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MiR-185-3p and miR-324-3p Predict Radiosensitivity of Nasopharyngeal Carcinoma and Modulate Cancer Cell Growth and Apoptosis by Targeting SMAD7

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Background: MiR-185-3p and miR-324-3p are 2 miRNAs that regulate nasopharyngeal carcinoma (NPC) radioresistance. This study tried to assess the clinical values of low miR-185-3p and low miR-324-3p expression in predicting response to radiotherapy (RT) and prognosis of NPC and to explore their new downstream targets.

Material/Methods: We recruited 80 patients with primary NPC. MiR-185-3p and miR-324-3p expression in the tumor tissues before and after RT or chemoradiotherapy (CRT) were determined. Overall survival and recurrence-free survival curves were estimated to assess the prognostic values of these 2 miRNAs. Their target was predicted using an online database and verified using dual luciferase assay, qRT-PCR, and Western blot analysis. In addition, the function of miR-185-3p/miR-324-3p-SMAD7 axis in NPC cells was investigated.

Results: The expression of miR-185-3p and miR-324-3p was significantly reduced after RT in radioresistant but not in radiosensitive cases. Although miR-185-3p and miR-324-3p are not independent prognostic indicators of overall survival of NPC, their low expression is still associated with poor overall survival and recurrence-free survival. In addition, miR-185-3p and miR-324-3p can modulate growth and apoptosis of NPC cells, partly via SMAD7.

Conclusions: Combined low miR-185-3p and miR-324-3p might be important markers for prediction of low response to RT/CRT and poor overall survival and recurrence-free survival. MiR-185-3p and miR-324-3p can modulate NPC cell growth and apoptosis, at least partly through targeting SMAD7.

MeSH Keywords: **MicroRNAs • Nasopharyngeal Neoplasms • Radiotherapy, Adjuvant**

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Background

Nasopharyngeal carcinoma (NPC) is a malignancy that is highly prevalent in southern China and Southeastern Asia [1]. Currently, radiotherapy (RT) is the primary treatment for patients with NPC due to high sensitivity [2]. For patients in advanced stages, cisplatin-based chemoradiotherapy (CRT) is usually administered [3]. However, the effect of RT or CRT heavily depends on the stage of the tumor. Generally, the 5-year survival rate of stage I/II ranges from 72% to 90%, but the rate dramatically drops to less than 55% in stage III/IV due to local recurrence and distant metastasis [4]. Development of radioresistance is a major cause of therapy failure, but the molecular mechanism of radioresistance of NPC is still poorly understood.

MiRNAs is a group of conserved endogenous small non-coding RNAs. They can negatively regulate gene expression through degrading or inhibiting translation of target mRNAs, thereby modulating downstream physiological processes [5–7]. Recent studies found aberrant expression of miRNAs is involved in the development of radioresistance in NPC. For example, miR-205 can modulate radioresistance of NPC by directly targeting PTEN [8]. MicroRNA-451 and miR-101 can enhance radiosensitivity of NPC cells by targeting Ras-related protein 14 (RAB14) and stathmin 1, respectively [9, 10]. Moreover, 2 recent studies demonstrated that miR-185-3p and miR-324-3p can regulate NPC radioresistance by directly and simultaneously targeting WNT2B, a well-known oncogene [11,12], but whether other targets are involved in their regulation in NPC is not clear. SMAD7 gene encodes an intracellular protein, which is a transforming growth factor (TGF)- β type I receptor antagonist [13]. Overexpression of SMAD7 is associated with development of scleroderma, pancreatic cancer, skin cancer, colon cancer and lung cancer [14,15], but its function and how it is regulated in NPC have not been explored yet.

Due to the significant modulating effects of these 2 miRNAs over NPC radioresistance, it is of great clinical value to further study their predictive and prognostic values, as well as other downstream targets. Therefore, this study tried to assess the clinical values of low miR-185-3p and low miR-324-3p expression in predicting response to radiotherapy and prognosis of NPC and to explore their new downstream targets.

Material and Methods

Tissue sampling, radiotherapy, and response assessment

This study was approved by the institutional Ethics Review Board of the People's Hospital of Zoucheng. We recruited 80 patients with primary NPCs from the hospital after obtaining their informed consent. Tumor staging was performed based

on the TNM staging system of the International Union against Cancer. None of the patients had previous RT or CRT before biopsy sampling.

