot

For western blots, proteins were separated using a 12% SDSPAGE gel and transferred to a nitro membrane (Bio-Rad, Shanghai, China). 5% skim milk was used to shield membranes from hatching with anti-SATB2 antibodies (Abeam, Shanghai, China) or anti-β-actin antibodies (Abeam, Shanghai, China). After extensive membrane cleaning, the secondary antibody (Abeam, Shanghai, China) was then added to the system. Immunoreactive protein bands were detected by the enhanced chemiluminescence (ECL) system.

Statistical Analysis

Statistical analysis using SPSS. Differences between groups were estimated using t test or repeated analysis of variance. The correlation coefficient is determined using the Pearson correlation coefficient. Student t test was used to determine the difference. The p value < 0.05 was considered statistically significant. Each experiment was repeated three times. The result value is represented by the mean S.D.

Results

The expression of miR-875-5p is significantly up-regulated in NSCLC tissues and cells

miR-875-5p was found to be a new prostate cancer suppressor (3, 4). However, its role in non-small cell lung cancer has not been studied. Therefore, in order to study the potential role of miR-875-5p in NSCLC, we used microarray data (5, 6) from the GEO database (GSE64591) to analyze miR-875-5p expression pattern in 100 patients with early (I ~ IIIA) NSCLC and normal controls. The results showed that the expression of miR-875-5p was

up-regulated in plasma with non-small cell lung cancer (Fold change = 1.755). We were surprised to find that miR-875-5p is up-regulated in NSCLC. To further investigate this finding, we used real-time quantitative polymerase chain reaction (qRT-PCR) to analyze lung cancer tissue and adjacent normal lung tissue samples from 23 NSCLC patients. The results confirmed that miR-875-5p was significantly up-regulated (refractive=3.125; p-value <0.001 in patients with non-small cell lung cancer, Student t-test). This suggested that miR-875-5p may play a role in NSCLC. (Figure 1, $P < 0.05$).

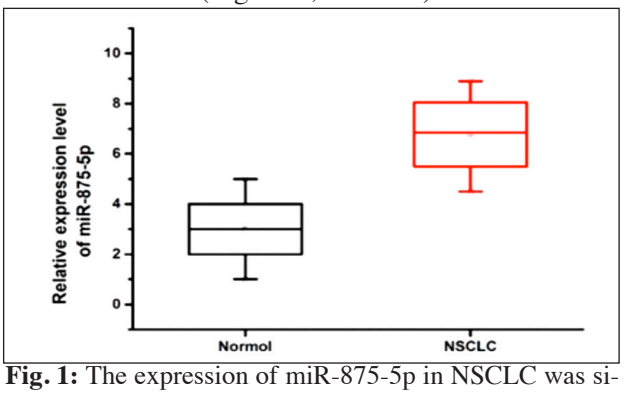

Fig. 1: The expression of miR-875-5p in NSCLC was significantly up-regulated.
**P <0.05, ***P <0.01, and the mean value of SD was calculated.*

Effect of miR-875-5p expression on proliferation and invasion of non-small cell lung cancer cells

To explore the effect of miR-875-5p expression on the biological behavior of non-small cell lung cancer cells, we selected A549 cells for functional increase and loss test. The miR-875-5p mimetic could increase the expression of miR-875-5p, and the miR-875-5p inhibitor could significantly inhibit the expression of miR-875-5p in A549 cells.

The results showed that miR-875-5p overexpression significantly increased cell growth rate in 3 and 4 day after transfection of A549 cells compared with the control group, but miR-875-5p inhibitors reduced the rate of cell growth. Colony formation experiments showed that miR-875-5p overexpression resulted in an increase in the number of cell colonies after transfection 14 days, whereas miR-875-5p inhibited cell colony formation in A549 cells compared to the control group. In addition, cell invasion assays showed that miR-875-5p overexpression increased cell invasion trend to control compared with after 48h of cell transfection, however, miR-875-5p had an opposite effect on A549 and H460 cytostatics. Later, we found that cell proliferation-associated proteins CDK2, EMT-related E-cadherin, and N-cadherin could be used to valuate the effects of miR-875-5p cell proliferation and EMT processes. We found that protein CDK2 and associated cell proliferation increased miR-875-5p overexpression in A549 cells. In addition, we also demonstrated that cadherin expression of EMT-related manufacturers decreased miR-875-5p mimics in A549 cells, whereas expression in N-cadherin H460 cells increased miR-875-5p. Thus, these results indicated that expression of miR-875-5p promoted cellular malignant behavior of NSCLC. (Figure 2).

