miR-219-5p inhibits proliferation and clonogenicity in chordoma cells and is associated with tumor recurrence

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Abstract. Chordoma is a rare malignant bone tumor that is usually localized to the skull base, vertebral column and sacrum. The transcription factor brachyury, which is encoded by the T gene, has a critical role in the development and progression of chordoma, although the mechanisms underlying brachyury regulation remain unclear. The aim of the current study was to identify and characterize microRNAs (miRs) that regulate brachyury expression in chordoma. MicroRNAs that target brachyury were predicted using miRanda and TargetScan. Using reverse transcription-quantitative polymerase chain reaction, miR-219-5p was shown to be significantly downregulated in chordoma tissues and the U-CH2 chordoma cell lines. A dual-luciferase reporter assay was used to validate the inhibitory effect of miR-219-5p on brachyury mRNA expression. The expression level of brachyury was downregulated in U-CH2 cells following transfection with miR-219-5p mimics and upregulated following transfection with the miR-219-5p inhibitor. The effects of miR-219-5p on the proliferation and clonogenicity of chordoma cells were assessed using cell counting kit-8, EdU and clone formation assays. These *in vitro* results indicated that miR-219-5p may have an important role in regulating the cell proliferation and clonogenicity of human chordoma cells, potentially by targeting brachyury. Furthermore, the associations between the expression levels of miR-219-5p and various clinicopathological factors were analyzed, and miR-219-5p expression was shown to correlate with tumor extent and recurrence. These results suggested that miR-219-5p functions as a tumor suppressor in chordoma and, therefore, that miR-219-50 may be a potential target for therapeutic intervention.

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Introduction

Chordoma is a rare malignant bone tumor with an incidence of ~0.1/100,000 individuals (1). It is thought to originate from notochordal remnants and is usually localized to the skull base, vertebral column and sacrum. Despite improvements in therapeutic modalities, the treatment of chordoma remains challenging because of its location and local recurrence (2). Therefore, understanding the molecular mechanisms underlying the development and progression of chordoma is crucial to identify novel therapeutic targets.

Brachyury, which is a transcription factor encoded by the T gene, is specifically expressed in chordomas and in the notochord (3). T gene duplication is common in familial chordoma, while gain of the T locus has also been found in sporadic cases (4,5). Silencing of brachyury using short hairpin RNA has been shown to result in decreased cell proliferation and induction of a senescent phenotype (5,6). It is well-documented that brachyury orchestrates several downstream pathways in chordoma; however, the mechanisms underlying brachyury regulation remain unclear (7).

MicroRNAs (miRNAs or miRs) are a class of small, non-coding, single-stranded RNAs that can regulate the process of translation by binding to the 3'-untranslated region (UTR) of mature mRNAs. Importantly, miRNAs have been shown to be involved in tumorigenesis, angiogenesis, invasion and metastasis (8-10). Furthermore, miRNAs may served as oncogenes or tumor suppressors, and have therefore been reported to be dysregulated in various human cancers, including gastric cancer (11), colorectal cancer (12), thyroid cancer (13) and squamous cell carcinoma (14). However, a role for miRNAs in the regulation of brachyury expression in chordoma has yet to be elucidated.

In the present study, it was first demonstrated that miR-219-5p was downregulated in chordoma tissue samples and the U-CH2 cell line. Therefore, whether brachyury is a direct target of miR-219-5p was evaluated. *In vitro* analyses showed that miR-219-5p inhibited chordoma cell proliferation and clonogenicity by downregulating brachyury. The associations between miR-219-5p expression and various patient clinicopathological factors were further analyzed and it was

shown that miR-219-5p expression was correlated with the tumor extent and recurrence.

Materials and methods

Patients and tissue samples. Tissue samples were obtained from 40 patients who had undergone surgery at Xuanwu Hospital, Capital Medical University (Beijing, China) between February 2008 and February 2013. Freshly received tissues from the operating room were immediately frozen with liquid nitrogen. All patients had confirmed pathological diagnosis according to the World Health Organization classification (15). A total of 40 skull base chordoma samples s and 10 non-neoplastic adjacent tissue samples (normal skeletal muscles) were used in accordance with the policies of the hospital's institutional review board. Written informed consent was obtained prior to initiation of the study from all patients. Medical records were reviewed to obtain clinical information for each chordoma case, including age, gender, tumor site, surgical approach and recurrence. The clinical findings of the 40 patients are listed in Table I.

Cell lines and reagents. The human chordoma cell line U-CH2 (ATCC, Manassas, VA, USA) was cultivated in Iscove's Modified Dulbecco's modified Eagle's medium (Thermo Fisher Scientific, Inc., Waltham, MA, USA) and RPMI-1640 medium (HyClone; GE Healthcare Life Sciences, Logan, UT, USA) at a ratio of 4:1 (vol/vol), supplemented with 10% fetal bovine serum (FBS; Seromed Biochrom, Berlin, Germany). Culture flasks were coated with rat tail type I collagen (BD Biosciences, San Diego, CA, USA) prior to use. The human embryonic kidney 293T, nasopharyngeal carcinoma CNE-2 and laryngeal carcinoma Hep-2 cell lines were obtained from the Institute of Basic Medical Sciences of the Chinese Academy of Medical Sciences (Beijing, China). 293T cells were cultured in RPMI-1640 medium containing 10% FBS. CNE-2 and Hep-2 cells were cultured in Dulbecco's Modified Eagle's Medium containing 10% FBS. All cell lines were cultured in a humidified atmosphere at 37˚C in 5% CO_2.

