Molecular Therapy
Methods & Clinical Development
Original Article

miR-1254 induced by NESG1 inactivates HDGF/DDX5-stimulated nuclear translocation of β-catenin and suppresses NPC metastasis

Chao Cheng,[1,5] Wenmin Li,[2,5] Xuemei Peng,[2] Xiong Liu,[3] Ziyan Zhang,[2] Zhen Liu,[4] Tongyuan Deng,[2] Rongcheng Luo,[2] Weiyi Fang,[2] and Xiaojie Deng[2]

[1]Department of Pediatric Otolaryngology, Shenzhen Hospital, Southern Medical University, Shenzhen, Guangdong, China; [2]Cancer Center, Integrated Hospital of Traditional Chinese Medicine, Southern Medical University, Guangzhou, China; [3]E.N.T. Department of Nanfang Hospital, Southern Medical University, Guangzhou, Guangdong, China; [4]Key Laboratory of Protein Modification and Degradation, School of Basic Medical Sciences, Affiliated Cancer Hospital and Institute of Guangzhou Medical University, Guangzhou, China

Nasopharyngeal carcinoma (NPC) is one of the most common malignant tumors in Chinese and other Southeast Asians. We aimed to explore the precise mechanism for NESG1 in NPC for understanding the pathogenesis of NPC. Transwell, Boyden assays, and wounding healing were respectively performed for cell metastasis. The microRNA (miRNA) microarray and luciferase reporter assays were designed to clarify NESG1-modulated miRNAs and miR-1254-targeted protein. Western blotting assays examined the pathways regulated by miR-1254, the (Hepatoma-Derived Growth Factor) HDGF/DDX5 complex, and NESG1. The chromatin immunoprecipitation (ChIP), electrophoretic mobility shift assay (EMSA), and co-immunoprecipitation (coIP) assays were used to explore the DNA-protein complex and protein-protein complex. NESG1 suppressed NPC migration and invasion via Wnt/β-catenin signaling. Further, miR-1254 was confirmed as a positive downstream modulator of NESG1 reducing metastatic abilities of NPC cells *in vivo* and *in vitro*. Transduction of HDGF significantly restored cell migration and invasion ability in miR-1254-overexpressing NPC cells. In clinical samples, miR-1254 expression was negatively correlated with HDGF and positively correlated with NESG1 expression. miR-1254 acts as an independent prognostic factor for NPC, which was induced by NESG1 to suppress NPC metastasis via inactivating Wnt/β-catenin pathway and its downstream EMT signals.

INTRODUCTION

Nasopharyngeal carcinoma (NPC) is one of the most common head-and-neck epithelial malignancies in southern China, which is also known as the Cantonese cancer.[1] The main etiologies of NPC are believed to be environmental factors, Epstein-Barr virus (EBV) exposure, and genetic factors.[2,3]

Previously, we identified a full-length NESG1 gene specifically expressed in human nasopharynx and trachea by screening different genes involving normal human nasopharyngeal and oral cavity mucosa of the soft palate.[4] A subsequent study showed that NESG1 might act as a tumor suppressor by inhibiting cell proliferation, migration, and invasion of NPC cells via modulating the cell cycle and the mitogen-activated protein kinase (MAPK) pathway.[5,6] Also, we verified that reduced expression of NESG1 is an unfavorable factor modulating cell growth in non-small cell lung cancer (NSCLC).[7] Our recent study revealed that NESG1 interacted with VPS33B to regulate the Epidermal Growth Factor Receptor (EGFR)/ phosphatidylinositol 3-kinase (PI3K)/AKT/c-Myc/P53/miR-133a signal, which suppresses NPC growth.[8] However, the detailed molecular basis for NESG1 to inhibit NPC metastasis is still unclear.

MicroRNAs (miRNAs) are small non-coding RNAs with 21 to 23 nucleotides that inhibit their targeted genes.[9] An increasing number of studies have demonstrated that miRNAs may play a significant role in tumor pathogenesis.[10,11] miR-486-3p acts as a tumor suppression factor in oral cancer.[12] Furthermore, many papers demonstrated that miRNAs could act as intermediate mediators participating in the modulation of some significant genes in tumor pathogenesis. For example, miR-139 inhibits hepatocellular carcinoma by directly targeting KPNA2.[13] miR-3188 targets Mechanistic Target Of Rapamycin Kinase (mTOR) in regulating NPC proliferation and chemosensitivity.[14] However, whether miRNAs involve in the pathogenesis of NPC induced by NESG1 remains to be studied.

In this study, we observed a reduced expression of miR-1254 in NPC, which is hypothesized to promote NPC pathogenesis. Further, miR-

Received 20 July 2020; accepted 1 February 2021;
https://doi.org/10.1016/j.omtm.2021.02.001.

[5]These authors contributed equally

Correspondence: Rongcheng Luo, Cancer Center, Integrated Hospital of Traditional Chinese Medicine, Southern Medical University, Guangzhou, China.
E-mail: luorc02@vip.163.com
Correspondence: Weiyi Fang, Cancer Center, Integrated Hospital of Traditional Chinese Medicine, Southern Medical University, Guangzhou, China.
E-mail: fangweiyi1975@163.com
Correspondence: Xiaojie Deng, Cancer Center, Integrated Hospital of Traditional Chinese Medicine, Southern Medical University, Guangzhou, China.
E-mail: sophiexiaojie@qq.com

