

Original Article

Inhibition of MiR-155 suppresses cell migration in nasopharyngeal carcinoma through targeting ZDHHC2

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Abstract: Overexpression of miR-155 in nasopharyngeal carcinoma (NPC) is partly driven by Epstein-Bar virus infection. However the role of miR-155 in NPC oncogenesis is unclear. This study showed that miR-155 inhibitor could inhibit the cell migration in NPC cell lines. ZDHHC2 was identified as a direct target of miR-155 and downregulation of ZDHHC2 prompted cell migration in NPC. Furthermore, reduced ZDHHC2 expression was associated significantly with metastasis and poor survival of NPC patients. Collectively, inhibition of miR-155 suppresses cell migration in NPC through targeting ZDHHC2. The potential of miR-155 and ZDHHC2 as therapeutic targets in NPC should be further investigated.

Keywords: Nasopharyngeal carcinoma, miR-155, ZDHHC2, cell migration

Introduction

Nasopharyngeal carcinoma (NPC) is a tumor arising from the mucosal epithelium covering the nasopharyngeal surface and is one of the most common malignancies in South-China, Southeast-Asia and North Africa [1]. Genetic alterations, Epstein-Barr virus (EBV) infection and other environmental factors were reported to be associated with risk for NPC [2, 3]. NPC has a obvious clinical-pathological behavior of loco-regional recurrence and metastasis, which is different from other types of head and neck cancers [4]. However to date the pathogenesis of NPC is not investigated clearly.

MicroRNAs is an abundant class of non-coding RNAs, typically 20-23 nucleotides in length and they are involved in many cellular processes including apoptosis, differentiation, proliferation, and metabolism [5]. In the microRNAs

family, microRNA 155 (miR-155) is located on chromosome 21 and transcribed from the B-cell integration cluster. MiR-155 plays important roles in many human tumors, including breast cancer [6-8], leukemia [9], melanoma [10], lymphoma [11-13], cervical cancer [14], hepatocellular carcinoma [15], pancreatic cancer [16], lung cancer [17, 18], colon cancer [6], and gastric adenocarcinoma [19]. We have also reported that miR-155 is upregulated in NPC, which is partly driven by EBV encoded LMP1 and LMP2A [20]. However the function of miR-155 in NPC is unclear yet.

Zinc finger, DHC-type containing 2 (ZDHHC2), also known as reduced expression associated with metastasis protein (REAM), is one member of DHC protein family of protein acyltransferases (PATs). It is located in chromosome 8p21.3-22 [21], where frequent loss of heterozygosity has been detected in various types of

metastatic cancers, including colorectal cancer [22], hepatocellular carcinoma [22], prostate cancer [23], non-small cell lung cancer [22], urinary bladder cancer [24], breast cancer [25]. Reduced ZDHHC2 was found to be associated with lymph node metastasis and poor prognosis in gastric adenocarcinoma [26], and the mRNA level of ZDHHC2 expression was significantly reduced in primary and metastatic foci of advanced colorectal cancer [21]. However the expression pattern of ZDHHC2 has not been investigated in NPC yet. In view of the proposed roles of miR-155 and ZDHHC2 in cancer, we aimed to investigate the function of miR-155 and ZDHHC2 and their potential relationship in NPC.

Materials and methods

Patients and tissue samples

The study was approved by the Ethics Committee of Sun Yat-sen University Cancer Center (Guangzhou 510060, China). All samples used in this study were anonymous and collected from patients for routine pathology use. The patient records/information was de-identified as well as anonymized, prior to analysis. No informed consent (written or verbal) was obtained for use of retrospective tissue samples from the patients in this study. In this retrospective study, archival formalin-fixed, paraffin-embedded (FFPE) tissue specimens from 124 primary NPC patients (29 females and 95 males; aged from 21 to 77 years; median, 48 years) who underwent radical radiotherapy with or without chemotherapy from 1999 and 2007 were obtained from the Sun Yat-sen University Cancer Center (Guangzhou, China, 510060). The disease stages of all patients were classified or reclassified according to the China 1992 NPC staging system [27]. Of the 124 primary NPC patients, 4 were classified as stage I, 26 as stage II, 64 as stage III, and 28 as stage IV, and 2 cases without stage information available.

Cell lines

Human NPC cell lines CNE1 (EBV negative, from Cancer Center, Sun Yat-sen University, China), TW03 (EBV negative, the generous gift of Prof. Chin-Tarng Lin, National Taiwan University Hospital) [28] were cultured in 1640 (Gibco USA) containing 10% fetal calf serum

(FCS), and Human Embryonic Kidney 293 T cells (from American Tissue Culture Collection, ATCC, Manassas, VA) were cultured in DMEM (Gibco USA) containing 10% fetal calf serum (FCS). All the cell lines were grown in a humidified incubator at 37°C with 5% CO₂.

MiRNA and siRNA transfections

Before transfection, 2×10⁵ cells per well were plated into 6-well plates and grown for one day in antibiotic-free medium containing 10% FCS. When the cell confluence was reached to 40% to 60%, cells were transfected with 100nM miR-155 Anti-miRTM miRNA Inhibitor (Cat#: AM12601, Ambion, USA), or Anti-miRTM miRNA Inhibitors-Negative Control #1 (Cat#: AM170-10, Ambion, USA), or 100 nM four individual ZDHHC2 specific siRNAs (Please see [Figure S1](#) for detailed siRNA sequence) and a negative control siRNA by using Lipofectamine 2000 (Invitrogen, USA) according to the manufacturer's instructions. Transfected cells were grown at 37°C for 6 h, followed by incubation with complete medium. For q-PCR and Western blot analysis, cells were harvested for RNA and protein respectively 48 h post-transfection.

Luciferase reporter assays

The whole 3'UTR sequences of ZDHHC2 were cloned to downstream from the luciferase reading frame in the plasmid pmiR-Glo-report-Vector (Genepharma, China). Mutations were made in these two putative miR-155 target sites (433-439 and 2334-2340). All inserts were sequenced in their entirety to verify polymerase fidelity. Luciferase reporter assays were performed by transiently transfecting HEK 293T cells respectively with 200 ng of with pmiR-Glo-ZDHHC2-3'UTR-wild, pmiR-Glo-ZDHHC2-3'UTR-433mut, or pmiR-Glo-ZDHHC2-3'UTR-2334mut, together with 30 nM miR-155 Pre-miRTM miRNA Precursor Molecules (Cat#: PM12601, Ambion, USA), or Pre-miRTM miRNA Precursor Molecules-Negative Control #1 (Cat#: AM17110, Ambion, USA) and 200 ng of pCMV-Renilla (internal control) using Lipofectamine 2000 (Invitrogen) respectively. Firefly and Renilla luciferase activities were measured consecutively by using Dual Luciferase Assay (Cat#: E1910, Promega, USA) 48 hr after transfection. Firefly luciferase values have been normalized to Renilla, and the ratio of firefly/renilla was presented.

