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ORIGINAL ARTICLE

Correlation between the expression of miR150 and FOXO4 and the local recurrence and metastasis of nasopharyngeal carcinoma after intensive radiotherapy

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Summary

Purpose: *To investigate the relationship between the expression of miR150 and FOXO4 in nasopharyngeal carcinoma (NPC) and the local recurrence and metastasis after intensive radiotherapy.*

Methods: *94 patients with NPC were selected in Hunan Provincial People's Hospital from May 2011 to May 2013. All patients received intensive radiotherapy. Thirty healthy controls were also included. The expression levels of miR150 and FOXO4 mRNA in blood lymphocytes were detected by RT-PCR. All patients with NPC were followed up for 36 months. Blood was drawn from patients to analyze the expression of miR150 and FOXO4. MiR150 inhibitor was used to treat NPC cells, and FOXO4 overexpression cell lines were established. Transwell invasion assay was performed to investigate the effects of miR150 expression inhibition and FOXO4 overexpression on cell invasion. Protein levels were detected by western blot.*

Results: *Compared with healthy controls, the levels of miR150 mRNA in NPC patients were significantly increased, while FOXO4 mRNA levels were significantly decreased ($p<0.05$). The levels of miR150 and FOXO4 were significantly correlated with distant metastasis and tumor recurrence ($p<0.05$). High expression level of miR150 or low expression level of FOXO4 significantly shortened the overall survival (OS) of patients ($p<0.05$). Cox's proportional hazards model showed that miR150 and FOXO4 were potential independent risk factors for NPC ($p<0.05$). The level of miR150 in patients with tumor recurrence was significantly higher than that in patients without tumor recurrence, while the level of FOXO4 in patients with tumor recurrence was lower than that patients without tumor recurrence ($p<0.05$). MiR150 expression inhibition or FOXO4 overexpression significantly reduced the invasion abilities of CNE1 and CNE2 cells and protein levels of matrix metalloproteinase2 (MMP2) and MMP9 ($p<0.05$).*

Conclusion: *MiR150 and FOXO4 are closely related to the metastasis and recurrence of NPC, and are independent prognostic factors for NPC. MiR150 and FOXO4 are of clinical significance in predicting NPC prognosis.*

Key words: *intensive radiotherapy, metastasis, FOXO4, miR150, nasopharyngeal carcinoma, recurrence*

Introduction

Nasopharyngeal carcinoma (NPC) is one of the most common malignant tumors of the head and neck and is common in the coastal areas of southern China with an annual incidence of about 30 to 80/100,000 [1,2]. However, it is rare in most Western countries, especially in Europe and the northern United States, the incidence is below 1/100,000 [3]. NPC is deep in the nasopharynx, and surgical operations may cause big trauma, and no tissue available after lesion removal to cover the wound. Therefore, the treatment of nasopharyngeal carcinoma is mainly radiotherapy [4]. Most clinical NPCs are undifferentiated or poorly differentiated squamous cell carcinomas with the characteristics

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of rapid growth. Patients with cervical lymph node metastasis account for 70 to 80% of new cases, and distant metastasis accounts for 4.2% [5]. Although NPC patients are sensitive to radiotherapy/chemotherapy, the failure rate is still high due to local recurrence and distant metastases, and 5-year survival rate is only 50-60% [6,7]. Molecular mechanisms of occurrence and development of NPC are still poorly understood, and so far effective target genes for prognosis or treatment are lacking.

MicroRNAs (miRNAs) are endogenous noncoding RNA molecules that regulate many important biological processes such as cell development, proliferation, differentiation, apoptosis, signal transduction and oncogenesis by regulating gene expression [8,9]. MiRNA chip analysis showed that a considerable number of miRNAs were abnormally expressed in tumor tissues. Among them, miR-150 is located on chromosome 19q13 and plays an indispensable role in the hematopoietic system [10]. Recent studies reported that miR-150 promoted the progression of cervical cancer by regulating FOXO4 [11]. As an important member of the FOXO family of tumor suppressor genes, FOXO4 can induce apoptosis by up-regulating the expression of a variety of pro-apoptotic genes [12]. In addition, recent studies showed that FOXO4 and β-catenin could inhibit the invasion and metastasis of tumor cells [13]. So far, correlation between the expression of miR-150 and FOXO4 in patients with NPC and prognosis still hasn't been well studied. The main purpose of this study was to explore the relationship between miR150 and FOXO4 expression and recurrence and metastasis of NPC, and to explore potential molecular mechanisms.

