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MicroRNA-300 targets hypoxia inducible factor-3 alpha to inhibit tumorigenesis of human non-small cell lung cancer

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Non-small cell lung cancer (NSCLC) is one of the most deadly human cancers. MicroRNA-300 acts as both tumor promoter and suppressor in different types of cancer. Here, we try to identify the function of microRNA-300 in human NSCLC. We compared MicroRNA-300 levels between tumor tissues versus paired adjacent non-tumor lung tissues from NSCLC patients, and in NSCLC versus normal lung cell lines. Effects of microRNA-300 on cell proliferation, invasion and migration were examined *in vitro*, and on tumor growth *in vivo* using a xenograft mouse model. Potential mRNA targets of microRNA-300 were predicted and underlying mechanism was explored. MicroRNA-300 expression was lower in both NSCLC tissues and cell lines. Overexpression of microRNA-300 inhibited proliferation, invasion and migration of NSCLC cells *in vitro*, and tumor growth *in vivo*. MicroRNA-300 could directly bind to the 3'-UTR of hypoxia inducible factor-3 alpha (HIF3α) mRNA, and inhibit both its mRNA and protein expressions. Restoring HIF3α expression could rescue the inhibitory effects of microRNA-300 on tumorigenesis of NSCLC both *in vitro* and *in vivo*. MicroRNA-300 is a tumor suppressor microRNA in NSCLC by downregulating HIF3α expression. Both microRNA-300 and HIF3α may serve as potential therapeutic targets in NSCLC treatment.

Key words: Non-small cell lung cancer, microRNA-300, tumor, xenograft, hypoxia inducible factor-3 alpha

Non-small cell lung cancer (NSCLC) is one of the most prevalent and aggressive cancer types, and also the most deadly human cancer worldwide [1, 2]. Metastasis of cancer is a complex process involving multiple biological behaviors including intravasation, circulation, migration and growth in secondary tissues and/or organs. Surgery, radiation and chemotherapy are the three main approaches for lung cancer treatment, while lung cancer patients have poor prognosis because of high metastatic potential, high recurrence and drug resistance incidents [3, 4]. Therefore, new therapeutic methods are needed for early detection of lung cancer, as well as for target based treatments.

MicroRNA (miRNA or miR) is a type of small non-coding RNA molecules, with a length of 20-25 nucleotides. They directly bind to complementary sequences in 3' untranslated regions (3'-UTR) of messenger RNAs (mRNAs), and subsequently degrade those target mRNAs or repress their translation [5, 6]. miRNAs have been widely investigated in many types of human cancers, while they act as both tumor suppressor and promoter in different types of cancers [7, 8].

In the context of our current study, we focused on the role of miR-300 in NSCLC, whose functions has been controversial in different types of cancer. miR-300 was found to be commonly upregulated in both glioma tissues and glioma stem-like cells, and enhance their self-renewal and reduce their differentiation, possibly through targeting leucine zipper tumor suppressor 2 [9]. In osteosarcoma, elevated miR-300 expression was correlated with larger tumor size and more advanced metastasis stage [10], and expression of miR-300 could also promote proliferation, invasion and epithelial-mesenchymal transition (EMT) of osteosarcoma cells by targeting the tumor suppressor gene bromodomain-containing protein 7 [11]. Similarly in both breast cancer and

gastric cancer, miR-300 was upregulated and promoted cancer cell proliferation and invasion by targeting p53 expression [12, 13]. These above reports supported the role of miR-300 as an oncogene miR. However, on the contrary, other studies suggested that miR-300 might also act as a tumor suppressor miR. For instance, miR-300 expression inhibited EMT and metastasis of human epithelial cancer by targeting Twist [14]. In pituitary tumor cells, miR-300, among several other miRs, could inhibit the expression of pituitary tumor transforming gene (PTTG1) and inhibit tumorigenesis, mediated by a p53/PTTG1 feedback loop [15]. In addition, in human oral squamous cell carcinoma, inhibition of miR-300 by Wnt1-inducible signaling pathway protein-1 resulted in lymphangiogenesis via upregulating vascular endothelial growth factor-C expression [16].

To the best of our knowledge, despite the above inconsistent roles reported for miR-300 in other cancers, unfortunately no study has investigated the function of miR-300 in NSCLC. In this study, we investigated the role of miR-300 in NSCLC, to be more precise, whether miR-300 is a tumor suppressor or promoter miRNA. Our results showed that miR-300 expression was significantly lower in both NSCLC patients tissues and NSCLC cell lines. Overexpression of miR-300 inhibited NSCLC cell growth both *in vitro* and *in vivo*. Furthermore, we identified that hypoxia inducible factor-3 alpha (HIF3α) mRNA is the direct molecular target of miR-300. Restoring HIF3α expression in the NSCLC cells could rescue the inhibitory effects exerted by miR-300 on tumorigenesis of NSCLC both *in vitro* and *in vivo*.

Patients and methods

Patients and tissues. 25 patients with newly diagnosed NSCLC were referred to Affiliated Hospital of Qingdao University and underwent radical surgery. The lung cancer tissues and paired adjacent non-tumor lung tissues were collected during the surgery. Tissue samples were placed in a cryovial tube and frozen in liquid nitrogen immediately for further analysis. No patients received radiation or chemotherapy before the surgery. The procedure was approved by the Ethics Committee of Affiliated Hospital of Qingdao University. All patients signed informed consents as well.