Bioinformatics analysis. miRNAs targeting the 3'-UTR of brachyury mRNA were predicted using miRanda (http://www.microrna.org/microrna/home.do) and TargetScan (http://www.targetscan.org).

Reverse transcription-quantitative PCR (RT-qPCR). Total RNA was isolated from tissue samples and cell lines using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) and was reverse transcribed using the TianScript RT kit (Tiangen Biotech Co., Ltd., Beijing, China). The reverse transcription reaction was performed in a total volume of 14.5 μl, which included 2.5 μl (1 μg) total RNA, 2 μl dNTP mix (2.5 mM), 2 μl specific reverse transcription primer (10 μM) and 8 μl RNase-free water. The reaction mixture was initially incubated at 70˚C for 5 min, followed by incubation on ice for 2 min. The tubes were centrifuged at 1,000 x *g* at 4˚C for 5 sec, after which, 4 μl 5X First-Strand Buffer, 0.5 μl RNasin (40 U/μl) and 2 μl reverse transcriptase (200 U/μl) were added to a total reaction volume of 21 μl. The reaction mixture was then incubated at 42˚C for 50 min, followed by incubation at 95˚C for 5 min. qPCR was performed on an ABI PRISM 7500 Sequence Detection system (Applied Biosystems; Thermo Fisher Scientific, Inc.) using SYBR FAST qPCR Master Mix (Kapa Biosystems, Inc., Wilmington, MA, USA), according to the manufacturer's protocol. The PCR conditions were as follows: 95˚C for 3 min, 60˚C for 20 sec; 40 cycles of 95˚C for 3 sec, 60˚C for 20 sec; and 95˚C for 15 sec, 60˚C for 15 sec and 95˚C for 15 sec. Each sample was run in triplicate. The relative expression of miRNA compared with U6 was calculated using the $2^{-\Delta\Delta Cq}$ method (16). The primer sequences, which were designed by Beijing Microread Genetics, Co., Ltd. (Beijing, China), were as follows: miR-219-5p stem-loop, 5'-GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGAAGAATTGC-3'; miR-219-5p forward, 5'-GGACGGTTGATTGTCCAAAC-3'; common loop reverse, 5'-TCGTATCCAGTGCAGGGTC-3'; U6 forward, 5'-CTCGCTTCGGCAGCACATATACT-3'; and U6 reverse, 5'-ACGCTTCACGAATTTGCGTGTC-3'.

Dual luciferase report vector construction and assay. The 3'-UTR fragment of brachyury mRNA containing the seed sequence of miR-219-5p was amplified by PCR using T gene forward and T gene reverse primers. The PCR product was then digested using *Xho* I and *Not* I (Takara Bio, Inc., Tokyo, Japan) and inserted into the pmiR-RB-Report™ dual-luciferase vector (Guangzhou RiboBio, Co., Ltd., Guangzhou, China), which contains the firefly luciferase gene *Luc* and *Renilla* luciferase gene *Rluc*, to construct the wild-type T-3'UTR (T-WT) reporter vector. The mutant 3'UTR of the T gene, containing mutated miRNA binding sites, was amplified using T-mut forward and T-mut reverse primers prior to splicing by overlap extension PCR. This was then inserted into the vector to construct the mutant T-3'UTR (T-Mut) reporter vector. The following primer sequences were used (Guangzhou RiboBio, Co., Ltd.): T forward, 5'-GATTACTCGAGAGCAGCAAGGCCCAGG-3' and T reverse: 5'-ATTGCGGCCGCGCATATTGCGTTTATTTTG-3'; and T-mut forward, 5'-GTAGCCAAACTGTTAGTGCAGAAAGCATTTTCTG-3' and T-mut reverse, 5'-GCTTTCTGCACTAACAGTTTGGCTACTTTGTCAA-3'. Upon reaching 80% confluence, U-CH2 cells were seeded into rat tail type I collagen-coated 96-well plates at a density of 3,000 cells per well (100 μl medium per well) in triplicate. After 24 h of cultivation, the cells were transiently co-transfected with 100 ng T-WT or T-Mut reporter vectors and 75 nM miR-219-5p mimics or non-target control (NC; Guangzhou RiboBio, Co., Ltd.) using Lipofectamine 2000 reagent (Invitrogen; Thermo Fisher Scientific, Inc.). After 72 h, firefly and *Renilla* luminescence were measured using a Modulus™ Microplate Fluorometer (Turner BioSystems, Sunnyvale, CA, USA) with the Dual-Glo® Luciferase Assay system (Promega Corporation, Madison, WI, USA), according to the manufacturer's protocol.

Cell counting kit (CCK)-8 and EdU assays. U-CH2 cells were seeded into rat tail type I collagen-coated 96-well plates at a density of 3,000 cells per well (100 μl medium per well) in triplicate. Subsequently, the cells were transfected with 75 nM miR-219-5p mimics, NC, miR-219-5p inhibitor or NC inhibitor (Guangzhou RiboBio, Co., Ltd.) using Lipofectamine 2000. After 24 h of cultivation, viable cells were

Table I. Clinical features of chordoma patients and relative expression levels of miR-219-5p.