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PCR assays

For miRNAs quantitative realtime PCR (qPCR) assay, total RNA from cell lines was isolated using Trizol reagent (Invitrogen) according to the manufacturer's instructions, then was treated with RNase free DNase I (Cat#: 047-16728001, Roche). The miR-155 quantitative realtime PCR assay was performed by TaqMan MicroRNA Assays (Cat#: 4373124, Applied Biosystems, USA) and RNU6B (Cat#: 4373381, Applied Biosystems, USA) was used as internal control. For mRNA qPCR assay, total RNA was extracted from cell lines using TRIZOL reagent (Invitrogen). After reverse transcription of the total RNA, the first-strand cDNA was then used as template for detection of ZDHHC2, expression by quantitative real time PCR (qPCR) with the SYBR Green I chemistry (Power SYBR Green PCR Master Mix, CAT#: 4367659, ABI Inc., USA). GAPDH was used as internal control. The primers were ZDHHC2 (Forward: TCT TAG GCG AGC AGC CAA GGA T and Reverse: CAG TGA TGG CAG CGA TCT GGT T); GAPDH (Forward: AGC CAC ATC GCT CAG ACA C and Reverse: GCC CAA TAC GAC CAA ATC C). The relative expression level was determined as $2^{-\Delta\Delta Ct}$. Data are presented as the expression level relative to the calibrator (control sample), with the standard error of the mean of triplicate measures for each test sample.

Western-blot assays

Cells were harvested and lysed with RIPA buffer (Upstate, USA) at 48 h post-transfection. Equal amounts of denatured protein sample were separated by SDS-PAGE and were then transferred electrophoretically to PVDF membranes (Pall, USA) for immunoblot analysis. Antibodies used for immunoblot analysis were against ZDHHC2 (1:1000 dilution, Cat#: AP 5592a, Abgent, CA) and an anti-GAPDH antibody (1:5,000 dilution, sc-32233, Santa Cruz, USA) was used as loading control. All protein bands were detected using an enhanced chemiluminescent (ECL) Western blot Kit (Cell Signaling Technology, USA).

Transwell assay

For the migration assay, 5×10^4 cells were plated in the top chamber with a non-coated membrane (24-well insert; 8- μ m pore size; BD Biosciences, USA). The cells were plated in

medium without serum, and medium supplemented with 10% serum was used as a chemoattractant in the lower chamber. The cells were incubated for 24 h. Cells that didn't migrate through the pores, were removed by a cotton swab. Filters were fixed with 90% ethanol, stained with 0.1% crystal violet, and photographed. Cell numbers were counted under microscope.

Wound-healing assay

The cultured cells were transfected with 100 nM miR-155 inhibitor or negative control. At 48 h post-transfection, the cells were allowed to reach confluence before dragging a 200 μ l sterile pipette tip through the mono-layer. The cells were then washed and allowed to migrate for additional 24 h. At 0, 6, 12 and 24 h post-wounding, images were captured. The experiment was performed in triplicate.

Methylthiazolyl blue tetrazolium (MTT) assay

Cell growth was determined using MTT (Sigma-Aldrich, USA) spectrophotometric dye assay. At 24 h post-transfection with miR-155 inhibitor or negative control, cells were seeded into 96-well plates (4×10^3 cells/well), when the cells were adhered (about culturing for 6 h), DDP was added to each well according to the concentration gradient (0 nM, 12.5 nM, 25 nM, 50 nM, 100 nM, 200 nM, 400 nM, 800 nM) and each concentration gradient was repeated thrice. After culturing for 48 h, MTT (10 μ L per well) was added. The mixtures were further cultured for 4 h and the liquids were discarded. To each well 100 μ L dimethylsulfoxide (DMSO) was added and the plate was shaken for 10 min. The absorbance at 570 nm was measured using an automatic ELISA reader. The inhibition rate (%) = $(1 - A_{\text{experimental group}} / A_{\text{control group}}) \times 100$.

Immunohistochemistry

In our study, primary antibodies against ZDHHC2 (1:200 dilution, Cat#: AP 5592a, Abgent, CA) were used. Briefly, tissue sections were dewaxed, incubated with hydrogen peroxide for 10 minutes, incubated in retrieval buffer solution for antigen recovery, blocked with normal serum for 10 minutes and incubated with a primary antibody for 60 minutes, followed by detection using a Catalyzed Signal Amplification