Cell culture. Human NSCLC cell lines H1299 and H1975, and normal human lung cell line Beas-2B were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were cultured in DMEM (Invitrogen-Gibco, Carlsbad, CA, USA) with 10% fetal bovine serum (FBS), 1% penicillin and streptomycin at 37°C under 5% CO_2.

MicroRNA assays. The relative expression level of miR-300 was measured using TaqMan advanced miRNA assay kit (478795_mir; Applied Biosystems, Carlsbad, CA, USA), and normalized with RNU48 control miRNA assay kit (P/N 001006; Applied Biosystems, Carlsbad, CA, USA). Stable expression of miR-300 and negative control (sequence 5'-UCA CAA CCU CCU AGA AAG AGU AGA-3') were introduced using the shMIMIC human lentiviral kit (VSH6185-202526750; GE Dharmacon, Lafayette, CO, USA). All assays were performed following manufacturer's instructions.

Wound healing assay. Around 10^5 cells per well were seeded into 6-well plates. After the cells reached 100% confluency, they were starved with DMEM without FBS and treated with 10 μg/ml mitomycin C (Sigma-Aldrich, St. Louis, MO, USA) for 2 h. A straight line wound was then made by a pipette tip. The cell debris was washed out by PBS three times. Thereafter, the cells were cultured for another 24 h in the presence of 10 μg/ml mitomycin C, then the distance of the remaining gap was measured.

Cell invasion assay. Cell invasion ability was measured using Matrigel-coated transwell inserts (8 μm pore size, BD Biosciences, San Jose, CA, USA) in 24-well plates. Briefly, 3×10^4 cells per well were suspended in DMEM without FBS and seeded onto the matrigel coated inserts. The lower chamber was filled with 1 mL medium supplemented with 10% FBS. The cells were incubated under 5% CO_2 at 37°C for 24 h, and stained with 15 μM 5-ethynyl-2'-deoxyuridine (EdU) for another 4 h at 37 °C. The inserts were removed and developed with EdU kit (Invitrogen, Carlsbad, CA, USA).

Cell proliferation assay. Around 10^4 cells per well were seeded into 12-well plates. Every 48 h for 8 days, cells were first fixed with 3.5% paraformaldehyde (PFA), and permeabilized with 0.2% Triton X-100, followed by staining with 15 μM EdU. Number of EdU-positive cells was then counted under a light microscope based on five random fields, and plotted as relative to the count on day 0.

Xenograft tumor model. The animal procedure was approved by the Ethics Committee of Affiliated Hospital of Qingdao University, and performed in the Animal Facility of Affiliated Hospital of Qingdao University according to "Guide for the Care and Use of Laboratory Animals" by the National Institutes of Health. 6 weeks old nude mice were used in our current study. 4×10^6 cancer cells stably expressing either control miRNA or miR-300 suspended with DMEM were inoculated subcutaneously. Tumor was measured every two days with a Vernier caliper, and tumor volume was calculated by the longest axis × shortest axis × shortest axis)/2 (mm^3). The day the tumor grew to a measurable size (140-160 mm^3) was marked as day 0.

RNA extraction and qRT-PCR. Total RNA was extracted using RNeasy MiniPrep Kit (Qiagen, Germantown, MD, USA). Reverse-transcription PCR was performed using Superscript II First-Strand Synthesis kit (Life Technologies, Carlsbad, CA, USA). The relative mRNA expression levels were normalized to the level of GAPDH mRNA. Sequences of primers used were *HIF3α* forward 5'-CAT GGA CTG GGA CCA AGA CA-3', reverse 5'-CGC AGG TAG CTG ATT GTG AG-3'; *GAPDH* forward 5'-AAT CCC ATC ACC ATC TTC CA-3', reverse 5'-TGG ACT CCA CGA CGT ACT CA-3'.

Luciferase reporter assay. Possible miR-300 recognition site or mutated sites on the 3'-UTR of HIF3α mRNA was

cloned into the downstream of firefly luciferase reporter, respectively, using the pMir-Report vector kit (Applied Biosystems). 10^5 per well of H1299 cells transduced with control miRNA or miR-300 were seeded in 24-well plates, and transfected with either wild type (Luc-wtHIF3α) or mutated (Luc-mtHIF3α) constructs, together with pMir-Report β-gal vector as internal control, using Lipofectamine 2000 (Invitrogen). Cell lysates were collected 24 h after transfection. The luciferase activities were measured and normalized to internal control β-gal activity, by Dual-luciferase reporter assay kit (Promega, Madison, WI, USA) according to manufacturer's instructions.

Western blot analysis. Cell lysates were extracted using RIPA buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, protease inhibitor cocktail). Equal amount of proteins was separated by SDS-PAGE and then subjected to standard Western blot analysis. Anti-HIF3α, anti-Ki67, anti-CD31 or anti-GAPDH antibodies (Cell Signaling, Danvers, MA, USA) were incubated respectively with the membrane at 4 °C overnight, and the bands were visualized using SuperSignal West Pico substrate (Pierce, Rockford, IL, USA).