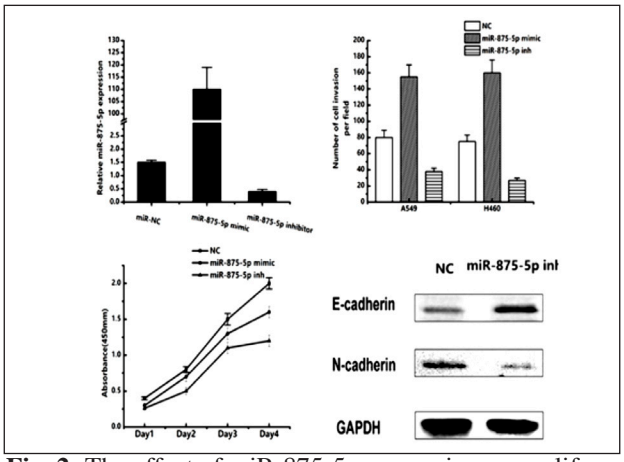

Fig. 2: The effect of miR-875-5p expression on proliferation and invasion of non-small cell lung cancer cells.
*CCK8 assay was used to assess cell growth after transfection of miR-NC and miR-875-5p; after transfection of A549 cells with miR-NC or miR-875-5p mimic, Western blot was used to detect the expression of E-cadheirn and N-cadherin. *P <0 .05, and the mean value of SD was calculated.*

SATB2 is a direct target of miR-875-5p in NSCLC

By searching for miRNAs (TargetScan and miRanda)[7,8] for some effective targeted prediction databases, we found that SATB2 was a potential target[9] for miR-875-5p. The 3-UTR sequence of the wild type (WT) SATB2 mRNA and the mutant (MUT) SATB2 mRNA containing the miR-875-5p binding site was inserted into the mir-glo vector for luciferase reporter gene detection. A549 cells were co-transfected with miR-875-5p mimic or miR-NC with three UTR SATB2-WT or three UTR SATB2-MUT luciferase reporter vectors.

The results showed that the luciferase activity was significantly decreased after co-transfection of miR-875-5p mimic and SATB2 3 UTR-WT reporter vectors, while there was no significant change in the activity of the enzyme after co-transfected with miR-875-5p mimic and SATB2 3 UTR-MUT

reporter vectors. Furthermore, we showed that transcriptional and protein-level expression of SATB2 inhibited miR-875-5p regulated by miR-875-5p mimetic. When the transcriptional level and protein expression of SATB2 miR-875-5p were down-regulated, the inhibitors were increased compared to control A549 cells. Therefore, these results indicated that SATB2 was a direct target of miR-875-5p. (Figure 3).

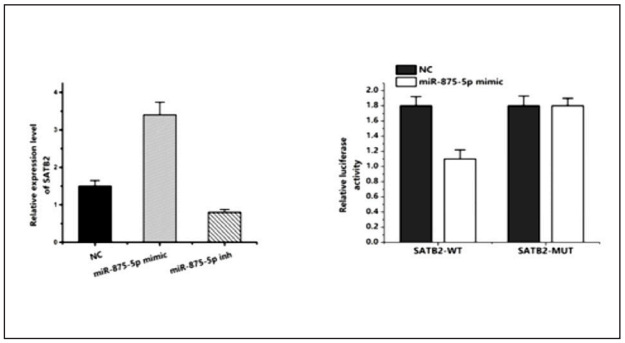

Fig. 3: SATB2 is the target of miR-875-5p in NSCLC. **SATB2 is a potential target of miR-875-5p. The 3-utr sequence of wild-type (WT) SATB2 mRNA containing the predicted miR-875-5p binding site and the 3-utr sequence of MUT SATB2 mRNA were inserted into the mir-glo vector, and A549 cells were co-transfected with miR-875-5p mimic or mir-nc and pmirglol-SATB2-2-utrAfter transfection with mir-nc miR-875-5p mimic and miR-875-5p inhibitor in A549 cells with -wt or pmirglol-SATB2-utr-wt luciferase activity vector, the relative mRNA and protein expression of SATB2 *P <0.05 were detected by qrt-pcr and western blot, and the mean values of SD were calculated.*

