CircCTDP1 promotes nasopharyngeal carcinoma progression via a microRNA-320b/HOXA10/TGFβ2 pathway

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Abstract. Circular RNAs have been reported to play a vital role in the development and progression of various types of cancer. However, the underlying molecular role of circular RNA CTDPI (circCTDP1) in the tumorigenesis of nasopharyngeal carcinoma (NPC) remains unknown. In the present study, circCTDP1 expression was found to be markedly upregulated in NPC tissues and cell lines (SUNE1, SUNE2 and 6-10B cell lines). Knockdown of circCTDP1 resulted in inhibition of proliferation, migration and invasion, and promoted apoptosis of NPC cells. Moreover, circCTDP1 directly interacted with microRNA (miR) -320b based on bioinformatics prediction and dual luciferase assay, and transfection with an miR-320b inhibitor reversed the effects of circCTDP1 knockdown on NPC cells. Furthermore, circCTDP1/miR-320b promoted NPC progression by regulating the expression of homeobox A10 (HOXA10). In addition, it was demonstrated that HOXA10 may exert its oncogenic role in NPC by regulating the expression of transforming growth factor β2 (TGFβ2). Taken together, these results revealed a novel regulatory mechanism, which may provide an improved understanding of NPC tumorigenesis and be useful in the development of potential targets for NPC therapy.

Introduction

Nasopharyngeal carcinoma (NPC) is a malignancy that occurs in nasopharyngeal epithelial tissues and has a specific ethnic and geographic distribution, with the highest incidence in Southeast Asia and North Africa (1). Despite great improvements in diagnosis and surgical treatment, the 5-year survival rate for advanced NPC is approximately 70%, which remains unsatisfactory (2,3). Currently, the main therapeutic methods for NPC are irradiation therapy, alone or combined with chemotherapy (4,5). However, the physical and psychological side effects of the treatments are severe. Therefore, an investigation of the molecular mechanisms of this disease is required to develop novel treatment strategies for patients with NPC.

Circular RNAs (circRNAs) are a class of non-coding transcripts that contain a ring structure (6). Increasing evidence indicates that the aberrant expression of circRNAs is associated with a variety of diseases, including cancer (7-9). Additionally, some circRNAs play vital roles in the development and progression of NPC. For example, circRNA ZNF609 (10), circRNA_0008450 (11) and circRNA_000543 (12) accelerate NPC tumorigenesis and metastasis. C ircular RNA c TdP1 (circcTdP1) was reported to function as a competitive endogenous RNA (ceRNA) for microRNA (miR)-29a-3p to regulate the expression of hyaluronan synthase 3, integrin subunit β1, DNA methyltransferase 3β and vascular endothelial growth factor A (VEGFA), and subsequently promote the growth and metastasis of bladder cancer (13). However, the molecular mechanisms of circcTdP1 action in the tumorigenesis of NPC are unclear.

miRNAs are a family of small non-coding RNAs of approximately 22 nucleotides in length, which regulate gene expression by complementary binding or complex mechanisms (14). In multiple studies, several miRNAs, such as miR-101 (15), miR-184 (16), miR-543 (17) and miR-449 (18), have been demonstrated to suppress proliferation, migration and invasion of NPC. miR-320b was also reported to act as a tumor suppressor during occurrence and progression in different types of cancer, including NPC. In a study by Li et al (19), the overexpression of miR-320b inhibited NPC cell proliferation and promoted apoptosis, while knockdown of miR-320b accelerated tumor growth and inhibited apoptosis. circRNAs can act as ceRNAs to regulate the development and progression of various cancers (20-22), which led to the hypothesis that circCTDP1 may promote NPC progression via miR-320b.

Homeobox A10 (HOXA10), a member of the homeobox gene family, plays a critical role in embryonic development (23). Abnormal expression of HOXA10 has been observed in several types of cancer, including endometrial carcinoma, ovarian cancer and breast cancer (24-26). Shen et al (27)
also reported that HOXA10 is upregulated in NPC tissues compared to normal tissues, and promotes NPC progression by binding to the promoter of Zic family member 2. However, the precise mechanism of the regulation of HOXA10 in NPC remains unclear.