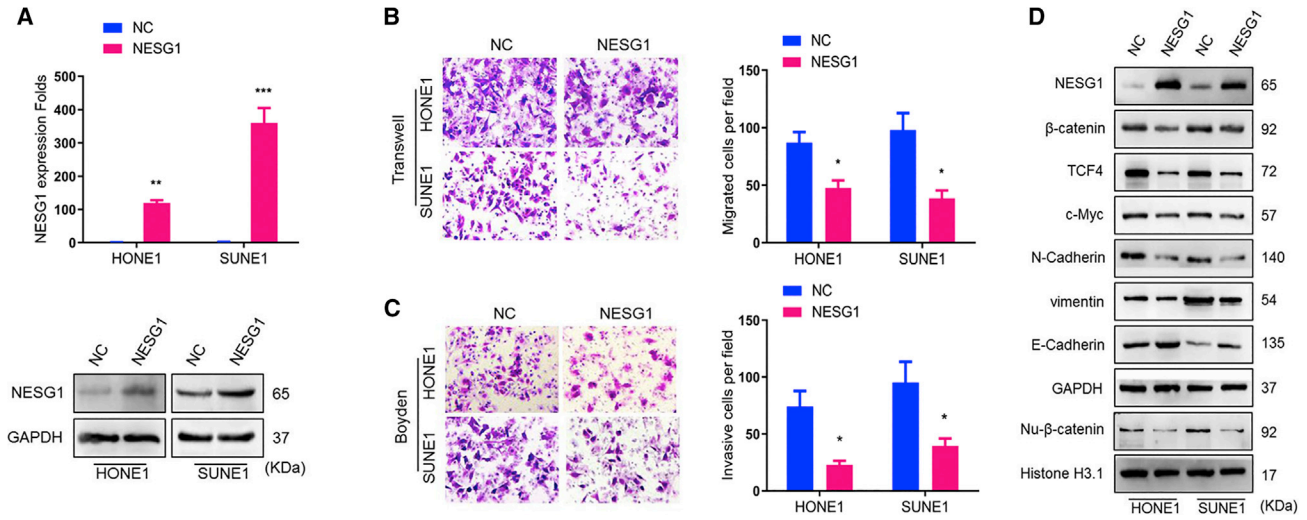

Figure 1. NESG1 suppresses NPC metastasis via inhibiting Wnt/β-catenin signaling

(A) Real-time PCR and western blot were used to examine the mRNA and protein levels of NESG1 after transfecting NESG1 plasmid. Student's t test, mean ± SD, $^{**}p < 0.01$, $^{***}p < 0.001$. (B and C) Transwell and Boyden assays for HONE1 and SUNE1 cells transfected with NESG1 plasmid compared with negative control. Original magnification, ×200; scale bar, 25 μm. $^{*}p < 0.05$. (D) Expression of β-catenin, Nu-β-catenin, TCF4, c-Myc, N-cadherin, and vimentin was inhibited, but E-cadherin were increased after HONE1 and SUNE1 cells were transfected with NESG1 plasmid. GAPDH and histone H3.1 served as a loading control.

1254, as a metastasis suppressor, was induced by NESG1 and targeted HDGF to suppress DDX5/β-catenin/TCF4 and its downstream Epithelial-mesenchymal transition (EMT) signal. Our data reveal a new NPC metastasis molecular mechanism, which provides a new clue for understanding the pathogenesis of NPC.

RESULTS

NESG1 suppresses NPC metastasis ability via inhibiting Wnt/β-catenin signaling

To verify the functional significance of NESG1 in NPC, we ectopically expressed NESG1 in the NPC cells HONE1 and SUNE1. The mRNA and protein levels were significantly upregulated in NESG1-overexpressed cells compared with respective control cells via quantitative real-time PCR and western blot analysis (Figure 1A). Consistent with previous research, NESG1 markedly inhibited the metastatic ability in NPC cells HONE1 and SUNE1 by the Transwell and Boyden assays (Figures 1B and 1C). Further, western blot analysis showed that ectopic expression of NESG1 suppressed the Wnt/β-catenin signaling pathway in HONE1 and SUNE1 cells. The expression levels of β-catenin, nuclear β-catenin, TCF4, c-Myc, N-cadherin, and vimentin were downregulated, while E-cadherin was upregulated (Figure 1D).

miR-1254 is induced by NESG1 through Wnt/β-catenin/c-Myc

To determine NESG1-targeted miRNAs in NPC, we used the miRNA qPCR assays on NESG1-overexpressing SUNE1 cells (Figure 2A). miR-1254 was confirmed to be a positive downstream modulator of NESG1 via quantitative real-time PCR in NESG1-overexpressing NPC cells (Figure S1A). Reduction of miR-1254 by a specific inhibitor in HONE1 and SUNE1 cells appeared to reverse the NESG1-suppressed EMT effect based on the Transwell and Boyden assays (Figure 2B).

To explore the regulating mechanisms of NESG1 on miR-1254 expression, we used PROMO and TFSEARCH bioinformatics software to predict the possible transcription factor-binding sites (TFBSs). Two c-Myc-binding motifs at 638 to 643 and 670 to 675 were identified inside the putative miR-1254 promoter region (Figure S1B). Quantitative real-time PCR analysis indicated that reduced c-Myc expression markedly upregulated miR-1254 expression in both cell lines (Figure S1C). Since c-Myc acts as the downstream factor activated by Wnt/β-catenin signaling, we chose c-Myc as the transcription factor of miR-1254. Chromatin immunoprecipitation (ChIP) assay was used to confirm the binding between c-Myc and miR-1254 promoter in HONE1 and SUNE1 cells (Figure 2C). We performed an electrophoretic mobility shift assay (EMSA) to determine whether the nuclear extracts of NPC cells bind to the predicted P1 and P2 sites (Figure 2D). Also, the wild-type miR-1254 promoter luciferase activity was attenuated when sites A and B were mutated in HEK293T cells (Figure S1D). These results demonstrated that the two c-Myc-bound miR-1254 promoter region sites were activated. Furthermore, ChIP assays showed that elevated NESG1 attenuated the binding between c-Myc and promoter regions of miR-1254 (Figure 2E).

Pathoclinical features of miR-1254 expression

The *in situ* hybridization assay was used to confirm the expression of miR-1254 in NPC tissues compared to nasopharynx (NP) tissues (Figure 3A; Table S4). Moreover, we observed that high miR-1254 expression was negatively correlated with clinical stage (I–II versus III–IV) and lymph node metastasis (N classification; N0–N1 versus N2–N3). The clinical characteristics of the NPC patients are summarized in Table S5. Further, survival analysis presented that NPC patients with high miR-1254 expression had longer survival times than those with low expression (Figure 3B). A multivariate analysis