Methods

Clinical data

A total of 94 patients with NPC were selected in Hunan Provincial People's Hospital from May 2011 to May 2013. All patients were diagnosed using imaging tests, biochemical examination and pathological confirmation. Their average age was 42±13.7 years. None of those patients was treated by radiotherapy and chemotherapy before enrollment. After enrollment, all patients were treated with intensive radiotherapy. All patients were followed up for 36 months or until death. At the same time, 30 healthy people were selected in the physical examination center to serve as control group. This study was approved ethics committee of Hunan Provincial People's Hospital and all patients signed informed consent.

RT-PCR

Lymphocytes were separated from peripheral blood drawn from each participant using a kit provided by Huangyuyang Biotechnology Co., Ltd (Beijing, China). Total RNA was extracted using Trizol reagent (Invitrogen, USA) according to the manufacturer's instructions. Then 2 μg RNA was used as template to synthesize cDNA using ® PrimerScript RT (Takara Corporation, Dalian, China). Target mRNA level was measured using a ReverTra Ace qPCR RT Kit (Toyobo, Cat No.: FSQ-101, Japan). Primers for each gene were synthesized by Sangon (Shanghai, China). Primer sequences were as follows: miR150: 5'-TCTCCCAACCCTTGTACCAGTG-3', Reverse, 5'-CAGTGCGTGTCGTGGAGT-3'; FOXO4: Forward, 5'-AGTCTGAGGTGCTGGCGGAG-3', Reverse, 5'-GGTGGTGGCGTATCAGAGGTG-3'; GAPDH: Forward, 5'-ATTGATGGATGCTAFGAGTATT-3', Reverse, 5'-AGTCTTCTGGGTGGCAGTGAT-3'. GAPDH was used as endogenous control. The relative mRNA level of each gene was calculated using 2-ΔCt method. ΔCt = Ct (target gene) - Ct (GAPDH).

Cell culture

Human nasopharyngeal carcinoma cells CNE1 and CNE2 cells were purchased from Cell Bank of Chinese Academy of Sciences (Wuhan, China). Cells were cultured with RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS), 100 μg/mL streptomycin and 100 IU/ml penicillin in an incubator (5% CO_2, 95% humidity).

MiR150 inhibitor treatment and FOXO4 overexpression plasmid construction and transfection

Before transfection, cells were inoculated into 6-well plates with 2×10^5 cells per well. Transfection was performed when cell confluency reached 40 to 60%. MiR150 inhibitor was designed and synthesized by Jima Industrial Co., Ltd, and transfection was performed at a concentration of 50 nM. Lentiviral vectors that stably overexpress FOXO4 were pGCSIL-GFP (Genechem, Shanghai, China). Transfection was performed using Lipofectamine 2000 reagent (Invitrogen, USA). After transfection, cells were cultured in complete medium for 48 hrs, and then were harvested for further analysis.

Transwell invasion assay

Transwell invasion assay was performed to evaluate cell invasion ability. Transwell plates were purchased from BD Biosciences, and 100 μL of 1% Matrigel was applied to an 8-μm pore size polycarbonate filter. After treatment, cells were digested, collected and mixed with serum-free RPMI 1640 medium to adjust the cell concentration to 1×10^6 cells/ml. Then, 120 μL of cell suspension was transferred to the upper chamber and 600 μL of RPMI 1640 complete medium containing 10% FBS was transferred to lower chamber. Cells were incubated in an incubator. After 48 hrs, the upper chamber was removed, and the membrane was fixed with 3% formaldehyde. DAPI staining was performed, and the stained cells were counted under a fluorescence microscope (200x).