In the present study, the molecular mechanism of the circCTDP1/miR-320b/HOXA10 transforming growth factor β2 (TGFβ2) axis was investigated with regard to tumorigenesis and the progression of NPC. The aim of the study was to provide a better understanding of NPC initiation and progression, which may help in the future development of diagnostic and therapeutic targets for NPC.

Materials and methods

Clinical specimens. A total of 32 paired NPC tissues and paracarcinoma tissues were obtained from 32 NPC patients (22 males and 10 females) with a median age of 47 years (range, 27-79 years) between August, 2016 and May, 2018. The patients all provided written informed consent for the use of their samples. All experimental protocols were approved by the Ethical and Scientific Committee of the Third Affiliated Hospital of Soochow University (Jiangsu, China).

Cell culture. A normal human nasopharyngeal epithelial cell line (NP69), 3 NPC cell lines (SUNE1, SUNE2 and 6-10B) and 293T cells were obtained from the American Type Culture Collection (ATCC; Manassas). The cell lines were cultured in RPMI-1640 medium, and supplemented with 10% fetal bovine serum. The 293T cells were cultured in Dulbecco's modified Eagle's medium (Gibco; Thermo Fisher Scientific, Inc.) with 10% FBS and 1% penicillin-streptomycin. All the cell lines were maintained at 37°C in a humidified atmosphere with 5% CO2.

Cell transfection. The short hairpin RNA (shRNAs) targeting circCTDP1 (shcircCTDP1; 5'-UCGAGAUGCCAGGCUCAA C-3') with negative control (shNC; 5'-UCUCCAGUGCCAGCU CAAC-3'), miR-320b mimics (5'-AAAGCUUGGGAGAGA GGCCAA-3') with negative control (miR-NC; 5'-AAUUCU CGGCAUGUCACUU-3') and miR-320b inhibitor (5'-UUG CCCUCUAACCCAGCUUUU-3') with negative control (inh-miR-NC; 5'-CAGUACUUUGGUGAGUACAA-3') were synthesized by GenePharma (Shanghai, China). The full length of HOXA10 was subcloned into pcDNA3.1 to overexpress HOXA10 with empty pcDNA3.1 serving as control. The pcDNA3.1 vector was bought from GenePharma (Shanghai). Transfection of the cells with shcircCTDP1 (10 nM) or shNC (10 nM) and the miR-320b mimics (10 nM) or miR-NC (10 nM) and miR-320b inhibitor (10 nM) or inh-miR-NC (10 nM) was conducted with Lipofectamine 2000 transfection reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. The efficiency of transfection was determined in each experiment using RT-qPCR 24 h post-transfection. All functional experiments were carried out 48 h post-transfection.

RT-qPCR. According to the manufacturer's instruction, total RNA was extracted from tissues and cell lines using TRIzol (Invitrogen; Thermo Fisher Scientific, Inc.). RNA was reverse transcribed to cDNA by using a Reverse Transcription Kit (Takara). The following thermocycling conditions were used for the qPCR: Initial denaturation at 95°C for 3 min; 40 cycles of 95°C for 5 sec and 60°C for 30 sec. A melt curve step from 65-95°C was performed in increments of 0.5°C per 5 sec. The relative expression levels were calculated by comparing to the expression of GAPDH or U6 using the 2-ΔΔCt method (28).