MiR-875-5p promotes cell proliferation and invasion by regulating SATB2 in non-small cell lung cancer

We further analyzed the mRNA expression of SATB2 in 41 NSCLC tissues and adjacent normal tissues. The results showed that the expression level of SATB2 in non-small cell lung cancer tissues was significantly lower than that of the corresponding tissues. The expression level of SATB2 in NSCLC cells was also decreased compared to BEAS 2B cells. To investigate whether SATB2 mediates tumor-promoting effects of miR-875-5p-induced proliferation and invasion of non-small cell lung cancer cells, we performed CCK8 and cell invasion assays. After transfection of pcDNA3.1-SATB2 plasmid into A549 cells, SATB2 was overexpressed. CCK8 results showed that miR-875-5p overexpression promoted cell proliferation, and SATB2 overexpression inhibited cell proliferation. However, co-transfection with miR-875-5p mimic and pcDNA3.1-SATB2 plasmids rescued the effect of miR-875-5p mimic on proliferation of A549 cells.

Cell invasiveness was enhanced when miR-875-5p was overexpressed, while SATB2 overexpression inhibited A549 cell invasiveness. However, co-transfection with miR-875-5p mimic and pcDNA3.1-SATB2 plasmids rescued the effect of miR-875-5p mimic on A549 cell invasion. Taken together, these findings suggested that miR-875-5p promotes cell proliferation and invasion by regulating the expression of SATB2 in NSCLC cells. (Figure 4).

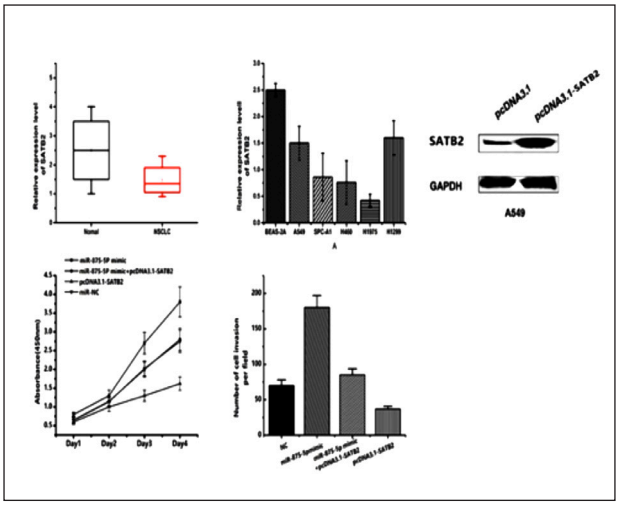

Fig. 4: miR-875-5p promotes proliferation and invasion of non-small cell lung cancer cells by regulating SATB2. *Expression of SATB2 in NSCLC tissues and adjacent normal tissues was detected by qrt-pcr. After transfection of mir-nc miR-875-5p mimic and miR-875-5p mimic plus pcdna3.1-SATB2 or pcdna3.1-SATB2 in A549 cells for internal control, mir-nc miR-875-5p was transfected into A549 cells by CCK8After mimics and miR-875-5p mimics were added with pcdna3.1-SATB2 or pcdna3.1-SATB2, cell growth was estimated using cell invasion assay *P <.05 and mean SD was set.*

Effect of miR-875-5p expression on the growth of NSCLC cells in vivo

To further investigate whether miR-875-5p expression affects tumor growth in vivo, we constructed a xenograft model using a stably transfected antagomiR-875-5p or control plasmid and injected subcutaneously into the left side of nude mice. The results showed that compared with the control group, the tumor size and growth volume of the antagomiR-875-5p group decreased, the growth rate slowed down, and the tumor weight was also reduced compared with the control group. Under inverted fluorescence microscopy, the apoptosis of the antagomiR-875-5p treated group with tissue sections were stained with an apoptosis kit was significantly higher than that of the control group. These results indicated that down-regulation of miR-875-5p expression in vivo could inhibit cell growth by regulating SATB2 expression.(Figure 5).