The patients were administered 2 Gy daily fractions, 5 days per week, for a total intended dose of 66–78 Gy. The dose of RT was adjusted according to the status of lymph node metastasis (50 Gy for patients with lymph node-negative invaded necks and 60–70 Gy for those with lymph node-positive invaded necks). For patients with advanced stage, additional chemotherapy using the cisplatin plus 5-fluorouracil (PF) regimen was given. The response to RT or CRT was assessed 1 month after treatment using fiber optic nasopharyngoscopy and MRI. The responses were assessed using the following standards: complete response (CR) (complete resolution of all assessable lesions), partial response (PR) (a reduction of 50% or more of the sum of the lesions and no progression of assessable lesions), no change (NC) (a reduction <50% or increase <25% in tumor size), and progressive disease (PD) (increase of 25% in tumor size or with new lesions). CR and PR were defined as radiosensitive, while NC and PD were defined as radioresistant. Patients were regularly followed up for 36 months.

Cell culture and transfection

Human undifferentiated non-keratinizing NPC cell line CNE-2 was obtained from the Experiment Animal Center of Sun Yat-Sen University and HEK-293T cells were obtained from ATCC. CNE-2 cells were cultured in RPMI 1640 and HEK-293T cells were cultured in Dulbecco's modified Eagle's (DMEM) medium supplemented with 10% heat-inactivated fetal bovine serum, 100 μ g/ml streptomycin, and 100 U/ml penicillin in an incubator with a humidified atmosphere and 5% CO₂ at 37°C.

To overexpress miR-185-3p or miR-324-3p in CNE-2 cells, the cells were transfected with 50 nM miR-185-3p or miR-324-3p mimic (Gene-Pharma, Shanghai, China) using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). SMAD7 lentiviral expression vectors (with 3'-UTR region) were purchased from GENECHM (Shanghai, China). Lentiviral particles were produced according to the manufacturer's instruction. Briefly, the lentiviral vectors and the packaging mix were co-transfected into HEK-293T cells. At 48 h after infection, the culture supernatant was collected and the viral titer was determined. To overexpress SMAD7, CNE-2 cells were treated with viral supernatants with the presence of 8 μ g/ml Polybrene (Sigma-Aldrich, St Louis, MO, USA).

QRT-PCR analysis of miR-185-3p and miR-324-3p expression

Total RNA from tissue samples was extracted using TRIzol reagent (Invitrogen) according to the manufacturer's instructions.

MiR-185-3p and miR-324-3p expression was quantified using TaqMan miRNA reverse transcription kit and TaqMan miRNA assay kits (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's protocol. MiRNA expression was normalized to that of rRNA U6 and quantified using the $2^{-\Delta\Delta CT}$ method.

Cell viability assay

Cell Counting Kit-8 (Beyotime, Shanghai, China) was used to determine cell viability according to the manufacturer's instructions. In brief, 1.5×10^3 cells were plated in 96-well plates. Cells with indicated transfection were cultured for 24, 48, and 72 h. Absorbance values at 450 nm were measured. Each experiment was performed in triplicate.

Dual luciferase reporter assay

Putative binding sites between miR-185-3p or miR-325-3p and 3'UTR of SMAD7 were predicted using MIRDB (<http://mirdb.org>). Five human SMAD7 3'UTR sequences with wild-type or mutant miR-185-3p binding sites (2 sites) and miR-325-3p binding sites (3 sites) were synthesized and cloned into pGL3 promoter vector, respectively. The recombinant plasmids were named as pGL3-SMAD7-WT1/MUT1, pGL3-SMAD7-WT2/MUT2, pGL3-SMAD7-WT3/MUT3, pGL3-SMAD7-WT4/MUT4, and pGL3-SMAD7-WT5/MUT5, respectively.

These luciferase reporter vectors (200 ng) were co-transfected into CNE-2 cells with miR-185-3p or miR-325-3p mimics (50 nM) or negative control miRNA (50 nM), respectively. At 24 h after transfection, luciferase activity was analyzed using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA). Firefly luciferase activity was normalized to that of Renilla luciferase.

Western blot analysis of SMAD7 expression

At 48 h after transfection, cells were lysed using a lysis buffer (Beyotime) and the protein concentration of the lysates was measured by BCA protein assay kit (Beyotime). The lysates were separated on 10% SDS-PAGE and then transferred onto a PVDF membrane. After blocking with 5% nonfat dry milk, the membranes were incubated with primary antibodies (anti-SMAD7(EPR622), 1:1000, ab124890, Abcam; anti- β -actin, 1:2000, ab8227, Abcam, Cambridge, MA, USA) overnight at 4°C. Membranes were washed and incubated with corresponding HRP-labeled secondary antibodies (anti-rabbit IgG, 1:10000, ab191866, Abcam). The blot signals were visualized using ECL Western blotting substrate (Promega).