Plasmid construction. HIF3α open reading frame was amplified from cDNA clone MGC:99497 using primers 5'-CCC GGG AAG CTT ATG GCG CTG GGG CTG CAG CGC-3' (forward) and 5'- CCC GGG GGT ACC TCA GTC AGC CTG GGC TGA GCC-3' (reverse), which was then cloned into pCMV2 vector using HindIII and KpnI following standard molecular cloning procedures.

Statistical analysis. All values were shown as mean ± SD. Student's t test or one-way ANOVA was performed for statistical comparison as appropriate. Tukey's post hoc test was performed after the significant ANOVA tests. Statistically significant were determined when $p<0.05$.

Results

Expression of miR-300 is reduced in NSCLC patient tissue and cell lines. To determine the expression of miR-300 in NSCLC, we measured endogenous levels of miR-300 in the lung cancer tissues vs. paired adjacent normal lung tissues from NSCLC patients. As shown in Figure 1A, the expression of miR-300 was significantly lower in cancerous tissue than the paired adjacent non-tumor lung tissues. Then we compared the expression of miR-300 between NSCLC cell lines and human normal lung cell lines. We found that miR-300 levels were also significantly reduced in NSCLC cell lines H1299 and H1975 compared to normal lung cell line Beas-2B (Figure 1B).

miR-300 inhibits proliferation, invasion and migration of NSCLC cells *in vitro*. Then we tried to understand the functions of miR-300 in the tumorigenesis of human NSCLC. Firstly, we made stable miR-300-overexpressing H1299 and H1975 cells using a lentiviral constitutive miRNA expression system (Figure 2A), which greatly inhibited proliferation of both the NSCLC cells (Supplementary Figure S1A, S1B). Transwell invasion assay was then performed with the above miR-300-overexpressing H1299 and H1975 cells. As shown in Figure 2B and 2C, after miR-300 overexpression, the abilities of invasiveness were significantly decreased in H1299 and H1975 cells. Wound healing assay with these overexpression cells was also performed to determine the changes in migration abilities as well. As shown in Figure 2D and 2E, the migration abilities of the miR-300-overexpressing cells were also significantly lower than control cells. Together, our results suggested that miR-300 might function as a tumor suppressor in NSCLC cell lines.

miR-300 inhibits the tumorigenesis of NSCLC in xenograft model. The nude mouse xenograft model was used to assess the *in vivo* functions of miR-300 in NSCLC

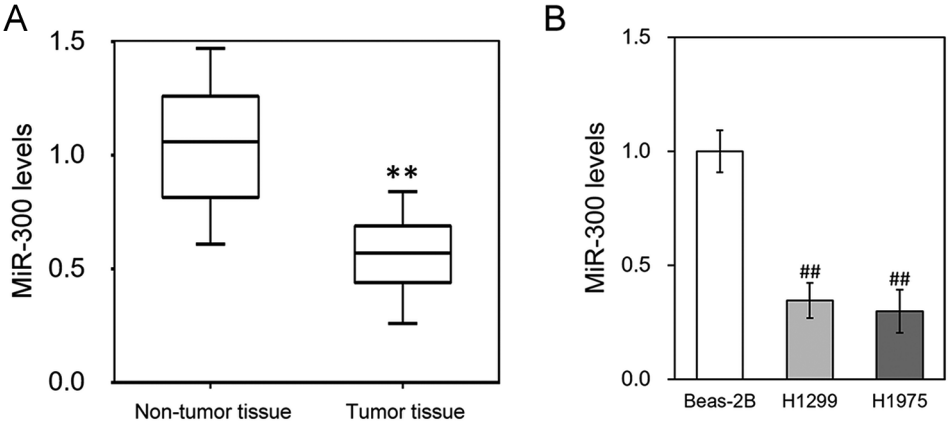

Figure 1. miR-300 is down-regulated in NSCLC tissue and cell lines. Relative levels of miR-300 were compared between cancerous and paired adjacent non-tumor lung tissues of NSCLC patients (n=25) (A), and among normal human lung cell line Beas-2B, and human NSCLC cell lines H1299 and H1975 (B). Values were expressed as mean ± SD from at least three independent experiments. ** $p<0.01$, compared to non-tumor tissue. ## $p<0.01$, compared to Beas-2B.

