ORIGINAL ARTICLE

MicroRNA-128 promotes apoptosis in lung cancer by directly targeting NIMA-related kinase 2

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Abstract

Background: MicroRNA-128 (miR-128) serves as a regulator by inducing cancer cell apoptosis, differentiation, the epithelial-to-mesenchymal transition process, and tumor growth by mediating different targets. NIMA-related kinase 2 (NEK2) is aberrantly expressed in lung cancer. The miR-128/NEK2 pathway has been reported to predict prognosis in colorectal cancer; however, the determination of a relationship between miR-128 and NEK2 in lung cancer has remained elusive. We explored the association between miR-128 and NEK2 in lung cancer.
Methods: MiR-128 and NEK2 expression were examined in 15 lung cancer tissues by real time-PCR. Lung cancer SK-MES-1 cells were transfected with miR-128 mimic, an inhibitor or a negative control. MiR-128 and NEK2 expression levels were detected using quantitative real time-PCR and Western blot. SK-MES-1 cell apoptosis was performed by flow cytometry.
Results: Compared to adjacent non-tumor tissues, miR-128 was downregulated and NEK2 was upregulated in 15 lung cancer tissues. Lung cancer SK-MES-1 cells transfected with miR-128 mimic induced a higher apoptotic rate than those transfected with the negative control. Dual luciferase assay further confirmed that NEK2 was a direct target of miR-128 in lung cancer, and transfection with miR-128 mimic could decrease the NEK2 protein level while the miR-128 inhibitor increased NEK2 expression. Finally, the apoptotic effect of lung cancer cells induced by miR-128 mimic could be reversed by NEK2 overexpression.
Conclusions: NEK2 was regulated by miR-128 in lung cancer and miR-128 induced lung cancer cell apoptosis by mediating NEK2 expression.

Introduction

Lung cancer is one of the most common malignancies and remains the leading cause of cancer-related death worldwide. Despite great achievements, such as targeted therapy, however, finding a radical cure for lung cancer still poses a challenge. Tumor development is a complicated and complex process that could be influenced by environmental or individual factors. Although tumor-related oncogenic factors have been extensively studied, the underlying mechanisms contributing to tumorigenesis remain elusive. Thus, determining such mechanisms is critical.

MicroRNAs (miRNAs), a class of small non-coding RNAs, participate in several cellular biological processes, including epithelial-to-mesenchymal transition,[1] apoptosis,[2] proliferation,[3] invasion and metastasis.[4,5] MiRNAs can present as oncogenes or tumor suppressors by binding to the 3′untranslational region (UTR) of target messenger (m)RNAs to regulate the post-transcriptional level. Emerging evidence has indicated that cancer development could be regulated in vitro or in vivo by inhibiting or overexpressing the level of some miRNAs.[6–8] Thus, miRNAs have the potential to be a promising therapeutic target for cancer treatment.

MiRNA-128 (miR-128) has been studied in different cancers, including lung,[9] breast,[10] bladder,[11] hepatocellular,[12] and gastric cancers,[13] and has been suggested as a potential therapeutic target for cancer. Recently, miR-128 has been

reported to be involved in different cancer biological processes. For example, miR-128 possessed an inhibitory effect on the epithelial-to-mesenchymal transition of osteosarcoma cells.[14] Shang *et al.* reported that miR-128 induced glioma cell apoptosis by regulating RhoE expression level.[15] However, the promoting or inhibitory effects of miR-128 depend on cancer types. In human primary osteosarcoma, miR-128 reportedly acted as an oncogenic miRNA promoting metastasis and predicting poor prognosis.[16] Therefore, the underlying mechanisms of miR-128 in different cancer types need to be further elucidated.