Western blot

Cells were harvested 48 hrs after transfection and washed with ice-cold PBS buffer. Then 200 μl of 1x RIPA lysis buffer was added to each sample. The cell lysate

was mixed with equal volume of SDS buffer, followed by 10% SDS-PAGE gel electrophoresis. Then, the proteins were transferred to PVDF membranes using Bio-Rad Trans-Blot® semi-dry transfer system at 25 V for 1 hr. The membranes were blocked with 5% skimmed milk at room temperature for 2 hrs. Then, the membranes were incubated with primary antibodies for MMP2 (1: 1000, Cat # sc-894, Santa Cruz, Santa Cruz, USA), MMP9 (1: 200, Cat #: sc- 21746, Santa Cruz), and GAPDH (1: 3000, Cat No.: sc-32233, Santa Cruz, USA) overnight at 4 °C. The next day, the membranes were washed, and incubated with horseradish peroxidase labeled anti-rabbit secondary antibody at room temperature for 1 hr. Protein bands were detected using an enhanced chemiluminescence system (ECLt, CST, USA).

Statistics

Statistical analyses were performed using SPSS (V15.0). Quantitative data were expressed as mean ± standard deviation (SD). Correlations between expression of miR150 and FOXO4 and the clinicopathological parameters of patients were analyzed by chi-square test. Kaplan-Meier method was used to plot survival curves, which were compared by Log-rank test. Cox's proportional hazards model was used to identify independent prognostic factors for NPC. Comparisons between two groups were performed by t-test, and comparisons among multiple groups were performed by one-way analysis of variance (ANOVA). $p<0.05$ was considered to be statistically significant.

Results

Expression of miR150 and FOXO4

The expression of miR150 and FOXO4 in blood of NPC patients and healthy controls was detected by RT-PCR. The expression level of miR150 in the NPC group was significantly higher than that in the control group, while FOXO4 level was significantly lower in the NPC group than that in the control group ($p<0.05$), and the differences were 2.75-fold and 4.09-fold, respectively ($p<0.05$;Figure 1).

Correlations between expression levels of miR150 and FOXO4 and clinicopathological features of NPC patients

As shown in Table 1, miR150 and FOXO4 expression levels were significantly correlated with distant metastasis and tumor recurrence in NPC patients ($p<0.05$), but were not correlated with gender, age, WHO pathological grade and TNM stage ($p>0.05$). In addition, Kaplan-Meier survival curves showed that high miR150 expression or low FOXO4

Table 1. Correlations between expression levels of miR150 and FOXO4 and clinicopathological features of NPC patients

Parameters	*miR-150*				*FOXO4*			
	n=94	*Low expression n=48*	*High expression n=46*	*p*	*n=94*	*Low expression n=56*	*High expression n=38*	*p*
Gender				0.313				0.286
Male	37	21	16		37	20	17	
Female	57	27	30		57	36	21	
Age, years				0.205				0.089
≤45	63	38	25		63	36	27	
>45	31	10	21		31	20	11	
WHO classification				0.347				0.094
I+II	37	18	19		37	17	20	
III+IV	57	30	27		57	39	18	
T stage				0.293				0.351
T1+T2	48	27	21		48	25	23	
T3+T4	46	21	25		46	31	15	
TNM stage				0.109				0.084
I+II	43	20	23		43	24	19	
III+IV	51	28	23		51	32	19	
Recurrence				0.036				0.015
Yes	28	6	22		28	10	18	
No	66	42	24		66	46	20	
Metastasis				0.014				0.039
Yes	33	8	25		33	9	24	
No	61	40	21		61	47	14	