N	Gender	Age (years)	Tumor site	Surgical approach	Initial/recurrent	miR
1	F	25	Clivus and CV	TA	Recurrent	0.451
2	M	25	Clivus	EEA	Recurrent	0.656
3	F	41	Clivus	EEA	Recurrent	0.600
4	F	13	Clivus and CV	TA and OCF	Recurrent	0.196
5	M	32	Clivus and SR	TA	Initial	0.960
6	M	62	Clivus	EEA	Initial	2.646
7	F	39	Clivus	TA	Recurrent	0.390
8	F	55	Clivus	EEA	Initial	0.210
9	M	34	Clivus	EEA	Initial	0.918
10	M	44	Clivus	EEA	Initial	6.881
11	F	52	Clivus	EEA	Initial	1.879
12	M	19	Clivus	EEA	Initial	0.554
13	F	15	Clivus	TA	Initial	0.230
14	M	50	Petroclival region	TA	Recurrent	0.342
15	M	62	Clivus	EEA	Initial	1.332
16	M	43	Clivus and ASB	EEA	Recurrent	0.292
17	M	23	Clivus	EEA	Initial	1.609
18	F	31	Clivus	TA	Recurrent	2.154
19	M	42	Petroclival region	EEA	Recurrent	0.662
20	M	33	Clivus	EEA	Recurrent	0.380
21	F	49	Clivus	EEA	Recurrent	0.898
22	F	32	Clivus	EOA	Initial	1.822
23	M	56	Clivus	EEA	Initial	1.589
24	M	51	Clivus and SR	EEA	Recurrent	0.820
25	F	59	Clivus	EEA	Initial	1.398
26	F	46	Clivus and CV	EOA	Initial	0.853
27	M	69	Clivus and CV	EOA	Initial	1.400
28	F	42	Clivus and ASB	EEA	Recurrent	0.143
29	F	16	Clivus	EEA	Initial	1.664
30	F	72	Clivus and CV	EOA	Initial	0.882
31	F	60	Clivus	EEA	Recurrent	1.526
32	F	66	Clivus	EEA	Initial	0.151
33	M	43	Clivus	EEA	Recurrent	1.058
34	M	22	Clivus	EEA	Initial	4.216
35	M	32	Clivus	EEA	Initial	1.211
36	M	42	Clivus and CV	EOA and OCF	Initial	0.189
37	M	47	Clivus and ASB	EEA	Recurrent	0.350
38	F	21	Clivus	EEA	Initial	2.359
39	F	28	Clivus	EEA	Recurrent	0.883
40	M	50	Clivus	EEA	Initial	0.418

CV, cervical vertebra; ASB, anterior skull base; SR, sellar region; TA, traditional approach; OCF, occipitocervical fusion; EEA, endoscopic endonasal approach; EOA, endoscopic transoral approach; miR, relative expression level of miR-219-5p.

evaluated daily for 4 days using CCK-8 assays (Dojindo Molecular Technologies, Inc., Kumamoto, Japan), according to the manufacturer's protocol. Briefly, 10 μl CCK-8 was added to each well and, after 1 h, the optical density (OD) of the samples was measured at an absorbance of 450 nm using a microplate spectrophotometer (Epoch 2; BioTek Instruments, Winooski, VT, USA). Each transfection was performed in triplicate. The impact of miR-219-5p on U-CH2 cell proliferation was also assessed using the Cell-Light™ EdU detection kit (Guangzhou RiboBio, Co., Ltd.), according to the manufacturer's instructions. Briefly, at 72 h following transfection, cells were incubated with 10 μM EdU for 2 h prior to fixation with 4% paraformaldehyde for 30 min and permeabilization using 0.5% Triton X-100 for 10 min. Proliferating cells were

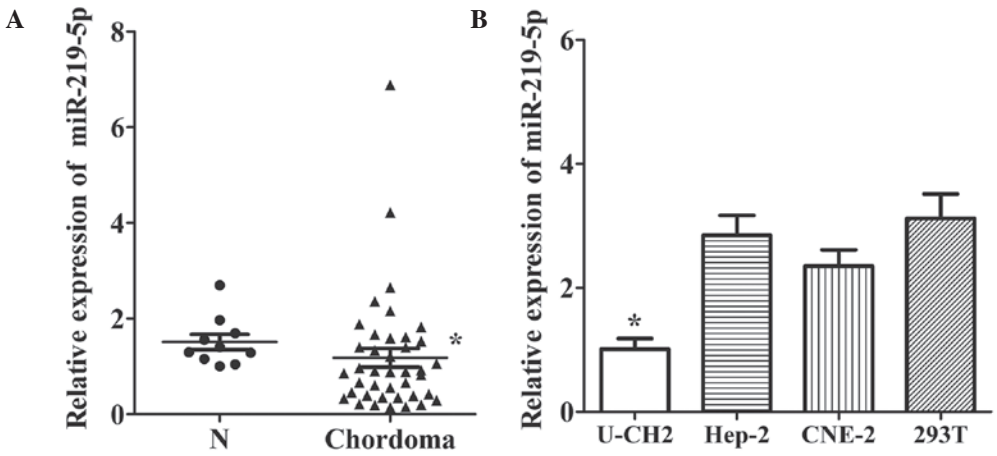

Figure 1. Relative expression of miR-219-5p in chordoma tumor tissues and cell lines. (A) miR-219-5p is downregulated in chordoma tissue samples compared with controls (*P<0.05). (B) Relative expression levels of miR-219-5p in U-CH2, Hep-2, CNE-2 and 293T cell lines (*P<0.01). N, non-neoplastic adjacent tissues.