NIMA-related kinase 2 (NEK2), a member of the Nek family of serine/threonine kinases, is structurally correlative to mitotic regulator NIMA and is enriched in centrosome.[17] In a variety of cancers, NEK2 is aberrantly expressed and is associated with drug resistance and poor prognosis.[18–22] Recent studies have indicated that NEK2 represents a promising therapeutic target for cancer treatment.[23,24] For instance, in a previous study, miR-128 played an essential role in inhibiting colorectal cancer cell proliferation by regulating NEK2.[25] High NEK2 expression was a tumor recurrence predictor for patients after hepatectomy treatment[26] and an NEK2 small interfering (si)RNA injection inhibited cancer cell proliferation and peritoneal dissemination of cholangiocarcinoma in a mouse model.[27] Furthermore, the combination of NEK2 siRNA and the chemotherapeutic agent cisplatin could enhance colorectal cancer cell apoptosis and death, suggesting an optional therapeutic approach for cancer treatment.[28–30] A previous study showed that in colorectal cancer, miR-128 inhibited NEK2 expression, while miR-128 methylation increased NEK2 expression, providing insight that the miR-128/NEK2 pathway may be applied to predict prognosis.[25] However, the association between NEK2 and miR-128 in lung cancer and the underlying mechanisms remain elusive.

In this study, we found that miR-128 mediated apoptosis of lung cancer cells while the apoptotic effect of miR-128 could be reversed by NEK2 overexpression in lung cancer cells. These results indicate that miR-128 and NEK2 could be prognostic biomarkers and therapeutic targets for lung cancer treatment.

Methods

Clinical samples

Fifteen lung cancer and adjacent non-tumor samples were collected and stored in −80°C before use. All patients signed written informed consent before tissue collection. The Medical Ethics Committee of the First Affiliated Hospital, College of Medicine, Zhejiang University approved the study, which was performed in accordance with the Helsinki Declaration.

Cell culture

Human lung cancer cell line SK-MES-1 was purchased from the Cell Bank of Type Culture Collection of Chinese Academy of Sciences (Shanghai, China) and cultured with RPMI 1640 medium supplemented with 10% fetal bovine serum (Gibco, Grand Island, NY, USA), 100 U/mL penicillin, and 100 μg/mL streptomycin (Gibco) in an atmosphere of 37°C, 5% CO_2.

Flow cytometry

With indicated treatment, cells were trypsinized and washed twice with phosphate buffered saline and suspended in binding buffer. The staining process was performed based on the manufacturer's instructions with a few modifications. Briefly, the cells were suspended in 100 μL binding buffer and stained with 5 μL PI and 5 μL Annexin V using an FITC Annexin V Apoptosis Detection Kit (BD Biosciences, San Jose, CA, USA). After incubation at room temperature for 20 minutes, cell apoptosis was performed using a FACScaliber Flow Cytometer (BD Biosciences).

Quantitative real time-PCR

MicroRNAs were extracted using a miRNeasy Mini Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. Reverse transcription was performed using a PrimeScript 1st Strand cDNA Synthesis Kit (Takara, Dalian, China). Quantitative real-time (RT)-PCR of miR-128 was applied with an ABI 7500 Real-Time PCR system using a SYBR kit (Qiagen). The primers for the miR-128 mimic, the inhibitor, and U6 small nuclear (sn) RNA were purchased from RiboBio (Guangzhou, China). The U6 snRNA was used as an internal control for miR-128 expression. MiR-128 expression was determined using the $2^{-\Delta\Delta ct}$ method.

Transfection

MiR-128 mimic, miR-128 inhibitor, and the negative control (NC) were purchased from RiboBio and transfected into SK-MES-1 cells using Lipofectamine 2000 (Invitrogen). Briefly, the miRNAs, NC, and Lipofectamine 2000 were mixed with opti-MEM (Gibco) for five minutes at room temperature, and then the Lipofectamine 2000 mixture was re-mixed with the miRNAs and NC for 30 minutes at room temperature. After 72 hours of transfection, the cells were collected for further study. Transfection of the NEK2-ORF plasmid was conducted following the same procedure.

Luciferase reporter assay

The wild-type 3′UTR binding site targeted by miR-128 and the mutated 3′UTR of NEK2 were amplified using the PCR method and then cloned into the luciferase reporter vector (Promega, Madison, WI, USA). Cells were co-transfected with luciferase reporter vector containing the wild-type 3′UTR or the mutated 3′UTR of NEK2 with miR-128 mimics, miR-128 inhibitor or the NC. After 48 hours of transfection, the luciferase activity was applied for dual luciferase assay (Promega) based on the manufacturer's instructions.

