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Nobiletin inhibited hypoxia-induced epithelial-mesenchymal transition of lung cancer cells by inactivating of Notch-1 signaling and switching on miR-200b

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Epithelial-mesenchymal transition (EMT) is an early step in the process of tumor metastasis. It is well known that tumor microenvironment affects malignancy in various carcinomas; in particular, that hypoxia induces EMT. Deregulated notch signaling also contributes a lot to the development of EMT in lung cancer. In this study, we investigated the use of Notch-1-inhibiting compound as novel therapeutic candidates to regulate hypoxia-induced EMT in lung cancer cells. According to previous screening, nobiletin was selected as a Notch-1 inhibitor. Hypoxia-induced EMT was characteristic of increased N-cadherin & vimentin expressions and decreased E-cadherin expressions. Treatment with nobiletin notably attenuated hypoxia-induced EMT, invasion and migration in H1299 cells, accompanied with reduced Notch-1, Jagged1/2 expressions and its downstream genes Hey-1 and Hes-1. Nobiletin treatment also promoted tumorsuppressive miR-200b level. Moreover, notch-1 siRNA prevented hypoxia-mediated cell migration and decreased Twist1, Snail1, and ZEB1/2 expressions, which are key EMT markers. Re-expression of miR-200b blocked hypoxia-induced EMT and cell invasion. Our findings suggest that downregulation of Notch-1 and reexpression of miR-200b by nobiletin might be a novel remedy for the therapy of lung cancer.

1. Introduction

Lung cancer is the leading cause of cancer-related death in the world and metastasis is the leading cause of death in lung cancer patients. Due to the dynamics of solid tumor growth, the oxygen pressure is inevitably lower than in the non-malignant tissues, leading to poor oxygen and nutrient deficiency (Salnikov et al. 2012). A hypoxic tumor environment plays a critical role in tumor metastasis and recurrence, providing a niche for cancer stem cells and causing a problem for NSCLC treatment. Tumor hypoxia has long been accompanied with poor outcome. Tumors with extensive hypoxia are more aggressive than better oxygenized tumors (Semenza 2010). Moreover, unsuccessful effects of anti-angiogenic therapies on tumor invasion were regarded to be greatly attributed to the generation of hypoxia.

Tumor hypoxia leads to induction of hypoxia-inducible transcription factors, e.g. HIF-1α and HIF-2α. Hypoxia and overexpression of HIF-1α and HIF-2α are known to play a key role in the sustenance and regulation of epithelial-to-mesenchymal transition (EMT) and metastatic phenotypes in lung cancer cells (Zhou et al. 2009; Lu and Kang 2010). Research demonstrated that deregulated activation of EMT promoted therapeutic resistance, invasion and metastasis (Singh and Settleman 2010; Xie et al. 2012). This process includes a loss of cell-cell attachments and apical-basal polarization, such as down-regulation of E-cadherin, which is the phenotype marker of epithelial cells, and up-regulation of N-cadherin and vimentin, which are the phenotype markers of mesenchymal cells (Jing et al. 2011). EMT-inducers, such as hypoxia or TGF-β, trigger changes in gene expression by complex signaling pathways. Several transcription factors such as zinc-finger E-box binding homeobox 1 (ZEB1) and ZEB2, Twist1, Snail1, and Snail2 have been shown to be crucial mediators in the converting of well-differentiated epithelial cells into motile mesenchymal cells.

Environmental signals regulate EMT through cell-cell contacts mediated by families of transmembrane receptors and ligands expressed on adjacent cells (Brabletz et al. 2005). The Notch axis is, particularly, a key participant in EMT, which is a conserved family of transmembrane receptors that adjusts critical cell fate (Artavanis-Tsakonas et al. 1999). Notch is known to be dis-regulated in various types of cancer, associated with unpleasant clinical outcomes (Ye et al. 2012; Espinoza and Miele 2013). It includes 4 Notch family members (Notch1–Notch4) and 5 Notch ligands–2 serrate-like ligands (Jagged1/2) and 3 Delta-like ligands (Dll1/3/4) (D'Souza et al. 2008; Li et al. 2011). Notch1 has been reported to regulate EMT in lung cancer. The EMT phenotype of chemo-resistant lung cancer cells can be reversed by knocking down Notch1 (Xie et al. 2012). Jagged1-induced Notch activation stimulates breast epithelial cells EMT *via* regulating Snail (Guerra et al. 2003). More importantly, it has been demonstrated that survival of non-small-cell lung cancer (NSCLC) cells in a hypoxic microenvironment depends on Notch-1 signaling both *in vitro* and *in vivo*, signifying that the inhibition of Notch may be an effective approach for lung cancer therapy.