Flow cytometric analysis. Flow cytometric analysis was performed using a FITC Annexin V Apoptosis kit (BD Biosciences) according to the manufacturer's instructions. Briefly, transfected SUNE2 and 6-10B cells were washed twice with PBS and resuspended in 1X Annexin V binding buffer containing 10 mM HEPES/NaOH (pH 7.4) (1x10⁶ cells/ml). Then the cells were mixed with FITC-Annexin V (5 µl) and propidium iodide for at 37°C for 20 min, and analyzed using a flow cytometer (BD Biosciences).
Bioinformatic prediction and luciferase reporter assay. StarBase (http://starbase.sysu.edu.cn) and TargetScan databases (http://www.targetscan.org) were used to predict the potential miRNAs that can bind to circCTDP1. A luciferase reporter assay was employed to investigate the regulatory relationship between circCTDP1 and miR-320b. circCTDP1 that contained the miR-320b binding site was cloned into the pSICHECK-2 vector (Promega Corporation) to construct wild-type circCTDP1. Mutant circCTDP1 that included a mutated version of the miR-320b binding site was also cloned into pSICHECK-2. Subsequently, miR-320b mimic or miR-NC and miR-320b inhibitor or miR-NC inhibitor were transfected into 293T cells that were transfected with wild-type circCTDP1 or mutant circCTDP1. The relationship between miR-320b and HOXA10 was confirmed using the same method. Luciferase activity was evaluated by Dual-Luciferase Reporter Analysis system (Promega Corporation). Firefly luciferase activity was normalized to Renilla (Promega Corporation) luciferase gene activity.
Western blot analysis. Following transfection for 48 h, proteins were extracted from transfected SUNE2 and 6-10B cells using radioimmunoprecipitation assay buffer (Beyotime Institute of Biotechnology). Protein concentration was measured with the bicinchoninic acid assay (Beyotime Institute of Biotechnology). Following denaturation, 10 µg protein/lane was separated by 10% SDS-PAGE. Proteins were transferred onto polyvinylidene difluoride (PVDF) membranes and blocked in 5% non-fat milk for 2 h at room temperature. The membranes were incubated with primary antibodies against HOXA10 (1:1,000; mouse monoclonal antibody, sc-271139; Santa cruz Biotechnology) and GAPDH (1:1,000; mouse monoclonal antibody, sc-47724; Santa Cruz Biotechnology) overnight at 4°C. Following primary incubation, membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (1:1,000; goat anti-mouse IgG, ab205719 and goat anti-rabbit IgG, ab205718; Abcam) for 2 h at room temperature. Protein bands were visualized using the Pierce ECL Western Blotting kit (Pierce; Thermo Fisher Scientific, Inc.). Protein expression was quantified using Image-Pro® Plus software (version 6.0; Media Cybernetics, Inc.). GAPDH was used as endogenous control for data normalization.

Xenograft experiment. Six male BALB/c nude mice (6 weeks old) were maintained under specific pathogen-free conditions and randomly divided into two groups, and were housed individually in microisolator ventilated cages (temperature, 26-28°C; 40-60% humidity and ventilation 10-15 times/h) with free access to water and food. Cells transfected with shNC or shcircCTDP1 were suspended in 100 µl PBS and injected subcutaneously into the mice. Tumors were examined every 5 days. On 30th day, the mice were sacrificed by cervical dislocation after deep anesthesia with 2% isoflurane (Baxter Healthcare Corporation) to obtain the tumors. The tumors were examined every 5 days. On 30th day, the mice were sacrificed by cervical dislocation after deep anesthesia with 2% isoflurane (Baxter Healthcare Corporation) to obtain the tumors. The tumors were photographed and tumor weights were measured. Tumor length (L) and width (W) were measured. Tumor volume was calculated using the formula: V = 1/2 x L x W². The animal experiments were approved by the Ethics Committee of the Third Affiliated Hospital of Soochow University.
Statistical analysis. Statistical analyses were performed with SPSS 18.0 software (SPSS, Inc.). Data were presented as mean ± SD. Comparisons of parameters between two groups were analyzed by a paired Student's t-test. Comparisons among multiple groups were performed using one-way ANOVA followed by Tukey's test. The correlation between gene
expression levels was analyzed by Pearson's correlation coefficient. Kaplan-Meier analysis and the log-rank test were used to estimate survival curves. Cut-off values were determined using Youden's index. P<0.05 was considered to indicate a statistically significant difference.

Results

CircCTDP1 is upregulated in NPC tissues and cell lines. Using RT-qPCR, the expression levels of circCTDP1 in clinical tissues (NPC specimens and paracarcinoma tissues) was examined. As presented in Fig. 1A, circCTDP1 was significantly upregulated in NPC tissues compared with normal tissues. Similarly, circCTDP1 expression in 3 NPC cell lines (SUNE1, SUNE2 and 6-10B) was markedly upregulated compared with expression in NP69 cells, a normal nasopharyngeal epithelial cell line (Fig. 1B). Moreover, Kaplan-Meier analysis showed that patients with high circCTDP1 expression exhibited a worse prognosis compared with patients with low circCTDP1 expression (Fig. 1C). These results suggest circCTDP1 may play a pivotal role in the progression of NPC.