Flow cytometry analysis of cell apoptosis

At 48 h after indicated transfection, CNE-2 cells were harvested and fixed in 70% ice-cold ethanol at 4°C for 24 h and then

stained with Fluorescein Active Caspase 3 Staining Kit (ab65613, Abcam). The proportion of cells with active caspase 3 was determined in a flow cytometer (FACSCalibur, BD Biosciences, San Jose, CA, USA). Data acquisition was performed using CellQuest 3.2 software (BD Bioscience). Each experiment was performed in triplicate.

Statistical analysis

All statistical analysis was performed using SPSS 18.0 software (IBM, Chicago, IL, USA). The paired Wilcoxon test was performed to compare miR-185-3p and miR-324-3p expression in the tumor samples before and after RT or CRT. The patients were divided into high/low miR-185-3p and high/low miR-324-3p expression groups according to the median level of miRNA expression. Pearson's χ^2 test was performed to compare the proportion of radioresistant cases among overall, low miR-185-3p expression, low miR-324-3p expression, and combined low miR-185-3p and low miR-324-3p expression groups. The relationship between miR-185-3p or miR-324-3p expression and clinicopathological characteristics was also assessed using Pearson's χ^2 test. Overall survival and recurrence-free survival curves were estimated using the Kaplan-Meier method. The log-rank (Mantel-Cox) test was used to assess the level of difference between the curves. Univariate analysis and multivariate analysis of the association between clinicopathological parameters and overall survival were performed using the Kaplan-Meier method (log-rank test) and the Cox proportional hazards model, respectively. Group comparison was performed using the unpaired *t* test. A 2-sided *P* value of <0.05 was considered statistically significant.

Results

The association between miR-185-3p/miR-324-3p and radioresistance and prognosis of NPC patients

To study the expression change of miR-185-3p and miR-324-3p in response to RT/CRT, the levels of these 2 miRNAs in tumor tissues before and after RT/CRT were quantified using qRT-PCR analysis. Pathological assessment showed that there were 60 radiosensitive and 20 radioresistant cases. In the radiosensitive cases, miR-185-3p and miR-324-3p were similar before and after therapy ($p=0.155$ and $p=0.368$, respectively) (Figure 1A, 1C). However, the expression of these 2 miRNAs was generally significantly reduced after therapy ($p<0.001$ in both groups) (Figure 1B, 1D). Considering the significantly decreased miR-185-3p and miR-324-3p expression in radioresistant NPC cases, we further explored whether low miR-185-3p and miR-324-3p expression can predict radioresistance. Compared with the whole patient group with 25% of radioresistant cases, the proportions of radioresistant cases in the low miR-185-3p and low miR-324-3p