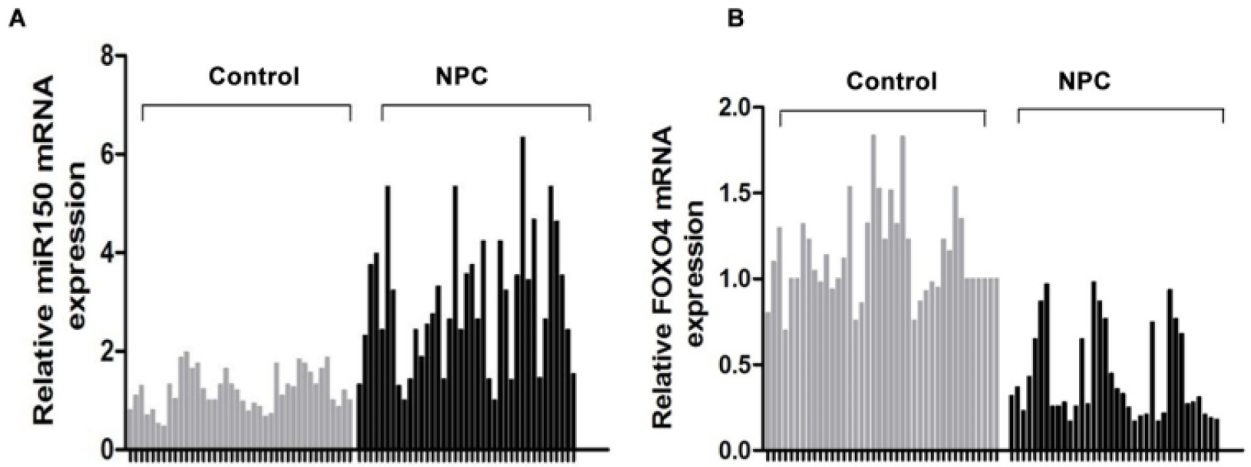

Figure 1. Expression of miR150 and FOXO4 mRNA in group and control group. RT-PCR results showed that expression level of miR150 in the NPC group was significantly higher than that in the control group **(A)**, while FOXO4 level was significantly lower in the NPC group than in the control group **(B)** ($p<0.05$).

Table 2. Cox's proportional hazards model to identify independent prognostic factors for NPC

Variables	*Subset*	*Hazard ratio (95% CI)*	*p*
miR-150expression alone	Low vs. High	1.641(1.034-2.082)	0.014
FOXO4expression alone	High vs. Low	2.72(2.405-3.288)	0.006
miR-150 and FOXO4expression	miR-150 High and/or FOXO4 low vs. miR-150 Low and FOXO4 high	2.247(1.488-3.410)	0.014
Gender	Male vs. Female	1.327(0.835-1.734)	0.431
Age, years	≤45 vs. >45	0.748(0.302-1.226)	0.082
WHO classification	I+II vs. III+IV	1.421(1.047-1.839)	0.046
T stage	T1+T2 vs. T3+T4	0.739(0.628-1.362)	0.285
TNM stage	I+II vs. III+IV	0.827(0.583-1.327)	0.551
Recurrence	Yes vs. No	2.741(2.430-3.431)	0.018
Metastasis	Yes vs. No	1.874(1.428-2.417)	0.008

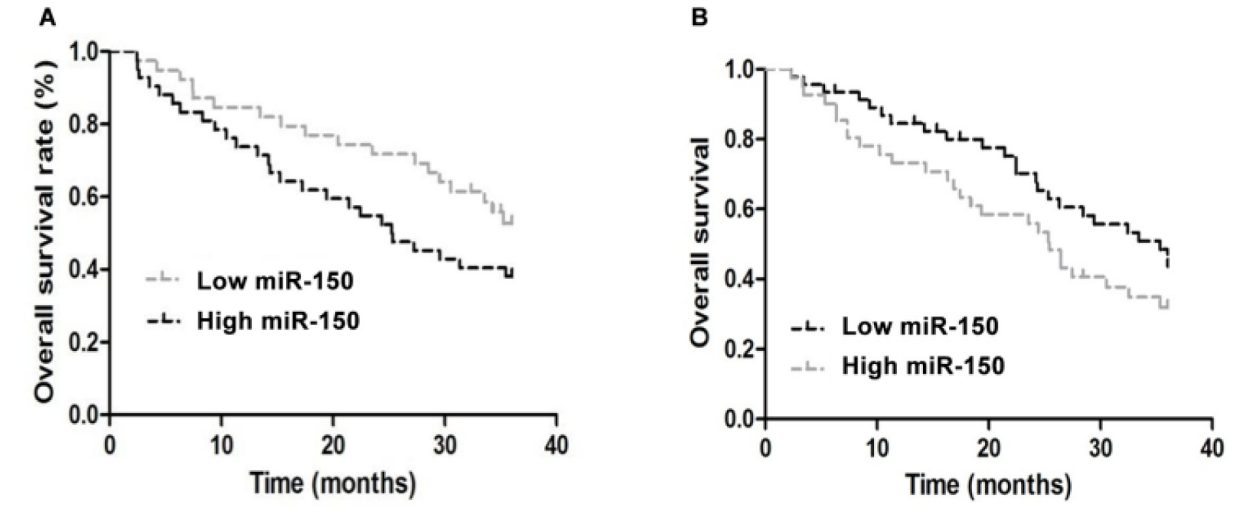

Figure 2. Correlation between miR150 and FOXO4 expression and OS. High expression level of miR150 or low expression level of FOXO4 were significantly correlated with shortened OS ($p<0.05$). **A:** Three-year survival rate of patients with low expression level of miR150 was higher than that of patients with high expression level of miR150 (58.23 vs 42.82%). **B:** 3-year survival rate of patients with high expression level of FOXO4 was higher than that of patients with low expression level of FOXO4 (45.76 vs 38.27%, $p<0.05$).

