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Original Article

MiR-22 decreases the migration of nasopharyngeal carcinoma by TRPM7

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Abstract: Nasopharyngeal carcinoma (NPC) is a highly metastatic head and neck cancer. Transient receptor potential melastatin 7 (TRPM7), a non-selective cation channel with predominant permeability for Ca^{2+} and Mg^{2+}, has been reported to regulate the development of NPC. Our results further confirmed overexpression of TRPM7 in NPC tissues by immunohistochemistry assay. MicroRNAs (miRNAs) are reported to play an important role in the progression and development of NPC. Our luciferase assay proved that TRPM7 was a direct target of miR-22. In addition, the knockdown of miR-22 increased the migration of NPC cells. On the contrary, the overexpression of miR-22 decreased the migration progression. In conclusion, we found miR-22 directly targeted TRPM7 to regulate the migration of NPC cells, which makes it a potential therapeutic target in NPC.

Keywords: miR-22, nasopharyngeal carcinoma, migration, TRPM7

Introduction

Nasopharyngeal carcinoma (NPC) is a familiar head and neck cancer derived from epithelium cells, which located in the nasopharynx [1]. As a multi-gene genetic disease, NPC occurs mainly in southern China, Southeast Asia, and North Africa. The metastatic potential of NPC at the early stages is via both lymph and blood vessels [2]. With the development of precise imaging and radiotherapy, the prognosis of NPC patients is much better [3]. However, distant metastases are still a major reason of the poor treatment in NPC patients [4].

Transient receptor potential cation channel subfamily M member 7 (TRPM7) belongs to the TRPM subfamily, a non-selective cation channel with predominant permeability for Ca^{2+} and Mg^{2+} [5], and involves in the calcium concentration in cells [6]. It has been reported that TRPM7 increases the migration of human nasopharyngeal carcinoma cell by mediating Ca^{2+} influx [6]. In addition, activation of TRPM7 channels is critical for the growth and proliferation of human head and neck squamous carcinoma [7]. What's more, the overexpression of TRPM7 correlated with metastasis and poor prognosis of NPC [5]. But the upstream of TRPM7 in NPC is unknown.

MicroRNAs (miRNAs) are endogenous ~22-nucleotide RNAs that play significance roles by matching the mRNAs of protein-coding genes to direct their suppression [8], which exit in many organisms. MiRNAs are concerned with some diseases and cell functions, containing proliferation and differentiation [9]. The expression of miRNAs is implicated in different tumors, such as NPC [2]. There are different expression levels of miRNA-22 (miR-22) in different types of cancers. It's reported that miR-22 was down regulated in clear cell renal cell carcinoma [10], colorectal cancer [11], hepatocellular carcinoma [12] and breast cancer [13], while the expression in prostate cancer was high [14]. It's demonstrated that the neuroprotective role of miR-22 is regulated by its target gene TRPM7 [15]. However, the effect of miR-22 in NPC is unknown.

Firstly, we took immunohistochemistry and found TRPM7 was highly expressed in NPC tissues. Besides, we carried out luciferase reporter assay to investigate miR-22 was the upstream of TRPM7. To further test the function of miR-22, we made research in vitro. And miR-22 is closely contacted with cancer development. In this study, we explored whether TRPM7 regulated NPC progression controlled by miR-22 and the biological role of miR-22 in NPC.