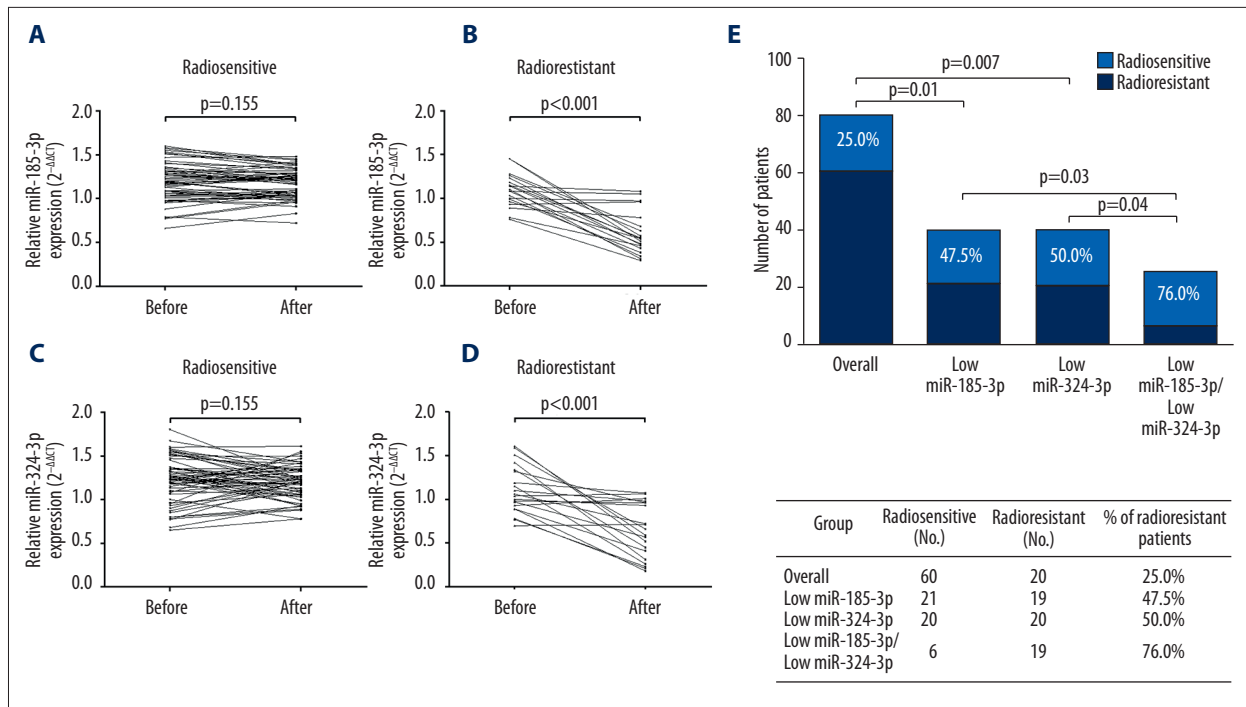


Figure 1. Association between miR-185-3p/miR-324-3p and radioresistance of NPC patients. (A, B). qRT-PCR analysis of miR-185-3p expression in tumor tissues of radiosensitive (n=60) and radioresistant (n=20) patients before and after RT or CRT. (C, D). qRT-PCR analysis of miR-324-3p expression in tumor tissues of radiosensitive (n=60) and radioresistant (n=20) patients before and after RT or CRT. (E) (Upper panel). Proportions of radioresistant cases in overall, low miR-185-3p, low miR-324-3p expression, and combined low miR-185-3p and low miR-324-3p groups, respectively. (Lower panel). Statistical data of the 4 groups.

expression groups were significantly higher (47.5% and 50%, respectively) (Figure 1E). Interestingly, the proportion of radioresistant NPC in the combined low miR-185-3p and miR-324-3p group (76.0%) was significantly higher than in the groups with low miR-185-3p or miR-324-3p alone (Figure 1E). Then, we studied the association of miR-185-3p or miR-324-3p expression with clinicopathological features in NPC. Statistical analysis showed that low miR-185-3p expression was significantly associated with EBV infection, advanced clinical stage (III+IV), higher grade of differentiation, and higher rate of radioresistance (Table 1), while low miR-324-3p expression was significantly associated with EBV infection, advanced clinical stage (III+IV), and higher rate of radioresistance (Table 2).

Because low miR-185-3p and miR-324-3p expression are closely associated with radioresistance, we then studied whether they are independent prognosis indicators of NPC. Univariate analysis showed that clinical stage (III+IV vs. I+II), radiosensitivity (Resistant vs. Sensitive), miR-185-3p (Low vs. High), and miR-324-3p (Low vs. High) were prognostic factors (Table 3). When applying multivariate analysis using the Cox proportional hazards model, we found that only clinical stage and radiosensitivity were independent prognostic factors for overall survival (Table 3). Although miR-185-3p or miR-324-3p cannot predict

poor prognosis independently, their combined low expression significantly affects radiosensitivity. Therefore, we decided to compare the prognostic value of miR-185-3p or/and miR-324-3p in overall and recurrence-free survival of NPC. Overall survival curves and recurrence-free survival curves in the groups with high/low miR-185-3p and miR-324-3p expression are shown in Figure 2. As expected, patients with low miR-185-3p or low miR-324-3p had significantly poorer overall survival (Figure 2A, 2B) and recurrence-free survival (Figure 2D, 2E). Patients with combined low miR-185-3p and miR-324-3p expression had poorer overall survival (Figure 2C) and recurrence-free survival (Figure 2F).

Both miR-185-3p and miR-324-3p can target 3'UTR of SMAD7 and modulate growth and apoptosis of NPC cells

Because miR-185-3p and miR-324-3p have well confirmed roles in radioresistance of NPC, we decided to further explore their downstream targets. Through prediction in MIRDB, we found that miR-185-3p has 2 putative binding sites, while miR-324-3p has 3 putative binding sites with 3'UTR of SMAD7 (Figure 3A). CNE-2 cells were first transfected with miR-185-3p or miR-324-3p for overexpression (Figure 3B). MiR-185-3p or miR-324-3p overexpression significantly reduced SMAD7 expression at both mRNA (Figure 3C up panel) and protein level (Figure 3C

Table 1. The association between miR-185-3p expression and clinicopathological features in NPC patients.