expression were significantly correlated with shortened OS (p<0.05;Figure 2). Three-year survival rate of patients with miR150 low expression was higher than that of patients with high miR150 expression (58.23 vs 42.82%), and 3-year survival rate of patients with high FOXO4 expression was higher than that of patients with low FOXO4 expression (45.76 vs 38.27%, p<0.05).

Cox's proportional hazards model to identify independent prognostic factors for NPC

As shown in Table 2, gender, age, WHO classification, TNM stage showed no significant correlation with OS (p>0.05), while expression of miR150 and FOXO4, distant metastasis and tumor recurrence were independent prognostic risk factors for NPC (p<0.05).

Comparison of expression level of miR-150 and FOXO4 in patients with and without NPC recurrence

As shown in Table 3, no significant differences in serum levels of miR150 and FOXO4 were found between patients with and without NPC recurrence (p>0.05). At 6 months and 1 year after treatment, serum levels of miR150 patients with NPC recurrence were significantly higher than in patients without NPC recurrence, while the levels of FOXO4 in patients with NPC recurrence were lower than in patients without NPC recurrence (p<0.05), suggesting miR150 and FOXO4 may participate in NPC recurrence.

Effects of miR150 expression inhibition and FOXO4 overexpression on invasion ability of CNE1 and CNE2 cells

MiR150 inhibitor was used to treat CNE1 and CNE2 cells, and FOXO4 overexpression CNE1 and CNE2 cell lines were established. Transwell invasion assay was performed to investigate the effects of miR150 expression inhibition and FOXO4 overexpression on cell invasion. As shown in Figure. 3, both miR150 expression inhibition and FOXO4 overexpression significantly reduced the invasion ability of NPC cells (p<0.05).

Table 3. Comparison of expression level of miR-150 and FOXO4 in patients with and without NPC recurrence

Gene	*Group*	*n*	*Pre-radiotherapy*	*Post-radiotherapy 6 months*	*Post-radiotherapy 1 years*
miR-150	Recurrence	28	1.03±0.16	1.76±0.25**	2.33±0.42***
	No recurrence	66	1.17±0.21	0.37±0.31	0.23±0.13
FOXO4	Recurrence	28	1.12±0.12	1.89±0.68**	2.52±0.85***
	No recurrence	66	1.07±0.19	0.48±0.23	0.26±1.53