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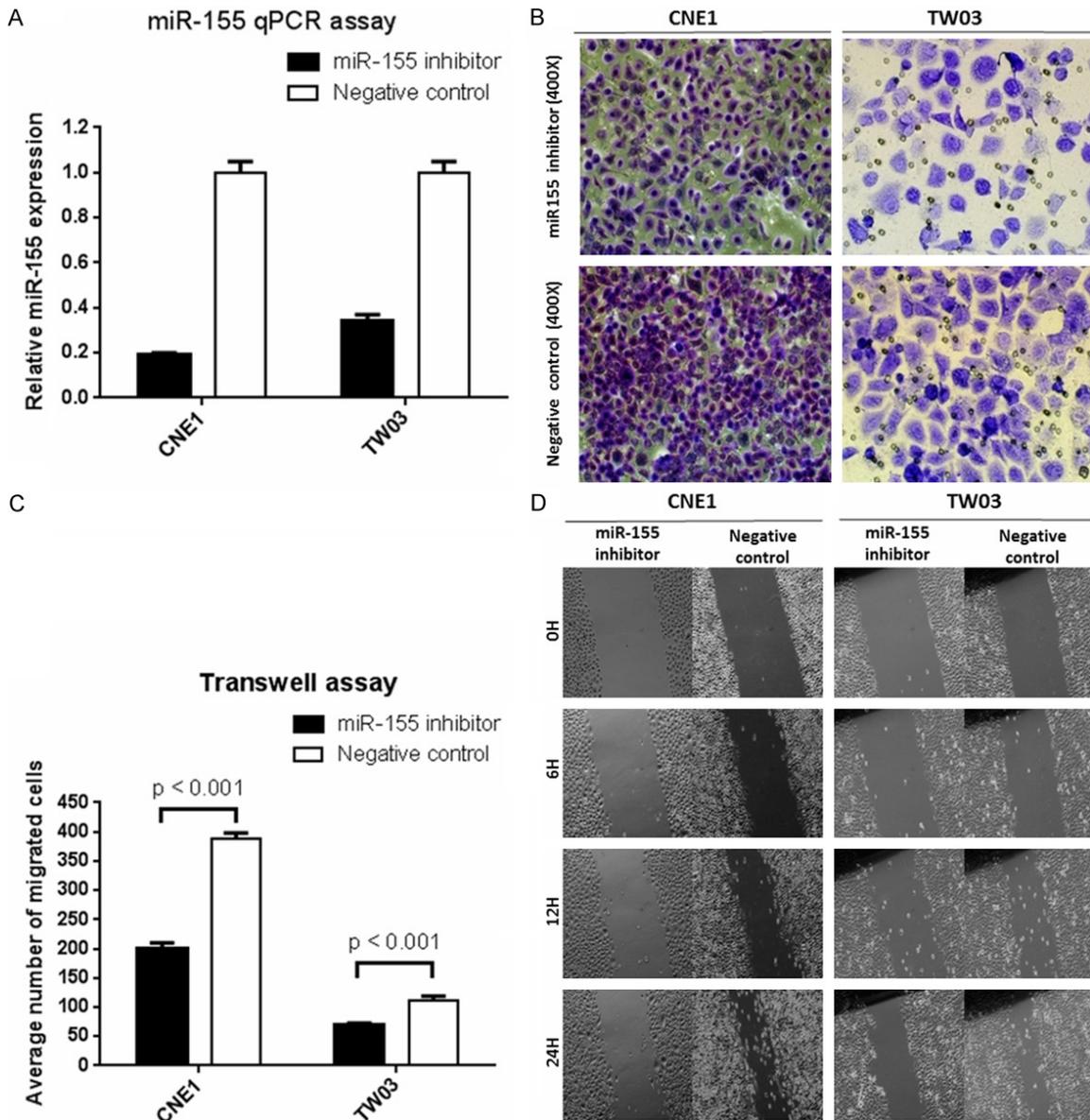


Figure 1. miR-155 inhibitor suppresses cell migration in nasopharyngeal carcinoma CNE1 and TW03 cells. A. The relative miR-155 expression in CNE1 and TW03 cells transfected with miR-155 inhibitor (100 nM) and inhibitor negative control (100 nM) by qPCR detection, and U6 was used as a loading control. B and C. The migrational ability of CNE1 cells and TW03 cells was analyzed by transwell migration assays at 48 h post-transfection with a miR-155 inhibitor (100 nM) and a negative control (100 nM). D. The migrational ability of CNE1 cells and TW03 cells was analyzed by wound healing assays at 48 h post-transfection with a miR-155 inhibitor (100 nM) and a negative control (100 nM).

Kit (DAKO, USA); signal was visualized using diaminobenzidine. Non-immune rabbit serum was substituted for the primary antibody as a negative control. The immunohistochemistry results were evaluated and scored by a senior pathologist without knowledge of the clinicopathological outcomes of the patients. A semi-quantitative estimation was made by using a composite score obtained by adding the values of the staining intensity and the relative abun-

dance of positive cells. The intensity was graded as 0 (no staining), 1 (weak staining), 2 (moderate staining) and 3 (strong staining). The abundance of the positive cells was graded from 0 to 3 (0.5% positive cells; 1. 5-25%; 2. 26-50%; 3. 51-75%; 4. >75%). A composite score greater than the median value was considered as high expression, and composite scores less than or equal to the median value were considered as low expression.

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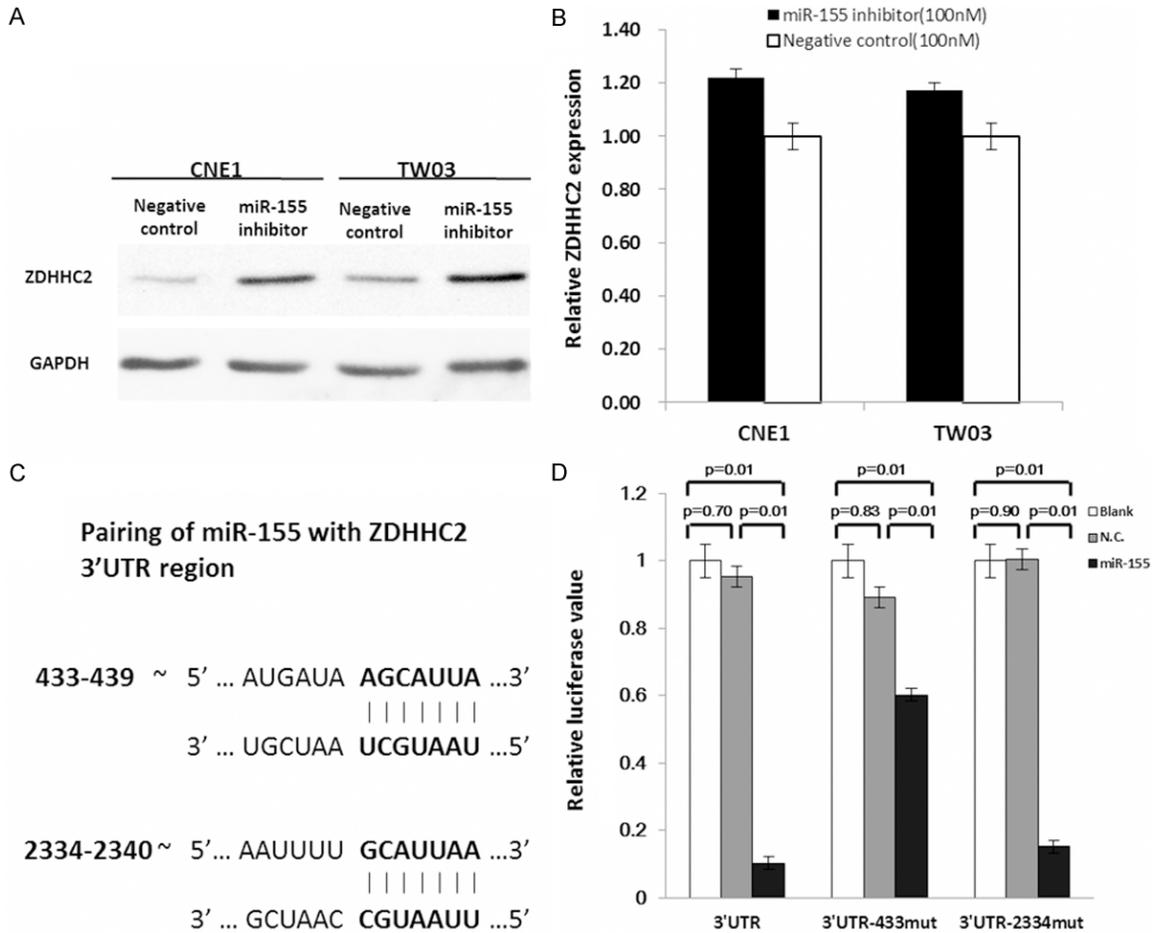