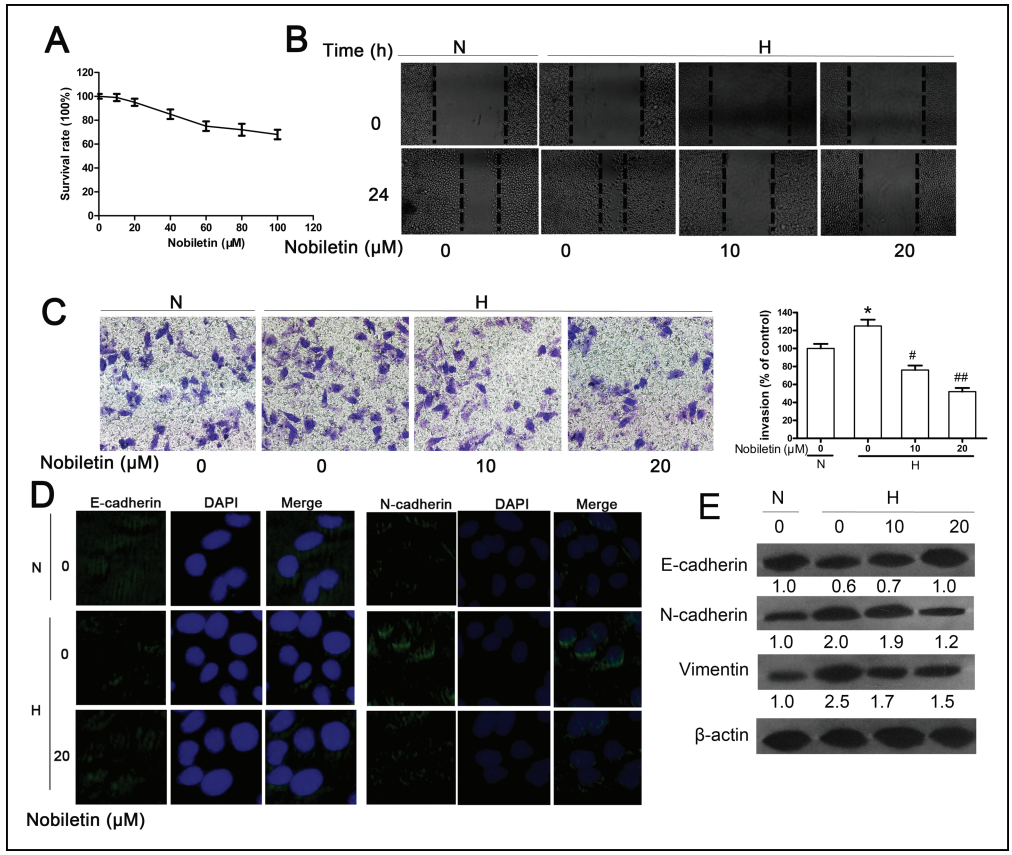

Fig. 1: Nobiletin inhibited cell migration and invasion, and EMT induced by hypoxia. (A) MTT assay on H1299 cells. Survival rates for H1299 cells after incubating with nobiletin for 24 h under hypoxia. (B) Wound healing assays for H1299 cells. After scraping the scratches, H1299 cells were treated with nobiletin (10, 20 μM) for 24 h under hypoxia; cells under normoxia was the control. (C) Invasion assays. H1299 cells were treated with nobiletin (10, 20 μM) for 24 h under hypoxia; the matrigel-coated inserts with 8 μm pores were used to examine the invasive ability. The number of invaded H1299 cells was counted from five random fields and presented (right panels) as mean ± S.E.M of three independent experiments. *P < 0.05 *vs.* the normoxia control; #P < 0.05, ##P < 0.01 *vs.* the hypoxia control. (D) H1299 cells were treated with nobiletin (10, 20 μM) for 24 h under hypoxia, and immunofluorescence staining was performed to assess expressions of N-cadherin and E-cadherin. (E) H1299 cells were treated with nobiletin (10, 20 μM) for 24 h under hypoxia, and EMT-related proteins were then analyzed by western blot analysis. β-actin was used as the loading control. Protein expressions were semi-quantified by densitometry analysis. N, normoxia; H, hypoxia; EMT, epithelial mesenchymal transition; DAPI, 4, 6-diamidino-2-phenylindole.