**compared with patients without NPC recurrence, p<0.01; ***compared with patients without NPC recurrence, p<0.001.

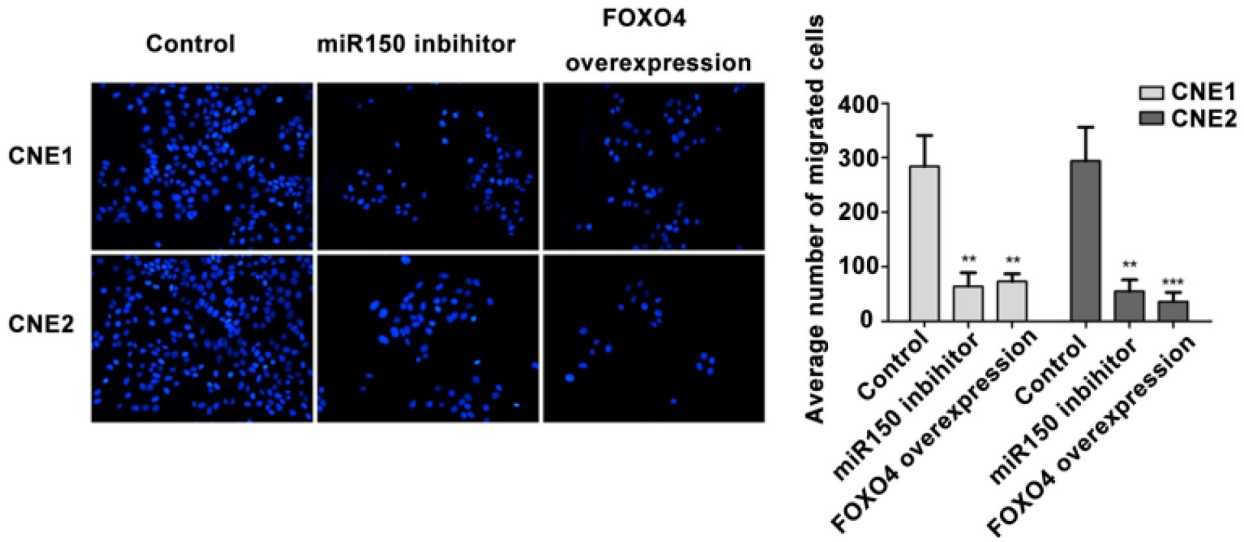

Figure 3. Effects of miR150 expression inhibition and FOXO4 overexpression on invasion ability of CNE1 and CNE2 cells. Both miR150 expression inhibition and FOXO4 overexpression significantly reduced the invasion ability of NPC cells. **compared with control group, p<0.01; ***compared with patients without NPC recurrence, p<0.001.

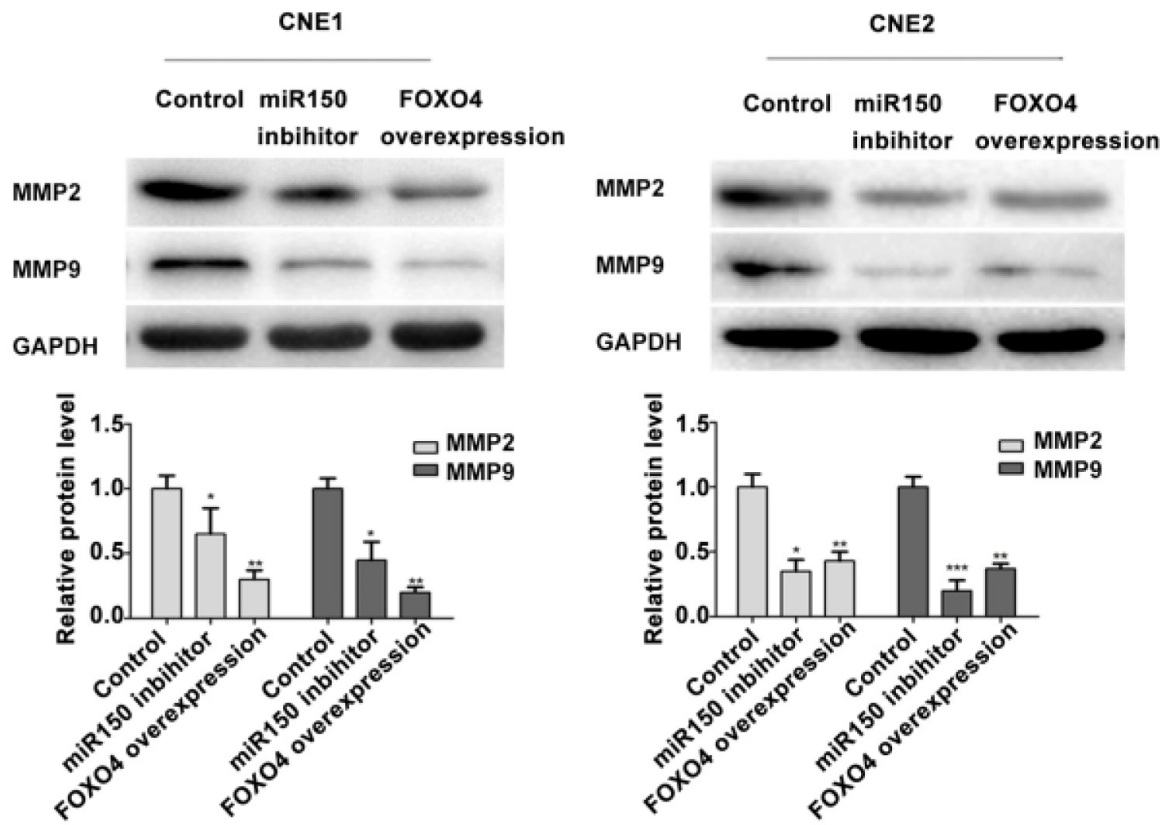

Figure 4. Effects of miR150 expression inhibition and FOXO4 overexpression on MMP2 and MMP9 expression. MiR150 expression inhibition and FOXO4 overexpression significantly reduced the expression levels of MMP2 and MMP9. *compared with the control group, p<0.05; **compared with the control group, p<0.01; ***compared with patients without NPC recurrence, p<0.001.