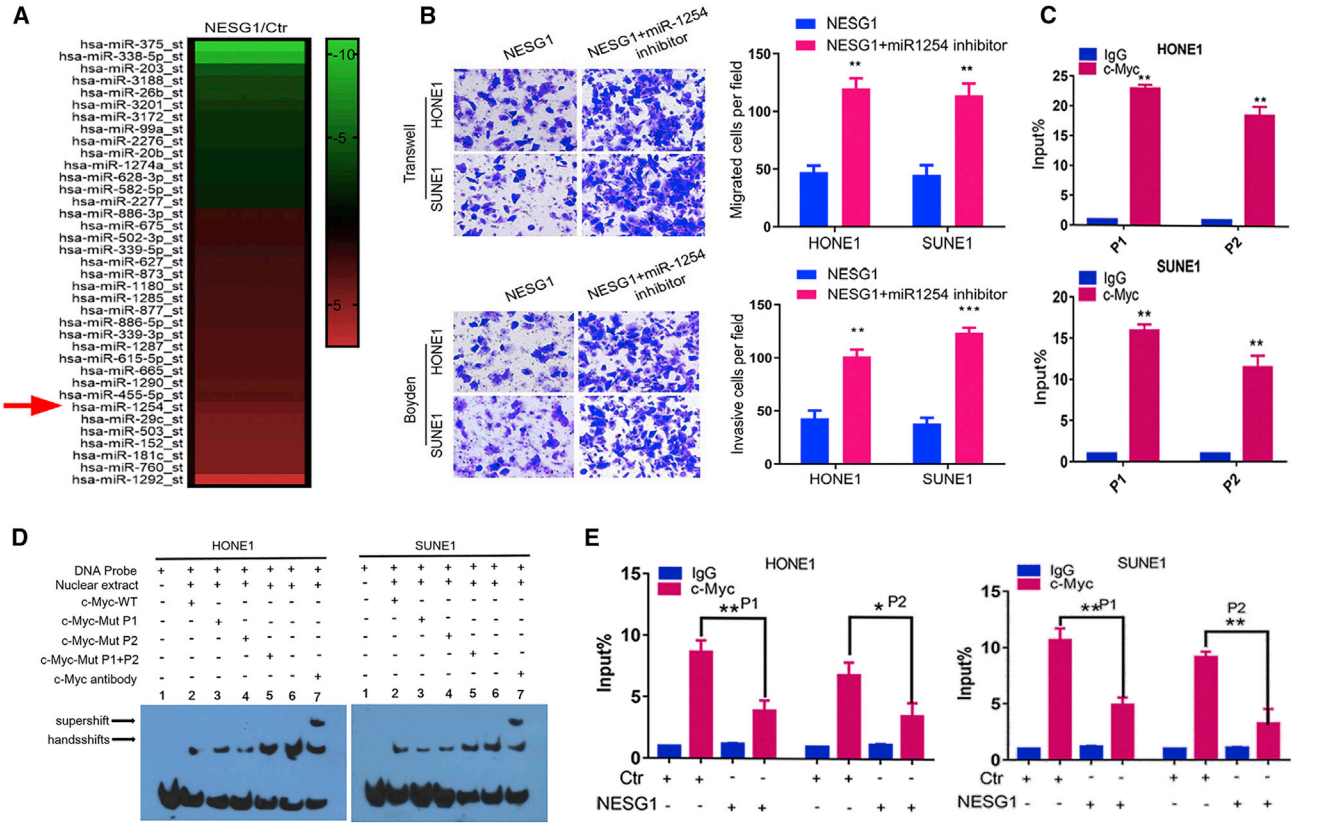

Figure 2. miR-1254 is induced by NESG1 through wnt/β-catenin/c-Myc

(A) Hierarchical cluster analysis of differentially expressed miRNAs between SUNE1-NESG1 and SUNE1-Ctr cells. Red represents upregulated miRNAs, and green represents downregulated miRNAs. (B) Transwell and Boyden assays for HONE1 and SUNE1 cells treated with NESG1 or both NESG1 and miR-1254 inhibitor. Original magnification, ×200; scale bar, 25 μm. **p < 0.01, ***p < 0.001. (C) Quantitative real-time PCR and PCR gel detected the binding sites P1, P2, and P1+P2; IgG antibody served as the negative control. **p < 0.01. (D) EMSA and supershift assay of c-Myc binding to miR-1254 promoter in HONE1 and SUNE1 cells. (E) ChIP experiment and the gel figures showing amplification of c-Myc-binding sites P1 and P2 in NESG1 overexpressing cell by quantitative real-time PCR and PCR gel; IgG antibody was used as the negative control. *p < 0.05,**p < 0.01.

showed that the level of miR-1254 expression was an independent prognostic factor for NPC (p = 0.007; Table S6). Further, levels of miR-1254 and NESG1 were significantly decreased in NPC tissues compared to NP tissues by quantitative real-time PCR analysis (Figure 3C). Moreover, NESG1 levels were positively correlated with miR-1254 expression (Figure 3D).

miR-1254 suppresses NPC metastasis *in vivo* and *in vitro*

To explore the biological role of miR-1254 in NPC, we inserted mimics and a specific inhibitor of miR-1254 in HONE1 and SUNE1 cell lines. The infection efficiency was examined by quantitative real-time PCR (Figure 4A). Compared with negative controls, the mimics of miR-1254 reduced cell migration and invasion, as measured by the Transwell and Boyden assays. The specific inhibitor of miR-1254 reversed the effect of the mimics (Figure 4B). Further, we constructed lentivirus carrying miR-1254 sequence, and its flanking control sequence established HONE1-Lv-miR-1254 and SUNE1-Lv-miR-1254 stable cell lines. Fluorescence microscope detection and quantitative real-time PCR confirmed the stable overexpression in these cells (Figures 4C and 4D). We inoculated LV-miR-1254-GFP HONE1 and SUNE1 cells under the liver capsules of nude mice. In the HONE1 group, eight of ten mice of NC group and two of ten mice of miR-1254 group intrahepatic and intestinal metastasis occurred. In the SUNE1 group, metastasis occurred in nine of ten mice in the NC group and three of ten mice in the miR-1254 group (Figures 4E and 4F).

miR-1254 directly targets HDGF

TargetScan and RNAhybrid algorithms were used to predict the direct target of miR-1254. HDGF was predicted to be a possible target (Figure 5A). Our previous study determined that HDGF could induce Wnt/β-catenin signaling in lung adenocarcinoma.[15] Therefore, we hypothesized that miR-1254 suppressed NPC metastasis by modulating HDGF. Overexpression of miR-1254 downregulated the expression levels of HDGF protein, whereas a miR-1254 inhibitor reversed the effect (Figure 5B). Meanwhile, we observed that levels of HDGF were elevated in NPC tissues compared to NP tissues (Figure 5C). HDGF levels were negatively correlated with NESG1 as well

