MicroRNA-145-5p modulates KLF5 and inhibits the migration and invasion in nasopharyngeal carcinoma

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Research Article

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Abstract

Human krüppel like factor 5 (KLF5) is a zinc finger transcription factor, which contributes to tumor suppression in nasopharyngeal carcinoma (NPC). However, the precise role of KLF5 in NPC remains poorly understood. In the present study, the association between KLF5 and miR-145-5p in NPC cells was elucidated. Cellular proliferation was detected using the WST-1 colorimetric assay. Cellular migration and invasion were studied using wound healing assays and the ECMatrix cell invasion assay, respectively. The protein expression was examined by western blot. Transfection was subsequently performed for generating NPC cells overexpressing KLF5 and miR-145-5p. The upregulation of KLF5 or miR-145-5p inhibited the migration and invasion of NPC cells. It was observed that miR-145-5p could induce the expression of the KLF5 protein in the NPC cell lines. Additionally, the activity of the migration marker, focal adhesion kinase (FAK), was suppressed following the overexpression of miR-145-5p and KLF5 in NPC cells. The results of this study indicated that miR-145-5p may repress the migration and invasion of NPC cells via KLF5, and could be a potential therapeutic target for NPC.

Introduction

Nasopharyngeal carcinoma (NPC), which develops in the nasopharyngeal epithelium, is one of the most common squamous cell carcinomas of the head and neck in East and Southeast Asia [1]. Compared to the other head and neck cancers, metastasis of the neck lymph node and distant metastasis are commonly observed in NPC [2], making its treatment a challenging one. The long-term survival rate of patients with NPC has improved with the advancement in intensity-modulated radiation therapy and adjuvant chemotherapy, however, local relapse and distant metastasis continue to remain the leading causes of mortality [3]. In Taiwan, according to the Cancer Registry Annual Report that was published by the Health Promotion Administration Ministry of Health and Welfare in December 2018, there were 1,518 new cases of NPC and 678 deaths due to NPC in 2016 alone [4]. Therefore, the molecular mechanisms underlying the tumorigenesis and malignant progression of NPC is important issue for effective diagnosis and therapy.

MicroRNAs (miRNAs) are 22–24 nucleotide non-coding RNAs that regulate gene expression by targeting the 3'-UTR of the target mRNA [5, 6]. The incidence of several human tumors is correlated with altered miRNA expression, and the abnormal expression of miRNAs has been observed in several malignancies [7]. In the recent years, an increasing number of studies are being conducted on the expression of microRNAs (miRNAs) in human malignancies. Several miRNAs have been shown to be involved in NPC pathogenesis through alteration of gene networks.

Krüppel like factor 5 (KLF5) is a zinc finger transcription factor that contributes to tumor suppression in NPC [8]. However, KLF5 has an oncogenic role in certain types of cancer, such as pancreatic cancer, breast cancer, cervical cancer and colorectal cancer [9–12]. The miR-493-5p represses the metastasis and proliferation of osteosarcoma cells by targeting KLF5 [13]. MiR-153 suppresses cellular invasion by inhibiting KLF5 in laryngeal squamous cell carcinoma [14]. Therefore, the molecular mechanisms

underlying the associations between the miRNAs and KLF5 in NPC are yet to be studied. The expression of miR-145-5p, a well-known tumor suppressor miRNA, is downregulated in several types of cancer, including NPC [15, 16]. Previous studies have demonstrated that miR-145-5p suppresses non-small cell lung cancer cell invasion [17]. Clinically, a lower expression of miR-145-5p has been associated with the poor prognosis of osteosarcomas [18].

In this study, we aimed to elucidate the mechanism by which KLF5 affects the development of NPC. The results demonstrated that KLF5 is modulated by miR-145-5p and regulates the tumorigenesis of NPC. The results of this study could aid the development of therapeutic strategies against NPC.

Materials And Methods

Cell culture

Nasopharyngeal carcinoma cells line HONE-1 and NPC-TW01 were kindly provided by Professor Chang-Shen Lin at Kaohsiung Medical University. Two cell lines were cultured in Dulbecco's Modified Eagle Medium (DMEM, Gibco™, Thermo Fisher Scientific, MA, USA) supplemented with antibiotic-antimycotic (Gibco™, Thermo Fisher Scientific, MA, USA) and 10% fetal bovine serum (FBS, Gibco™, Thermo Fisher Scientific, MA, USA) at 37°C in a 5% CO_2 air atmosphere.

Cell transfection

NPC cells were transfected with synthetic hsa-miR-145-5p mimics (mirVana® miRNA mimic, Thermo Scientific™, MA, USA) to overexpress miR-145-5p. Scramble form of miRNA was used as a control (mirVana® miRNA mimic, Thermo Scientific™, MA, USA). KLF5 overexpression plasmids, GFP-KLF5 and CMV-KLF5 were purchased from Sino Biological, Wayne, PA, USA. Cell transfections were performed using TurboFect (Thermo Scientific™, MA, USA) according to the manufacturer’s protocol.