Western blot

Cells were lysed with radioimmunoprecipitation assay buffer at 4°C for 30 minutes and then boiled for 10 minutes mixed with $1 \times$ loading buffer. The cell lysates were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred with polyvinylidene fluoride membrane (Millipore, Billerica, MA, USA). After incubation with 5% non-fat milk for one hour, the membranes were incubated with primary antibodies as follows: NEK2 antibody (AP8074c, Abgent, San Diego, CA, USA), glyceraldehyde 3-phosphate dehydrogenase antibody (ab8245, Abcam, Cambridge, UK), and BCL-2 associated X (Bax) antibody (#5023S), B-cell lymphoma 2 (Bcl-2) antibody (#15071S), and cleaved Caspase-3 (#9664S) purchased from Cell Signaling Technology (CST, Danvers, MA, USA), followed by incubation with horseradish peroxidase-conjugated secondary antibodies. Glyceraldehyde 3-phosphate dehydrogenase was used as an internal control.

Statistical analysis

Data were analyzed using SPSS version 19.0 (IBM Corp., Armonk, NY, USA) and shown as the mean ± standard deviation. The student's *t*-test was performed to analyze the differences between samples and *P* value <0.05 was considered statistically significant. All experiments were repeated in triplicate.

Results

Downregulation of miR-128 and upregulation of NIMA-related kinase 2 (NEK2) in human lung cancer samples

We used 15 lung cancer and adjacent non-tumor samples to elucidate the expression of miR-128 in lung cancer. As shown in Figure 1a, the miR-128 expression level was lower in lung cancer than adjacent non-tumor samples ($P < 0.05$). We also examined the NEK2 expression level using real-time-PCR and found that the NEK2 expression level was higher in tumor than in adjacent non-tumor samples (Fig. 1b). These data indicated that miR-128 is downregulated and that NEK2 is upregulated in lung cancer samples.

MiR-128 was regulated in lung cancer cell line SK-MES-1 by transfection

To investigate the role of miR-128 in lung cancer cell line SK-MES-1, we transfected the miR-128 mimic, miR-128 inhibitor, and the NC. After 72 hours of transfection, we applied quantitative RT-PCR to detect the relative miR-128 expression level. As shown in Figure 2, the miR-128 expression level in the miR-128 mimic group was statistically higher than in samples transfected with the NC (**$P < 0.01$), while in the miR-128 inhibitor group, the miR-128 expression level was significantly lower than samples transfected with the NC (*$P < 0.05$). These data confirmed the overexpression or inhibition of miR-128 in the SK-MES-1 cell line.
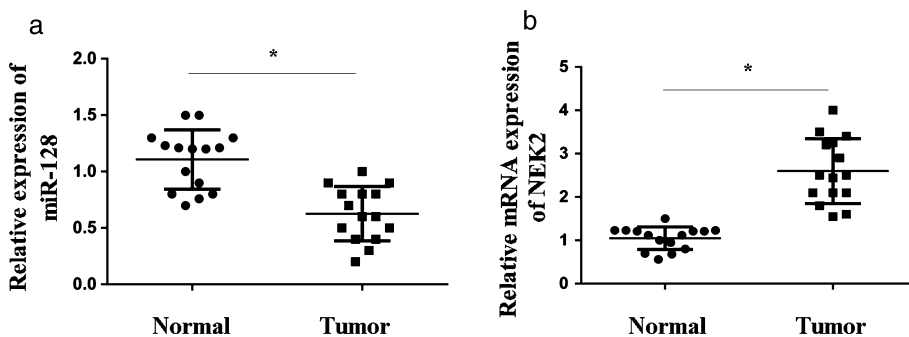