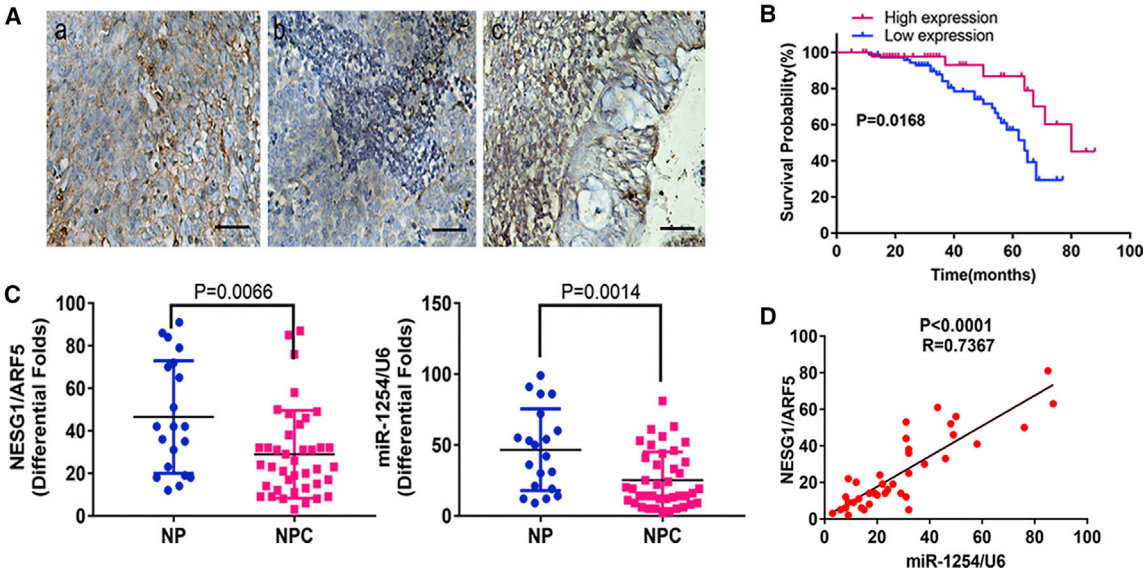

Figure 3. Pathoclinical features of miR-1254 expression

(A) miR-1254 expression in NPC and NP samples; (a) strong expression of miR-1254 in NPC samples; (b) weak staining of miR-1254 in NPC samples; and (c) positive expression of miR-1254 in NP samples (original magnification, ×400). Scale bar, 30 mm. (B) Kaplan-Meier survival analysis of overall survival of 120 NPC patients (log-rank test, p = 0.0168). (C) Decreased expression of NESG1 and miR-1254 was detected in NPC specimens compared to nasopharyngeal tissues by quantitative real-time PCR, normalized to the ARF5 and U6, respectively. Student's t test, mean ± SD, p = 0.0066; p = 0.0014. (D) miR-1254 expression was positively correlated with expression of NESG1 mRNA in NPC tissues.

as miR-1254 in the same NPC tissues (Figure 5D). Co-transfection of mimics of miR-1254 and HDGF 3′ UTR wild-type (WT) vector decreased the luciferase reporter activity compared with the vector group (Figure 5E, lanes 1 and 2), while miR-1254 inhibitor had the opposite effect (Figure 5E, lanes 3 and 4). These effects were absent while co-transfecting both mutated HDGF reporter and the mimics , or inhibitor of miR-1254 (Figure 5E, lanes 5 and 6). As shown in Figure 5F, the mRNA level of Ago2 binding between miR-1254 and HDGF in SUNE1 cells was increased. Further, the enrichment of HDGF mRNA in Ago2-immunoprecipitation (Ago2-IP) was also increased by miR-1254 (Figure 5G). Inserting HDGF plasmid into miR-1254-overexpressing NPC cells enhanced cell metastasis based on the Transwell and Boyden assays (Figure 5H). Moreover, HDGF notably restored the β-catenin/Transcription Factor 4 (TCF4) transcriptional activity in the miR-1254-overexpressing cells (Figure 5I) and reversed miR-1254-modulation of β-catenin, TCF4, c-Myc, E-cadherin, N-cadherin, and vimentin (Figure S2A).

miR-1254 promotes the suppressive effect of NESG1 by regulating HDGF/DDX5/β-catenin signaling

Endogenous (Figure 6A) and exogenous (Figure 6B) coIP showed an interaction of DDX5 with HDGF and β-catenin. Nuclear colocalization of HDGF and DDX5 proteins was observed by immunofluorescence (IF) (Figure 6Ca). Subsequently, the nuclear extract experiment showed that the β-catenin nuclear protein was upregulated in DDX5-overexpressed cells compared with the control group (Figure S2B) and co-localized with DDX5 after DDX5 was overexpressed (Figure 6Cb). Inserted DDX5 plasmid revered the metastasis ability of the NESG1 or miR-1254-overexpressing SUNE1 cells (Figures S3A and S3B). In subsequent investigations, we observed that HDGF suppression resulted in the downregulated mRNA and protein levels of DDX5 (Figure 6D). A previous study suggested that the β-catenin/TCF4 complex could induce the transcription of DDX5 promoter. The schematic graph of the promoter region of DDX5 with TCF4 is shown in Figure S2C.[15] Consistent with the previous study, knockdown HDGF or β-catenin reduced the TCF4 combination with DDX5 promoter in NPC cells (Figure 6E). Moreover, the suppression of DDX5 not only decreased the protein expression levels of HDGF, β-catenin, TCF4, and c-Myc (Figure 6F) but also reduced HDGF mRNA (Figure 6G). Further, DDX5 knockdown attenuated β-catenin/TCF4 transcriptional activity in HDGF-overexpressing cells (Figure S2D). Overexpression of miR-1254 suppressed HDGF and DDX5 levels simultaneously (Figure S2E). Western blot analysis showed that the expression levels of HDGF, DDX5, β-catenin, nuclear β-catenin, TCF4, c-Myc, N-cadherin, and vimentin were upregulated and E-cadherin was downregulated after treatment with the specific inhibitor of miR-1254 into NESG1-overexpressing NPC cells (Figure S2F).