Nobiletin, a naturally occurring polymethoxy flavonoid from *Citrus depressa*, is shown to inhibit tumor cell invasive activity not only by suppressing the gene expression of MMPs but also by enhancing TIMP-1 production in human fibrosarcoma cells (Sato et al. 2002). Studies also show that nobiletin has the potential to inhibit metastasis of breast cancer *via* reducing expressions of CXC chemokine receptor type 4 (CXCR4) and MMP9 (Baek et al. 2012). Moreover, nobiletin could cause p53-mediated cell cycle arrest and apoptosis through manipulating the ratio of Bax/Bcl-2 protein levels in lung cancer cells (Luo et al. 2008). Nobiletin could also notably inhibit the levels of MMP2/9 and pAkt in Akt1-cDNA-transfected cells accompanied with an obvious reduction in cell migration. Nobiletin inhibits the migration of various cell lines; however, the molecular mechanisms by which nobiletin works under hypoxia is not well understood. Recently, we found that nobiletin treatment diminished Notch-1 expression in lung cancer cells under hypoxia, which led us to explore whether Notch axis could contribute to nobiletin-induced inhibitory effects on EMT of lung cancer cells.

2. Investigations and results

2.1. Effect of nobiletin on cell migration and invasion, and EMT induced by hypoxia

In an attempt to exclude the influence of cell viability by nobiletin, we performed MTT assays of H1299 cells. Cells were treated with nobiletin (0–100 μM) under hypoxia for 24 h. The non-cytotoxic concentrations of nobiletin used for this study were identified. According to the MTT assays (Fig. 1A), 5, 10, and 20 μM were selected for further study, which exhibited no significant toxicity on the cell viability.

Wound healing capacity of the cancer cells was reduced by nobiletin treatment, which can be augmented by hypoxia (Fig. 1B). In line with the wound healing assay, the results of the migration assay showed that H1299 cells under hypoxia displayed more potent invading ability through the Matrigel than the normoxic cells (Fig. 1C, $P < 0.05$). These data proposed an inhibiting effect of nobiletin on the invasiveness and motility of NSCLC cells.

Hypoxia has been reported to lead to EMT of cancer cells. The cells under hypoxia demonstrated a spindle shape with pseudopodia formation, morphologically different from the normoxic cells. EMT is accompanied with the loss of E-cadherin. We carried out an immunofluorescence assay to examine the protein levels of N-cadherin and E-cadherin in H1299 cells under hypoxia. The decrement of E-cadherin and increment of N-cadherin were observed under hypoxic conditions (Fig. 1D). Likewise, hypoxia exposure resulted in lower E-cadherin expression than the normoxic cells as revealed by the western blot assays, whereas the expression of the mesenchymal markers N-cadherin and vimentin were up-regulated (Fig. 1E). Nobiletin treatment at 20 μM prevented hypoxia-induced EMT, as revealed by increased expression of the epithelial phenotype marker E-cadherin, and decreased expression of N-cadherin and vimentin.

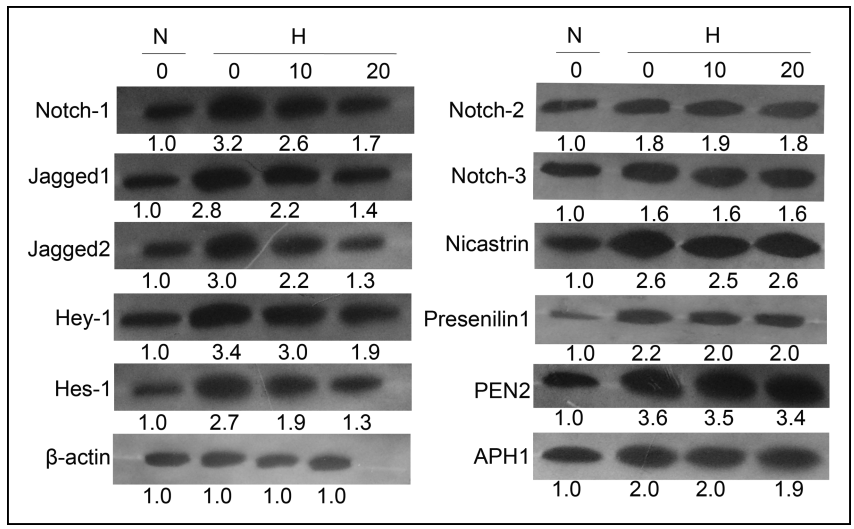

Fig. 2: Nobiletin inhibited Notch signaling in H1299 cells under hypoxia. H1299 cells were treated with nobiletin (10, 20 μM) for 24 h under hypoxia, and the expressions of Notch-1/2/3, its ligand Jagged1/2, target genes Hes-1&Hey-1 and four subunits of γ-secretase were measured by western blot analysis. β-actin was used as the loading control. Protein expressions were semi-quantified by densitometry analysis. N, normoxia; H, hypoxia.