Cell proliferation assay

Cell proliferation was analyzed by Premix WST-1 Cell Proliferation Assay System (TaKaRa, Mountain View, CA, USA), at least, triplicate samples according to the manufacturer’s protocol. Briefly, HONE-1 ($1x10^3$ cells/well) and NPC-TW01 ($1x10^3$ cells/well) cells, after transfection, were seeded in 96-well plates in 100 µL medium and were incubated at 37°C. After incubation for 24, 48, and 72h, 10 µL of WST-1 solution was added to each well and incubated for 1 h. Finally, cell proliferation was analyzed by measuring absorbance at 450 nm in a spectrophotometer.

Wound-healing assay

Cell migration was estimated by wound-healing assay. Briefly, NPC cells were counted and seeded in 2-well culture-inserts (ibidi, Gräfelfing, Germany) after transfection. After 24 h of incubation, the insert was removed to create the gap. The gap of NPC cells was photographed at different time points using a phase contrast microscope. The wound healing area was analyzed by Image J software, and wound closure percentage was calculated as reported by Ayman Grada el. [19].

Cell invasion assays

Cell invasion ability was measured by QCM ECMatrix cell invasion assay (Merck, Darmstadt, Germany) according to the manufacturer's protocol. Briefly, NPC cells were transfected and seeded (1.5×10^5) into the upper chamber with serum-free medium and incubated onto the lower chamber having serum-containing medium for 24 h at 37°C. The cells migrated through the ECM layer and clung to the bottom of the polycarbonate membrane. Invaded cells were incubated with cell detachment buffer, and then lysed and stained with CyQuant GR® dye (Merck, Darmstadt, Germany). Finally, the fluorescence was measured using a fluorescence plate reader through 480/520 nm filter set.

Protein lysate preparation and Western blot analysis

The cell lines were plated in 6 cm dish using a density of 2×10^5 cells and were allowed to grow to 60% confluence. Forty-eight hour after transfection of microRNA mimics, the cell lines were washed twice with cold PBS, lysed in cell lysis buffer (Cell signaling) for 10 min and scraped. The extracts were centrifuged at 13500 g for 10 min at 4°C. Protein concentrations were measured and equalized using Bio-Rad protein assay (Bio-Rad Laboratories) according to the manufacturer's instructions. Equivalent amounts of protein (30 μg) were then separated by SDS-PAGE and then transferred to polyvinylidene difluoride membranes (PVDF). The PVDF membranes were blocked for 1 h in blocking buffer (1X Tris-buffered saline, 5% nonfat dry milk, and 0.1% Tween 20), which was subsequently replaced by the primary antibody in blocking buffer, overnight at 4°C. After incubation, the membranes were washed three times in washing buffer (1X Tris-buffered saline and 0.1% Tween 20). Primary antibody was detected using AffiniPure Mouse or Rabbit Anti-Human IgG (Jackson ImmunoResearch, USA) and visualized with Immobilon™ Western Chemiluminescent HRP Substrate (Merck Millipore, USA). The bands were scanned and quantified using Image J software.

RNA extraction and real-time PCR analyses

Total RNA was extracted from NPC cells using Trizol reagent (Invitrogen, Carlsbad, CA, USA) and then reverse transcribed to cDNA with reverse transcriptase and random primers. Real-time PCR was performed by StepOnePlus™ Real-Time PCR System (Applied Biosystems™, Thermo Fisher Scientific, MA, USA) using SYBR Green master mixture (Thermo Fisher Scientific, MA, USA). The following primers were used in the Real-Time-PCR experiment: KLF5 sense, 5'-CCCTTGCACATACACAATGC-3'; KLF5 antisense 5'-GGATGGAGGTGGGGTTAAAT-3'; GAPDH sense, 5'-GCACCACCAACTGCTTAGCA-3'; and GAPDH antisense, 5'-TCTTCTGGGTGGCAGTGATG-3'. The relative levels of KLF5 mRNA are expressed as the inverse log of the ΔΔCq value and normalized with the internal control gene, GAPDH [20].

Dual-luciferase reporter assay

For dual-luciferase reporter assay, NPC cells were seeded in a 96-well plate and co-transfected with pmirGLO dual-luciferase miRNA target expression vector (catalog number: E1330, Promega, Madison, USA) using TurboFect reagent (Thermo Scientific™, MA, USA). The samples were wild-type KLF5, scramble control and miR-145-5p mimics. Renilla and Firefly luciferase activities were measured 48h post-transfection using the dual-luciferase reporter assay kit (catalog number: E2920, Promega, Madison, USA) and Microplate Luminometer (BioTeck, Winooski, VT, USA).