stained with 100 μl 1X Apollo643 for 30 min, after which the cells were stained with 100 μl 1X Hoechst 33342. Fluorescent cells were counted using the automated Acumen eX3 Laser Scanning Cytometer (TTP Labtech, Ltd., London, UK).

Clone formation assay. U-CH2 or 293T cells were inoculated into rat tail type I collagen-coated 24-well plates at a density of 300 cells per well. After 18 h, the cells were transfected with 75 nM miR-219-5p mimics, NC, miR-219-5p inhibitor or NC inhibitor using Lipofectamine 2000, according to the manufacturer's protocol. Subsequently, the cells were cultured in a humidified chamber at 37˚C containing 5% CO_2, with culture medium changed every 2 days. After 5 days of cultivation, the cells were stained with 20 μM Calcein-AM (Invitrogen; Thermo Fisher Scientific, Inc.) for 45 min. Cell clusters >200-μm in size were considered colonies and were counted using the automated Acumen eX3 Laser Scanning Cytometer.

Western blotting. At 72 h following transfection, U-CH2 cells were lysed in ice-cold radioimmunoprecipitation assay lysis buffer (Beyotime Institute of Biotechnology, Haimen, China). The protein concentration was estimated using a bicinchoninic acid protein assay (Beyotime Institute of Biotechnology). A total of 50 μg protein was separated by 10% SDS-PAGE and transferred onto polyvinylidene difluoride membranes (EMD Millipore, Billerica, MA, USA). The membranes were blocked with 5% non-fat dry milk in 0.1% Tween-20 at 4˚C for 1 h, then incubated with primary antibodies against brachyury (1:1,000; ab20680; Abcam, Cambridge, MA, USA) or β-actin (1:2,000; sc-130656; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) overnight at 4˚C. Membranes were washed with TBS and incubated with alkaline phosphatase-conjugated goat anti-rabbit secondary antibody (1:3,000; BA1011; Wuhan Boster Biological Technology, Ltd., Wuhan, China). Respective bands from these blots were observed using the BCIP/NBT Color Development Substrate (Promega Corporation). The experiments were repeated three times. The mean densities of the bands were analyzed using Quantity One 4.6.2 software (Bio-Rad Laboratories, Inc., Hercules, CA, USA) and normalized to that of β-actin.

Statistical analysis. Statistical analyses were performed using SPSS software, version 21.0 (IBM SPSS, Armonk, NY, USA) and GraphPad Prism, version 5.0 (GraphPad Software, Inc., La Jolla, CA, USA). Differences in miR-219-5p expression were assessed using non-parametric tests (Mann-Whitney U test) due to the heterogeneity of variances of the residuals when applying analysis of variance. The relative luminescence ratios, ratio of cell proliferation and colony formation, and densities of bands were analyzed using Student's t-tests. Two-sided Fisher's exact test was used to identify differences between categorical variables. P<0.05 was considered statistically significant.

Results

miR-219-5p is downregulated in chordoma tissues and the U-CH2 cell line. Using miRanda and TargetScan, 16 miRNAs targeting brachyury mRNA were predicted, including miR-23a, miR-219-5p, miR-221, miR-222 and miR-448. Of these, RT-qPCR demonstrated that the expression of miR-219-5p was downregulated in chordoma tissue compared with normal skeletal muscle (Fig. 1A). Subsequently, the expression levels of miR-219-5p in U-CH2 cells were compared with CNE-2, Hep-2 and 293T cells (Fig. 1B). These results suggest that the expression of miR-219-5p is significantly decreased in chordoma tissues and the U-CH2 cell line.

miR-219-5p directly binds to the 3'-UTR of brachyury mRNA. The miR-219-5p seed sequence in the 3'-UTR of brachyury mRNA was predicted using miRanda (Fig. 2A). The wild-type and mutant T-3'-UTR fragments, containing the miR-219-5p target site, were inserted into the reporter vectors and validated by DNA sequencing (Fig. 2B). Dual-luciferase reporter assays showed that, when miR-219-5p was overexpressed, the luciferase activity in the wild-type T-3'-UTR reporter vector was significantly reduced (Fig. 2C). However, luciferase activity in the reporter vector containing the mutant T-3'-UTR was not affected by miR-219-5p. These results suggest that miR-219-5p may negatively regulate brachyury expression by directly binding to the GACAAUC sequence in the 3'-UTR of T gene mRNA.