2.2. Effect of nobiletin on Notch signaling in NSCLC cells

Accumulating evidence has confirmed the important role of Notch-1 signaling in EMT (Sahlgren et al. 2008). To further explore the molecular mechanism involved in hypoxia-induced EMT inhibition, the expressions of Notch-1, its ligand Jagged1/2 and downstream target genes Hes-1, Hey-1 in hypoxic cells were measured by western blot analysis. After nobiletin treatment for 24 h, the increment in Notch-1, Jagged1/2, Hey-1 and Hes-1 protein levels induced by hypoxia was almost blunted. Notch-2 and Notch-3 expressions were not significantly influenced by hypoxia exposure or/and nobiletin treatment (Fig. 2).

The Notch receptor is activated after binding to one of 5 Delta or Jagged ligands that are expressed on neighboring cells. Upon ligand binding, Notch undergoes a series of cleavages, and the final cleavage is carried out by a presenilin-dependent γ-secretase protease complex, causing translocation of Notch intracellular domain (ICD) into the nucleus (Capaccione and Pine 2013). γ-Secretase is a multi-protein complex, characteristic of the proteolytic ability. The catalytic subunits (Nicastrin, APH1, Presenilin1, and PEN2) have been known to be crucial for activity (Eliasz et al. 2010). We next examined whether activation of the Notch-1 pathway under hypoxia would be affected by nobiletin (Fig. 2). The expressions of the four