MiRNA target-predicting and bioinformatics

For predicting miRNA and mRNA direct interaction, we used miRNA target-predicting tools. The prediction tools contained Targetscan [21], miRDB [22], Miranda [23] and mirDIP [24]. The levels of KLF5 and miR-145-5p in head and neck squamous cell carcinoma (HNSC) tissues were derived from the TCGA dataset of UALCAN [25]. The ratio of KLF5 mRNA expression in nasopharyngeal carcinoma and nasopharynx (cancer vs. normal) was derived from Oncomine database.

Statistical analysis

Experiments were performed independently at least three times. The results are shown as mean ± SD. All statistical analyses were performed using the SPSS 19.0 (IBM SPSS, Armonk, NY, USA) and Microsoft Office Excel for PC. Statistical significance of dissimilarity between groups was confirmed by two-tailed student's *t-test*. *P*-values less than 0.05 were regarded as statistically significant.

Results

KLF5 overexpression modulates the proliferation, migration and invasion of NPC cells

A previous study demonstrated that the expression of KLF5 is downregulated in NPC [8]. In this study, analysis with the TCGA dataset revealed that the levels of KLF5 in head and neck squamous cell carcinoma (HNSC) tissues were reduced in tissues from tumor stages and nodal metastasis of HNSC (Fig. 1a and 1b). Furthermore, KLF5 mRNA expression was lower in nasopharyngeal carcinoma in comparison to nasopharynx tissues (Fig. 1c). In order to identify the role of KLF5 in the development of NPC, KLF5 was overexpressed by transfecting the GFP-KLF5 vector in HONE-1 and NPC-TW01 cells (Fig. 2a). The overexpression of KLF5 was found to inhibit the proliferation of NPC-TW01 cells (Fig. 2b). Wound healing assay confirmed the suppression of wound closure in cells overexpressing KLF5, in comparison to the control cells (Fig. 2c). As the invasive cells in the cell invasion assay were analyzed using a green fluorescent dye, we transfected the NPC cell lines with the CMV-KLF5 vector instead of the GFP-KLF5 vector. Cell invasion assay showed the inhibitory effects of CMV-KLF5 on NPC cell invasion (Fig. 2d and 2e). These results indicated that KLF5 acts as a tumor suppressor, and represses the proliferation, migration, and invasion of NPC cells.

MiR-145-5p suppresses the proliferation, migration, and invasion of NPC cell lines

In order to elucidate the molecular mechanism underlying the modulatory activity of KLF5 on the function of NPC cells, we examined the possible upstream miRNA regulators of KLF5 by using four different miRNA target-predicting tools, and finally selected miR-145-5p as the possible candidate miRNA (Fig. 3a). We analyzed the expression of miR-145-5p in HNSC and tumor adjacent normal tissues by TCGA dataset. The data indicated that the level of miR-145-5p was significantly downregulated in HNSC as compared to the normal tissues (Fig. 3b). Furthermore, the levels of miR-145-5p were reduced in tissues from caner stages and nodal metastasis of HNSC, in comparison to that of the normal tissues (Fig. 3c). In order to evaluate the biological function of miR-145-5p in NPC cells, we examined cellular proliferation, migration, and invasion by transfecting with miR-145-5p mimics. As depicted in Fig. 2d, the overexpression of miR-145-5p inhibited the proliferation of NPC cells, compared to that of the scrambled control. MiR-145-5p effectively repressed the migration and invasion of both the NPC cell lines, in comparison to that of the control cells (Fig. 3e-g).

KLF5 expression is upregulated by miR-145-5p in NPC cells

The association between KLF5 and miR-145-5p was further validated by examining the endogenous levels of the KLF5 protein in NPC cells following transfection with miR-145-5p mimics. Western blot analysis revealed that the miR-145-5p mimics induced the expression of KLF5 (Fig. 4a and 4b). The mRNA expression of KLF5 was analyzed by real-time PCR. However, the mRNA level of KLF5 did not change after transfection with miR-145-5p mimics (Fig. 4c). The 3'-UTR of the KLF5 mRNA contains a predicted binding site for miR-145-5p (Fig. 4d). We employed dual-luciferase reporter assays for analyzing whether miR-145-5p directly targeted KLF5. However, the reporter with the wild-type KLF5 3'-

UTR either showed a slightly decreased luciferase activity or had no effects on the NPC cells following transfection with miR-145-5p mimics, in comparison to that of the scrambled control (Fig. 4e and 4f). Using Ingenuity Pathway Analysis (IPA), we identified a gene regulatory mechanism between miR-145-5p and KLF5 (Fig. 4g). These findings suggested that miR-145-5p regulates the expression of KLF5, not by directly targeting the 3'-UTR of the KLF5 mRNA, but by other molecular mechanisms.