Effects of miR150 expression inhibition and FOXO4 overexpression on MMP2 and MMP9 expression

The expression of MMP2 and MMP9 was detected to investigate the molecular mechanism of miR150 and FOXO4 in regulating the invasion ability of CNE1 and CNE2 cells. As shown in Figure 4, both miR150 expression inhibition and FOXO4 overexpression significantly reduced the expression levels of MMP2 and MMP9 in CNE1 and CNE2 cells ($p<0.05$), indicating that MMP2 and MMP9 may be involved in the process of miR150 and FOXO4 in regulating NPC cell invasion.

Discussion

The occurrence of NPC is a multi-step process with various internal and external factors involved [14]. Those factors may include abnormal activation of oncogenes and genes involved in tumor metastasis, and inactivation of tumor suppressor genes [14]. Therefore, detecting the expression of those genes may provide references for the diagnosis and prognosis of NPC [15]. The main finding of this study was that miR150 and FOXO4 were associated with NPC prognosis.

MiR150 is a potential oncogene discovered recently. Expression of miR150 shows different characteristics in different tumor types. Previous studies have shown that miR150 is downregulated in pancreatic, esophageal, colon and liver cancers, whereas it is upregulated in gastric, breast and non-small cell lung carcinomas [16]. Our data show that miR150 expression was significantly upregulated in NPC patients. Aberrant expression of miR150 may regulate the development and progression to tumors by regulating the expression of oncogenes or tumor suppressor genes. Huang et al. have shown that overexpression of miR150 promotes the growth of human breast cancer cells and inhibits tumor cell apoptosis through P2X7 receptor [17]. Using the luciferase reporter system, Cao et al. demonstrated that miR150 directly inhibited transcription and translation of p53 at the 3'UTR locus [18].

FoxO4 protein as a tumor suppressor gene has been proved to play its role by inhibiting tumor cell proliferation, promoting apoptosis, protecting cell DNA damage and reducing oxidative stress [19]. Numerous studies have shown that FOXO4 transcription factors play a pivotal role in

the downstream of the PI3K-Akt signaling pathway. Phosphorylation of FOXO4 activates its tumor suppressor function [20]. Most importantly, Li et al. showed that miR150 could directly decrease FOXO4 expression level, thereby inhibiting p27, FasL, BIM and PRB activation and increasing cyclin D1, so as to affect the cell cycle and survival [21].

In this study, we found 46 cases of NPC patients with high expression level of miR150 and 56 cases with low expression level of FOXO4. Further clinical data analysis showed that miR150 and FOXO4 expression levels were significantly correlated with lymph node metastasis and recurrence. In addition, patients with high expression of miR150 or low expression of FOXO4 have a low 3-year survival rate, indicating that elevated miR150 expression or decreased FOXO4 expression are important prognostic factors for NPC. To further explore the relationship between miR150 and FOXO4 expression and invasion and metastasis in NPC, miR150 inhibitor was used to treat NPC cells, and FOXO4 overexpression cell lines were established. Compared with the control group, miR150 expression inhibition or FOXO4 overexpression significantly reduced the invasion abilities of NPC cells. Western blot results showed that miR150 expression inhibition or FOXO4 overexpression significantly reduced protein levels of MMP2 and MMP9, which is consistent with the findings reported by Xu et al. [22], since FOXO4 can inhibit epithelial-mesenchymal transition (EMT) in lung cancer cells, while MMPs promote EMT. Therefore, these results reflect the opposite functions of miR150 and FOXO4 in NPC metastasis. However, the molecular mechanism remains to be further studied.

In summary, our results suggest that downregulation of miR150 and overexpression of FOXO4 are two important prognostic factors for poor prognosis in NPC patients. Downregulation of miR150 and overexpression of FOXO4 can induce tumor recurrence and metastasis by upregulating MMP-2 and MMP-9. MiR150 and FOXO4 may serve as novel therapeutic targets for patients with NPC.

Conflict of interests

The authors declare no conflict of interests.

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