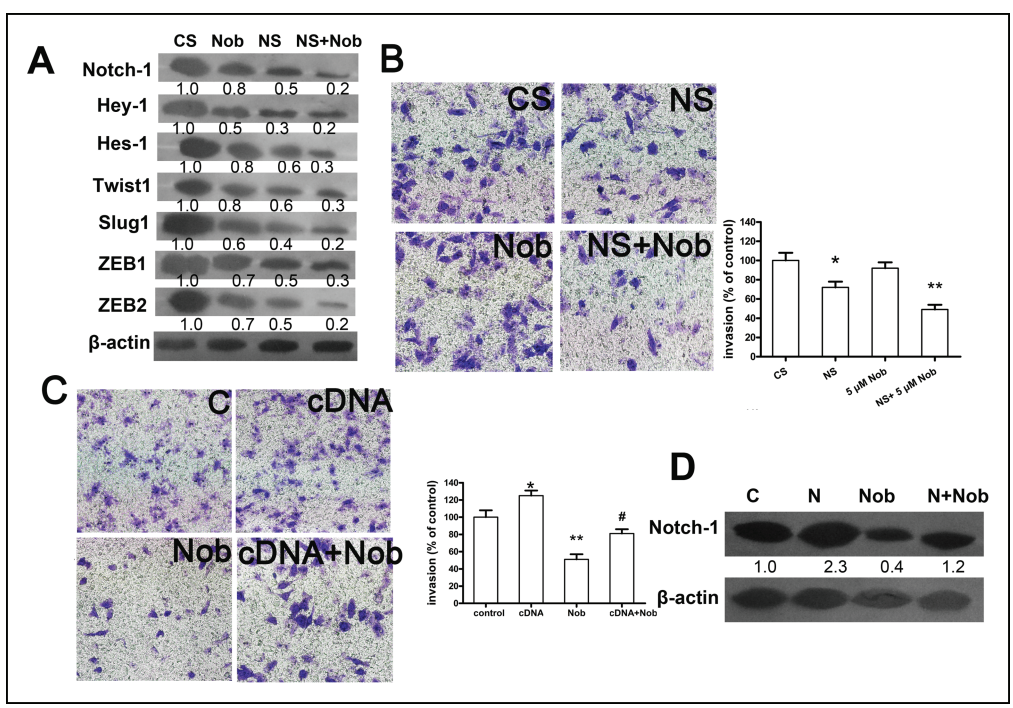

Fig. 3: Notch-1 siRNA inhibited Notch-1 signaling and decreased expressions of EMT markers. (A) Notch-1 siRNA-transfected H1299 cells were incubated with nobiletin (20 μM) under hypoxia for 24 h, and cell lysates were measured by western blot analysis. β-actin was used as the loading control. Protein expressions were semi-quantified by densitometry analysis. (B) Notch-1 siRNA plus nobiletin further reduced cell invasion in H1299 cells. Invaded H1299 cells was counted from five fields and presented (right panels) as mean ± S.E.M. $^{*}P<0.05$ *vs.* CS; $^{\#\#}P<0.01$ *vs.* NS. (C) Notch-1 overexpression by cDNA transfection abolished nobiletin-prompted migration inhibition. Notch-1 cDNA-transfected H1299 cells were incubated with nobiletin (20 μM) for 24 h. Invaded H1299 cells was counted from five fields and presented (right panels) as mean ± S.E.M. $^{*}P<0.05$, $^{**}P<0.01$ *vs.* C; $^{\#}P<0.05$ *vs.* Nob. (D) Notch-1 cDNA-transfected H1299 cells were incubated with nobiletin (20 μM) for 24 h under hypoxia and cell lysates were measured by western blot analysis. Protein expressions were semi-quantified by densitometry analysis. For (A) & (B), CS, control siRNA; NS, Notch-1 siRNA; Nob, 20 μM nobiletin; NS + Nob, Notch-1 siRNA + 20 μM Nob. For (C) & (D), C, control; cDNA, Notch-1 cDNA; Nob, nobiletin; cDNA + Nob, Notch-1 cDNA + nobiletin.

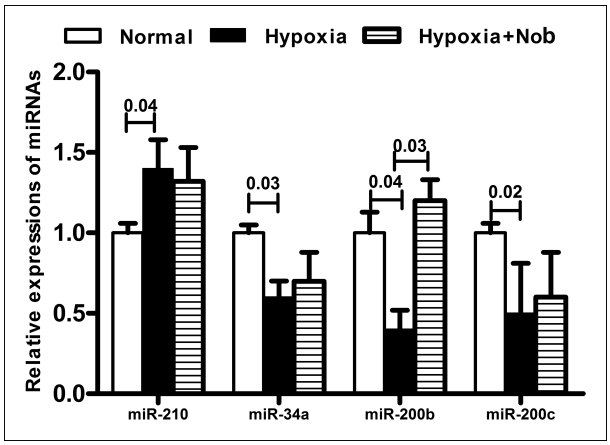

Fig. 4: Nobiletin increased the expression of miR-200b on H1299 cells. Real-time reverse transcription-PCR analysis following nobiletin (20 μM) treatment for 24 h was carried out as described in the Materials and methods. Mean ± S.E.M. from three independent experiments. Nob, nobiletin.

components of the γ-secretase complex were not influenced by nobiletin. The γ-secretase complex was not involved in the down-regulation of the Notch pathway mediated by nobiletin. Herein, we conclude that nobiletin inhibits the expression but not the activation of Notch-1.

2.3. Effect of Notch-1 deficiency on cell invasion and EMT in H1299 cells

To verify the key role of Notch-1 in invasion and EMT in hypoxic NSCLC cells, we carried out knockdown of Notch-1 by siRNA in H1299 cells. Reduced expressions of Notch-1, Hey-1, Twist1, Snail1, ZEB1, ZEB2 and Hes-1 as well as a decline in cell invasion were observed (Fig. 3A, 3B). Treatment of Notch-1 siRNA plus nobiletin (5 μM) further prevented cell invasion in H1299 cells (Fig. 3B). Compared with single nobiletin treatment, combination of nobiletin and Notch-1 siRNA inhibited Notch-1 activity to a more significant extent as revealed by western blot assays.

Afterwards, Notch-1 complementary DNA-transfected cells were constructed to achieve Notch-1 overexpression status. In Notch-1 complementary DNA-transfected H1299 cells, a significant increase was obtained in both Notch-1 expression and cell motility than the negative control. Additionally, the inhibitory effect of nobiletin on invasion was attenuated by Notch-1 overexpression after 24 h incubation, to a certain extent (Fig. 3C, 3D). These data confirmed the possible role of Notch-1 in nobiletin's effect.

2.4. Effect of nobiletin on the expression of miRNAs in NSCLC cells

It has been demonstrated that a specific set of miRNAs are up-regulated by hypoxia (Chan and Loscalzo 2010). Here, we examined whether miRNA molecules could regulate the gene expression of Notch-1 in hypoxic H1299 cells, since several types of miRNA have been identified as key regulators of Notch-1 signaling (Kashat et al. 2012). As shown in Fig. 4, oncogenic miR-210 was increased, whereas tumor-suppressive miR-34a and miR-200b/c was reduced in H1299 cells under hypoxia. Furthermore, we found that miR-200b expression was threefold higher in the H1299 cells treated with nobiletin compared to untreated H1299 cell under hypoxia. However, miR-210, miR-200c and miR-34a expressions were not affected by nobiletin treatment.