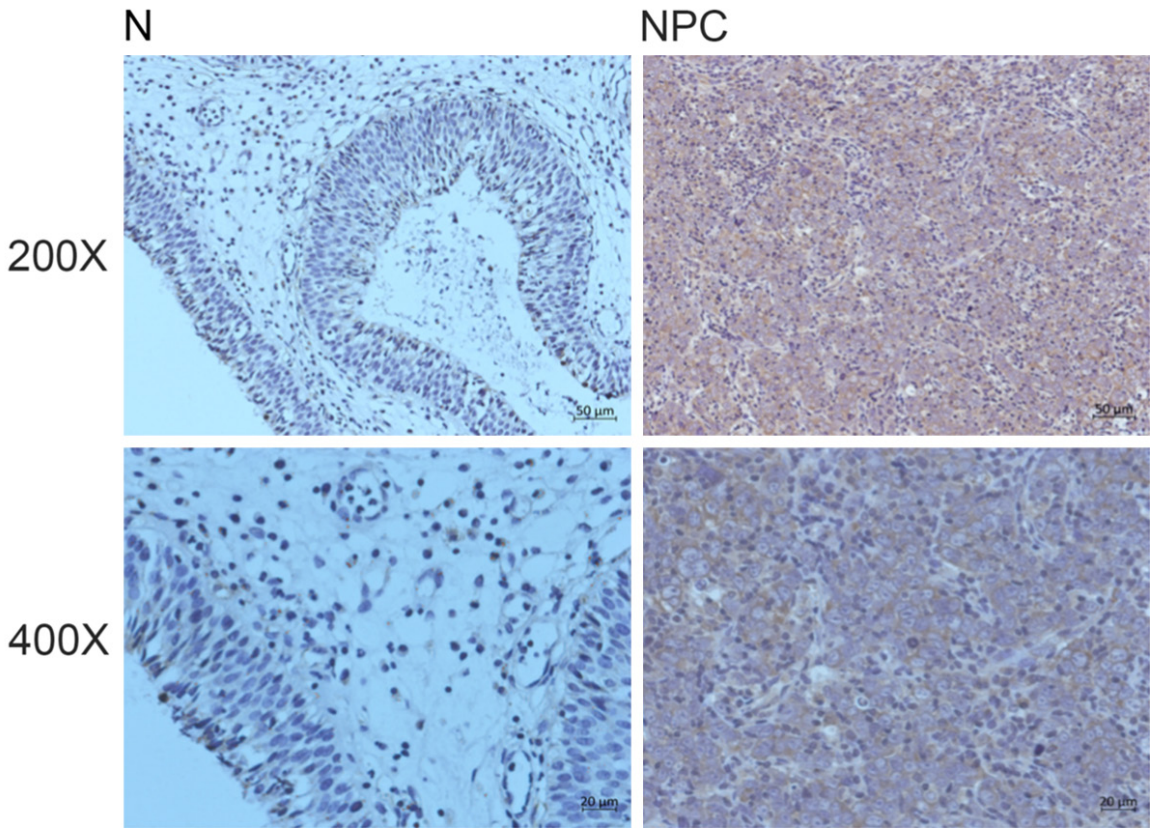

Figure 1. The expression of TRPM7 in NPC. Left, the low expression of TRPM7 was found in noncancerous tissues (N). Right, high expression of TRPM7 was observed in nasopharyngeal carcinoma (NPC) tissues.

Table 1. The expression of TRPM7 in noncancerous nasopharyngeal tissues (N) and NPC patients

Type	Total	High expression (n%)	P
N	4	0%	
NPC	14	100%	<0.0001***

Statistical analyses were performed by the Pearson χ^2 test. ***P<0.0001 was considered significant.

Materials and methods

Cell lines and cell culture

The CNE2 cells were maintained in RPMI-1640 (Gibco BRL, Grand Island, NY, USA) supplemented with 10% FBS (Gibco). All cells were incubated in an atmosphere of 37°C containing 5% CO_2. Before we tried the transfection, CNE2 cells were incubated on a 6-well plate overnight and cultured to 30-50%. The morpholino (Mo) and precursor (Pre) were transfected into CNE2 cells in vitro using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. The Mock group was transfected with Lipofectamine 2000 and the control group was incubated by medium.

Immunohistochemistry

Slides of paraffin-embedded nasopharyngeal carcinoma tissue sections were incubated with TRPM7 antibody (Santa, 1:100). IHC was performed with DAB Detection Kit (Enhanced Polymer) (ZSGB-BIO, China), color development with DAB kit (ZSGB-BIO, China), and counterstaining with hematoxylin. TRPM7 expression level was scored based on staining intensity and distribution using the immunoreactive score (IRS). IRS = SI (staining intensity) * PP (percentage of positive cells). SI was determined as 0 = negative; 1 = weak; 2 = moderate; and 3 = strong. PP was defined as 0, <1%; 1, 1%-10%; 2, 11%-50%; 3, 51%-80%; and 4, >80% positive cells. Then the IRS ranges from 1 to 12 for each section: 0- (negative); 1-4+ (weak positive); 5-8++ (moderate positive); 9-12+++ (strong positive). When evaluating TRPM7 expression, tumors with a staining score of >6 were considered positive. Then the scores of 10-12 were considered

Table 2. Clinical information of patients in the NPC study, related to **Figure 1**