MiR-145-5p and KLF5 inhibits the activity of focal adhesion kinase (FAK) in NPC cell lines

Previous studies have demonstrated that FAK signaling plays a crucial role in modulating cell proliferation, migration, and invasion in NPC [26-29]. In order to elucidate the mechanism by which miR-145-5p and KLF5 affects cellular function in NPC, the molecules related to the activation of FAK were analyzed. Western blot analysis revealed that the overexpression of miR-145-5p or KLF5 suppressed the activation of FAK in NPC cells (Fig. 5a and 5b). These results suggested that the effect of miR-145-5p on cellular migration, and invasion in NPC could be mediated by the KLF5/FAK pathway.

Discussion

The results of the present study demonstrated that the upregulation in KLF5 expression repressed the function of the NPC cells. The overexpression of miR-145-5p increased the levels of the KLF5 protein, and inhibited the proliferation, migration, and invasion of the NPC cell lines. Our study also revealed that both miR-145-5p and KLF5 suppresses the activity of FAK.

Certain miRNAs have been reported to modulate the development and progression of NPC [30-32]. In this study, we examined the role of miR-145-5p, which is downregulated in several cancers, including NPC [7,16,33]. We verified that miR-145-5p plays a significant role in the proliferation, migration, and invasion of the NPC cells. These data are consistent with the results of previous studies reporting the role of miR-145-5p as a tumor suppressor miRNA in NPC [34,35]. Additionally, other studies have demonstrated that miR-145-5p suppresses the expression of KLF5 in gastric cancer and hepatocellular carcinoma [36,37]. Interestingly, the results of our study demonstrated that the overexpression of miR-145-5p increases the levels of the KLF5 protein. We identified miR-145-5p as a possible candidate miRNA that directly targets KLF5, by using miRNA target-predicting tools. However, the results of the dual-luciferase assay demonstrated that miR-145-5p may not directly target the 3'-UTR of the KLF5 mRNA. Additionally, the study by Place *et al.* demonstrated that miRNAs can target the promoter sequences of genes and induce gene expression [38]. Other hypotheses have revealed that long non-coding RNAs that cause gene silencing, can be targeted by miRNAs and thereby increase gene expression [39]. Using IPA, we identified WW Domain Containing E3 Ubiquitin Protein Ligase 1 (WWP1), which may reduce the expression of KLF5 (Fig. 4g). WWP1 is an E3 ubiquitin ligase which can bind to KLF5 and trigger the ubiquitin-mediated proteasomal degradation of KLF5 [40]. According to the IPA prediction pathway, it is possible that c-Myc and SOX9 induced the expression of WWP1. Moreover, previous studies have demonstrated that c-Myc and SOX9 can promote cell migration and invasion in

NPC cells [41,42]. These studies suggest that miR-145-5p might indirectly induce the expression of KLF5 by post-transcriptional regulation or by inhibiting protein degradation. However, these speculations require further clarification in the future.

The KLF5 protein in humans is also known as IKLF or BTEB2, and is an important member of the KLF family. KLF5 has opposing roles and either acts as a transcriptional repressor or activator of cellular function, or as a regulator in numerous tumors [43]. KLF5 has been verified to act as an oncogene in gastric cancer, breast cancer, leukemia, bladder cancer, and ovarian cancer. It has been demonstrated that KLF5 acts as a tumor suppressor in prostate cancer and esophageal cancer [44,45]. In this study, we found KLF5 as tumor suppressor inhibiting NPC cell migration and invasion. One limitation of this study is that we used only two NPC cell lines. Novel NPC cell lines from other sources need to be used in further studies. The other limitation of our study is that it only explores the antitumor function of KLF5 *in vitro*. The application of the suppressive function of the KLF5-based strategy to the development of antitumor therapeutics requires further validation by *in vivo* studies, and using NPC tissues from human subjects.

Furthermore, the increase in the expression levels of KLF5 or miR-145-5p downregulated the activity of FAK in NPC cells. FAK is a multi-functional regulator in several tumors, including colon, prostate, breast, thyroid, liver, gastric, and ovarian cancers [46]. The FAK signaling pathway has been studied in numerous tumors and has been found to regulate cellular proliferation, migration, invasion, and metastasis [47-49]. However, the role of FAK in NPC is not clear. Using IPA we identified potential molecular networks between KLF5 and FAK (data not shown). Therefore, the suppression in FAK activity by miR-145-5p and KLF5 in NPC requires further exploration by molecular pathway prediction studies. Taken together, the present study is the first to report the role of miR-145-5p in NPC cells by modulating the KLF5/FAK pathway. The results of this study further indicated that miR-145-5p-based therapeutics and KLF5 activators could serve as potential therapeutic strategies for NPC.

Declaration Of Conflicting Interests

The authors declare they have no conflict of interest.