Parameters	Case number	miR-185-3p expression				P value
		High	%	Low	%	
Gender						
Male	56	26	33%	30	38%	0.33
Female	24	14	17.5%	10	12.5%	
Age						
≤45	33	16	20%	17	21.3%	0.82
>45	47	24	30.0%	23	28.8%	
Smoking						
Y	43	24	30%	19	23.8%	0.26
N	37	16	20.0%	21	26.3%	
EBV infection						
Y	68	30	38%	38	47.5%	0.02
N	12	10	12.5%	2	2.5%	
Clinical stage						
I+II	59	38	47.5%	21	26.3%	0.0003
III+IV	21	2	2.5%	19	23.8%	
Histological grade						
Differentiated	18	5	6.3%	13	16.3%	0.04
Undifferentiated	62	35	43.8%	27	33.8%	
Radiosensitivity						
Sensitive	60	39	48.8%	21	26.3%	0.0008
Resistant	20	1	1.3%	19	23.8%	

Bold indicates significant values.

down panel) in CNE-2 cells. To further verify the binding, we reconstructed dual luciferase plasmids with wild-type or mutant miR-185-3p or miR-324-3p binding sequences. Transfection of miR-185-3p or miR-324-3p mimics could inhibit the luciferase activity of plasmids carrying wild-type sequences, but not the plasmids with mutant sequences (Figure 3D, 3E). Functionally, both miR-185-3p and miR-324-3p can inhibit growth of CNE-2 cells (Figure 3F) and also partly abrogate SMAD7-induced higher growth rate (Figure 3G). An inverse trend was observed in flow cytometry analysis of cell apoptosis. Both miR-185-3p and miR-324-3p can promote apoptosis of CNE-2 cells (Figure 3H) and also partly abrogate SMAD7-induced lower apoptosis rate (Figure 3H). These results suggest that both miR-185-3p and miR-324-3p can target 3'UTR of SMAD7 and modulate growth and apoptosis of NPC cells.

Discussion

Intrinsic and acquired radioresistance is a major obstacle in NPC therapy. The development of radioresistance is a complicated

process, involving multiple dysregulated physiological and pathological processes, such as overexpression of DNA repair proteins [16], autophagy [17], angiogenesis [18], and cancer stem cells [19]. However, the exact molecular mechanisms underlying radioresistance are still not fully understood.

During recent years, the roles of miRNAs in regulating fundamental cellular processes, such as cell proliferation, differentiation, and apoptosis, have been widely reported. In NPC, some miRNAs are considered as potential prognostic indicators. For example, a recent study confirmed that combining miR-BART7 and miR-BART13 levels produces a 90% predictive value for the presence of NPC, while these 2 miRNAs significantly diminished after radiotherapy [20]. More importantly, several studies directly demonstrated that miRNAs are involved in regulation of radiosensitivity in NPC. For example, miR-205 can modulate radioresistance of NPC by directly targeting PTEN [8]. MicroRNA-451 and miR-101 can enhance the sensitivities of NPC cells by targeting Ras-related protein 14 (RAB14) and stathmin 1, respectively [9,10]. MiR-185-3p and miR-324-3p can regulate NPC radioresistance by directly and

Table 2. The association between miR-324-3p expression and clinicopathological features in NPC patients.

Parameters	Case number	miR-324-3p expression				P value
		High	%	Low	%	
Gender						
Male	56	29	36%	27	34%	0.63
Female	24	11	13.8%	13	16.3%	
Age						
≤45	33	15	19%	18	22.5%	0.50
>45	47	25	31.3%	22	27.5%	
Smoking						
Y	43	22	28%	21	26.3%	0.82
N	37	18	22.5%	19	23.8%	
EBV infection						
Y	68	28	35%	40	50.0%	0.01
N	12	12	15.0%	0	0.0%	
Clinical stage						
I+II	59	38	47.5%	21	26.3%	0.0003
III+IV	21	2	2.5%	19	23.8%	
Histological grade						
Differentiated	18	6	7.5%	12	15.0%	0.11
Undifferentiated	62	34	42.5%	28	35.0%	
Radiosensitivity						
Sensitive	60	40	50.0%	20	25.0%	0.003
Resistant	20	0	0.0%	20	25.0%	

Bold indicates significant values.