Patient	Yr. DX.	Gender DX.	Age DX.	Pathological type (Low differentiated squamous cell carcinoma: 1; others: 2)	Clinical stage
1	2007.08	W	69	1	3
2	2009.07	W	43	1	2
3	2008.06	M	60	1	1
4	2010.06	W	45	1	2
5	2010.04	M	63	1	3
6	2010.05	M	63	1	3
7	2007.08	M	50	1	4
8	2006.08	W	48	1	4
9	2010.12	M	70	1	1
10	2012.06	M	64	1	1
11	2010.07	W	49	1	1
12	2010.07	W	52	1	2
13	2012.10	M	50	1	4
14	2012.03	W	72	1	1

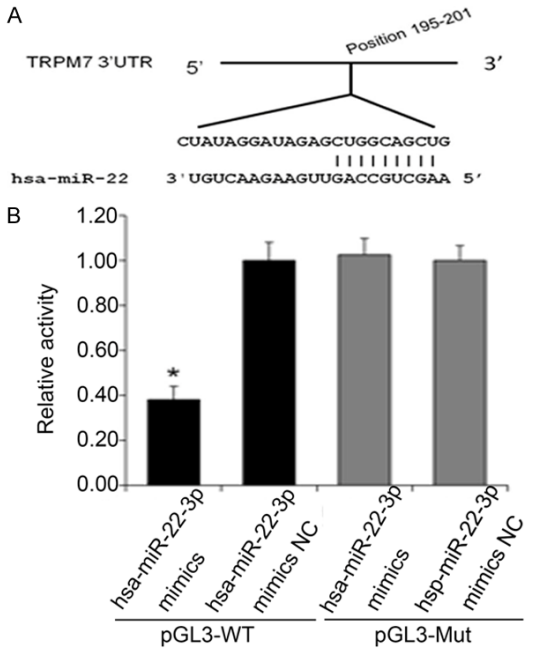

Figure 2. TRPM7 is the target gene of miR-22. A. The predicted 3'UTR region of miR-22 in TRPM7. B. Transfected the wild-type (wt) or mutant (mut) TRPM7 3'-UTR luciferase reporter plasmid of hsa-miR-22-3p mimics (mimics) or negative control miRNA (NC) to HEK293 cells, and detected the relative luciferase activity. *P<0.05.

to be high expression, and 6-9 were considered to be low expression. Ten different fields of each tumor were used for IRS evaluation. Noncancerous tissue was used as controls.

Luciferase reporter assay

HEK293 cells were seeded in the inner wells of 24-well plates. When the cell confluence reach 70%, we cotransfected the cells using Lipofectamine 2000 (Invitrogen) with 80 ng of the pGL3 vector plasmid harboring the wild-type or mutant 3'-UTR of TRPM7 and 4 ng of the pRL-TK. The miR-22 mimics or mimics NC (Biomics Biotechnologies Co. Ltd. (Nantong, China) was cotransfected at a final concentration of 50 pmol. Luciferase activities were measured consecutively 48 h post-transfection using the Dual-Luciferase reporter assay system (Promega, Southampton, UK) basing on the manufacturer's instructions. Luminescence signals served as a measure for reporter activity normalized for transfection efficiency.

Quantitative real-time PCR (qRT-PCR) analysis

MiR-22 expression in NPC and N tissues was measured with SYBR qRT-PCR. We extracted the total RNA of tissues with TRIzol (Invitrogen). Then 1 μg of total RNA was used to synthesize the cDNA of miR-22. Subsequently, 25 μl reactions were mixed, which contains 2 μl mRNA cDNA, 12.5 μl of SYBR Green Mix and 200 nM of primers, to 1 cycle of 95°C for 10 min, and then 40 cycles of 95°C for 20 sec, 62°C for 30 sec and 72°C for 30 sec. U6 was used to normalized the expression of miR-22. The primers of miR-22 were designed and synthesized by Biomics Biotechnologies Co., Ltd. (Nantong, China). Sequences of primers were shown below: Forward primer ACACTCCAGCTGGGAAGCUGCCAGUUGAAG, reverse primer TGGTGTCGTGGAGTCG.