2.5. Effect of reexpression of miR-200b on EMT and cell invasion in H1299 cells

We transfected mimics into H1299 cell to investigate the essential role of miR-200b in EMT and cell invasion. The transfection of miR-200b mimics indeed improved miR-200b levels, and reduced Notch-1 expressions in hypoxic H1299 cells (Fig. 5A, 5B). As expected, re-expression of miR-200b considerably diminished invasion and EMT of H1299 cells under hypoxia (Fig. 5B, 5C), implying that renewal of miR-200b is related to the modulation of cell invasion and EMT in NSCLC cell. These data suggest that the biologic function of nobiletin on EMT and invasion is greatly attributed to inducing miR-200b expression. Besides, knocking down Notch-1 by siRNA in H1299 cells under hypoxia also resulted in up-regulated miR-200b expressions (Fig. 5D), and these effects are similar with nobiletin treatment as depicted in Fig. 4.

3. Discussion

Pathogenesis of cancer is a multistep process of alterations in miRNA, tumor-suppressor genes, or oncogenes in lung cancer. Recently, emerging evidence reveals that miR-200, a potential tumor suppressor, could modulate lung cancer EMT. In our study, we confirm that the anti-metastasis effect of nobiletin was closely related to Notch-1 signaling. Furthermore, the activity of miR-200b as a tumor suppressor was confirmed. miR-200b is also a key player in the inhibition of EMT by nobiletin. Herein, we propose that nobiletin suppresses EMT *via* the Notch-1/miR-200b loop in H1299 cells.

The EMT process, which plays a critical part in the advance of NSCLC metastasis, modulated the differentiation of tubular epithelial cells into myofibroblasts. Besides, EMT is regarded as one of the crucial causes of drug resistance (Thomson et al. 2005). Seeking effective drugs that target EMT has attracted worldwide attention. E-Cadherin, N-cadherin, and vimentin are proteins directly related to EMT. In this study, we showed that nobiletin supressed EMT, invasion and migration of lung cancer cells under hypoxia. In H1299 cells, nobiletin increased the expression of E-cadherin and inhibited the expression of N-cadherin and vimentin. Clinical data have demonstrated that 30% of NSCLC cases have increased Notch activity. High Notch1 expression correlates with poor prognosis in NSCLC (Donnem et al. 2010). Notch signaling pathway molecules were overexpressed in lung cancer and Hes-1 was nearly positively correlated with HIF-1α in lung cancer tissues, which positively correlates with hypoxia. Notch increases lung cancer stem cell self-renewal and induces an EMT phenotype (Xie et al. 2013). We found that nobiletin inhibited the expression of Jagged1/2, Notch-1, Hey-1 and Hes-1 induced by hypoxia but had no noteworthy influence on the expression of γ-secretase complex, which implied that nobiletin inhibited the expression but not the activation of Notch-1. Moreover, transient Notch1 inactivation meaningfully repressed EMT and invasion of H1299 cells through matrigel-coated Transwell inserts. Notch1 siRNA transfection plus nobiletin exhibited more profound inhibiting effect, all of which indicated that nobiletin functioned *via* the hypoxia-induced Notch-1 pathway.

MicroRNAs (miRNAs) are small noncoding RNAs that take part in posttranscriptional gene regulation (Lee and Ambros 2001). Reduced miR-200 levels have been confirmed in lots of tumor specimens such as prostate, breast, and pancreatic cancer (Zhang et al. 2007; Gao et al. 2014). Overexpression of miR-200 prevents tumor cells from differentiating into an epithelial phenotype and abolishes their migrating capacity in mice (Gibbons et al. 2009). Disease recurrence correlates well with low levels of miR-200b in lung cancers at early-stage (Patnaik et al.