Table 3. Univariate and multivariate analysis of clinicopathological factors for overall survival.

Clinicopathological	Univariate analysis (N=80)			Multivariate analysis (N=80)		
	Hazard ratio (95% CI)	P value		Hazard ratio (95% CI)	P value	
Gender (Male vs. Female)	1.527 (0.567–4.112)	0.403				
Age (>45 vs. ≤45)	1.414 (0.599–3.335)	0.429				
Smoking (Yes vs. No)	0.935 (0.412–2.12)	0.873				
EBV infection (Yes vs. No)	3.84 (0.518–28.495)	0.188				
Clinical stage (III+IV vs. I+II)	31.994 (10.26–99.764)	<0.0001		8.013 (1.811–35.458)	0.006	
Histological grade (Undifferentiated vs. Differentiated)	0.588 (0.242–1.432)	0.242				
Radiosensitivity (Resistant vs. Sensitive)	32.54 (10.701–98.947)	<0.0001		6.784 (1.108–41.536)	0.038	
miR-185-3p (Low vs. High)	6.337 (2.151–18.668)	0.001		0.705 (0.119–4.172)	0.700	
miR-324-3p (Low vs. High)	28.797 (3.874–214.042)	0.001		4.268 (0.401–45.431)	0.229	

CI – confidence interval. Bold indicates significant values.

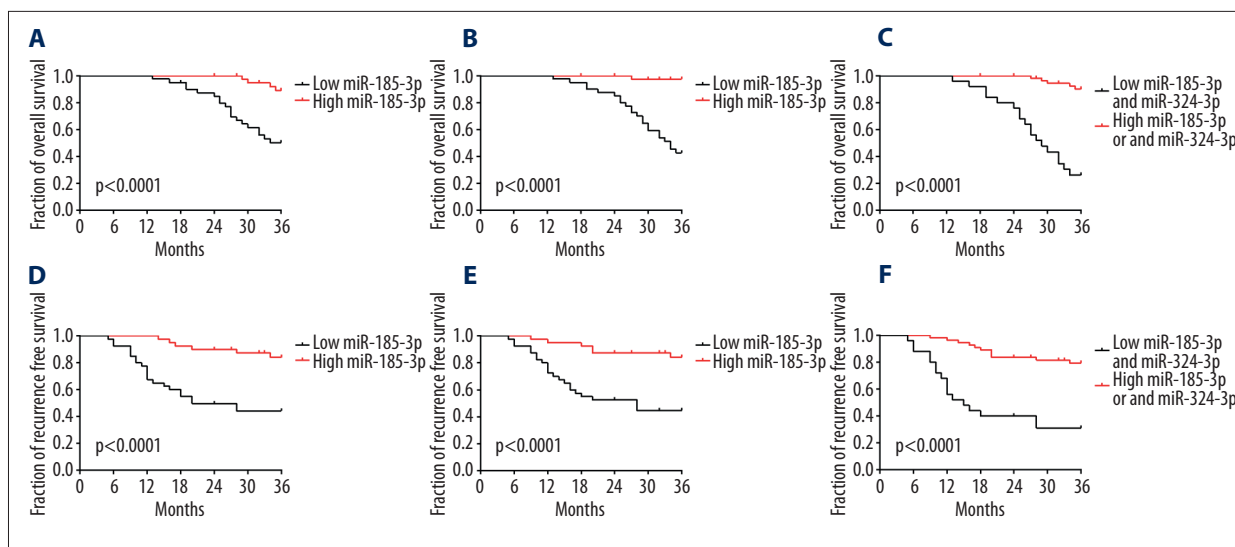


Figure 2. Association between miR-185-3p and miR-324-3p expression and prognosis of NPC patients. Kaplan-Meier curves for overall survival (A–C) and recurrence-free survival (D–F) in NPC patients divided according to median miR-185-3p or/and miR-324-3p expression. Patients with low miR-185-3p (A, D) or low miR-324-3p (B, E) had significantly poorer overall survival (A, B) and recurrence-free survival (D, E). Patients with combined low miR-185-3p and miR-324-3p expression had poorer overall survival (C) and recurrence-free survival (F).