Western blot

We lysed the transfected cells using RIPA Lysis Buffer containing protease inhibitor PMSF on ice. Twenty micrograms of total protein samples were separated on 10% SDS-PAGE gel and transferred to PVDF membrane (Millipore, Billerica, MA, USA). And we blocked the membrane in 5% non-fat dry milk in Tris-buffered

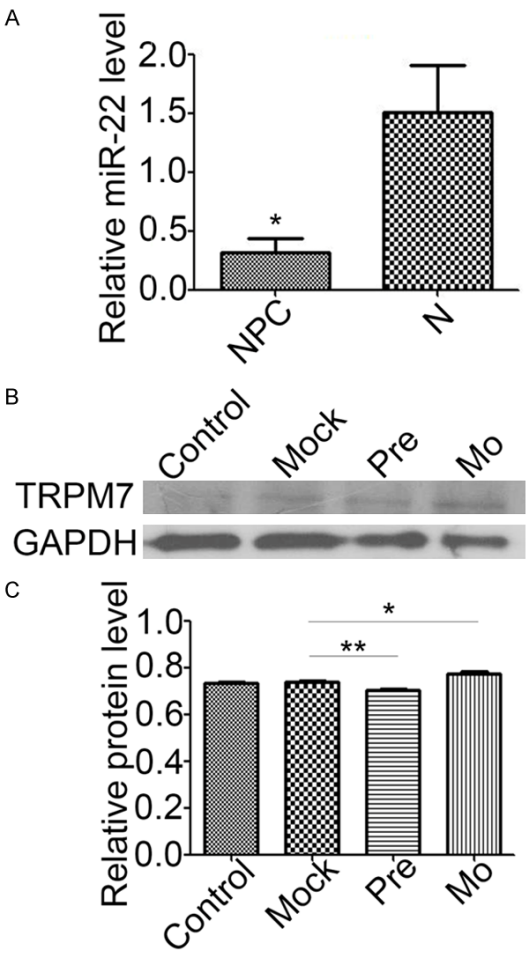

Figure 3. Expression of miR-22 in NPC and N tissues and the transfected efficiency of miR-22 to CNE2 cells. A. The expression levels of miR-22 in nasopharyngeal carcinoma (NPC) tissues and noncancerous tissues (N) were measured by RT-qPCR, U6 was used as an internal control for miR-22. Data are the means ± SD. *P<0.05. B, C. Western blot analysis of TRPM7 in CNE2 cells transfected with the morpholino and precursor of miR-22. The data shown were tested at least three independent experiments. Data were analyzed by Student's t-test. *P < 0.05, **P<0.01.

saline (TBST) for 1 h at room temperature. Then the membrane was incubated with primary antibody overnight at 4°C, next incubating it with HRP-tagged secondary antibody at room temperature for 1 h. Immunoreactivity was detected by enhanced chemiluminescence system (ECL, Cell Signaling Technologies). Data were obtained using Image J software, and GAPDH was used as the loading control. We used anti-TRPM7 (1:500; Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-GAPDH (1:5000; Cusabio, Wuhan, China), anti-Mouse IgG (1:5000; Sangon Bio Co. Ltd, Shanghai, China). All the experiments were repeated at least three times.

Wound healing assay

CNE2 cells were transfected with either morpholino or precursor and control of miR-22 on six-well plates. After the cells reached 90% confluence, wounds were made by a 100-ul pipette tip. The plate was incubated at 37°C. Wound healing was observed by microscope every 12 hours, and the migration distance was measured by the wound distance measure at 0 h.

Transwell assay

The migration activities of CNE2 cells were assayed using transwell cell culture chambers. After treating cells with Mo-miR22 and Pre-miR22 for 36 h, adding 200 ul of cells (30000 cells) in FBS free RPMI Medium modified to the top transwell chamber (8-um pore size, coring, China). The bottom chamber contained 500 ul of complete medium. The migrated cells were fixed with methanol anhydrous and stained with crystal violet after 20 h. Cells were counted in 3 random fields and captured the images with a digital camera through the Olympus microscope.

Cell cycle analyses

We transfected CNE2 cells with morpholino, precursor of miR-22, and harvested the cells after 48 h. Then the cells were fixed in 70% ethanol at -20°C overnight. Next, incubating the cells with 1% TritonX-100 10 minutes, with that added 1 mg/ml RNase A for 20 minutes and stained with PI/RNase Staining Buffer (BD Biosciences, San Diego, CA). Cell Quest acquisition and analysis programs were used after that.

Statistical analyses

Statistical analysis was performed using the GraphPad Prism 5. All the same experiments were carried out at least three times, data were analyzed by t tests and nonparametric tests shown as mean ± SEM.