Figure 2. ZDHHC2 is the direct target of miR-155. A. The expression of ZDHHC2 was analyzed by Western-blot at 48 h post-transfection in CNE1 cells and TW03 cells. The miR-155 inhibitor could upregulate ZDHHC2 expression in protein level. B. In q-PCR detection, the ZDHHC2 mRNA was not changed significantly after transfection. GAPDH was used as a loading control. C. Pairing of miR-155 with ZDHHC2 3'UTR region. D. Overexpression of miR-155 by the miR-155 mimic resulted in a significant decrease in luciferase signals of pmir-Glo-ZDHHC2-3'UTR, pmir-Glo-ZDHHC2-3'UTR-433-mut and pmir-Glo-ZDHHC2-3'UTR-2334-mut transfected HEK293 cells.

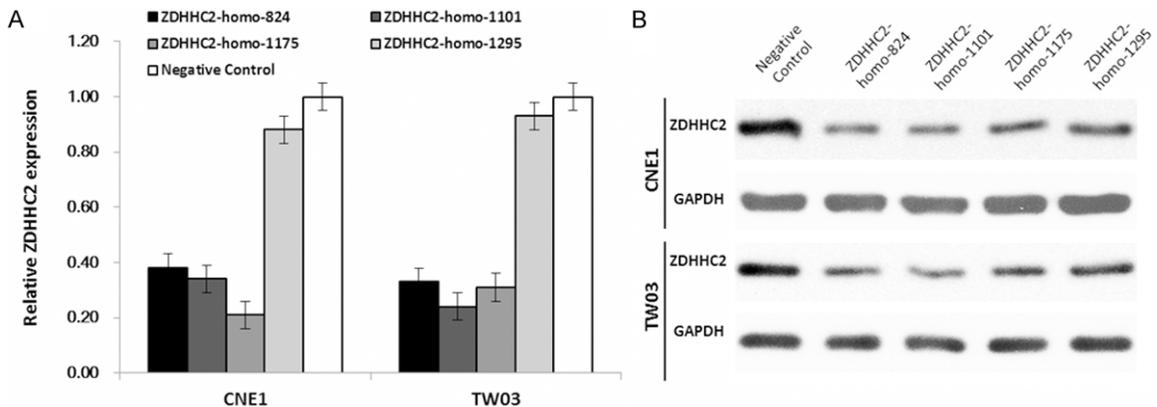


Figure 3. The inhibition effect of four ZDHHC2 siRNAs in nasopharyngeal carcinoma CNE1 and TW03 cells. A. The relative ZDHHC2 expression were analyzed by q-PCR detection in CNE1 and TW03 cells at 48 h post-transfection with a negative control, ZDHHC2-homo-824, ZDHHC2-homo-1101, ZDHHC2-homo-1175 and ZDHHC2-homo-1295 respectively. GAPDH was used as a loading control. B. The expression of ZDHHC2 was analyzed by Western-blot at 48 h post-transfection in CNE1 cells and TW03 cells, and GAPDH was used as a loading control.

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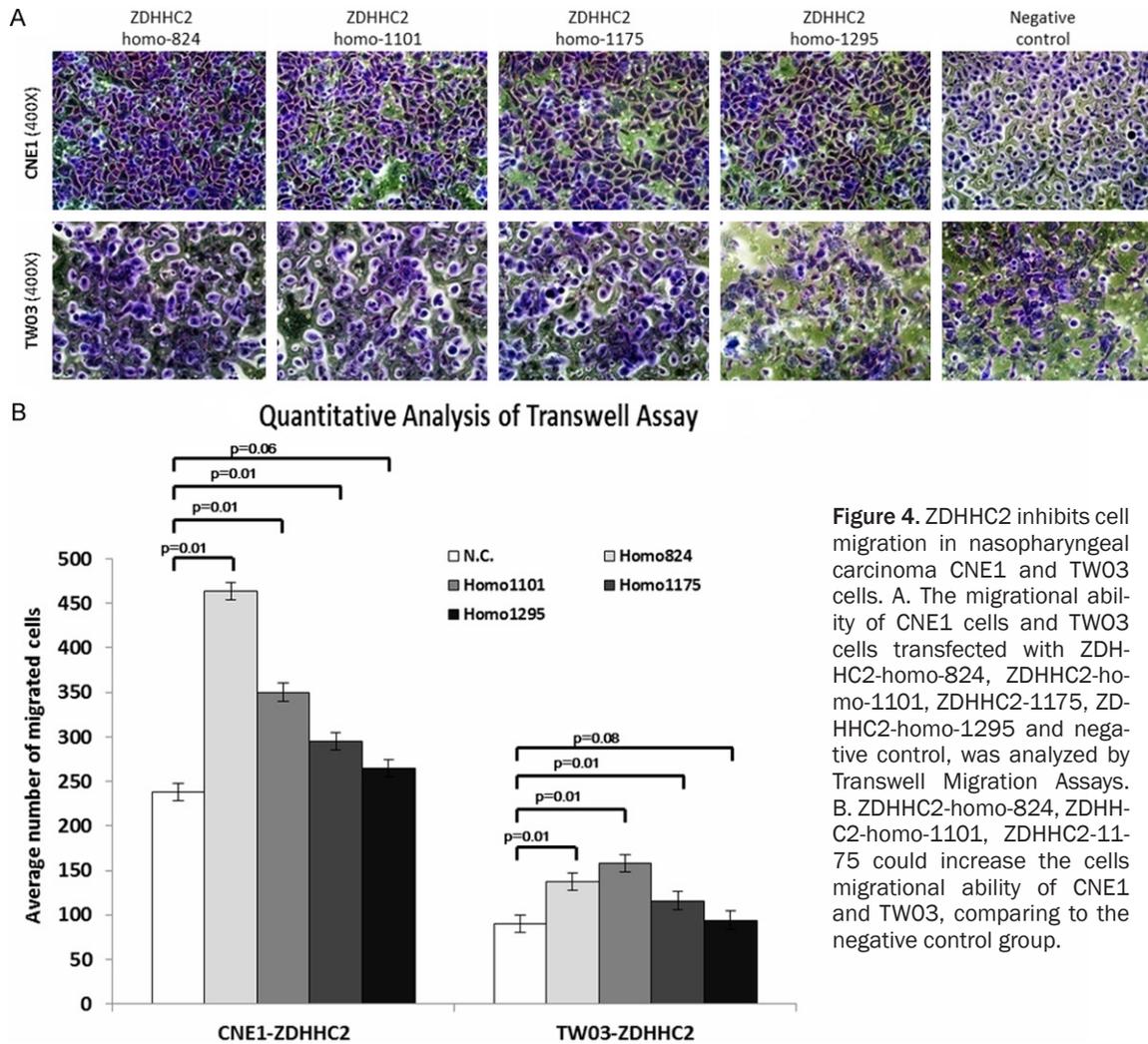