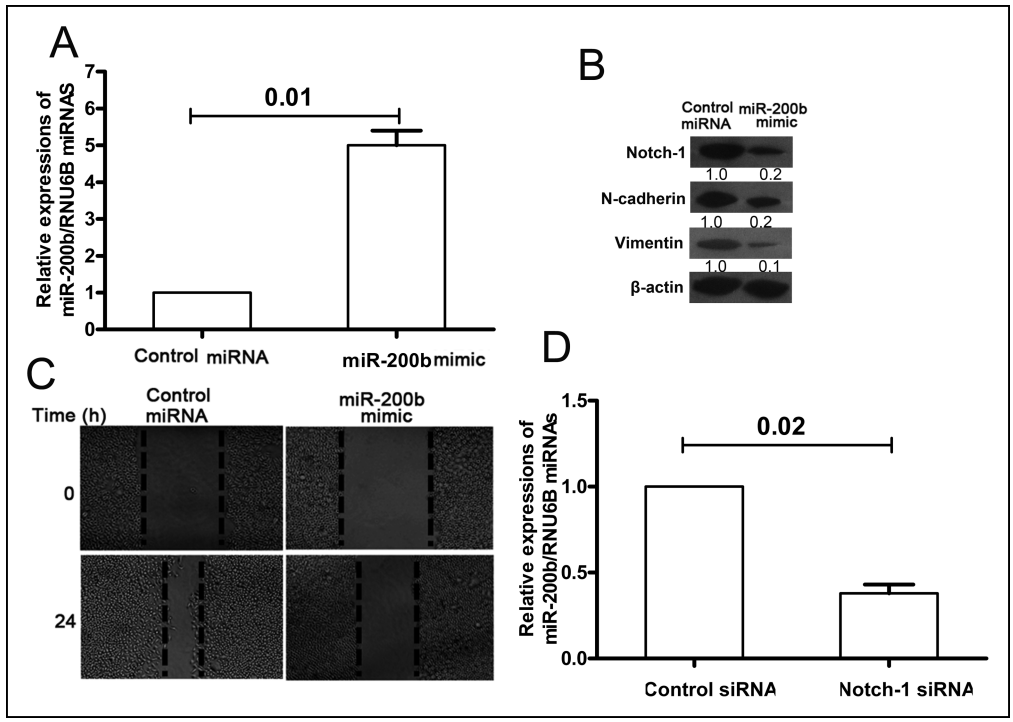

Fig. 5: miR-200b mimic inhibited EMT and cell invasion in H1299 cells under hypoxia. (A) The miR-200b levels was detected in H1299 cells transfected with miR-200b mimics with RNU6B as a control mRNA. (B) The expressions of Notch-1, N-cadherin and vimentin were examined by western blot assays. β-actin was used as the loading control. Protein expressions were semi-quantified by densitometry analysis. (C) Wound healing assays for H1299 cells. Decreased invasion was observed in H1299 cells transfected with miR-200b mimics. (D) Knocking down Notch-1 by siRNA in H1299 cells under hypoxia promoted miR-200b expressions with RNU6B as a control mRNA. Mean ± S.E.M. from three independent experiments.

2010). A new correlation between EMT and Notch has been recognized through miR-200 in which its expression is manipulated by ZEB1 and ZEB2 (Yang et al. 2011), which are crucial transcription factors in the EMT process. Our study proved for the first time that oncogenic miR-210 was up-regulated and tumor-suppressive miR-34a and miR-200b was down-regulated in NSCLC cells under hypoxia. Here, we revealed that siRNA Notch-1 could promote miR-200 expression, which is in line with nobiletin-mediated up-regulation of miR-200 accompanied with the suppression of EMT and cell migration. Furthermore, reexpression of miR-200b inhibited Notch-1 expression as well as EMT and cell invasion, suggesting a negative mutual feedback loop between miR-200b and Notch-1. We concluded that miR-200b might take part in nobiletin-induced EMT suppression, probably *via* directly manipulating Notch-1 expression.

For the first time, our results showed that nobiletin repressed the translation and transcription of Notch-1, Jagged1/2, Hey-1 and Hes-1 in hypoxic H1299 cells. The treatment of Notch-1 siRNA plus nobiletin almost blocked migration and significantly inhibited expression of downstream gens of Notch-1 in H1299 cells under hypoxia. Moreover, miR-200b was also involved in the inhibitory effect of nobiletin on EMT process. Taken together, the experimental results suggest that nobiletin might be a potential candidate for interference with hypoxia-induced EMT and tumor invasion by blunting the Notch pathway and switching on miR-200b.

4. Experimental

4.1. Reagents

Nobiletin, trypsin for cell culture, Tween 20, bovine serum albumin (BSA), sodium dodecyl sulfate (SDS), fetal bovine serum (FBS), penicillin, streptomycin, 4',6-diamidino-2-phenylindole (DAPI) and MTT [3-(4,5-dimethythiazol- 2-yl)-2,5-diphenyl tetrazolium bro-mide] were obtained from Sigma (St. Louis, MO). Cell culture media (RPMI-1640) were acquired from Gibco (Grand Island, NY). Trizol and Lipofectamine 2000 were obtained from Invitrogen (Carlsbad, CA). The other chemicals and reagents used were of analytical grade.

4.2. Cell culture

The human NSCLC cell line, H1299, was acquired from the American Type Culture Collection (ATCC, Manassas, VA). H1299 cells were grown in RPMI1640 medium supplemented with 10% FBS and 1% penicillin and streptomycin. All the following experiments were carried out under hypoxia (1% O_2, 5% CO_2, 94% N_2), unless otherwise specified. Cells were treated with nobiletin (0–100 μM) dissolved in DMSO. Cells were periodically tested for mycoplasma infection and were found to be negative.