Results

TRPM7 expression increased in NPC

We tested the expression of TRPM7 in NPC tissues by immunohistochemical analysis in NPC samples obtained from the patients. Consistent

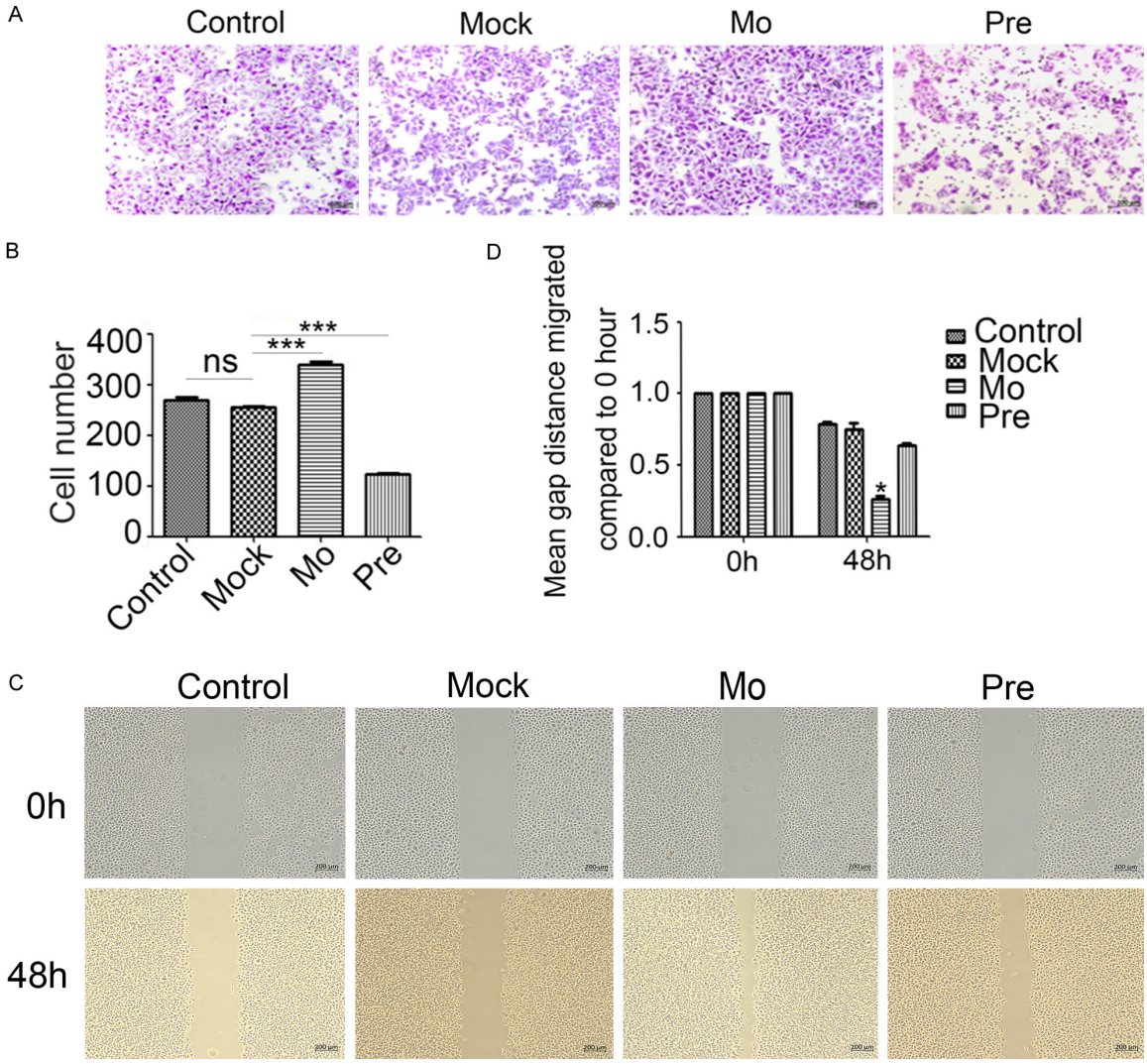

Figure 4. MiR-22 decreases the migration of CNE2 cell. A. Transwell assays were used to test the migratory capability effects of miR-22 on CNE2 cells. B. The cells that migrated through the members were counted, ***P<0.001. C. Cells were transfected until confluence and the mobility were measured by wound-healing assays. Representative images of the wound-healing were shown at 0 h and 48 h. D. The relative migration distance of cells was measured. The data were means ± SEM, *P<0.05. All experiments were carried out at least three times.

with previous study [5], we also found TRPM7 is higher in NPC tissues than in noncancerous nasopharyngeal tissues (N) (**Figure 1**). Furthermore, we found that 100% of high expression of TRPM7 in NPC patients and 0% of high expression of TRPM7 in noncancerous nasopharyngeal patients (**Table 1**). We showed the demographic and clinic information of samples in (**Table 2**). Taken together, we confirmed the overexpression of TRPM7 in NPC.