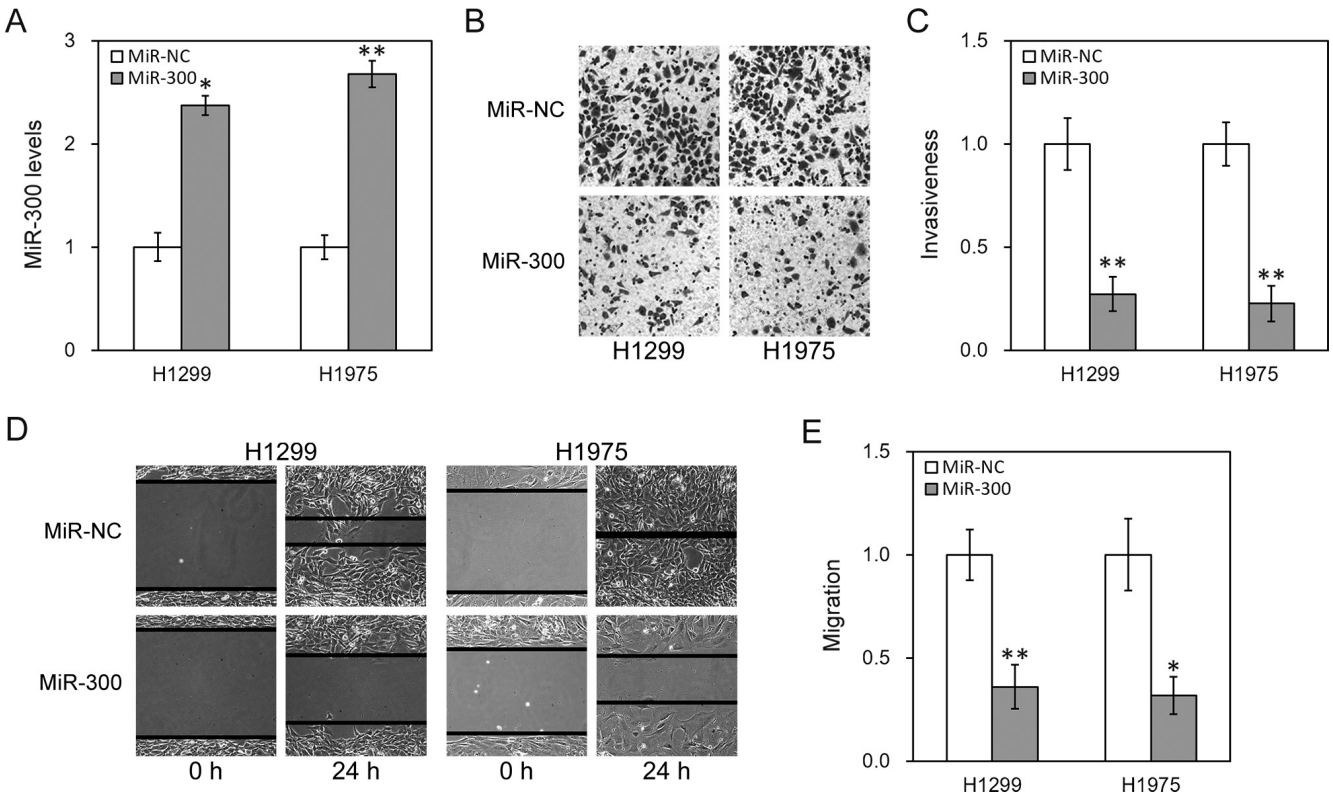

Figure 2. miR-300 inhibits invasion and migration of human NSCLC cell lines *in vitro*. (A) Levels of miR-300 were examined in NSCLC cell lines H1299 and H1975 transduced with either control miRNA (miR-NC) or miR-300. These cells were then subjected to Transwell invasion assay (B and C), as well as wound healing assay (D and E). Values were expressed as mean ± SD from at least three independent experiments. Representative images of the assays were shown in (B) and (D), respectively. * $p<0.05$, ** $p<0.01$, compared to respective miR-NC group.

tumorigenesis. 6 weeks old nude mice were equally divided into four experimental groups, with 10 mice in each group. H1299 or H1975 stably expressing either negative control miRNA or miR-300 were inoculated subcutaneously. Tumor growth was measured at indicated time points as shown in Figure 3. Our results showed that tumor growth from miR-300-overexpressing NSCLC cell lines H1299 and H1975 was much slower than those from miR-NC-expressing cells (Figure 3A, 3B). The data from these i*n vivo* mouse xenografts is consistent with our *in vitro* findings, further suggesting that miR-300 functions as a tumor suppressor miRNA in human NSCLC.

miR-300 directly binds 3'-UTR of HIF3α mRNA and represses its expression in human NSCLC cells. To identify the possible targets of miR-300, we conducted an *in silicon* analysis using the miRanda method [17, 18]. The region on the 3'-UTR of HIF3α mRNA was highly ranked as potential complementary sequences to miR-300 (Figure 4A). Therefore we started to investigate whether HIF3α is involved in the miR-300 inhibitory function in NSCLC cells.

We introduced wild type 3'-UTR recognition site of HIF3α, as well as its mutated form, to the downstream of a luciferase reporter gene open reading frame (Figure 4B). H1299 cells stably expressing either miR-NC or miR-300 were transfected with these luciferase constructs, together with β-gal internal control, and the luciferase activities were subsequently measured as normalized to the internal control β-gal activities (Figure 4C). Indeed, the luciferase activity of the wild type miR-300 recognition site transfected miR-300-overexpressing H1299 cells was greatly reduced comparing with that of control miR-NC-expressing H1299 cells. While the activity of luciferase with mutated recognition site remained unchanged in H1299 cells expressing either miR-NC or miR-300. These results validated that the 3'-UTR of HIF3α mRNA was directly targeted by miR-300.