Knockdown of circCTDP1 suppresses NPC progression. To investigate the involvement of circCTDP1 in NPC, shRNAs targeting circCTDP1 were designed. RT-qPCR indicated that the expression of circCTDP1 in SUNE2 and 6-10B cells was decreased after cells transfected with shcircCTDP1 (Fig. 1D). Subsequently, an MTT assay revealed that cell viability was markedly reduced when NPC cells were transfected with shcircCTDP1 (Fig. 1E). Moreover, flow cytometric analysis revealed that circCTDP1 knockdown significantly promoted apoptosis in SUNE2 and 6-10B cell lines (Fig. 1F). To further explore the effect of circCTDP1 on the migration and invasion of NPC cells, wound healing and cell invasion assays were performed. As presented in Fig. 1G and H, the migration and invasion abilities of the shcircCTDP1 cells were reduced relative to shNC cells. Moreover, an in vivo xenograft experiment revealed that depletion of circCTDP1 reduced the tumor growth rate in mice (Fig. II-K). A statistically significant difference in tumor volume was observed between the two groups and the maximum tumor volume was 1,153 mm³. These data indicate that circCTDP1 knockdown suppresses proliferation, mobility and promotes apoptosis of NPC cells.

miR-320b inhibitor rescues the effects of circCTDP1 knockdown on NPC cells. Kaplan-Meier survival analysis showed that NPC patients with low miR-320b expression had a shorter OS time compared with those patients with high miR-320b expression (Fig. 2A). Using starBase bioinformatic analysis software, circCTDP1 was suggested to bind to miR-320b via complementary base pairing (Fig. 2B). A dual luciferase reporter assay showed that a marked decrease in the luciferase activities following co-transfection of the cells with miR-320b mimics and wild-type circCTDP1 expression vector, but not with the mutant-type circCTDP1 vector (Fig. 2C). To investigate whether the effects of circCTDP1 on NPC were mediated by miR-320b, the miR-320b inhibitor was transfected into shcircCTDP1-expressing SUNE2 and 6-10B cells. RT-qPCR analysis demonstrated that the introduction of miR-320b inhibitor reduced miR-320b expression in NPC cells transfected with shcircCTDP1 (Fig. 2D). The proliferation of shcircCTDP1-transfected SUNE2 and 6-10B cells was increased when transfected with miR-320b inhibitor, as revealed by MTT assay (Fig. 2E). Furthermore, flow cytometry showed that knockdown of circCTDP1 promoted apoptosis, whereas the cell apoptotic rate was reduced by co-transfection with miR-320b inhibitor (Fig. 2F). Similarly, transfection with miR-320b inhibitor partly reversed the shcircCTDP1-mediated reduction of the migration and invasion abilities of NPC cells (Fig. 2G and H). Therefore, it was concluded that miR-320b antagonizes circCTDP1-regulated NPC progression in vitro.