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Figures

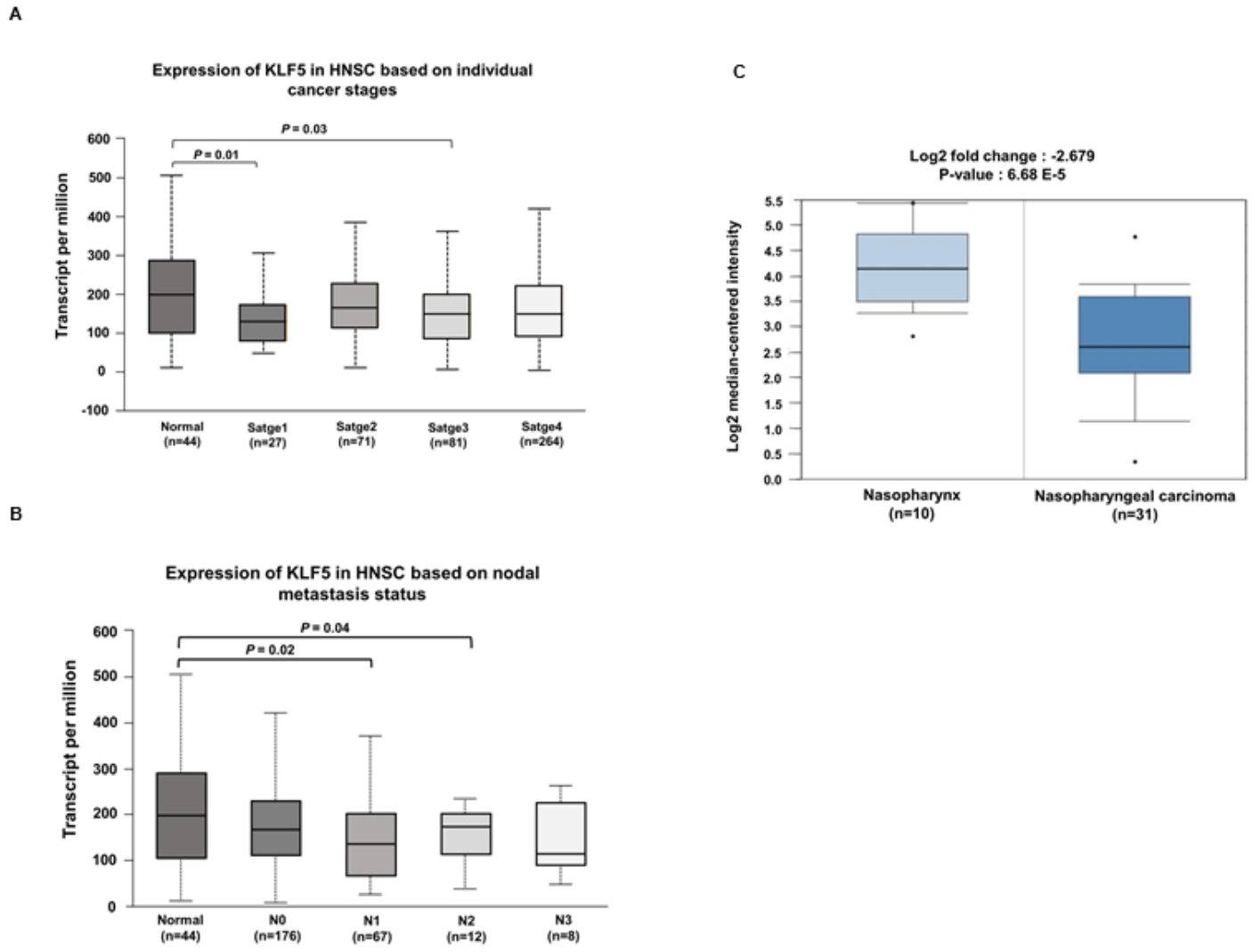

Figure 1

The downregulated mRNA expression of KLF5 in HNSC and NPC tissues. a, b. Boxplot of expression of KLF5 in tissues of normal people and HNSC individual cancer stages and nodal metastasis status of patients from TCGA HNSC datasets. c. The fold change of KLF5 in cohort of Sengupta Head-Neck on nasopharyngeal carcinoma was extracted from the Oncomine database.