We then investigated whether endogenous HIF3α mRNA can be targeted by miR-300. We measured the mRNA level of HIF3α in both H1299 and H1975 cells, and the results showed that overexpression of miR-300 could significantly repress HIF3α mRNA expression (Figure 5A). Protein expression of HIF3α was also consistently reduced in miR-300-overexpressing NSCLC cells, compared to cell lines expressing miR-NC (Figure 5B). Thus, our results showed that endogenous HIF3α mRNA was indeed a target of miR-300, which might be involved in the miR-300 tumor suppression function in NSCLC cells.

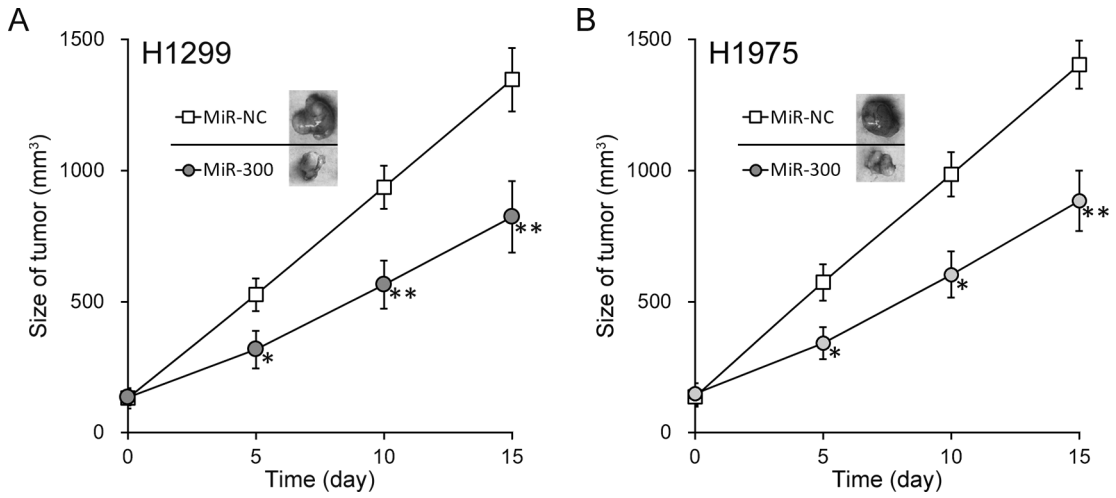

Figure 3. miR-300 inhibits tumorigenesis of NSCLC xenografts *in vivo*. Human NSCLC cell lines (A) H1299 and (B) H1975 stably expressing either control miRNA (miR-NC) or miR-300 were injected into mice (n=10 each) to establish xenograft model. Size of the tumor xenograft was measured at indicated days post injection. Representative images of the tumor on day 15 were also shown. Values were expressed as mean ± SD. * $p<0.05$, ** $p<0.01$, compared to miR-NC group on respective time points.

Re-introduction of HIF3α bypasses the inhibitory effect of miR-300 on tumorigenesis in human NSCLC cells. To further test whether HIF3α is the main contributor of miR-300 function in human NSCLC, we made plasmid expressing HIF3α without the 3'-UTR under CMV promoter and an empty vector as control, and introduced them separately into miR-300-overexpressing H1299 and H1975 cells (Supplementary Figure S2A). As shown in Figure 6A and 6B, both invasion and migration capabilities of miR-300-overexpressing H1299 and H1975 cells were markedly increased after re-introduction of HIF3α expression. In addition, in the mouse xenograft model, re-introduction of HIF3α accelerated tumor growth from miR-300-overexpressing cells (Figure 6C, 6D), as well as upregulated expressions of tumor growth markers including Ki67 and CD31 (Supplementary Figure S2B). Taken together, our results clearly demonstrated that miR-300 could function a tumor suppressor miRNA in human NSCLC by targeting HIF3α.

Discussion

HIF3α was first characterized in 1998 [19], and remained as the least studied member of the HIF family. The methylation status of HIF3α genomic region was correlated with childhood obesity and alanine aminotransferase levels [20]. The splicing variant HIF3α4 was found to be epigenetically silenced in hypervascular malignant meningioma, and restoring HIF3α4 expression disrupted angiogenesis of these malignant meningiomas [21]. In addition, in colorectal cancer, HIF3α was able to promote tumor growth by activating the JAK-STAT3 signaling pathway [22]. However, the molecular mechanisms underlying the involvement of HIF3α in human NSCLC remains unclear.