MiR-22 is the upstream of TRPM7

To explore the function further, we tried to explore the upstream of TRPM7. The 3'UTR of TRPM7 contained a predicted binding site for miR-22 by the prediction software *microRNA.org-Targets and Expression* (**Figure 2A**). Also, it's been reported that TRPM7 was the target gene of miR-22 in Parkinson's disease [15]. We confirmed the target role by Luciferase Reporter Assay, and TRPM7 bound the 3'UTR region of miR-22 (**Figure 2B**).

MiR-22 decreases the migration of CNE2 cells

We carried out quantitative real-time PCR (qRT-PCR), and found the expression of miR-22 in NPC tissues was lower than N tissues (**Figure 3A**). Subsequently, we examined the function of miR-22 in NPC cells. We investigated the effect of miR-22 on cell migration with transwell migration assay and wound-healing assay. CNE2 cells were transfected with miR-22 mor-

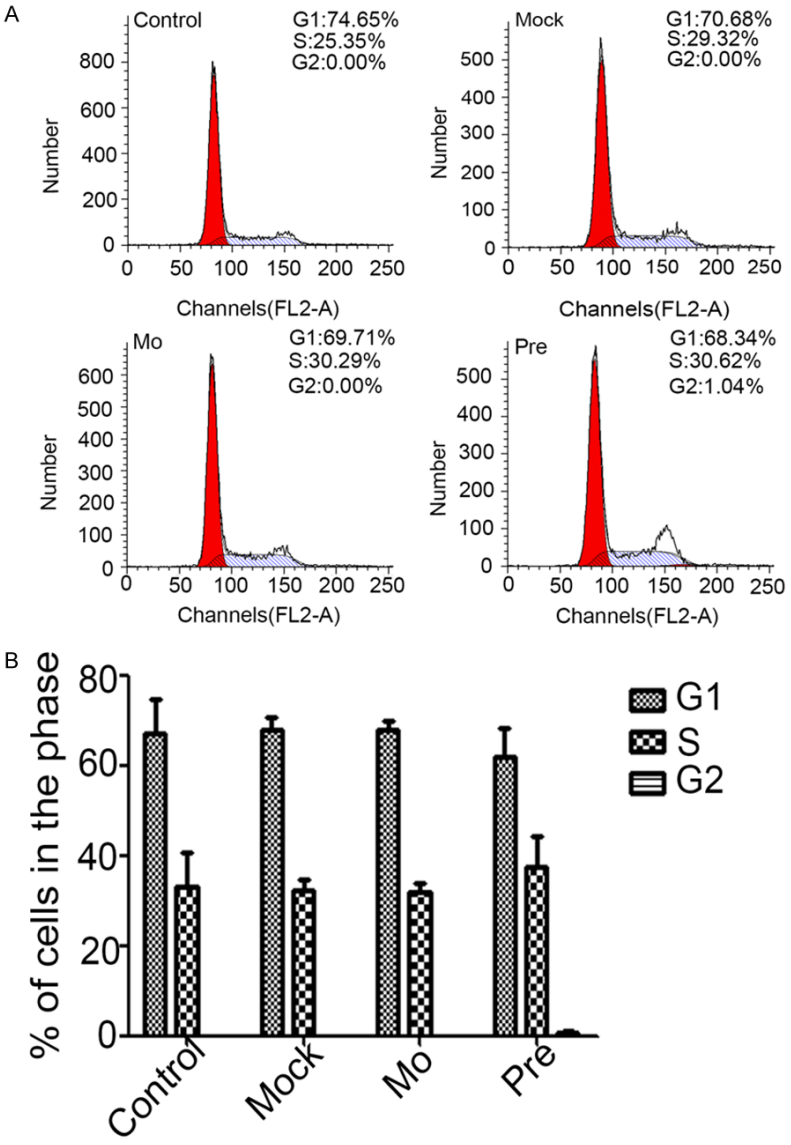

Figure 5. Relationship of miR-22 with proliferation in CNE2 cells. A. Flow cytometry was used to analysis the CNE2 cells with miR-22 knockdown and overexpression. B. The percent of cells in each phase was shown as the columnar. Data are mean± SEM. P>0.05.