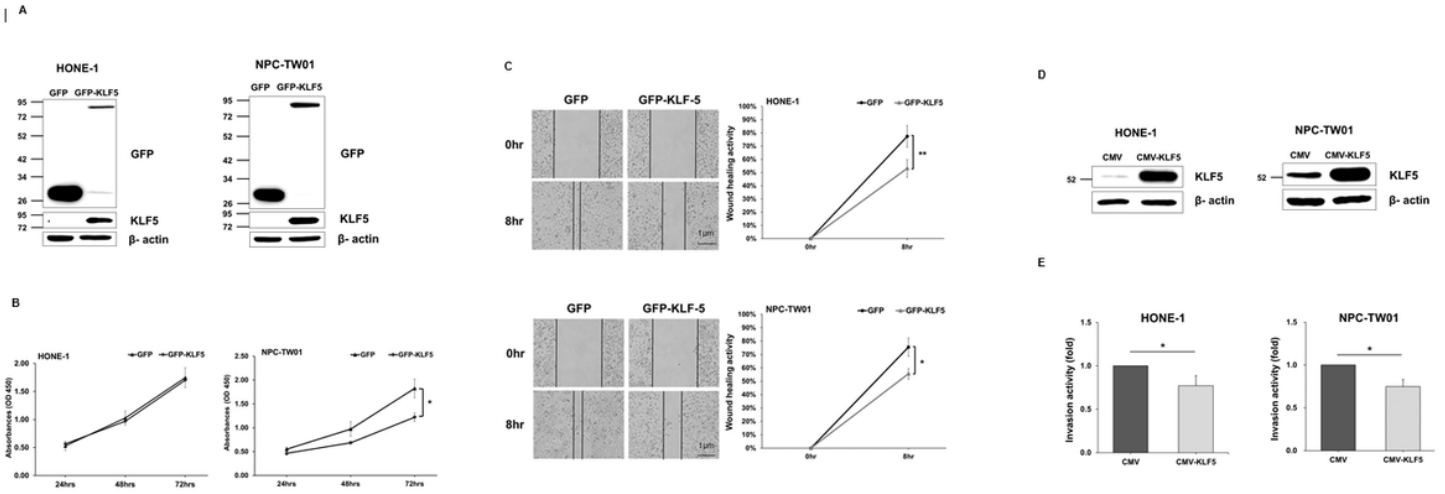

Figure 2

Overexpression of KLF5 mediates the proliferation, migration and invasion of NPC cells. a. Western blot analysis of GFP and KLF5 protein levels in HONE-1 and NPC-TW01 after transfection of GFP and GFP-KLF5 vectors. b. Proliferation rates of HONE-1 and NPC-TW01 cells at 24 to 72hrs time points after transfection with GFP and GFP-KLF5, n = 3. c. HONE-1 and NPC-TW01 cells were transiently transfected with GFP and GFP-KLF5 and analyzed by wound healing assays, n = 3. d. Western blot analysis of KLF5 levels in HONE-1 and NPC-TW01 after transfection of CMV and CMV-KLF5 vectors. e. HONE-1 and NPC-TW01 cells were transiently transfected with CMV and CMV-KLF5 and analyzed by invasion assays, n = 3. Results were shown as mean ± SD; *P < 0.05, **P < 0.01.