Figure 4. ZDHHC2 inhibits cell migration in nasopharyngeal carcinoma CNE1 and TW03 cells. A. The migrational ability of CNE1 cells and TW03 cells transfected with ZDHHC2-homo-824, ZDHHC2-homo-1101, ZDHHC2-1175, ZDHHC2-homo-1295 and negative control, was analyzed by Transwell Migration Assays. B. ZDHHC2-homo-824, ZDHHC2-homo-1101, ZDHHC2-1175 could increase the cells migrational ability of CNE1 and TW03, comparing to the negative control group.

Statistical analysis

All the data in this study was analyzed by SPSS 13.0 software. The association between ZDHHC2 and clinical-pathological parameters were assessed by Chi-Square test. Kaplan-Meier analysis and log-rank tests were used to compare the difference in survival curves. It was considered as significant differences when $P < 0.05$.

Results

Inhibition of miR-155 suppresses cell migration in nasopharyngeal carcinoma

To investigate the function of miR-155 in NPC, miR-155 inhibitor (Ambion, USA) and miRNA inhibitor control (Ambion, USA) were transiently transfected to two EBV negative NPC cell lines

CNE1 and TW03 respectively, and then both transwell assay and wound healing assay were performed to determine if miR-155 could influence the cell migration ability in NPC cells. miR-155 was reduced by 81%, and 66% in CNE1 and TW03 cells respectively by miR-155 inhibitor by qPCR detection at 48 hours posttransfection (**Figure 1A**). The CNE1 and TW03 cells transfected with miR-155 inhibitor (Ambion, USA) and miRNA inhibitor control (Ambion, USA) were used for transwell migration assays at 48 hours posttransfection. The average number of migrated CNE1 cells transfected with miR-155 inhibitor was 201 ± 9 , which was significantly less than that (388 ± 10) of CNE1 cells transfected with inhibitor control ($P < 0.001$) (**Figure 1B** and **1C**). The average number of migrated cells was 69 ± 4 and 112 ± 7 , respectively, in miR-155 inhibitor and inhibitor control treated