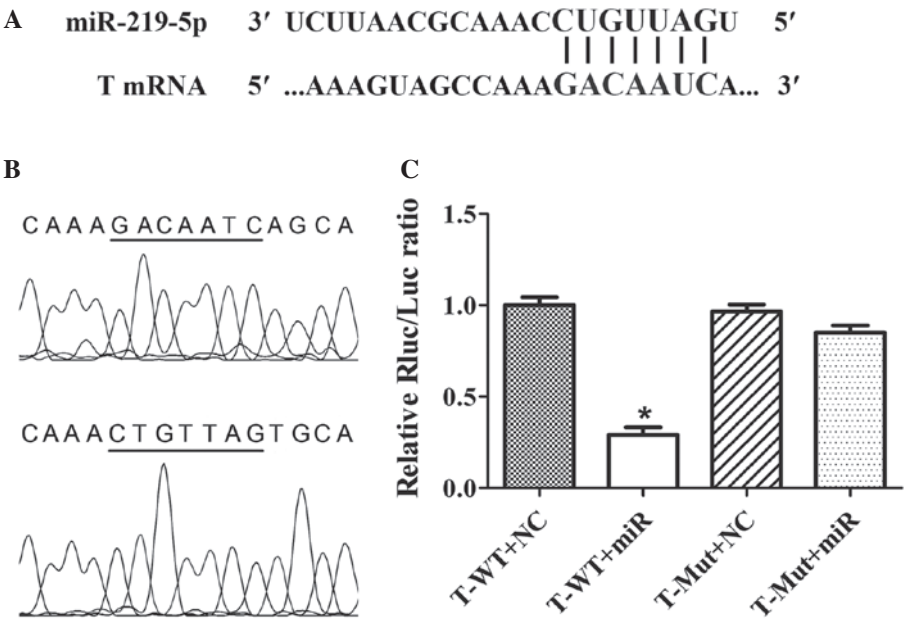

Figure 2. Brachyury is a direct target of miR-219-5p. (A) The binding site of miR-219-5p in the 3'-UTR of the T gene. (B) DNA sequencing of T-WT and T-Mut vectors validated that the binding site of miR-219-5p (GACAATC) was mutated to CTGTTAG in the T-Mut vectors. (C) Dual-luciferase assays confirmed the direct regulation of miR-219-5p on T-3'UTR in U-CH2 cells (*P<0.01). miR, miR-219-5p mimics; 3'-UTR, 3'-untranslated region; T-WT, wild-type T-3'UTR reporter vector; T-Mut, mutant T-3'UTR reporter vector; NC, normal control.