Figure 1 The expression level of microRNA (miR)-128 and NIMA-related kinase 2 (NEK2) in lung cancer samples compared to adjacent non-tumor samples. (**a**) miR-128 expression and (**b**) NEK2 expression were compared between lung cancer and paired adjacent non-tumor tissue samples. U6 small nuclear RNA was used as an internal control for the normalization of miR-128 expression and glyceraldehyde 3-phosphate dehydrogenase was used as internal control for the normalization of NEK2 expression. The experiment was repeated in triplicate. *$P < 0.05$.
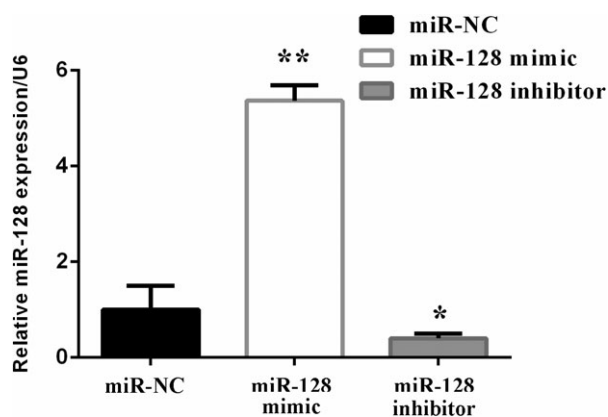
Figure 2 The relative expression of microRNA (miR)-128 in SK-MES-1 cells transfected with miR-128 mimic, inhibitor or negative control (NC). MiR-128 expression was significantly increased after transfection of the miR-128 mimic into SK-MES-1 cells compared to the NC, ** $P < 0.01$. MiR-128 expression was significantly decreased after transfection of the miR-128 inhibitor into SK-MES-1 cells compared to the NC, * $P < 0.05$. The experiment was repeated in triplicate.

MiR-128 promoted apoptosis in SK-MES-1 cells

To further identify whether miR-128 could induce SK-MES-1 cells apoptosis, we used flow cytometry to detect the apoptotic rate of SK-MES-1 cells transfected with miR-128 mimic or NC. As shown in Figure 3a,b, SK-MES-1 cells transfected with miR-128 mimic displayed a significantly higher apoptotic rate than those transfected with the NC (** $P < 0.01$). Moreover, we performed Western blot to detect the apoptosis-related proteins in SK-MES-1 cells transfected with miR-128 mimic or the NC, and results showed that apoptotic protein Bax and cleaved Caspase-3 were upregulated in the miR-128 mimic group while anti-apoptotic protein Bcl-2 was decreased in the miR-128 mimic group (Fig. 3c). Meanwhile, the miR-128 inhibitor had an opposite effect on apoptosis, and transfection with the miR-128 inhibitor could induce the upregulation of anti-apoptotic protein Bcl-2 and downregulation of pro-apoptotic proteins Bax and cleaved Caspase-3 (Fig. 3d). These data indicated that miR-128 could induce SK-MES-1 cell apoptosis.

NEK2 was a direct target of miR-128 in SK-MES-1 cells

To explore the underlying mechanism for the function of miR-128 in SK-MES-1 cells, we used the public database TargetScan (http://www.targetscan.org/) to predict the potential targets of miR-128. We found that the 3′UTR of NEK2 mRNA possessed the complementary sequence to

miR-128 (Fig. 4a). To verify this prediction, dual-luciferase reporter assay was performed to detect whether NEK2 was a direct target of miR-128 in SK-MES-1 cells. As shown in Figure 4b, luciferase activity decreased significantly in SK-MES-1 cells transfected with miR-128 mimic and NEK2 containing wild-type 3′UTR. However, the luciferase activity was not altered statistically in SK-MES-1 cells transfected with miR-128 mimic and NEK2 containing mutant 3′ UTR. Moreover, we performed Western blot to detect the NEK2 protein level, as shown in Figure 4d. The NEK2 protein level decreased in SK-MES-1 cells transfected with the miR-128 mimic compared to those transfected with the NC. Furthermore, miR-128 inhibition greatly increased the luciferase activity when SK-MES-1 cells were transfected with the miR-128 inhibitor and NEK2 containing wild-type 3′UTR; however, the luciferase activity did not alter significantly when the cells were transfected with miR-128 inhibitor and NEK2 containing mutant 3′UTR (Fig. 4c). Western blot results showed that transfection of the miR-128 inhibitor could increase NEK2 protein expression (Fig. 4e). These data indicated that NEK2 was a direct target of miR-128 in lung cancer cell line.