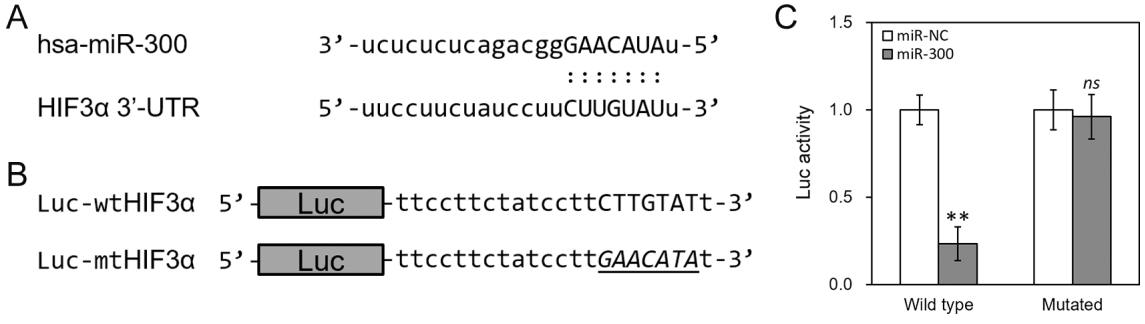

Figure 4. miR-300 directly recognizes 3'-UTR of HIF3α mRNA. (A) Possible miR-300 recognition site on the 3'-UTR of *HIF3α* mRNA. (B) Wild type site of *HIF3α* mRNA 3'-UTR and its mutated version (underscored) were cloned downstream of luciferase reporter open reading frame (Luc). (C) Luciferase activities of constructs Luc-wtHIF3α or Luc-mtHIF3α were measured in H1299 cells stably expressing either control miRNA (miR-NC) or miR-300. Values were expressed as mean ± SD from at least three independent experiments. ** $p<0.01$, *ns* not significant, compared to respective miR-NC group.

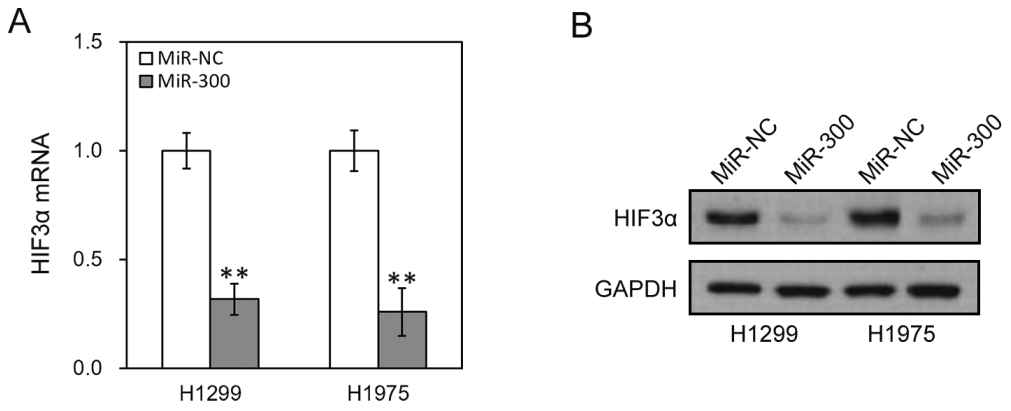

Figure 5. miR-300 inhibits expressions of both HIF3α mRNA and protein in human NSCLC cells. Human NSCLC cell lines H1299 and H1975 stably expressing either control miRNA (miR-NC) or miR-300 were subjected to analysis of HIF3α mRNA (A) and protein (B) levels. Values were expressed as mean ± SD from at least three independent experiments. ** $p<0.01$, compared to respective miR-NC group.