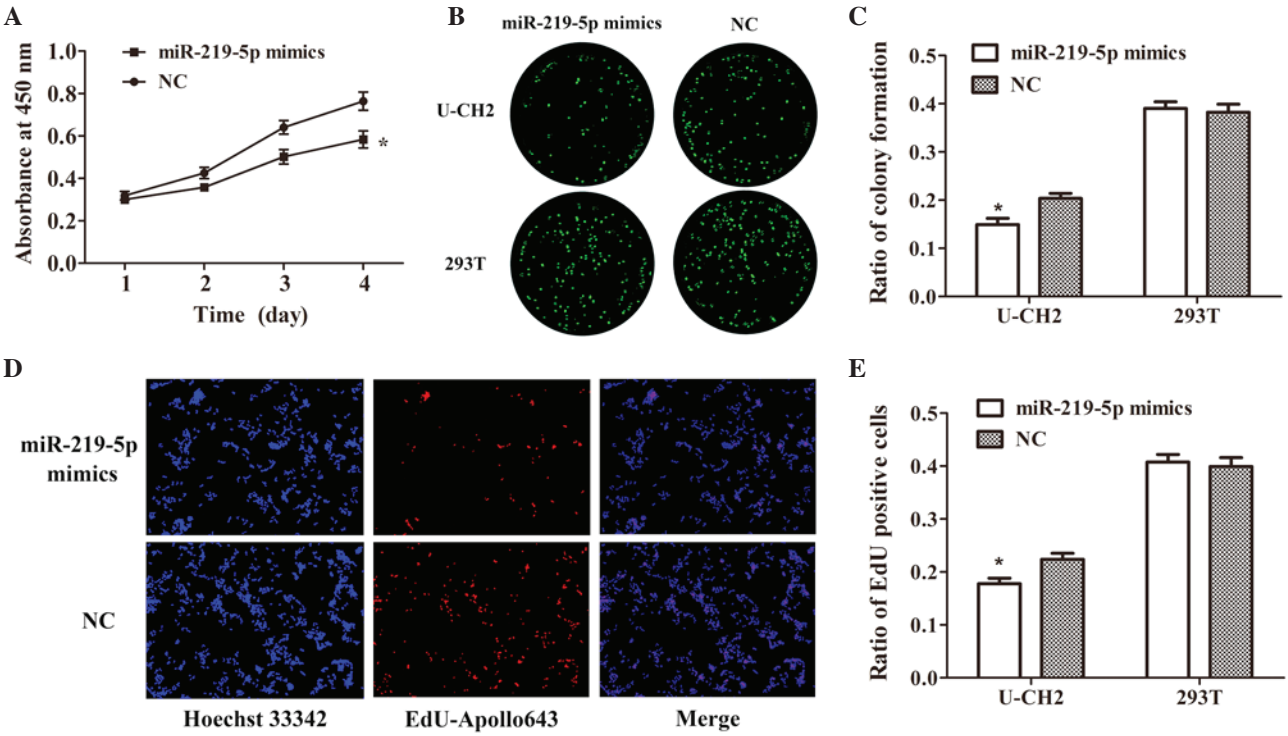

Figure 3. miR-219-5p overexpression suppressed the proliferation and colony formation of chordoma cells. (A) Cell counting kit-8 assays showing reduced cell growth activity in U-CH2 cells transfected with miR-219-5p mimics compared with NC (*P<0.01). (B) Acumen eX3 laser scanning cytometer imaging showing colonies stained with Calcein-AM (green). (C) miR-219-5p mimics reduced U-CH2 cell colony formation compared with NC, while 293T cell colony formation was unchanged (*P<0.05). (D) Acumen eX3 laser scanning cytometer imaging showing cells stained with Hoechst 33342 (blue) and proliferating cells labeled with EdU-Apollo643 (red). (E) Proliferation of U-CH2 cells declined following transfection with miR-219-5p mimics compared with NC, while the proliferation of 293T cells was unchanged (*P<0.05). miR, miR-219-5p mimics; NC, normal control.

Effect of miR-219-5p overexpression on cell proliferation and colony formation in chordoma cells. To confirm the biological function of miR-219-5p in chordoma, CCK-8, EdU and clone formation assays of U-CH2 cells transfected with miR-219-5p mimics or NC were performed. As a comparison, the effect of miR-219-5p on proliferation and colony formation was also measured in 293T cells. Significantly reduced cell growth activity (P<0.01; Fig. 3A), colony formation (P<0.05;

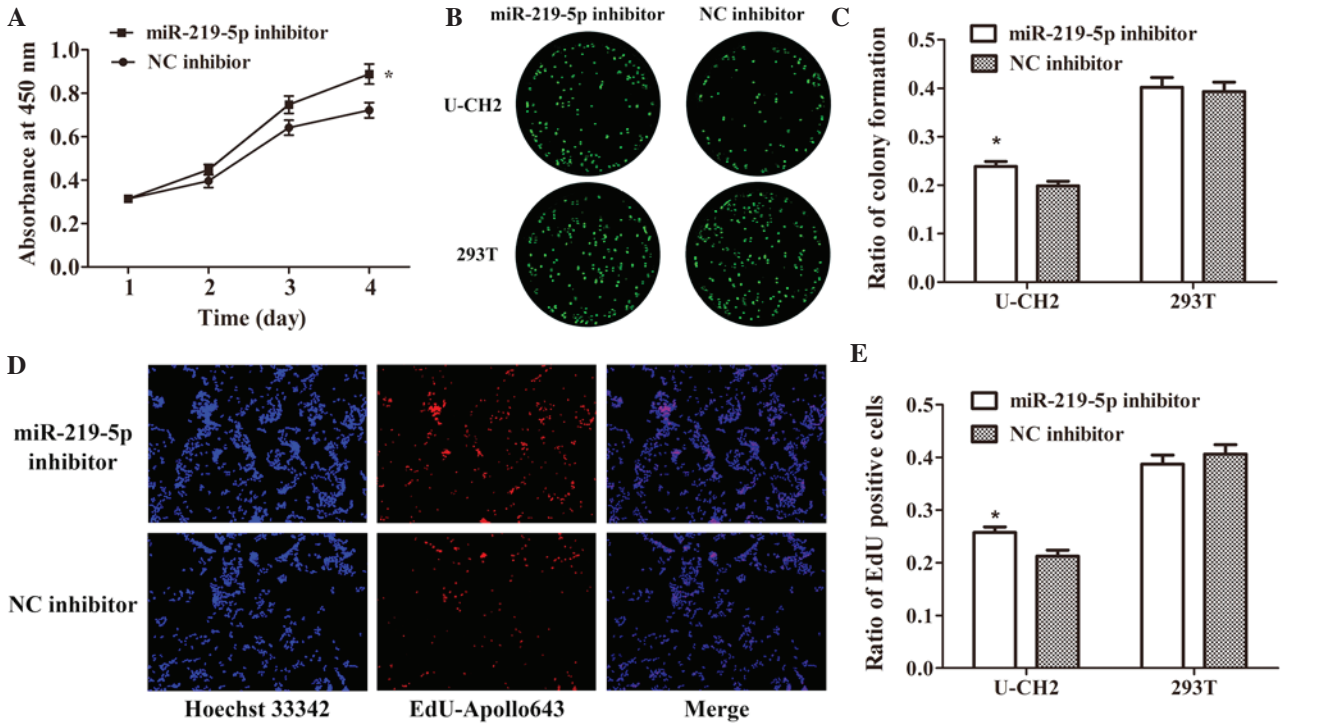

Figure 4. Inhibition of miR-219-5p promoted the cell proliferation and colony formation of U-CH2 cells. (A) Cell counting kit-8 assays demonstrated that transfection with miR-219-5p inhibitor increased U-CH2 cell growth activity compared with NC (*P<0.05). (B) Acumen eX3 laser scanning cytometer imaging showing colonies stained with Calcein-AM (green). (C) miR-219-5p inhibitor increased U-CH2 cell colony formation compared with NC inhibitor, while 293T cells were unaffected (*P<0.05). (D) Acumen ex3 laser scanning cytometer imaging showing cells labeled with Hoechst 33342 (blue) and proliferating cells labeled with EdU-Apollo643 (red). (E) Proliferation of U-CH2 cells increased following transfection with miR-219-5p inhibitor compared with NC inhibitor, while 293T cells were unaffected (*P<0.05). NC, normal control.