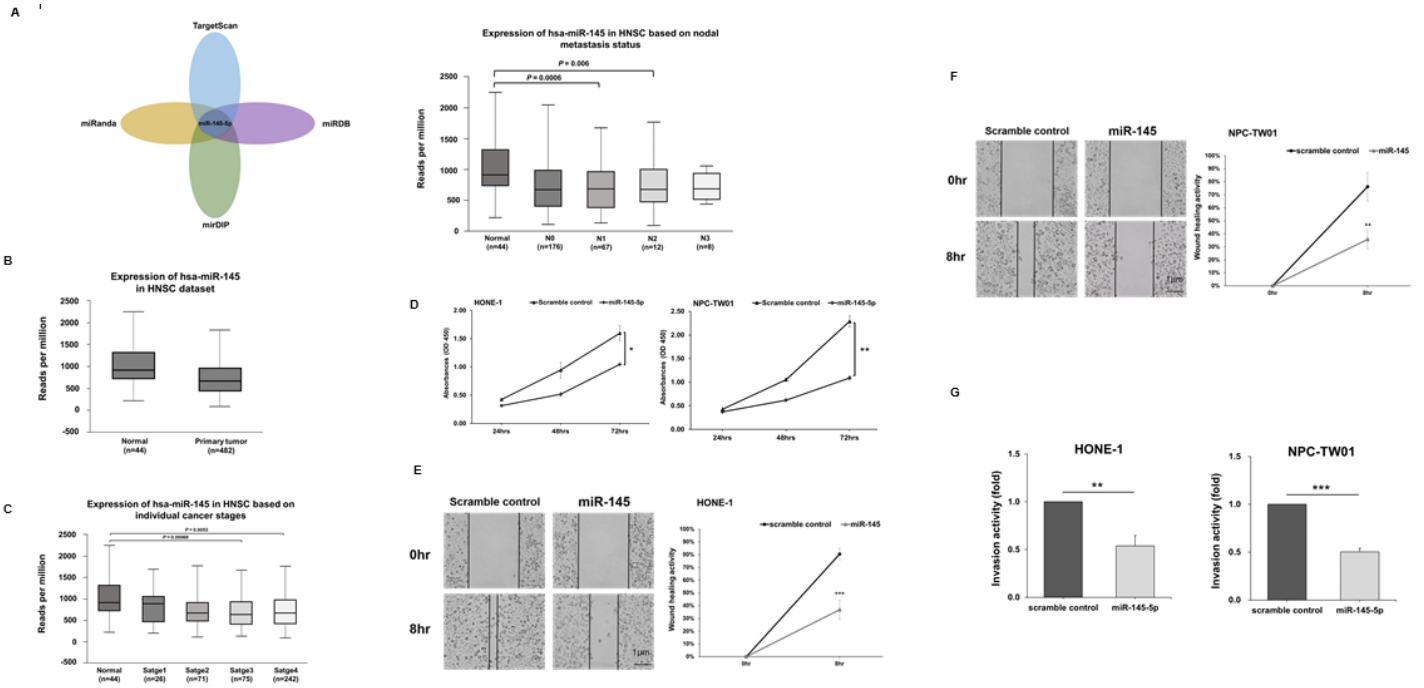

Figure 3

MiR-145-5p represses the proliferation, migration, and invasion of NPC cell lines. a. The schematic illustration described that the putative miR-145-5p may direct target to KLF5 from four prediction databases: Targetscan, miRDB, mirDIP and miRanda. b. Boxplot of expression of hsa-miR-145 in tissues of normal people (n=44) and HNSC patients (n=482) from TCGA HNSC data sets, P = 0.02. c. Boxplot of expression of hsa-miR-145 in tissues of normal people and HNSC individual cancer stages and nodal metastasis status of patients from TCGA HNSC datasets. d. Proliferation rates of HONE-1 and NPC-TW01 cells at 24 to 72hrs time points after transfection with either scramble control and miR-145-5p mimics, n = 3. e and f. HONE-1 and NPC-TW01 cells were transiently transfected with scramble control and miR-145-5p mimics and analyzed by wound healing assays, n = 3. g. HONE-1 and NPC-TW01 cells were transiently transfected with scramble control and miR-145-5p mimics and analyzed by invasion assays, n = 3. Results were shown as mean ± SD; *P < 0.05, **P < 0.01, ***P < 0.001.