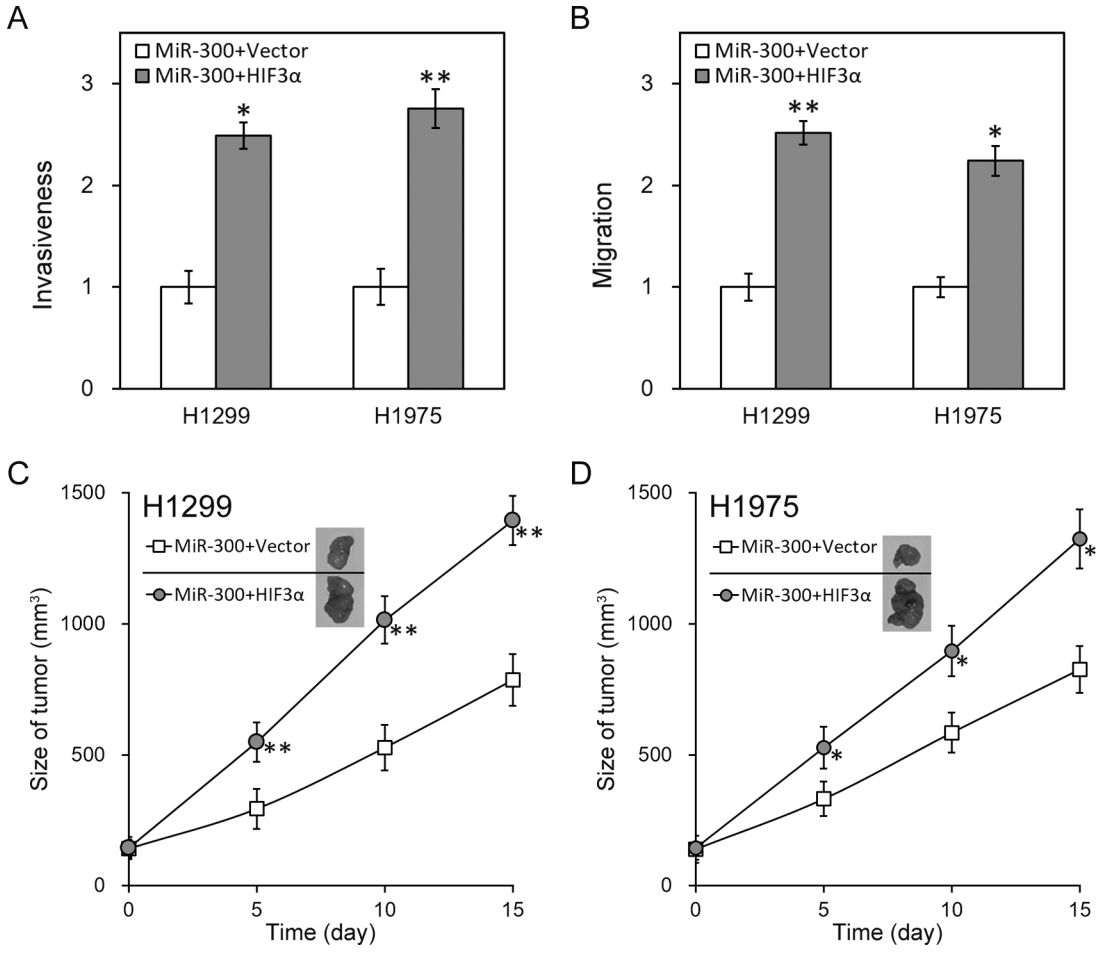

Figure 6. Re-introduction of HIF3α bypasses inhibitory effect of miR-300 on growth of human NSCLC cell lines *in vitro* and *in vivo*.
miR-300-expressing human NSCLC cell lines H1299 and H1975 were transfected with either empty vector or plasmid expressing HIF3α in the absence of its 3'UTR. (A and B) Cells were subjected to (A) cell invasion assay and (B) wound healing assay. Values were expressed as mean ± SD from at least three independent experiments. (C and D) Cells were injected into mice (n=10 each) to establish xenograft model. Size of the tumor xenograft was measured at indicated days post injection. Representative images of the tumor on day 15 were also shown. * $p<0.05$, ** $p<0.01$, compared to respective miR-300+vector group.

In the current study, we presented data revealing the molecular mechanism implicating both miR-300 and HIF3α in the tumorigenesis of NSCLC. Our results showed that endogenous miR-300 levels were significantly lower in both the NSCLC tumor tissues from patients and human NSCLC cell lines. Overexpression of miR-300 can significantly inhibit proliferation, migration and invasion capabilities of human NSCLC cell lines *in vitro*, and the tumor growth in a mouse xenograft model *in vivo*. Furthermore, we identified sequences in the 3'-UTR of HIF3α mRNA as a direct target of miR-300 in NSCLC cells. Expression of miR-300 greatly reduced expression of both HIF3α mRNA and protein. Finally, re-introducing HIF3α expression into miR-300-overexpressing NSCLC cells strongly restored the inhibitory effects of miR-300 on tumorigenesis both *in vitro* and *in vivo*. The above results clearly demonstrate that miR-300 acts as a tumor suppressor miRNA in human NSCLC by inhibiting HIF3α expression. Interestingly, HIF3α was first implicated in cancer as a dominant-negative regulator of HIF1, where it was found to be downregulated in renal cell carcinoma and was able to inhibit tumor progression [23, 24]. This reported synergistic action between HIF1 and HIF3α, together with our results suggesting HIF3α as a tumor suppressor miR, warrants further studies into potential involvement of HIF1 (or hypoxia in general) in the actions of miR-300/HIF3α in NSCLC, and other cancers.

Besides HIF3α, other members of the HIF family, or hypoxia itself, are widely reported to be involved in lung cancer [25]. Expression of HIF1α can be induced by hyperthermia in lung cancer through the AKT/ERK signaling pathways [26], and HIF1α expression was also found to be correlated with tumor proliferation and anti-apoptotic pathway in surgically resected lung cancer [27]. Particularly in NSCLC, HIF1α could promote tumor invasion by inhibiting expression of semaphorin 4B [28], and mediate the transcription regulation of cyclooxygenase-2 by thioredoxin-1 [29]. On the other hand, another HIF member, HIF2α, also plays important roles in lung cancer possibly by directly targeting plasminogen activator inhibitor-1 [30, 31]. Two HIF2α alleles, rs4953354 and rs13419896, were also shown to act as potential biomarker in lung cancer [32, 33]. In a recent study, HIF2α also played important roles in tyrosine kinase inhibitor-resistant NSCLC cells [34].

Given the fact that these HIF members are highly similar proteins, it's not surprising that they might be collectively involved in human diseases including cancer. For example, differential expressions of HIF1α, HIF2α and HIF3α were observed in patients with chronic obstructive pulmonary disease [35] and laryngeal carcinoma [36]. In addition, HIF1α, but not HIF2α, was found to regulate splicing of HIF3α in human tissues and cancer cells [37]. But in the case of hepatocellular carcinoma, expression of HIF3α was shown to be associated with HIF2α, instead of with HIF1α [38], and both HIF1α and HIF3α participated in a regulatory feedback loop that functioned to promote metastasis [39]. All the above studies suggested that complex genetic and/or direct interactions among the three HIF proteins, and it would be of particular importance to further explore whether such interactions also affect tumorigenesis of NSCLC. Last but not least, another question remains in our study, as well as raised by numerous other studies involving HIF3α in human cancers, is that, how is HIF3α able to exhibit almost completely opposing roles (tumor suppressor vs. oncogene) in different types of cancers. We speculate that the answer may lie in its upstream regulatory pathways. In this context, results in our study clearly identify and demonstrate miR-300 as one HIF3α regulator. We speculate that precise titration of endogenous HIF3α levels by other factors, including but not limited to miR-300, could synergize with existing HIF family proteins to contribute to a more complex hypoxia response. More mechanistic investigations are currently underway to test this hypothesis.