NEK2 reversed the apoptotic effect of miR-128 in SK-MES-1 cells

After confirming that NEK2 was a direct target of miR-128 and was regulated by miR-128 in SK-MES-1 lung cancer cells, we further explored whether the apoptotic effect of miR-128 could be reversed by NEK2. As shown in Figure 5a, transfection with the miR-128 mimic induced a higher apoptotic effect on SK-MES-1 cells compared to the negative group; however, the apoptotic effect of miR-128 could be reversed by co-transfection with the miR-128 mimic and NEK2 plasmid without 3′-UTR (NEK2-ORF), and NEK2-ORF overexpression was confirmed by Western blot (Fig. 5b). These results suggested that NEK2 is a functional mediator of miR-128 on SK-MES-1 lung cancer cell apoptosis.

Discussion

Cancer development is a complex process that is mediated by a variety of factors and mounting evidence has indicated that miRNAs play critical roles in carcinogenesis in a variety of cancers.[31–34] MiRNA involvement in cancer development has been widely reported.[13,35] For instance, Wu *et al.* showed that miRNA-128 largely impeded colorectal carcinoma cell metastasis by directly targeting insulin receptor substrate 1 and the downstream protein kinase B signaling pathway. Moreover, miRNA-128 also inhibited colorectal cancer cell proliferation compared to those transfected with NC miRNA.[36] Furthermore, miRNA-128
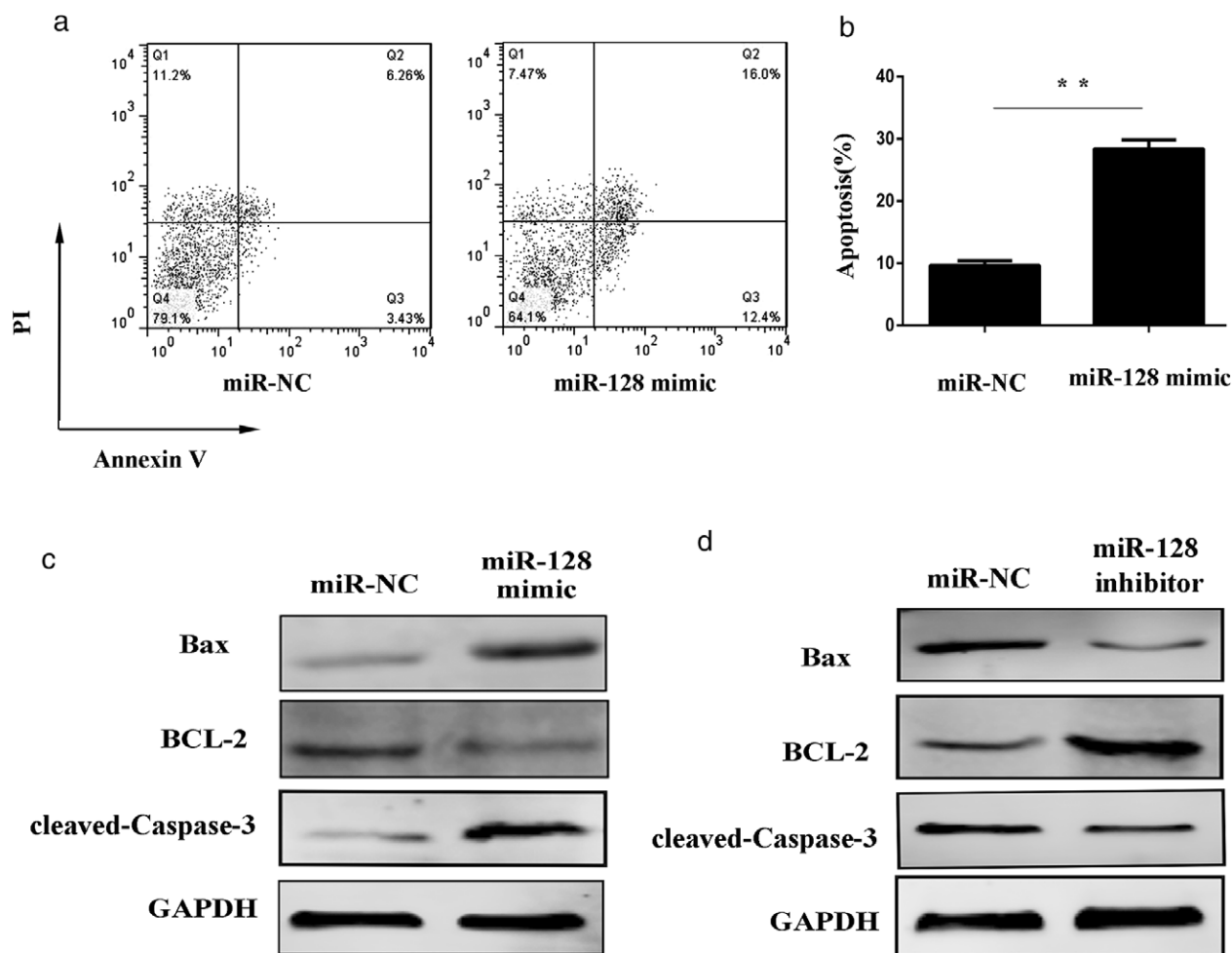
Figure 3 MicroRNA (miR)-128 induced SK-MES-1 cell apoptosis. (**a**,**b**) Flow cytometry showed that the miR-128 mimic induced higher SK-MES-1 apoptosis than in samples transfected with the negative control (NC), **$P < 0.01$. (**c**) Western blot showed that the miR-128 mimic upregulated pro-apoptotic proteins BCL-2 associated X (Bax) and cleaved Caspase-3 expression and downregulated B-cell lymphoma 2 (Bcl-2) expression. (**d**) Western blot showed that the miR-128 inhibitor upregulated BCL-2 expression and downregulated Bax and cleaved Caspase-3 expression. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal control. The experiment was repeated in triplicate.