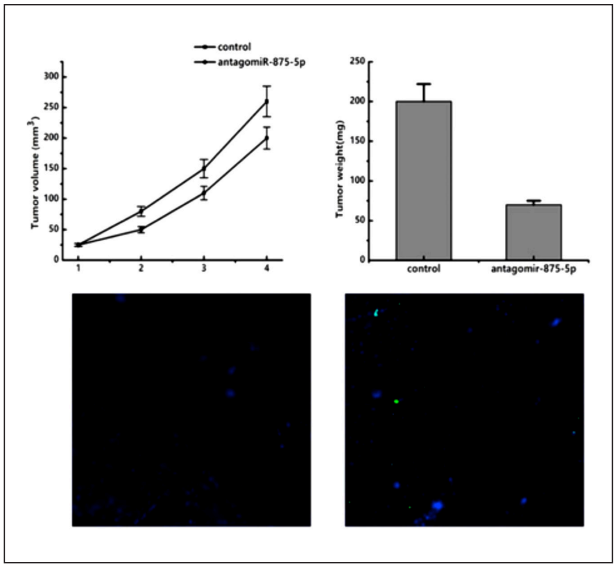

Fig. 5: Effect of miR-875-5p expression on the growth of NSCLC cells in vivo
Axenograft model with stable transfection antagomir -875-5p or the influence of control to the left of the plasmid into nude mice subcutaneously and at different time points according to tumor size and tumor weight antagomir - 875-5p group was obviously lower than the control group since 2 weeks, tumor volume of antagomir - 875-5p group was obviously lower than the control group TUNNEL dyeing tissue biopsies, green fluorescent antagomir - 875-5p group was obviously better than the control group $^{*}P < 0.05$.

Discussion

Mirna plays an important regulatory role in the pathogenesis of a variety of cancers[10]. miR-875-5p is a novel tumor-associated miRNA[11] recently discovered in prostate cancer. However, the relationship between miR-875-5p and non-small cell lung cancer has not been reported. In this study, we analyzed the expression pattern of miR-875-5p and its predicted target genes in 389 NSCLC patients and NSCLC cell lines. The results indicated that miR-875-5p expression was up-regulated (Fold change = 3.125; p-value < 0.001, Student t-test) possibly by targeting SATB2. Western blot and luciferase assay showed that SATB2 was the target gene of miR-875-5p. The results showed that miR-875-5p directly bond to the 3'utr region of SATB2 to inhibit its expression. The SATB2 gene encode is a DNA-binding protein[12] that specifically binds to the nuclear matrix attachment region and is involved in transcriptional regulation and chromatin remodeling[13]. SATB2 inhibits proliferation and invasion of colorectal cancer, gastric cancer, colorectal cancer, and NSCLC cells[14]. Therefore, we further investigated whether miR-875-5p can promote proliferation and invasion of NSCLC cell lines. Its showed that miR-875-5p promoted the proliferation, migration and invasion of H157 and A549 NSCLC cells, which confirmed our hypothesis and suggested the carcinogenic effect of miR-875-5p in NSCLC. Since miR-875-5p is a tumor suppressor of prostate cancer[15]. We were very surprised to find that miR-875-5p is an onco-miRNA in NSCLC. But this is not the first time that mirna has found conflicting effects in different cancers[16]. For example, miR-590 is a tumor suppressor in colorectal cancer and breast cancer, and is an onco-miRNA[17] in t cell acute lymphoblastic leukemia and lung adenocarcinoma.

This means that the role of miRNAs in cancer is very complex. Mirna not only promotes or inhibits cancer. They participate in a complex network and play different roles in different environments. At the same time, this study again revealed the heterogeneity of tumors at the molecular level. Taken together, our study demonstrated that miR-875-5p was up-regulated in NSCLC and promotes proliferation, migration and invasion of NSCLC cells via SATB2. This new miR-875-5p/SATB2 axis deepens our understanding of the NSCLC mechanism. Since miR-875-5p inhibitors inhibit the proliferation, migration and invasion of non-small cell lung cancer cells, miRNAs are becoming therapeutic targets in the context of molecular network research. In the future, miR-875-5p may become a potential therapeutic target for NSCLC therapy.

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