simultaneously targeting 3'-UTR and 5'-UTR of WNT2B, respectively [11,12]. These 2 studies also found that the expression of WNT2B protein gradually increases with increasing irradiation doses in cell studies [11,12]. In fact, WNT2B is an important member of the WNT signaling pathway, which is significantly upregulated in several types of cancer, such as gastric, colorectal, and breast cancers [21]. Although the mechanism of WNT2B in regulating radioresistance is still to be determined, through downregulating WNT2B, miR-185-3p and miR-324-3p can at least inhibit irradiation-induced epithelial-mesenchymal transition (EMT), which is an important mechanism of acquired radioresistance [11,12]. However, these 2 studies were largely based on *in vitro* studies and the clinical correlation results were based on limited tissue samples, without prognostic information provided. Therefore, although the regulative effect of these 2 miRNAs on radiosensitivity were verified in NPC cells, the prognostic value of miR-185-3p and miR-324-3p need to be explored in clinical settings. In addition, whether other targets are involved in their regulation over NPC development should be further studied.

In the current study, we compared the expression trend of both miR-185-3p and miR-324-3p in 80 NPC patients before and after RT or CRT. qRT-PCR analysis confirmed significantly lower miR-185-3p and miR-324-3p expression in tumor tissues in the radioresistant cases, but not in the radiosensitive patients. More importantly, we found that combined low miR-185-3p and miR-324-3p can better predict radioresistance than either miR-185-3p or miR-324-3p alone. We also found that although miR-185-3p and miR-324-3p are not independent

prognostic indicators of overall survival of NPC, they are still associated with poor overall survival and recurrence-free survival. Patients with combined low miR-185-3p and miR-324-3p had even poorer overall survival and recurrence-free survival.

Regarding the new downstream target, through searching and comparison in online databases, we found SMAD7 is a highly possible target of miR-185-3p and miR-324-3p. Although the role of SMAD7 in tumorigenesis is paradoxical, it is overexpressed and plays an oncogenic role in several types of cancer. For example, in pancreatic cancer, overexpression of SMAD7 contributed to continued activation of the PAI-1 promoter by TGF- β 1, which enhances the tumorigenicity of the cancer cells [22]. SMAD7 was overexpressed in stem-like side populations of epithelial ovarian carcinoma (EOC) cells, which grow in an epithelial pattern. It acts as an inhibitory element targeting TGF- β -stimulated epithelial-mesenchymal transition, thereby maintaining epithelial growth of EOC cells [23]. In colorectal cancer (CRC), overexpression of SMAD7 is associated with increased phosphorylation of the cyclin-dependent kinase (CDK)2 and accumulation of CRC cells in S phase. Silencing of Smad7 inhibits the growth of CRC cell lines both *in vitro* and *in vivo* [15]. Although the role of SMAD7 in these cancers is gradually being recognized, it is still unclear how it is regulated. Previous studies found that miR-367 targets SMAD7 in pancreatic ductal adenocarcinoma cells [24]. MiR-106b-25 cluster targets SMAD7 in human breast cancer [25], while miR-25 functions as a potential tumor suppressor in colon cancer by targeting Smad7 [26]. However, the function of SMAD7 in NPC and how it is regulated in NPC is still not understood. In