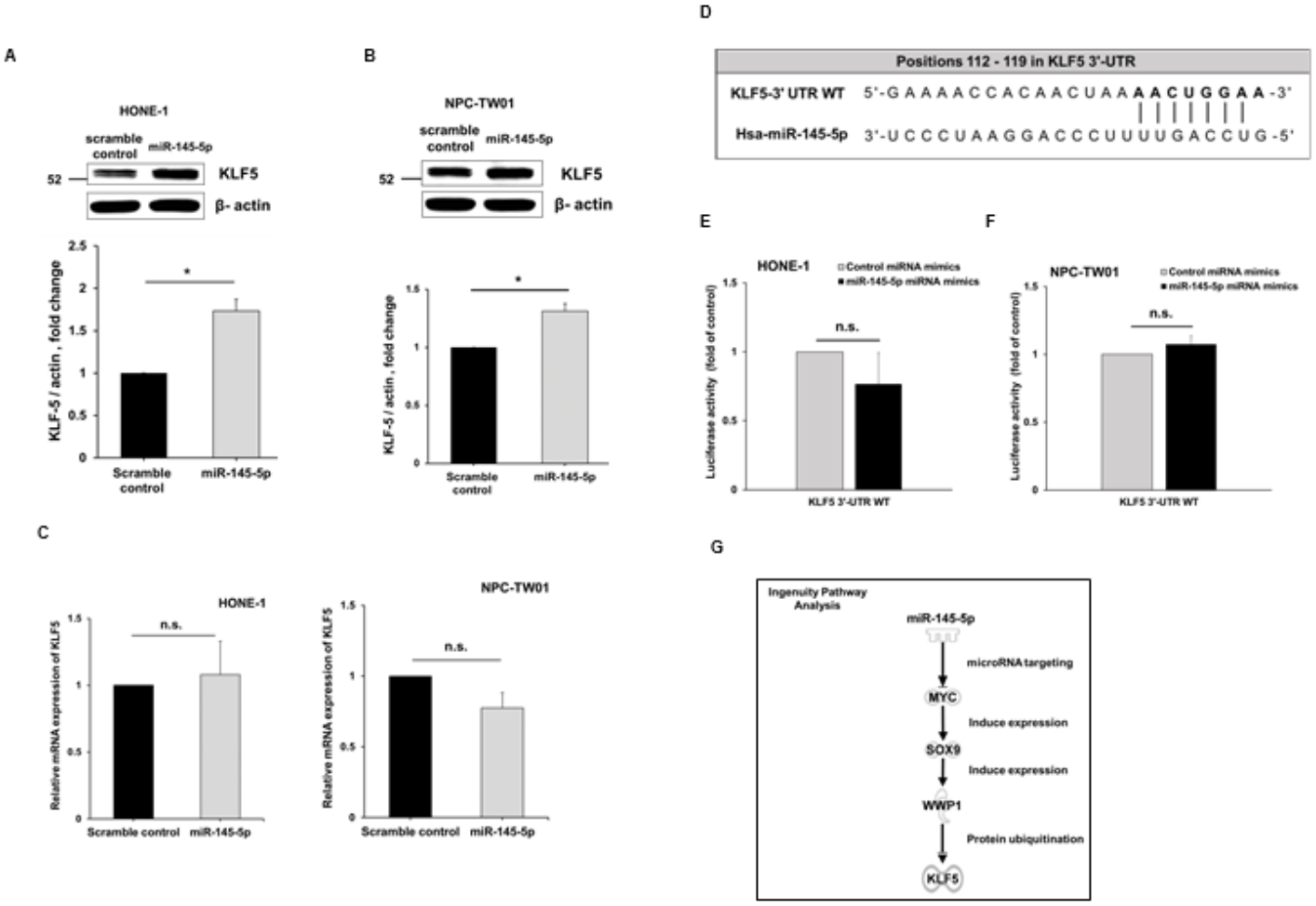

Figure 4

MiR-145-5p up-regulates KLF5 protein expression. a and b. Western blot analysis of KLF5 levels in HONE-1 and NPC-TW01 after transfection of scramble control and miR-145-5p mimics, n = 3. c. MiR-145-5p could not regulate KLF5 mRNA expression after transfection with scramble control or miR-145-5p mimics in HONE-1 and NPC-TW01 cells. n = 3. d. Complementarity between the seed sequence of hsa-miR-145-5p and the targeted sequence in 3'-UTR of KLF5 mRNA predicted by TargetScan. e and f. Relative luciferase

activity of wild-type (WT) KLF5 3'-UTR luciferase reporters in HONE-1 and NPC-TW01 cells with co-transfection of miR-145-5p mimic or scramble control, n = 3. g. Ingenuity Pathway Analysis (IPA) showed predicted interactions between miR-145-5p and KLF5. Results were shown as mean ± SD; *P < 0.05. ns, not significant.

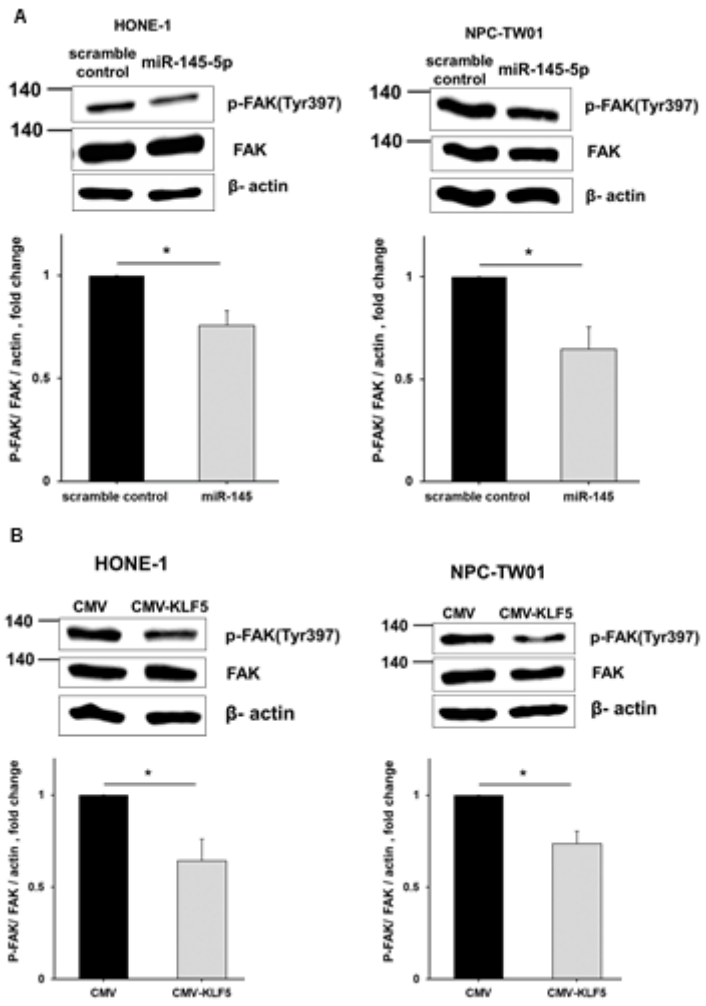

Figure 5

MiR-145-5p and KLF5 represses phosphor-FAKTyr397 expression in NPC cells. a. MiR-145-5p down-regulated phospho-FAKTyr397 protein expression after transfection with scramble control or miR-145-5p mimics in HONE-1 and NPC-TW01 cells, n = 3. b. Western blot analysis was used to detect phospho-FAKTyr397 and FAK expression in HONE-1 and NPC-TW01 cells after transfection with CMV or CMV-KLF5 vector as indicated, n = 3. Results were shown as mean ± SD; *P < 0.05.