DISCUSSION

NPC is a malignant carcinoma with high invasiveness and metastasis potential. Although the sensitivity of NPC to radiotherapy and chemotherapy have significantly improved the local control, distant metastasis remains a challenging problem.[16,17] Searching for the new molecular mechanisms of NPC metastasis will provide important

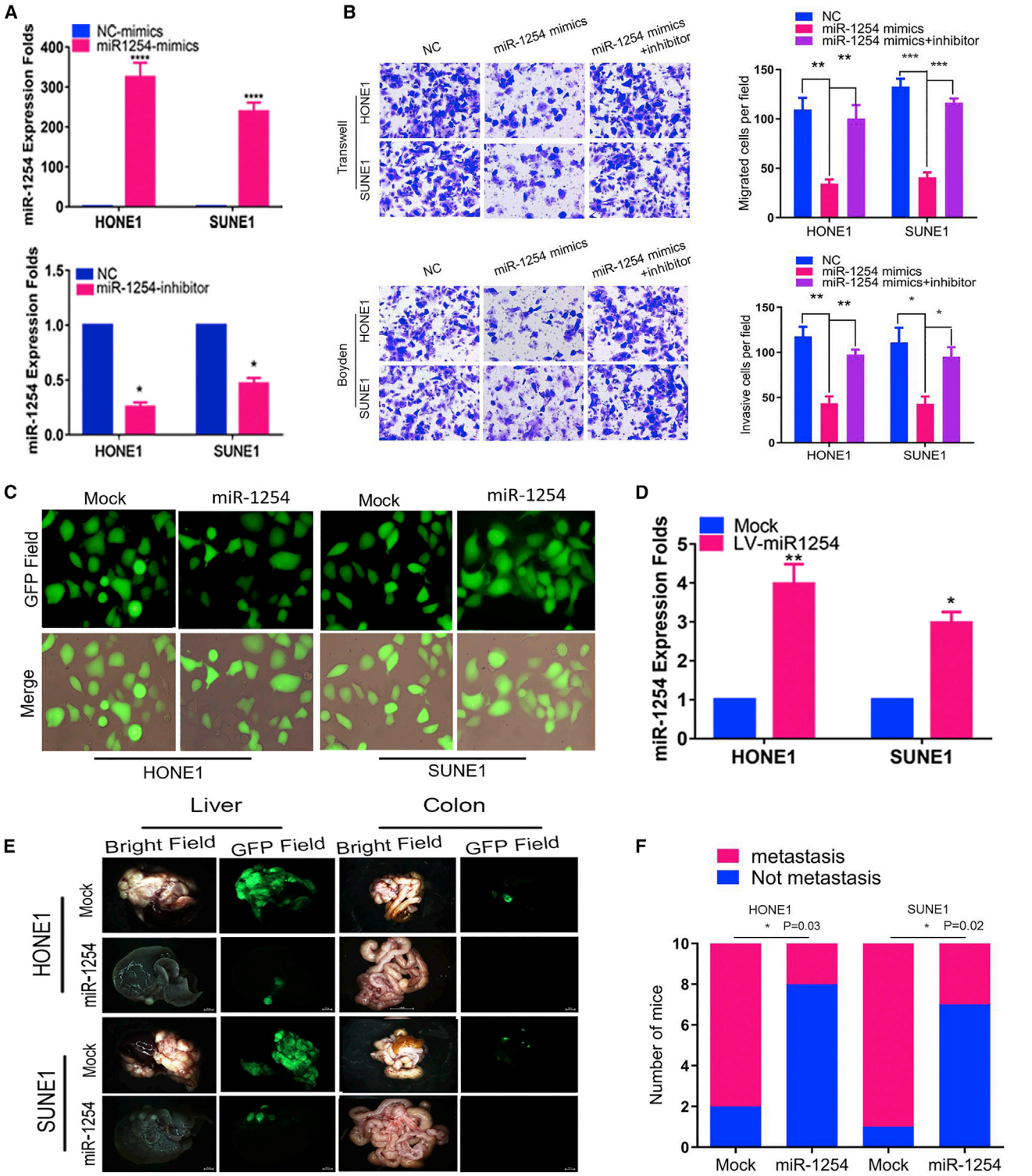

Figure 4. miR-1254 suppresses NPC metastasis *in vivo* and *in vitro*

(A) miR-1254 expression was detected in NESG1-overexpressing HONE1 and SUNE1 cells by real-time PCR. Student's t test, mean ± SD, *$p < 0.05$, normalized to U6. (B) Transwell and Boyden assays for HONE1 and SUNE1 cells transfected with either miR-1254 mimics or inhibitor compared with negative control. Original magnification, ×200; scale bar, 25 μm. *$p < 0.05$, **$p < 0.01$,***$p < 0.001$. (C) Fluorescence microscope detection confirmed the stable overexpression in HONE1 and SUNE1 cells after

(legend continued on next page)

insights into the etiology of NPC and improve the treatment strategies for tumor metastasis.[18]

In previous studies, we cloned and revised a new gene NESG1[4] in human nasopharyngeal mucosa. Subsequent investigations indicated that NESG1 acted as a tumor suppressor in NPC, NSCLC, and colorectal cancer.[5–8] The precise mechanism for its suppressive role in NPC metastasis remained unclear.

It is well known that dysregulation of Wnt/β-catenin and its downstream EMT signaling participates in the promotion of tumor metastasis.[19,20] In this study, we observed that NESG1 suppressed migration and invasion by reducing the levels of nuclear β-catenin, TCF4, and its downstream EMT signals, such as the c-Myc gene. These investigations further supported NESG1 as a tumor metastasis suppressor in NPC.

To deeply explore the molecular basis of NESG1 in suppressing NPC migration and invasion, we used a miRNA array to detect NESG1-modulated miRNAs in NESG1-overexpressing NPC SUNE1 cells and the control cells. Interestingly, miR-1254 was markedly induced by NESG1 in NPC. However, the molecular basis of NESG1-stimulated miR-1254 is still undetermined.

In previous studies, transcription factors have been shown to mediate the modulation of miRNAs.[21–23] c-Myc serves as an important oncogenic transcription factor that is overexpressed in most human malignancies, including NPC,[24,25] and modulates the expression of some miRNAs in tumors.[26,27] Besides its canonical function as a transcriptional activator, MYC also suppressed various genes, being involved in processes such as cell cycle progression and cell adhesion.[28,29] Subsequently, we used bioinformatics software to predict that there were binding sites of c-Myc in the miR-1254 promoter. In line with this prediction, we confirmed that c-Myc pre-transcriptionally modulated miR-1254 expression in NPC.

Also, c-Myc acts as a downstream transcription factor activated by wnt/β-catenin signaling.[24] Interestingly, we found NESG1 suppressed β-catenin/c-Myc signal and, therefore, stimulated miR-1254 expression in NPC.