4.3. Cell viability assay

The cytotoxicity effect of nobiletin on H1299 cells was determined by MTT assay (Song et al. 2012a). Exponentially growing cells (1×10^4 cells/well) were seeded in 96-well plate, and treated with nobiletin (0–100 μM) under hypoxia for 24 h. Subsequently, the medium containing MTT was removed. DMSO was used to dissolve the crystals. After mixing, the optical density (OD) was measured at 570 nm using a spectrum microplate reader (Molecular Devices, Sunnyvale, CA, USA).

4.4. Immunofluorescence microscopy

After treatment with nobiletin (20 μM) for 24 h under hypoxia, H1299 cells were fixed with 4% paraformaldehyde, permeabilized in 0.5% Triton X-100 and then blocked with bovine serum albumin. Next, cells were incubated overnight with anti-E-cadherin and anti-N-cadherin antibodies at 4 °C. Secondary antibodies were DyLight 728-conjugated IgG (Thermo Scientific). After counterstaining with DAPI, the cells were visualized with a fluorescence microscope (Zeiss Axio Observer A1).

4.5. Cell invasion assays

Cell invasion was assessed using 24-well Matrigel inserts with 8 mm pores (Corning, Cambridge, Ma). After treatment with nobiletin (10, 20 μM) for 24 h under hypoxia, H1299 cells were detached by trypsin, and suspended. A total of 2×10^5 cells were plated at the top of the Matrigel-coated insert in serum-free RPMI1640, with 10% FBS–RPMI1640 in the bottom chamber as chemoattractant. After 24 h under hypoxic conditions, cells that had moved through the pores were stained with crystal violet and counted under a microscope (Song et al. 2012b). All experiments were performed in triplicate.

4.6. Wound healing assay

After the cells reaching 90% confluency in 24-well plates, scratch the layer with a 10 μL pipette tip to create the wound. The cells were then washed and incubated without or with nobiletin for 24 h under hypoxia, and then photographed under the microscope.

4.7. Western Blotting

After H1299 cell had been treated with nobiletin for 24 h under hypoxia, cell lysates were subjected to SDS-PAGE and blotted onto PVDF membranes (Millipore, Bedford, MA) (Huang et al. 2014). Notch-1, Notch-2, Notch-3, Hey-1, Jagged1/2, Hes-1, Twist1, Snail1, ZEB1/2, E-cadherin, N-cadherin, vimentin, PEN2, APH1, nicastrin, presenilin1, and beta-actin antibodies (Cell Signaling Technology, Beverly, MA).

4.8. siRNA for Notch1

Notch1 siRNA (Santa Cruz, CA) includes 5′-CACCAGUUUGAAUGGUCAAtt-3′ and 5′-UUGACCAUUCAAACUGGUGtt-3′. H1299 cells were transfected with Lipofectamine 2000 and RNA primer pair. After 72 h of transfection, cells were kept under hypoxia for 24 h to determine the influence of Notch1 knockdown on EMT. The H1299 cells transfected with scrambled siRNA or Lipofectamine 2000 (mock transfection) were regarded as negative controls under hypoxia (Du et al. 2012).

4.9. Plasmids and transfections

The Notch1 gene was amplified by PCR and inserted into the reporter vector pCMV6-Entry/Notch-1 from OriGene Technologies Inc. (Rockville, MD). Plasmid transfections were carried out with Lipofectamine 2000 as previously described (Kang et al. 2013). Fresh medium was added 6 h after transfection, and proteins were harvested 24 h post-transfection.

4.10. miRNA analysis

Total miRNA was isolated using mirVana TM miRNA isolation kit (Ambion Inc, Grand Island, NY), as previously described (Subramaniam et al. 2012). Total miRNA from H1299 cells were exposed to reverse transcription with hexanucleotide primers. The cDNA was used to complete Real-time PCR. The changes in pri-miRNA were depicted as fold change relative to control. Pri-miR-200b: 5′-TGGCAGTGTCTAGCTGATTG-3′ and 5′-GGCAGTATACTGCTGATCGCTT-3′.

4.11. Transfection of miR-200b mimics

H1299 cells were transfected with miR-200b mimics or a negative control miRNA mimic obtained from Dharmacon (Chicago, IL), using Lipofectamine 2000 as previously described (Du et al. 2012). The cells were collected 72 h after transfection for further western blot analysis.

4.12. Statistical analysis

Statistical analysis was performed using SPSS 16.0 (Chicago, IL, USA). The statistical significance was examined using the one-way analysis of variance (ANOVA) followed by Dunnett's test. $P < 0.05$ was considered to indicate a statistically significant difference.

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