Fig. 3B and C) and proliferation (P<0.05; Fig. 3D and E) were observed in U-CH2 cells transfected with miRNA-219-5p mimics compared with NC. For 293T cells, there was no significant difference between the cells transfected with miR-219-5p mimics and NC in terms of colony formation (Fig. 3B and C) or cell proliferation (Fig. 3E). These results suggest that overexpression of miR-219-5p is able to suppress cell proliferation and colony formation in U-CH2 cells.

Effect of miR-219-5p knockdown on cell proliferation and colony formation in UCH-2 cells. The effect of inhibiting miR-219-5p on U-CH2 and 293T cells was examined. The results showed that downregulating the expression of miR-219-5p by transfection with miR-219-5p inhibitors significantly increased U-CH2 cell growth activity (P<0.05; Fig. 4A), colony formation (Fig. 4B and C), and proliferation (Fig. 4D and E), as compared with cells transfected with NC inhibitor. There was no significant difference in cell proliferation or colony formation between the 293T cells transfected with miRNA-219-5p inhibitor and NC inhibitor (Fig. 4). These results indicate that downregulation of miR-219-5p promotes U-CH2 cell proliferation and colony formation *in vitro*.

miR-219-5p regulates the expression of brachyury in U-CH2 cells. U-CH2 cells were transfected with miR-219-5p mimic, NC, NC inhibitor or miR-219-5p inhibitor, and the effects of miR-219-5p on the protein expression levels of brachyury were analyzed using western blotting (Fig. 5). The results demonstrated that the protein expression levels of brachyury were significantly reduced in miR-219-5p mimic-transfected U-CH2 cells compared with cells transfected with NC (P<0.01), but were increased in miR-219-5p inhibitor-transfected U-CH2 cells compared with cells transfected with NC inhibitor (P<0.05). These results suggest that miR-219-5p may regulate the protein expression of brachyury in U-CH2 cells.

miR-219-5p is correlated with tumor extent and recurrence. Among the 40 patients with skull base chordoma, 23 patients did not receive treatment prior to surgery. Five patients experienced tumor recurrence following the primary surgery and had to be operated on again. Twelve patients underwent the primary surgery at centers other than Xuanwu hospital. There was no significant relationship between the relative expression level of miR-219-5p and patient age (P=1.000) and gender (P=0.755; Table II). Compared with the initial tumors, the expression levels of miR-219-5p were significantly lower in recurrent tumors (P=0.022). The expression levels of miR-219-5p were downregulated in 12 of the 13 chordomas where the tumor had gone beyond the clivus. No statistically significant relationship was observed between surgical approaches and the miR-219-5p level (P=0.210).

Discussion

Owing to the intricate anatomic location, large tumor size and extensive bleeding during operation, complete surgical resection of chordoma is extremely difficult (2). A better understanding of the molecular mechanisms involved in

Table II. Correlations between miR-219-5p expression and various clinicopathological characteristics of skull base chordoma.

Features	miR-219-5p (n, %)		Total (n)	P-value
	Downregulation (<1)	Upregulation (>1)		
Gender				0.755
Male	12 (57.1)	9 (42.9)	21	
Female	12 (63.2)	7 (36.8)	19	
Age (years)				1.000
≤40	10 (58.8)	7 (41.2)	17	
>40	14 (60.9)	9 (39.1)	23	
Tumor site				0.005
Clivus	12 (44.4)	15 (55.6)	27	
Beyond clivus	12 (92.3)	1 (7.7)	13	
Initial/recurrent				0.022
Initial	10 (43.5)	13 (56.5)	23	
Recurrent	14 (82.4)	3 (17.6)	17	
Surgical approach				0.210
Endoscopic	18 (54.5)	15 (45.6)	33	
Traditional	6 (85.7)	1 (14.3)	7	

Data are presented as n (%).

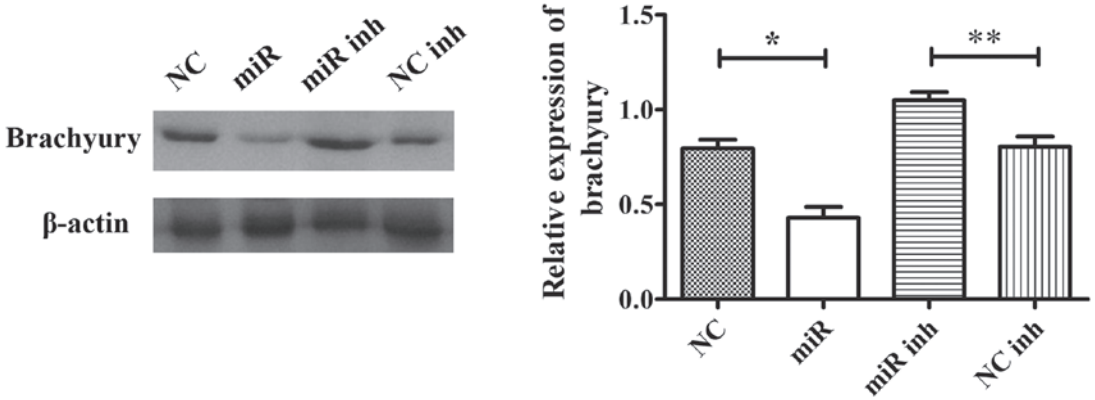

Figure 5. Western blot analysis of brachyury expression in U-CH2 cells followign transfection with miR or miR inh. Brachyury expression was downregulated following transfection with miR (*P<0.01) and upregulated after transfection with miR inh (**P<0.05), as compared with NC and NC inh, respectively. miR, miR-219-5p mimics; miR inh, miR-219-5p inhibitor; NC inh, NC inhibitor.