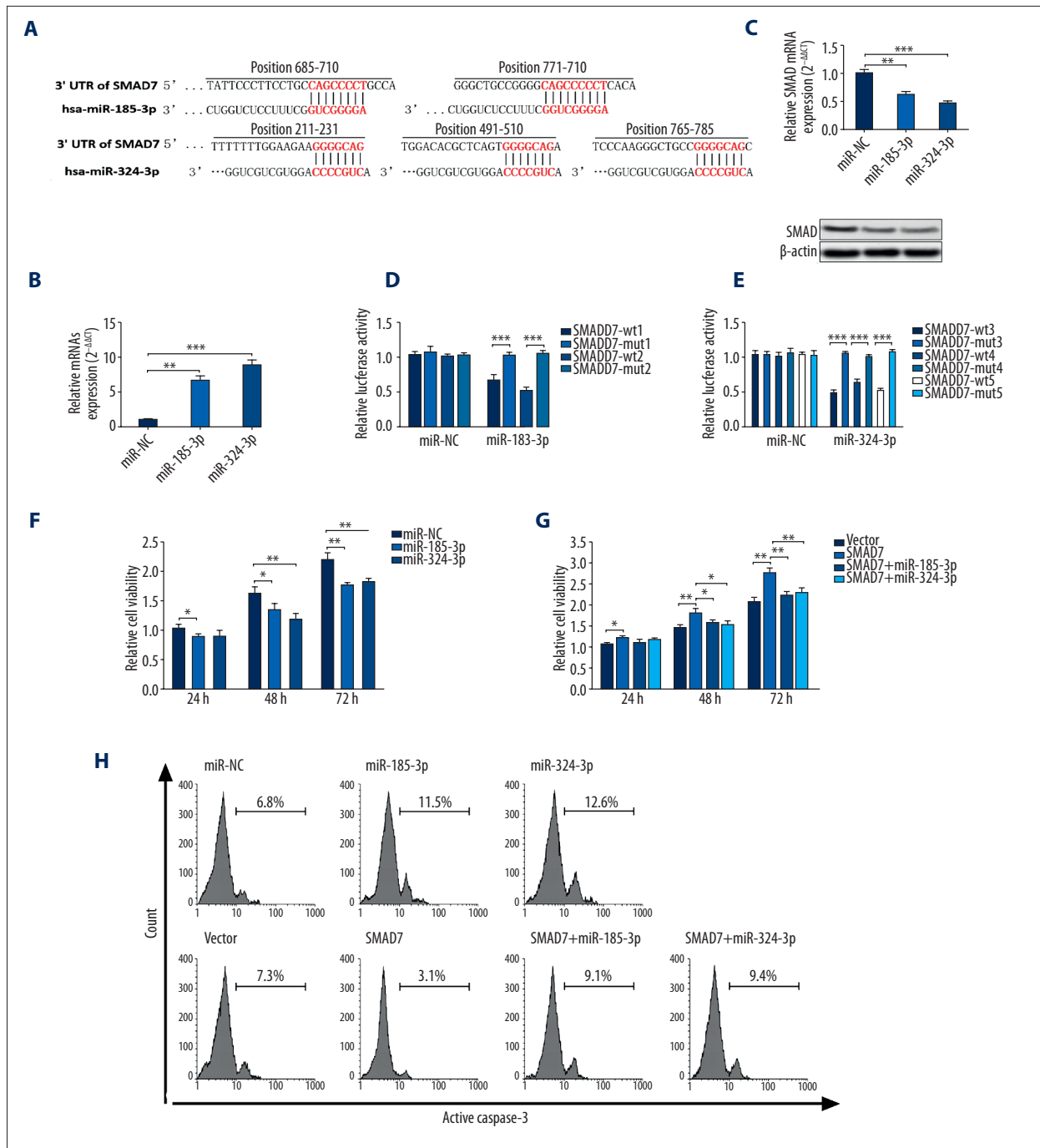


Figure 3. miR-185-3p and miR-324-3p can target 3'UTR of SMAD7 and modulate growth and apoptosis of NPC cells. **(A)** Predicted binding sites between miR-185-3p/miR-324-3p and 3'UTR of SMAD7. **(B)** CNE-2 cells were transfected with 50 nM miR-185-3p or miR-324-3p mimics for overexpression. **(C)** qRT-PCR analysis of SMAD7 expression at both mRNA (upper panel) and protein level (lower panel) in CNE-2 cells with miR-185-3p or miR-324-3p overexpression. **(D, E)** Dual luciferase assay of relative firefly luciferase activity in CNE-2 cells co-transfected with miR-183-3p **(D)** or miR-324-3p **(E)** and the reconstructed plasmids carrying wild-type or mutant miR-185-3p or miR-324-3p binding sequence. **(F, G)** CCK-8 assay of relative cell proliferation in CNE-2 cells with only miR-185-3p or miR-324-3p overexpression **(F)** or the cells with SMAD7 overexpression or combined SMAD7 and miR-185-3p or miR-324-3p overexpression **(G)**. **(H)** Representative images of flow cytometry analysis of apoptotic CNE-2 cells with only miR-185-3p or miR-324-3p overexpression or the cells with SMAD7 overexpression or combined SMAD7 and miR-185-3p or miR-324-3p overexpression. *, ** and *** donate significance at 0.05, 0.01 and 0.001 levels, respectively.

this study, reported, for the first time, that both miR-185-3p and miR-324-3p can target 3'UTR of SMAD7. MiR-185-3p and miR-324-3p can modulate NPC cell growth and apoptosis, at least partly through targeting SMAD7.

Conclusions

This study revealed the clinical significance of low miR-185-3p and miR-324-3p in RT of NPC. Combined low miR-185-3p

and miR-324-3p might be important markers for prediction of low response to RT/CRT and poor overall survival and recurrence-free survival. MiR-185-3p and miR-324-3p can modulate NPC cell growth and apoptosis, at least partly through targeting SMAD7.

Conflicts of interest

The authors confirm no conflicts of interest.

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