could promote lung cancer cell cycle arrest and chromosomal instability in a Mitomycin C-dependent manner.[37] In other studies, miRNA-128 overexpression has been associated with cancer cell migration, invasion, and proliferation, and silencing of miRNA-128 increased vascular endothelial growth factor C expression, thus resulting in enhanced metastasis and cancer cell growth.[11,38] These studies provide insight for understanding the progress and development of cancers. The in-depth study of miRNAs and the illustration of miRNA-related mechanisms indicate that the application of miRNAs may be a promising therapeutic agent for cancer.

Previous studies have documented that miR-128 is involved in cancer apoptotic process. In human embryonic kidney HEK293T cells, miR-128 induced cell apoptosis, cell cycle changes, and regulated apoptosis-related protein Bax

by binding to Bax 3′UTR.[39] In another report, miR-128 knockdown attenuated myocardial ischemia/reperfusion injury-induced cardiomyocyte apoptosis through the peroxisome proliferator activated receptor gamma pathway.[40] Consistent with these findings, we demonstrated that miR-128 was involved in cell apoptosis and that miR-128 overexpression induced lung cancer cell apoptosis by targeting NEK2. The miR-128 mimic also activated the apoptotic pathway, represented by an increase in Bax and cleaved Caspase-3 and downregulation of Bcl-2, while the miR-128 inhibitor increased Bcl-2 and decreased Bax and cleaved Caspase-3. Therefore, our data provide evidence that miR-128 may represent a therapeutic option for lung cancer treatment.