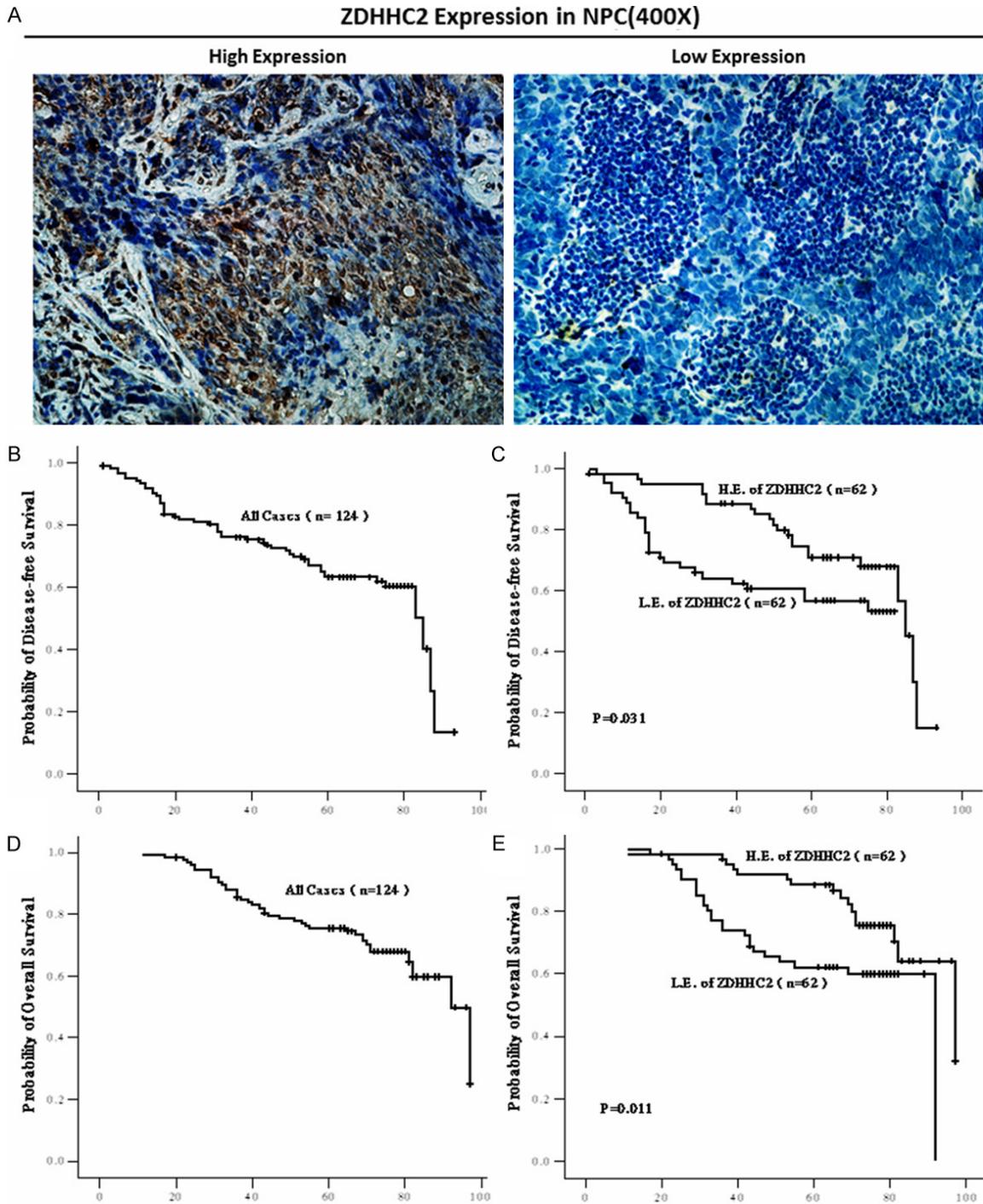


Figure 5. Low expression of ZDHHC2 predicts poor prognosis of nasopharyngeal carcinoma patients. A. Two representative images show high and low expression of ZDHHC2 in NPC tumor cells. B. Kaplan-Meier curves for disease-free survival (DFS) of the 124 NPC patients; C. Kaplan-Meier curves for DFS in NPC patients with low level and high level ZDHHC2 expression; D. Kaplan-Meier curves for overall survival (OS) of the 124 NPC patients; E. Kaplan-Meier curves for OS in NPC patients with low level and high level ZDHHC2 expression.

TW03 cells. There was statistically difference ($P < 0.001$) (Figure 1B and 1C). Notably, wound healing assay further demonstrated that miR-155 inhibitor could reduce the cell migration

ability in NPC CNE1 and TW03 cells (Figure 1D). Moreover MTT assay indicated that miR-155 could also inhibit cell proliferation in NPC CNE1 and TW03 cells (Figure S2).

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Table 1. Correlation between ZDHHC2 expression and clinical-pathological parameters of NPC

Parameters	Cases (n)	Missing (n)	ZDHHC2 Expression		Sig.
			L.E. (n)	H.E. (n)	
Gender		0			
Female	29		16	13	0.138
Male	95		46	49	
Age		0			
≤48 y	64		34	30	0.111
>48 y	60		28	32	
T stage		4			
T1+T2	50		30	20	0.035*
T3+T4	70		31	39	
N stage		4			
N0	29		14	15	0.161
N1+N2+N3	91		47	44	
TNM stage		2			
I+II	30		18	12	0.086
III+IV	92		44	48	
Recurrence		0			
No	85		39	46	0.062
Yes	39		23	16	
Metastasis		0			
No	95		42	53	0.011*
Yes	29		20	9	

ps: L.E.: Low Expression; H.E.: High Expression; * *P* value < 0.05.

ZDHHC2 is the direct target of miR-155

We found that ZDHHC2 protein was increased in miR-155 inhibitor transfected CNE1 and TW03 cells, compared with inhibitor control transfected cells (**Figure 2A**), but ZDHHC2 mRNA was not changed significantly (**Figure 2B**). Analyzing the sequence of ZDHHC2 3'UTR region by TargetScan [29], we found two potential miR-155 target sites in ZDHHC2 3'UTR region (base sequence 433-439 and 2334-2340) (**Figure 2C**).

To test whether ZDHHC2 responds to miR-155 through direct 3'UTR interactions, we cloned the wild type of 3'UTR of ZDHHC2 (3'UTR), and two mutated potential miR-155 target sites in ZDHHC2 3'UTR region (3'UTR-433mut and 3'UTR-2334mut) respectively into a reporter plasmid downstream of luciferase. The luciferase reporter assays were performed by tran-

siently transfecting HEK 293T cells respectively, with pmiR-Glo-ZDHHC2-3'UTR, pmiR-Glo-ZDHHC2-3'UTR-433mut, or pmiR-Glo-ZDHHC2-3'UTR-2334 mut, together with miR155 mimic (Ambion, USA), mimic control (negative control), or blank and pCMV-Renilla (internal control). After 48 hr transfection, a dual-luciferase reporter assay system (Promega, USA) was used to detect luciferase expression. MiR155 mimic could downregulate the ratio of luciferase significantly in pmiR-Glo-ZDHHC2-3'UTR group ($P=0.01$), pmiR-Glo-ZDHHC2-3'UTR-433mut group ($P=0.01$) and pmiR-Glo-ZDHHC2-3'UTR-2334 mut group ($P=0.01$), compared to negative control siRNA transfected groups and blank groups (**Figure 2D**). Taken together, these data confirmed that miR-155 could directly target ZDHHC2-3'UTR region through the sites of ZDHHC2-3'UTR 433~439 and 2334~2340 base sequence.

Inhibition of ZDHHC2 promotes cell migration in nasopharyngeal carcinoma

To investigate the function of ZDHHC2 in NPC, four ZDHHC2 specific siRNAs (ZDHHC2-homo-824, ZDHHC2-homo-1101, ZDHHC2-homo-1175 and ZDHHC2-homo-1295) and negative control were transiently transfected to two EBV negative NPC cell lines CNE1 and TW03, and then both transwell assay and wound healing assay were performed to determine if ZDHHC2 could influence the cell migration ability in NPC cells. Besides ZDHHC2-homo-1295, the other three ZDHHC2 siRNAs (ZDHHC2-homo-824, ZDHHC2-homo-1101 and ZDHHC2-homo-1175) could knock down both ZDHHC2 mRNA and ZDHHC2 protein significantly in NPC CNE1 and TW03 cells (**Figure 3A** and **3B**). The average number of migrated CNE1 cells transfected with ZDHHC2-homo-824, ZDHHC2-homo-1101 and ZDHHC2-homo-1175 was 464 ± 7 , 350 ± 6 , 295 ± 7 , which was significantly more than that of CNE1 cells transfected with ZDHHC2-homo-1295 and negative control (264 ± 4 and 237 ± 5) ($P=0.015$) (**Figure 4A** and **4B**). Similar results was found in NPC TW03 cells (**Figure 4A** and **4B**).