In prior investigations, miR-1254 has been reported to have a dual role in tumor progression. It serves as a tumor suppressor in cervical cancer, gastric cancer, and oral squamous cell carcinoma[30–32] and exerts oncogenic effects in breast cancer and lung cancer.[33,34] In this study, we investigated the expression of miR-1254 in NPC. The data showed that miR-1254 levels were significantly decreased in NPC tissues compared to NP tissues. High miR-1254 expression was negatively correlated with clinical stage and lymph node metastasis but positively correlated with overall survival time and acted as an independent prognostic factor for NPC. Moreover, we also observed that miR-1254 was positively correlated with NESG1 expression in NPC. Reduced miR-1254 was shown to be an unfavorable factor in NPC pathogenesis. These data suggested that miR-1254 might function as a tumor suppressor, which was similar to the role of NESG1 in NPC.

To confirm this hypothesis, we further explored the function and molecular basis of miR-1254 in NPC. Consistent with our hypothesis, we observed that miR-1254 suppressed NPC migration, invasion, and metastasis. In mechanism analysis, we initially combined bioinformatics analyses with a luciferase reporter and RNA IP (RIP) assays and validated HDGF as the target of miR-1254. HDGF is a multifunction oncogene participating in carcinogenesis of various cancers.[35–37] Anti-HDGF, by its neutralizing antibody, repressed cancer stem cells (CSCs) to prevent relapse of NSCLC by improving sensitivity to chemotherapy, suggesting its significance in tumor pathogenesis.[37] In a prior study, we verified that elevated nuclear expression of HDGF is a potential unfavorable prognostic factor for NPC patients.[38] Another study indicated that HDGF interacted with DDX5 oncogene and induced nuclear translocation of β-catenin to stimulate TCF4 and its downstream EMT signal and thus promoted lung adenocarcinoma metastasis.[15] In subsequent investigations, we found that overexpressed HDGF reversed miR-1254-mediated suppression by activating DDX5/β-catenin/TCF4-stimulated EMT signal. These data demonstrated that similar to NESG1, miR-1254 functioned as a tumor-metastatic suppressor in NPC.

To determine whether miR-1254 mediated NESG1 suppression in NPC, we used miR-1254 inhibitor in NESG1-overexpressed NPC cells. The data indicated that NPC migration and invasion abilities were increased. Molecular basis revealed that suppression of miR-1254 markedly restored the activation of HDGF/DDX5/β-catenin/TCF4 and its downstream EMT signal.

Conclusions

These data demonstrated that miR-1254 participated in NESG1-induced suppression in NPC. A growing number of studies have suggested that ectopic miRNA expression could display antitumor effects. Our findings may offer a new therapeutic strategy or a potential biomarker for NPC metastasis through adjusting miR-1254 expression.

MATERIALS AND METHODS

Cell culture

The NPC cells HONE1 and SUNE1 (resource, the Cancer Center of Sun Yat-sen University, Guangzhou, China) were cultured in 10% fetal calf serum (FCS; Invitrogen)-RPMI-1640 (Hyclone) with a humid atmosphere of 5% CO_2 at 37°C. HEK293T cells (resource, Cancer Research Institute of Southern Medical University, Guangzhou,

transfection by mock or LV-miR-1254. Scale bar, 15 μm. (D) Real-time PCR showing the effectiveness of HONE1 and SUNE1 cells after transfection with mock or LV-miR-1254; U6 used as an internal control. Student's t test, mean ± SD, *$p < 0.05$, **$p < 0.01$. (E) Fluorescent images are shown for four representative mouse models. (F) Graph describes the numbers of mice that occur metastasis or not; Fisher's exact test, *$p < 0.05$.