Emerging evidence has documented that NEK2 is an important carcinoma biomarker for prognosis and serves
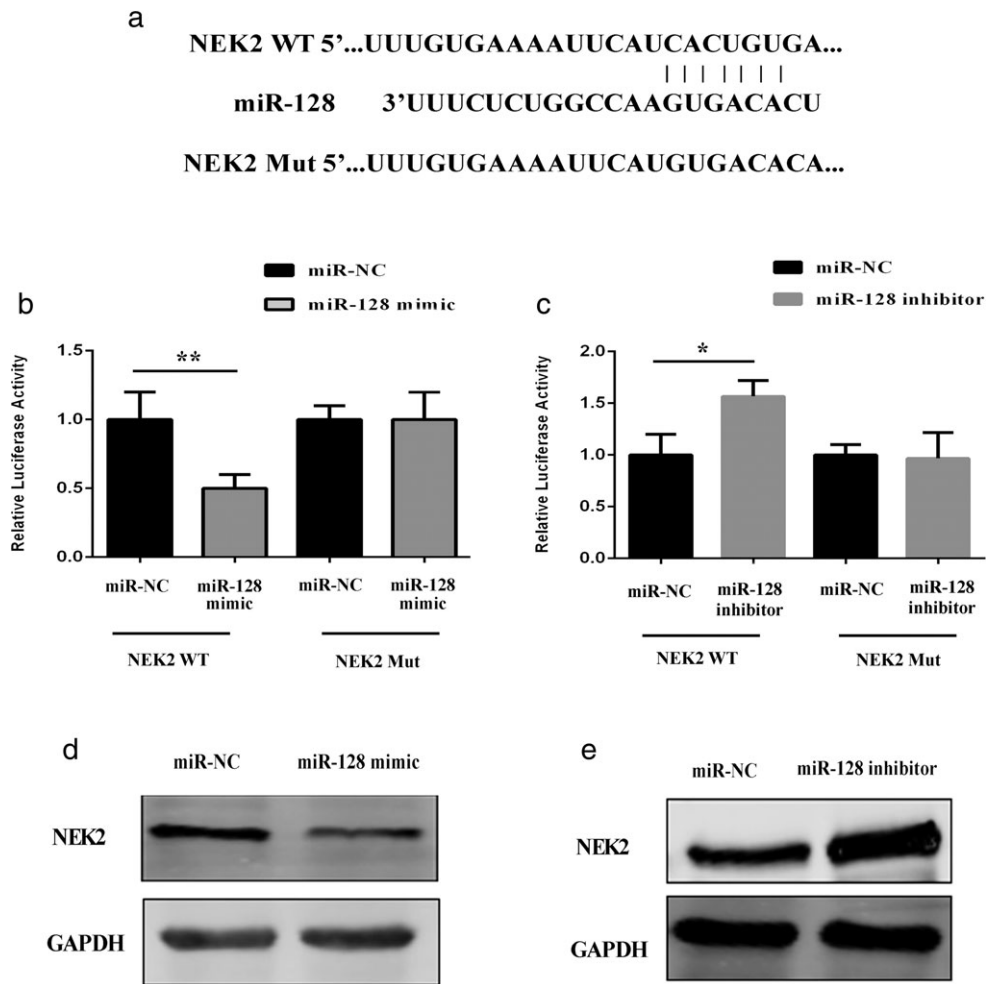
a

NEK2 WT 5'...UUUGUGAAAAUUCAUCACUGUGA...

| | | | | | |

miR-128   3'UUUCUCUGGCCAAGUGACACU

NEK2 Mut 5'...UUUGUGAAAAUUCAUGUGACACA...

Figure 4 MicroRNA (miR)-128 regulated NIMA-related kinase 2 (NEK2) expression by directly targeting the 3′ untranslated region (UTR) of NEK2 messenger RNA. (**a**) MiR-128 and its putative binging site of 3′UTR of wild-type (WT) NEK2 and the mutant *+ (Mut) 3′UTR of NEK2 was generated as indicated. (**b,d**) Dual luciferase reporter assay showed that the miR-128 mimic decreased the relative luciferase activity of 3′UTR of WT NEK2 but not the Mut 3′UTR of NEK2, **$P < 0.01$. Western blot showed that transfection with the miR-128 mimic decreased NEK2 protein levels. (**c,e**) Dual luciferase reporter assay showed that the miR-128 inhibitor increased the relative luciferase activity of 3′UTR of WT NEK2, but not the Mut 3′UTR of NEK2, *$P < 0.05$. Western blot showed that transfection with the miR-128 inhibitor increased the protein level of NEK2. The experiment was repeated in triplicate. **$P < 0.01$, *$P < 0.05$. NC, negative control.