Reduced expression of ZDHHC2 correlates with metastasis and poor prognosis of nasopharyngeal carcinoma

To investigate the association between ZDHHC2 expression and clinicopathological param-

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Table 2. Univariate and multivariate analysis of various prognostic parameters for OS in NPC

Variables	Univariate analysis		Multivariate analysis	
	HR (95% CI)	P	HR (95% CI)	P
Gender (F/M)	1.055 (0.515, 2.163)	0.884	0.650 (0.283, 1.495)	0.311
Age (>48 y/≤48 y)	3.890 (1.905, 7.944)	0.001	7.474 (3.189, 17.514)	0.001
T stage (T3+T4/T1+T2)	0.915 (0.479, 1.749)	0.789	1.561 (0.612, 3.985)	0.352
N stage (N1+N2+N3/N0)	1.070 (0.506, 2.263)	0.858	0.825 (0.357, 1.906)	0.653
TNM stage (III+IV/I+II)	1.211 (0.575, 2.550)	0.614	1.155 (0.380, 3.504)	0.780
Recurrence (Yes/No)	1.561 (0.830, 2.937)	0.167	0.777 (0.371, 1.630)	0.505
Metastasis (Yes/No)	6.036 (3.225, 11.295)	0.001	11.638 (4.995, 27.117)	0.001
ZDHHC2 (H.E./L.E.)	0.441 (0.231, 0.845)	0.014	0.213 (0.095, 0.480)	0.001

ps: L.E.: Low Expression; H.E.: High Expression.

eters of NPC patients, paraffin-embedded tissues section (n=124) with histopathologically confirmed NPC were examined using immunohistochemistry. The ZDHHC2 was observed predominantly in the cytoplasm of NPC tumor cells, and low expression of ZDHHC2 was observed in 50% (62/124) of NPC cancer patients (**Figure 5A**). Interestingly, reduced ZDHHC2 expression was associated significantly with metastasis (P=0.011) and T stage (P=0.035) (**Table 1**). No significant association was seen between ZDHHC2 expression and age, gender, N stage, TNM stage and recurrence (**Table 1**).

To investigate the prognostic value of ZDHHC2 expression in NPC patients, disease free survival (DFS) and overall survival (OS) analysis were performed in these 124 NPC cases, and the five-year DFS rate was 65.7% for these patients (**Figure 5B**). The five-year DFS rate was 55.4% for patients with low ZDHHC2 expression (n=62), and 73.4% for patients with high ZDHHC2 expression (n=62), which was a significant difference (P=0.031, **Figure 5C**). The five-year OS rate was 77.9% for these 124 NPC patients (**Figure 5D**). The patients with the low levels of ZDHHC2 expression (n=62) had a poorer prognosis than the patients with high levels of ZDHHC2 expression (n=62) (P=0.011, **Figure 5E**). Univariate and multivariate analyses were performed to compare the impact of ZDHHC2 expression and other clinicopathological parameters on prognosis. Univariate analyses showed that 3 factors, including ZDHHC2 expression (P=0.014), metastasis status (P=0.001) and age (P=0.001) were prognostic predictors of OS in NPC patients. Multivariate analyses indicated the relative risk of death in patients with high ZDHHC2 expression tumors

was lower than that of patients with low ZDHHC2 expression tumors (HR=0.213, 95% CI=0.095-0.480). ZDHHC2 expression had a significant, independent predictive value for survival of NPC patients (P=0.001). Moreover metastasis status (P=0.001) and age (P=0.001) were also independent prognosis predictors for NPC patients (**Table 2**).

Discussion

MiR-155 is considered as a typical multifunctional microRNA, which is involved in many other different biological processes including inflammation, haematopoiesis and immunity. MiR-155 also plays a pivotal role in many human cancers, and many studies have reported on the role of miR-155 in cancer development either as an oncomiR or as an oncosuppressor-miR [30]. Expression of miR-155 was upregulated in many human cancers with high proliferative activity and decreased apoptotic capability, including breast cancer [8, 31], B-cell lymphoma [12], hepatocellular carcinoma [15], cervical cancer [32], colon cancer [33] and lung cancer [34]. Moreover, upregulation of miR-155 has been also reported to promote the migration and invasion in many human cancers, including colorectal cancer [35], breast cancer [36], pancreatic cancer [37] and hepatocellular carcinoma [38]. However on the other hand, miR-155 was found to be downregulated and acted as an oncosuppressor-miR in adult Burkitt lymphoma [39], acute myeloid leukaemia [40], gastric cancer [41, 42], ovarian cancer [43] and melanoma [44]. In the present study, we report that miR-155 inhibitor could suppress cell migration as well as cell proliferation in NPC CNE1 and TW03 cells *in vitro*. These data proposed that miR-155 acts as an

oncomiR in NPC. In our previous study, we have reported that upregulation of miR-155 in NPC is partly driven by EBV encoded LMP1 and LMP2A [20]. Hence as an oncomiR, miR-155 may play an important role in oncogenesis of EBV encoded LMP1 and LMP2A in NPC.

MiRNAs could regulate about 30% of all protein-encoding genes of the human genome [45], and each miRNA could have many direct target transcripts while each transcript could be regulated by one or more miRNAs [46]. At present, several direct targets of miR-155 have been identified in many human cancers. Loss of C/EBPbeta (CCAAT/enhancer binding protein, beta) mediated by miR-155 shifts the TGF-beta response from growth inhibition to epithelial-mesenchymal transition, invasion and metastasis in breast cancer [36], and miR-155 could promote the proliferation of breast cancer cells through down-regulation of SOCS1 (Suppressor of cytokine signaling 1) [8] and FOXO3a (forkhead Forkhead box O3) [31]. MiR-155 affected pancreatic cancer cell invasiveness and migration by modulating the STAT3 signaling pathway through SOCS1 [37]. MiR-155 has also been reported to be involved in the development of lymphoma by targeting SMAD5 (SMAD family member 5) [47] and SHIP1 (inositol polyphosphate-5-phosphatase) [48]. In our study, we found that miR-155 inhibitor could increase endogenous ZDHHC2 protein expression in NPC CNE1 and TW03 cells, and luciferase reporter assay was performed to identify ZDHHC2 as direct targets of miR-155. This is the first report that ZDHHC2 is a direct target of miR-155. Moreover knockdown of ZDHHC2 could promote cell migration in NPC CNE1 and TW03 cells, but didn't affect the cell proliferation in NPC (data was not shown). These data suggested that miR-155 regulated cell migration in NPC through targeting ZDHHC2 and besides ZDHHC2, there should be some other targets of miR-155 which has not been identified in NPC.