In conclusion, our current study has demonstrated for the first time that, miR-300 is a tumor suppressor miR in human NSCLC. The suppressive effect of miR-300 is mediated by its direct binding to the HIF3α mRNA 3'-UTR, which subsequently represses its expression. Our results strongly suggest that both miR-300 and HIF3α could be further evaluated as potential targets for clinical NSCLC treatments.

Supplementary information is available in the online version of the paper.

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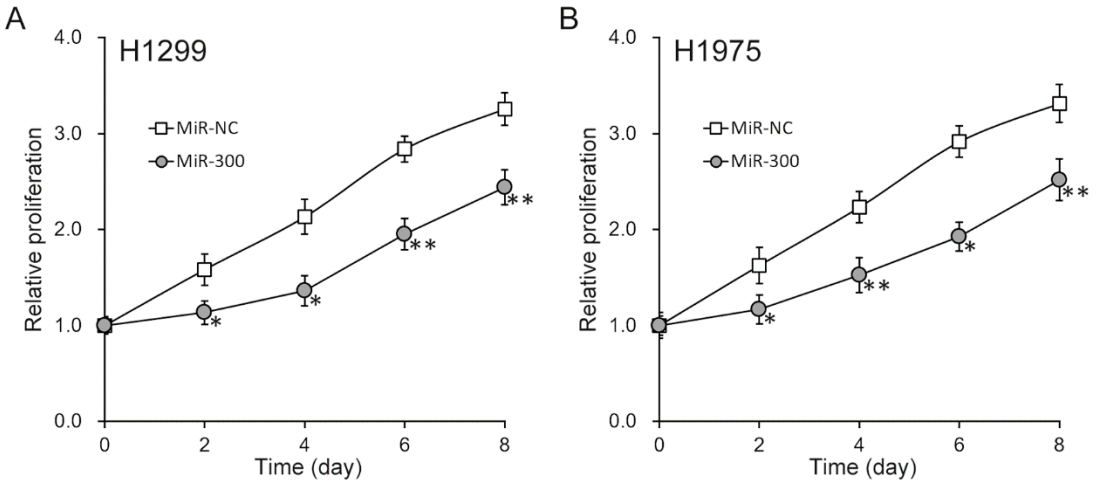

Figure S1. MiR-300 inhibits proliferation of human NSCLC cell lines *in vitro*.

Proliferation of human NSCLC cell lines (A) H1299 and (B) H1975 stably expressing either control miRNA (miR-NC) or miR-300 analyzed every 48 h for 8 days, and plotted as relative to day 0. Values were expressed as mean ± SD. * $p<0.05$, ** $p<0.01$, compared to miR-NC group on respective time points.

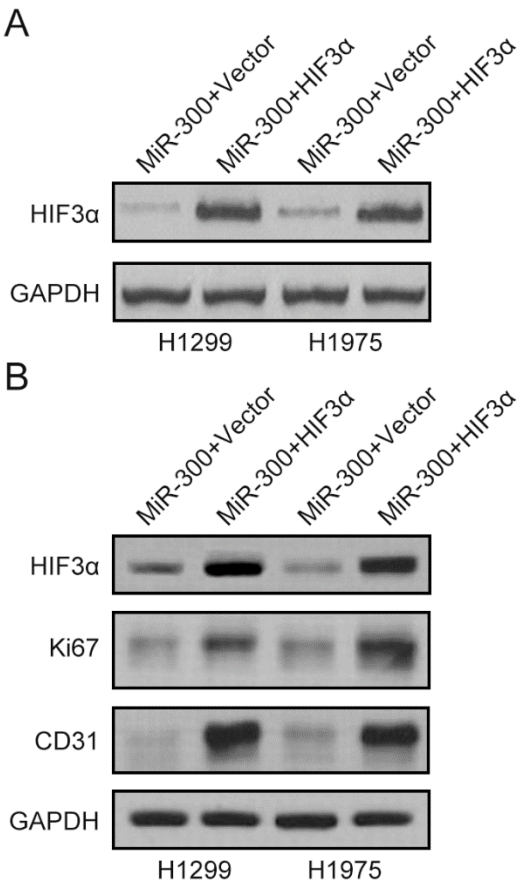

Figure S2. Expressions of HIF3α and tumor growth markers after re-introduction of HIF3α in the miR-300-expressing xenograft tumors *in vivo*.

(A) Protein levels of HIF3α were examined in the miR-300-expressing cells before being inoculated into the mice. (B) On day 8 after re-introduction of HIF3α into the miR-300-expressing xenograft tumors, protein levels of HIF3α, Ki67 and CD31 were examined by Western blot, with GAPDH as loading control.