pholino (Mo) and precursor (Pre), respectively. The Mock group was transfected with Lipofectamine 2000 and the control group was incubated by medium. Firstly, we tested the transfected efficiency by western blot analysis. As the overexpression and knockdown of miR-22 were efficiency (**Figure 3B**, **3C**), they were used for the follow-up studies. We investigated the effect of miR-22 on cell migration with transwell migration assay and wound-healing assays. Interestingly, the knockdown of miR-22 increased the cell number in the transwell chambers, while the overexpression of miR-22 showed the opposite phenomenon, compared with the mock group (**Figure 4A**, **4B**). In the wound-healing assays, the mean gap distance migrated of Mo group was obviously decreased compared to 0 hour, compared with other groups, which means the knockdown miR-22 truly increasing the migration of CNE2 cells (**Figure 4C**, **4D**). All the data supported that miR-22 might reduce cell migration.

Relationship of miR-22 with proliferation in CNE2 cells

To further explore the potential biological influences of miR-22 in NPC, we investigated the cell experiments about cell cycle. We found that the G1 phase, S phase and G2 phase of CNE2 cells had no obvious differences in morpholino and precursor groups, compared with control group (**Figure 5A**). The percents of cells in the phases were showed separately (**Figure 5B**). We considered miR-22 had poor relationship with proliferation in CNE2 cells.

Discussion

Although radiotherapy and chemotherapy of NPC have been widely applied to patients and good therapeutic effects have been shown, the ratio of metastasis is still high [16]. NPC is a complex biological process characterized by migration and proliferation [17]. Therefore, it's important to identify the mechanism of the NPC metastasis, progression and prognosis to contribute to the early diagnosis, supporting prognosis prediction. In this study we demonstrated that miR-22 decreased migration of CNE2 cells, which was regulated by TRPM7.

TRPM7 is a nonselective cationic channel, which is overexpression in plenty of malignancies [18]. TRPM7 Ca^{2+} and Mg^{2+} channels regulate

the migration of human cancers [19], including NPC [6]. Studies have reported TRPM7 plays a variety of functional roles in the proliferation and migration in NPC progression [7]. But the mechanisms of TRPM7 in NPC have not been fully clarified.

MiRNAs show great relationship with novel therapeutic for the treatment of cancers [20]. Evidences show that miR-22 is associated with cancer development, such as colorectal cancer [21], hepatic cancer [22], chronic heart failure [23] and non-small cell lung cancer [24]. However, there is still poor known on miR-22 and its targets in NPC.

In the current study, we used IHC to explore the expression of TRPM7 in NPC samples and found the overexpression of TRPM7 in NPC tissues compared with the noncancerous nasopharyngeal tissues (**Figure 1**). By activation, blockade, and knockdown of ion channels, Jianpeng Chen found TRPM7 regulate the metastatic process of NPC by mediating Ca^{2+} influx [5, 6]. And TRPM7 channels controlled the release of Ca^{2+} via calcium-induced calcium release (CICR) mechanism [6]. But the upstream control of TRPM7 is poorly known. Interestingly, we found TRPM7 was the target gene of miR-22 by analysis software, basing on the effects of miR-22 on cancers. Our results showed the target binding of TRPM7 and miR-22 (**Figure 2**). Further, the qRT-PCR assay showed the expression of miR-22 in NPC tissues was lower than N tissues (**Figure 3A**). In order to explore the role of miR-22 in vitro, we transfected the precursor and morpholino of miR-22 respectively, and found the interesting differences in transwell and wound-healing assays (**Figure 4**). The knockdown of miR-22 increased the migration of CNE2 cells, while the overexpression of miR-22 decreased the process. But the proliferation of CNE2 cells showed poor relationship with miR-22 (**Figure 4**). It's still need to explore the expression of miR-22 in NPC cells in the future.

In summary, we have indicated that miR-22 inhibited the migration activity in NPC cell. TRPM7 was verified as a direct target of miR-22, while the expression in NPC tissues was high. The results represented that miR-22/TRPM7 pathway might become an important novel therapeutic strategy for NPC.

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Disclosure of conflict of interest

None.

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