tumor progression may assist the development of novel therapeutic strategies for this malignant disease. Accumulating evidence indicates that miRNAs may have an important role in the progression of human chordoma (17-21). For example, reduced expression of miR-1 has been reported in chordoma, and miR-1 is suggested to suppress the growth of chordoma cells by inhibiting Met and histone deacetylase 4 (17). Further studies have shown that miR-1 inhibits chordoma cell migration and invasion and correlates with clinical prognosis (18,19). Another study confirmed that miR-608 and miR-34a were significantly downregulated in chordoma and may influence chordoma malignancy by targeting epidermal growth factor receptor (EGFR), B-cell lymphoma-xL and Met (20). Furthermore, some miRNAs are reported to impact the pathogenesis of chordoma by regulating the mitogen-activated protein kinase (MAPK) signaling pathway (21). In the present study, the relative expression level of miR-219-5p in 40 skull base chordoma specimens and different cell lines were evaluated. Our data suggested that miR-219-5p was downregulated in chordoma tissues and the U-CH2 cell line. In addition, the exogenous overexpression of miR-219-5p inhibited the proliferation and clonogenicity of U-CH2 cells by targeting brachyury, while suppression of miR-219-5p expression had the opposite effect.

Brachyury is a master regulator in chordoma that binds directly to ~100 targets and indirectly influences the expression of >60 other genes, including genes regulating the cell cycle, growth factors and cytokines, as well as extracellular matrix genes (22). Efforts have been made to clarify the mechanisms underlying the regulation of brachyury in chordoma. In the

genera *Ascidians* and *Xenopus,* as well as zebrafish, brachyury has been shown to be regulated by fibroblastic growth factor receptors via the RAS/RAF/MAPK kinase/extracellular signal-regulated kinase-ETS2 signaling pathway, but genetic alterations of these genes have not been detected in human chordoma (23). The P63 gene can regulate brachyury expression in mouse embryonic fibroblasts and murine-derived osteosarcomas, but not in human chordomas (24). In the present study, the inhibitory effect of miR-219-5p on brachyury, predicted by miRanda and TargetScan, was validated by the dual-luciferase reporter assay. *In vitro* experiments showed that miR-219-5p regulated the protein expression of brachyury in chordoma cells, and affected cell proliferation and clonogenicity. The results of the present study suggest that the loss of regulation of miR-219-5p on brachyury may be an important mechanism in the initiation and progression of chordoma.

A review of the literature demonstrated that miR-219-5p expression is consistently dysregulated in various tumors (25-29). Downregulation of miR-219-5p has been reported in glioblastoma (25). The exogenous overexpression of miR-219-5p in glioma cell lines could inhibit cell proliferation, growth and migration by targeting EGFR and inhibiting the receptor tyrosine kinase pathway (26). In addition, low expression of miR-219-5p has been associated with higher clinical stages and recurrence rates of meningioma (27). The level of miR-219-5p was reduced in hepatocellular carcinoma. In a previous study, miR-219-5p inhibited cell proliferation *in vitro* and resulted in the arrest of the cell cycle at the G1/S transition by inhibiting the expression of glypican-3 (28). miR-219-5p was also downregulated in papillary thyroid carcinoma and had a critical role in cell growth by inhibiting estrogen receptor-α (29). These findings, together with the results of the present study, indicate that miR-219-5p may exert a tumor suppressor role in the carcinogenesis of various tumors.

It remains controversial as to whether brachyury is a prognostic indicator for chordoma. Kitamura *et al* (30) reported that the expression of brachyury was an independent prognostic indicator in skull base chordoma, and was associated with a poor prognosis. Zhang *et al* (31) analyzed a chordoma tissue microarray and determined that brachyury was not a prognostic indicator for chordoma, although the specimens were mainly derived from mobile spine or sacrum chordoma. Expression of miR-219-5p has been correlated with the recurrence rate of meningioma (27) and overall survival time of hepatocellular carcinoma patients (28). The present study demonstrated that miR-219-5p expression was associated with tumor extent and recurrence, although no definitive conclusion could be drawn owing to the small sample size of the present study. As a regulator of brachyury, the prognostic value of miR-219-5p in skull base chordoma requires further investigation.

In summary, the present study identified that miR-219-5p was downregulated in chordoma tissues and the U-CH2 cell line, and was associated with extensive tumor and recurrence. Furthermore, miR-219-5p exhibited the ability to inhibit cell proliferation and clonogenicity of chordoma by targeting brachyury. These findings suggested that miR-219-5p may act as a tumor suppressor in human chordoma, and it may be a potential target for therapeutic intervention in chordoma.

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