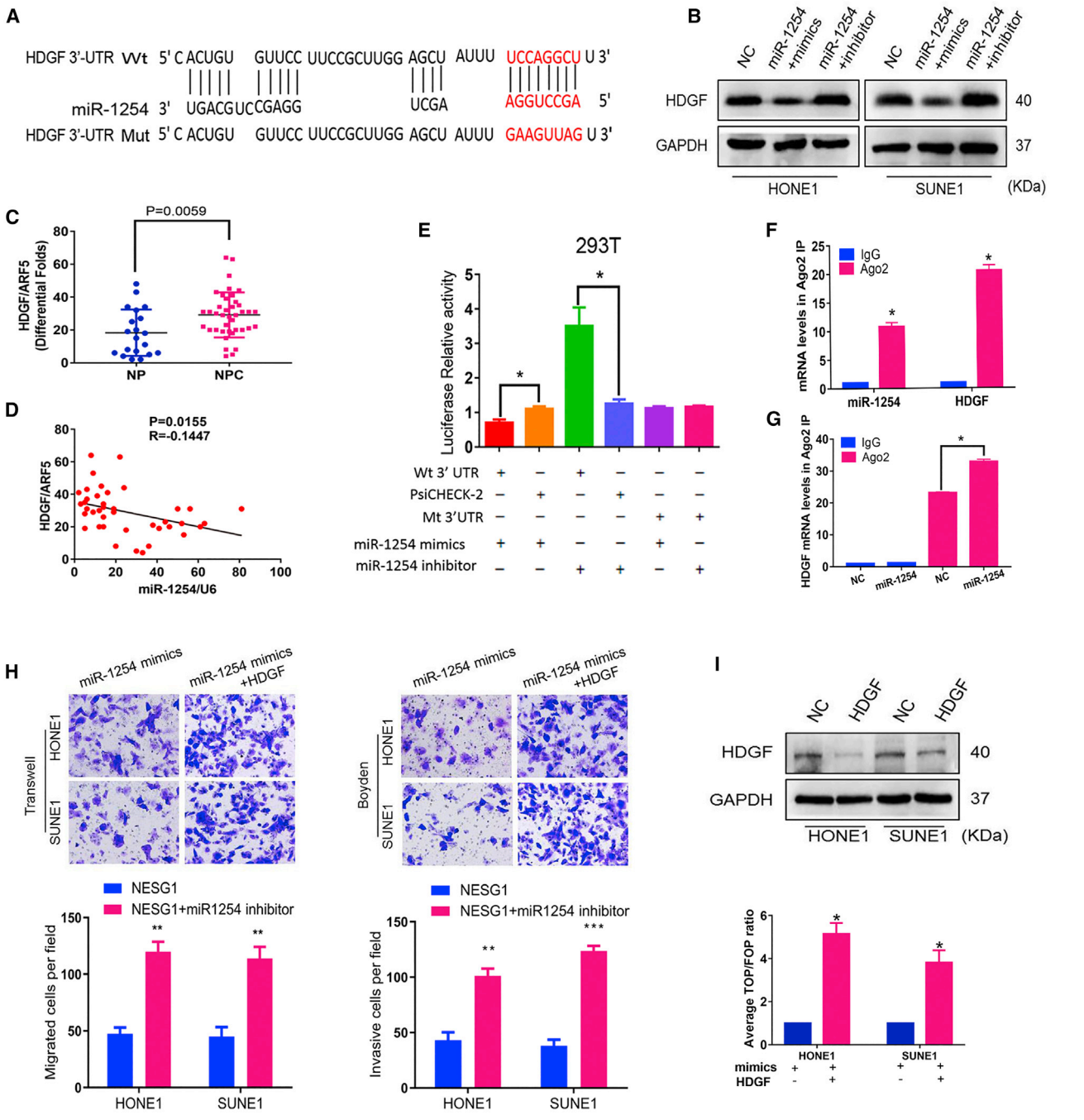

Figure 5. miR-1254 directly targets HDGF

(A) Graph showed the binding sequence of the 3′ UTR region of HDGF and seed sequence of miR-1254. (B) HDGF protein levels in miR-1254-overexpressing/suppressing NPC cells by western blot. (C) Increased expression of HDGF was detected in NPC specimens compared to nasopharyngeal tissues by quantitative real-time PCR, normalized to the ARF5; Student's t test, mean ± SD, p = 0.0059. (D) miR-1254 expression was negatively correlated with expression of HDGF mRNA in NPC tissues. (E) miR-1254 directly targets HDGF as confirmed by dual-luciferase reporter assay. One-way ANOVA and Dunnett's multiple comparison test. *p < 0.05. (F) miR-1254 and HDGF mRNA were increased in Ago2-IP. (G) HDGF mRNA were increased in miR-1254 binding-Ago2 complex after miR-1254 was overexpressed. Student's t test, *p < 0.05. (H) Transwell and Boyden assays of miR-1254-overexpressing cells or treated with HDGF plasmid and mimics. Scale bar, 50 μm, Student's t test, one-way ANOVA, *p < 0.05, **p < 0.01. (I) HDGF levels were detected by western blotting after transfection with with plasmid in HONE1 and SUNE1 cells, and HDGF elevated the TOP/FOP luciferase activity in miR-1254-overexpressing HONE1 and SUNE1 cells. Student's t test, *p < 0.05.

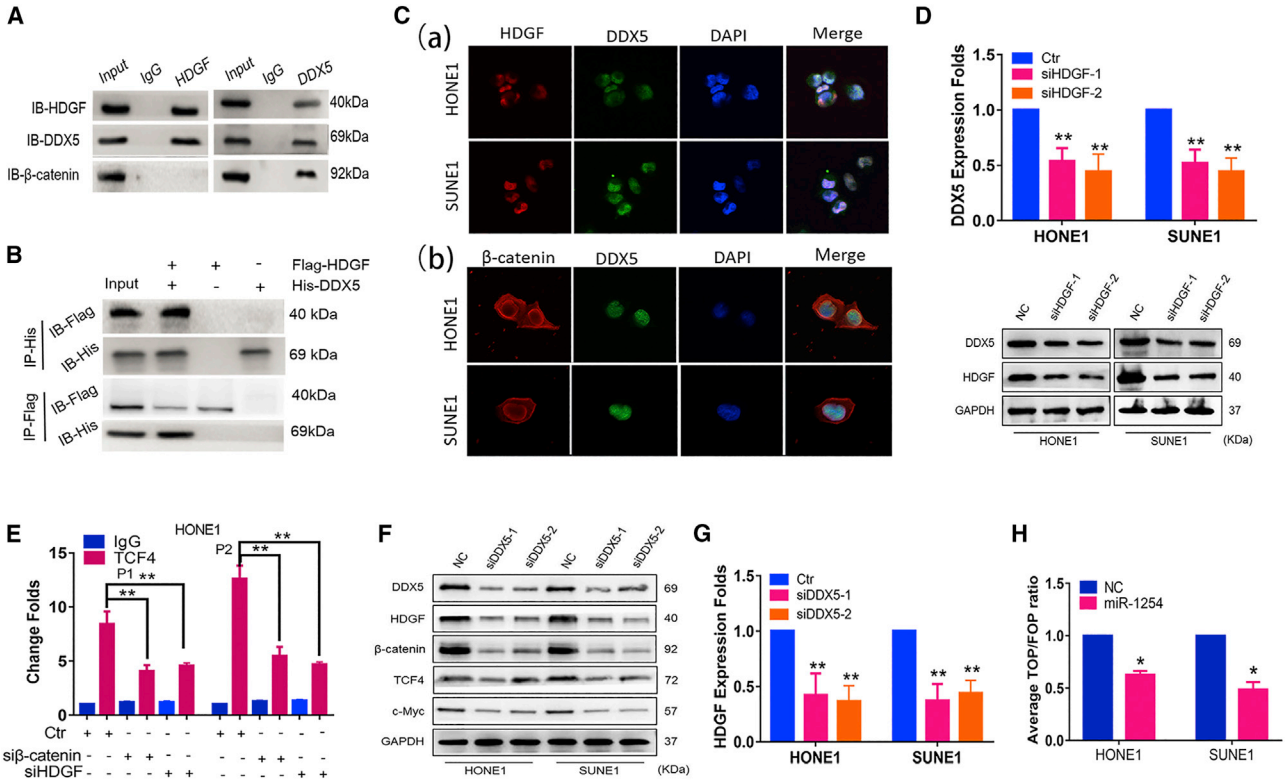

Figure 6. miR-1254 promotes the suppressive effect of NESG1

(A and B) Endogenous (A) and exogenous (B) coIP detected the interaction of HDGF and DDX5 in HONE1 cells. (C) Nuclear co-localization of HDGF protein and DDX5 protein, DDX5, and β-catenin in HONE1 and SUNE1 cells by immunofluorescence staining. Scale bar, 25 μm. (D) DDX5 mRNA and protein expression was reduced after HDGF knockdown by western blot and quantitative real-time PCR. Student's t test, $^{**}p < 0.01$. (E) β-catenin and HDGF siRNA reduced binding of TCF4 with DDX5. Student's t test, $^{**}p < 0.01$. (F) DDX5 siRNA decreases HDGF, β-catenin, TCF4, and c-Myc expression. (G) DDX5 siRNA decreases HDGF mRNA expression levels. One-way ANOVA and Dunnett's multiple-comparison test, $^{**}p < 0.01$. (H) The TOP/FOP luciferase activity was decreased in miR-1254-overexpressing HONE1 and SUNE1 cells. Student's t test, $^{*}p < 0.05$.