ZDHHC2 originally named as reduced expression associated with metastasis protein (REAM), has been proposed as a putative tumor/metastasis suppressor gene. The mRNA level of ZDHHC2 expression was significantly reduced in primary and metastatic foci of advanced colorectal cancer [21] and reduced of ZDHHC2 was found to be associated with metastasis and poor prognosis in gastric ade-

nocarcinoma [26] and hepatocellular carcinoma [49, 50]. In the present study, reduction of ZDHHC2 expression was observed in 50.0% (62/124) of NPC patients, and was associated significantly with metastasis ($P=0.011$) and T stage ($P=0.035$) of NPC patients. Furthermore, our results indicated that ZDHHC2 expression had a significant, independent predictive value for survival of NPC patients ($P=0.001$). Hence, our results also proposed that ZDHHC2 was a putative tumor/metastasis suppressor in NPC, which were consistent with previous studies of ZDHHC2. As one member of DHH protein family of protein acyltransferases (PATs), ZDHHC2 is directly involved in the palmitoyl transfer reaction [51]. CKAP4/p63, a cell surface receptor for antiproliferative factor (APF) [52], has been identified as a substrate of ZDHHC2 related to its tumor/metastasis suppressor function [53]. Moreover tetraspanins CD9 was also identified as the substrate of ZDHHC2 related to its putative tumor/metastasis suppressor [54]. It is possible that hypopalmitoylation of both CKAP4 and CD9 may increase tumor or metastatic behavior.

Taken together, inhibition of miR-155 suppresses cell migration in NPC through targeting ZDHHC2, and downregulation of ZDHHC2 is associated with metastasis and poor prognosis of NPC patients. Further studies are needed to validate and clarify the mechanism of miR-155 and its emerging targets in NPC, and the potential of miR-155 and ZDHHC2 as therapeutic targets for NPC should be further investigated.

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Disclosure of conflict of interest

None.

Abbreviations

NPC, Nasopharyngeal Carcinoma; ZDHHC2, Zinc finger, DHH-type containing 2; EBV, Epstein-Bar virus.

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RNA Oligo of ZDHHC2 siRNA		
N.C.	sense	5' - UUC UCC GAA CGU GUC ACG UTT - 3'
	antisense	5' - ACG UGA CAC GUU CGG AGA ATT - 3'
homo-824	sense	5' - CUC CGU CUG UGA UAA AUG UTT - 3'
	antisense	5' - ACA UUU AUC ACA GAC GGA GTT - 3'
homo-1101	sense	5' - CAA GCC AAG UUC CAU AUU ATT - 3'
	antisense	5' - UAA UAU GGA ACU UGG CUU GTT - 3'
homo-1175	sense	5' - CAG CUU GGG UUU CAG UAA ATT - 3'
	antisense	5' - UUU ACU GAA ACC CAA GCU GTT - 3'
homo-1295	sense	5' - GGA UCC UGA ACA AGC AUC UTT - 3'
	antisense	5' - AGA UGC UUG UUC AGG AUC CTT - 3'

Figure S1. The sequence of four ZDHHC specific siRNAs.

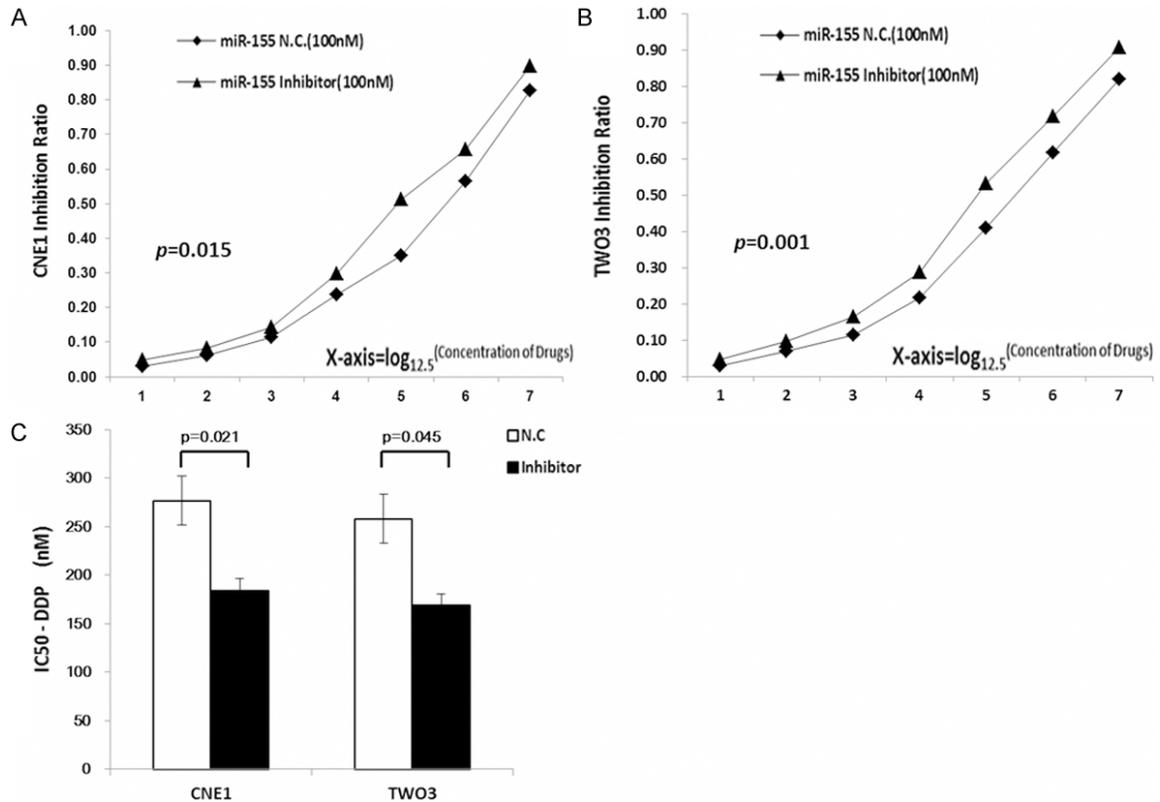


Figure S2. miR-155 inhibitor inhibits cell proliferation in nasopharyngeal carcinoma CNE1 and TW03 cells. The ability of cell proliferation was measured by MTT assay in CNE1 cells (A) and TW03 cells (B), which transfected with miR-155 inhibitor (100 nM) or inhibitor negative control (100 nM), The curve of inhibition ratio to the drug DDP showed that miR-155 inhibitor could reduce the ability of cell proliferation. (C) The IC₅₀-DDP have significant decrease in in CNE1 cells and TW03 cells transfected with miR-155 inhibitor (100 nM), compared to the cells transfected with negative control (100 nM).