Figure 5 NIMA-related kinase 2 (NEK2) mediates the apoptotic effect of miR-128 on SK-MES-1 cells. (**a**) Flow cytometry showed the early apoptosis rate of co-transfection of the miR-128 mimic and NEK2 ORF and miR-128 mimic compared to the negative control, *$P < 0.05$. (**b**) Western blot showed the successful overexpression of NEK2 ORF in SK-MES-1 cells. The experiment was repeated in triplicate. *$P < 0.05$.
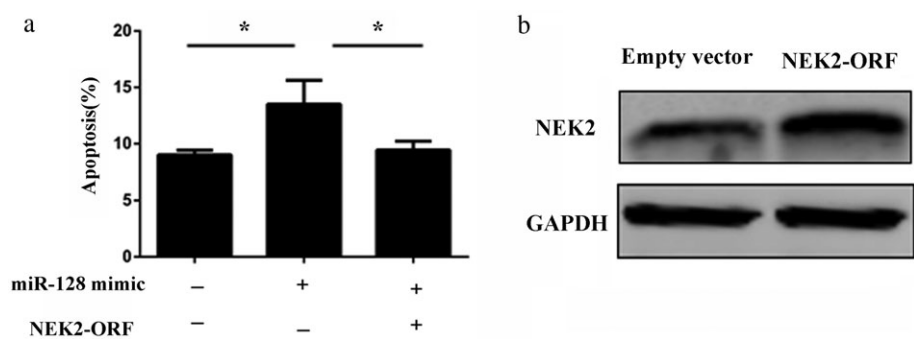
as a therapeutic target.[23,24,41] A recent report showed that NEK2 ectopic expression not only promotes hepatocellular cancer progression, but also activates drug resistance independent of protein phosphatase-1/protein kinase B and Wnt/β-catenin pathway activation.[42] Zhou *et al.* reported that the aberrant expression of NEK2 induced drug resistance mainly through activation of the efflux pumps in several cancers.[43] NEK2 also played a pivotal role in regulating cancer cell apoptosis.[43,44] MiRNA therapies have provided new insight into cancer treatment, and studies have demonstrated that the miRNA/NEK2 pathway has potential for therapeutic treatment.[18,25] However, the role of NEK2 regulation by miRNAs in lung cancer to induce apoptosis needs to be elucidated. In our study, we conducted a gain-of-function experiment to show the regulatory axis between miR-128 and NEK2 in lung cancer and our results showed that NEK2 overexpression could reverse the pro-apoptotic effect of miRNA-128. We also found that miRNA-128 directly targeted the 3′-UTR of NEK2 mRNA and regulated NEK2 protein expression in lung cancer cells. Thus, our study results indicate that miRNA-128 promotes lung cancer cell apoptosis by downregulating NEK2 expression.

In this study, we only sampled 15 lung cancer tissues, thus, a larger group of clinical lung cancer tissues are needed to confirm the clinical significance of our results. The underlying mechanism of miRNAs during lung cancer progression needs to be further elucidated.

In conclusion, our results reveal that miR-128 induces lung cancer cell apoptosis and regulates apoptosis-related proteins Bax, cleaved Caspase-3, and Bcl-2 expression. NEK2 was a direct target of miR-128 in lung cancer cells and NEK2 overexpression could reverse the pro-apoptotic effect of miR-128. Our results provide evidence to support the potential therapeutic strategy of using miR-128 in lung cancer to target NEK2.

Acknowledgment

Disclosure

No authors report any conflict of interest.

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