China) was grown in 10% newborn calf serum (NCS)-Dulbecco's modified Eagle's medium (Invitrogen) with a humid atmosphere of 5% CO_2 at 37°C.

Tissue specimens

40 NPC and 20 non-cancerous nasopharyngeal samples were kindly provided by Nan Fang Hospital, China. 120 NPC and 31 paraffin-embedded NP specimens were collected in Nan Fang Hospital, China. Before using these clinical materials for research purposes, consent of the patient and the approval of the Ethics Committee of Nan Fang Hospital were obtained.

Ago2 IP

RIP assays were performed with the EZ-Magna RIP RNA-binding protein IP kit (Millipore) according to the manufacturer's protocol as previously described.[36]

In situ hybridization

In situ hybridization was performed as previously described.[36] Tissue sections were hybridized with DIG-labeled probes after being dewaxed in xylene and treated with 3% H_2O_2. They were further incubated in blocking solution and then phosphatase-conjugated with sheep anti-DIG Fab fragments. Positive staining of miR-1254 was observed by adding AP substrate.

Lentivirus infection and cell transfection

Lentiviral vector with hsa-miR-1254 precursor and mock sequence were designed by GeneChem (Shanghai, China). The infection was applied according to the manufacturer's instructions with lentiviral vector. miR-1254 mimics and its inhibitors and small interfering RNAs (siRNAs) for HDGF, DDX5, and c-Myc were bought from RiboBio Technologies. (Guangzhou, China; Table S1). Plasmids for HDGF, NESG1, and DDX5 were purchased from Vigenebio (Guangzhou, China). Cells were transfected according to the manufacturer's protocol.

Quantitative real-time PCR

RNA was isolated from NPC cell lines, tissues, and normal NP tissues by Trizol (Takara Bio, Shiga, Japan). Quantitative real-time PCR was performed as described previously. U6 and ARF5 genes were used as

miRNA and gene internal controls, respectively. Specific sense primers for miR-1254, c-Myc, HDGF, U6, and ARF5 are shown in Table S2.

Western blotting

Western blot was performed as previously described. The resource of antibodies are presented in Table S3. The images were recorded with Molecular Imager (Bio-Rad, USA). All the figures correspond to the locations of molecular weight markers.

Immunofluorescent staining

The coverslips with NPC cells were blocked with 0.3% Triton X-100 fixed after fixed. Subsequently, the coverslips were incubated with primary monoclonal antibodies against HDGF, DDX5, and β-catenin and then Alexa Fluor 596 goat anti-rabbit immunoglobulin G (IgG) and Alexa Fluor 488 goat anti-mouse IgG. The coverslips were stained with DAPI and observed by an LSM80 fluorescent microscope (ZEISS, Germany).

miRNA array

Total RNA was isolated from SUNE1 NESG1-overexpressing and NC cells. The miRNA array was as described previously. Statistical analysis was carried out using the open-source R software.

TCF and luciferase reporter activity assay

293T cells were respectively transfected with TOPFLASH and FOPFLASH vector, psiCHECK-2 vectors, HDGF 3′ UTR WT/mt vectors, pGL3-basic luciferase reporter vector, c-Myc-binding site mutation vectors (primer sequences are listed in Table S2) with miR-1254 mimics or inhibitor. Luciferase activity was measured were examined by the dual-luciferase reporter agent (Promega, USA) after being harvested as previously described.[36]

CoIP

CoIP was carried out as previously described. The total protein was incubated with antibodies and then mixed with the beads. Finally, the mixed sample was eluted and boiled. Immune mixtures were analyzed by western blot.

Migration and invasion assay

Cells were added to the upper chamber with serum-free culture medium and filled the lower chamber with the culture medium (10% fetal bovine serum [FBS]). After 10–15 h, the filter was fixed, stained, and imaged as previously described.[36]

Animal experiments

All animal experiments were approved by the Animal Care and Use Committee of Southern Medical University. 4- to 5-week-old female mice (BALB/c, nu/nu) were used. 1×10^6 cells were injected into the liver membrane. Metastatic liver nodules were captured by stereo microscope (ZECC, USA). Further staining was observed by fluorescent microscope (Nikon, Japan).

EMSA

The EMSA was performed as previously described.[36] The binding mixture was incubated in specific oligonucleotide competitor or not with the biotin-labeled wild-type probe. Signals were recorded using a gel imaging system (BIOTOP, Guangzhou, China). The probe sequences are shown in Table S2. EMSA analysis was performed at Biosense Bioscience.

Statistical analysis

SPSS 21.0 was used for statistical analyses. Data are expressed as mean ± SD and independent experiments were repeated at least three times. Student's t test was used for two groups, and one-way analysis of variance (ANOVA) analysis was used for multiple groups. The Kaplan-Meier method was performed by survival analysis. All statistical tests were statistically significant differences at a $p < 0.05$.

SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.omtm.2021.02.001.

ACKNOWLEDGMENTS

This study was supported by the National Natural Science Fund of China (nos. 81071632 and 30870973); the Nature Science Key Fund of Guangdong Province (no. 2015A030311005); the Guangdong Science and Technology Project (no. 2016A020215233); the Nature Science Fund of Guangdong Province (nos. 2016A030313628, 2016A030313526 and 2017A030313701); the National Funds of Developing Local Colleges and Universities (no. B16056001) and Shenzhen Key Laboratory of Viral Oncology (ZDSYS 201707311140430).

AUTHOR CONTRIBUTIONS

Conceptualization, C.C., W.L., and X.D.; methodology, X.P., X.D., and W.F.; investigation, C.C., X.L., X.P., Z.Z., T.D., Z.L., R.L., W.F., and X.D.; writing – original draft, X.P.; writing – review & editing, R.L., W.F., and X.D.; funding acquisition, W.F.; resources, W.F.; supervision, X.D.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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