miR-320b directly targets HOXA10. Using the TargetScan database, it was found that miR-320b could target HOXA10 as they have complementary DNA regions (Fig. 3A). To further examine the binding between miR-320b and HOXA10, a dual luciferase reporter assay was performed. Compared with NC cells, the results showed that the miR-320b mimic significantly reduced the luciferase activity of the wild-type HOXA10 3'UTR, whereas the miR-320b inhibitor increased luciferase activity. On the other hand, there was no effect on cells transfected with a mutant 3'-UTR of HOXA10 (Fig. 3B). Furthermore, transfection with miR-320b mimic significantly suppressed the expression of HOXA10, while miR-320b inhibitor markedly enhanced the HOXA10 expression (Fig. 3C). Taken together, these data indicate that miR-320b directly targets HOXA10 and negatively regulates its expression.
circCTDP1/miR-320b regulates the progression of NPC via HOXA10. Based on the aforementioned results, miR-320b was indicated to directly target HOXA10. Therefore, it was hypothesized that HOXA10 may act as a critical factor in the progression of NPC. This hypothesis was supported by the analysis of Kaplan-Meier survival curves and the expression levels of HOXA10 in NPC patients. The results showed a significant correlation between HOXA10 expression and patient prognosis. Furthermore, the expression of HOXA10 was found to be regulated by circCTDP1 and miR-320b in NPC cell lines. The data were presented as mean ± SD (*P<0.05; **P<0.01).
Figure 4. Continued. circCTDP1 upregulates the expression of HOXA10 via targeting miR-320b. (G) Cell invasion assay of SUNE2 and 6-10B cell lines (NC, shcircCTDP1, shcircCTDP1 plus HOXA10, miR-320b mimic, and miR-320b mimic plus HOXA10). (H) Wound healing assay of SUNE2 and 6-10B cell lines (NC, shcircCTDP1, shcircCTDP1 plus HOXA10, miR-320b mimic, and miR-320b mimic plus HOXA10). (I) The circCTDP1 expressions were positively correlated with HOXA10 expressions within included NPC tissues. The miR-320b expressions were negatively correlated with HOXA10 expressions within included NPC tissues. The data were presented as mean ± SD (*P<0.05; **P<0.01).

Figure 5. HOXA10 regulates the expression of TGFβ2 in NPC cells. (A) RT-qPCR and western blot analysis shows the expression levels of TGFβ2 in SUNE2 and 6-10B cell lines transfected with shNC and shHOXA10. (B) RT-qPCR and western blot analysis shows the expression levels of TGFβ2 in SUNE2 and 6-10B cell lines transfected with pcDNA3.1 and pcDNA3.1-HOXA10. The data were presented as mean ± SD (*P<0.05).
circCTDP1/miR-320b axis in the development and progression of NPC. Firstly, Kaplan-Meier survival analysis showed that patients with NPC who exhibited a high HOXA10 expression had a shorter OS time compared with patients with a low HOXA10 expression (Fig. 4A). RT-qPCR analysis further demonstrated that the expression of HOXA10 was markedly reduced in NPC cells transfected with shHOXA10, but was notably increased in NPC cells transfected with HOXA10 overexpression plasmid (Fig. 4B). In addition, HOXA10 was overexpressed in shcircCTDP1- and miR-320b-mimic-transfected cell lines. As presented in Fig. 4C and D, the expression level of HOXA10 was significantly increased in shcircCTDP1+HOXA10 and miR-320b+HOXA10 groups. Subsequently, an MTT assay was performed to examine cell proliferation. As shown in Fig. 4E, HOXA10 could restore the attenuated cell proliferation of shcircCTDP1- or miR-320b-transfected SUNE2 and 6-10B cells. Furthermore, it was found that the overexpression of HOXA10 inhibited apoptosis and enhanced cell mobility in shcircCTDP1- or miR-320b-mimic-transfected SUNE2 and 6-10B cells (Fig. 4F-H). Moreover, there was a positive correlation between the expression of circCTDP1 and HOXA10 in NPC tissues, whereas there was a negative correlation between the expression of miR-320b and HOXA10 (Fig. 4I). Based on these results, the effects of shcircCTDP1 and miR-320b mimic on NPC cells were neutralized by overexpression of HOXA10, therefore suggesting that circCTDP1/miR-320b/HOXA10 may be an important signaling pathway involved in the progression of NPC.

HOXA10 regulates the expression of TGFβ2 in NPC cells. TGFβ2 is a vital modulator of tumor invasion and motility. RT-qPCR and western blot analysis showed that knockdown of HOXA10 decreased the expression of TGFβ2 in SUNE2 and 6-10B cells, whereas overexpression of HOXA10 increased the expression of TGFβ2. These results demonstrated that HOXA10 could regulate the expression of TGFβ2 in NPC cells (Fig. 5A and B).

Discussion

NPC is one of the most life-threatening tumors worldwide which is mainly ascribed to tumor recurrence and distant metastasis (29,30). Thus, developing novel prognostic markers for NPC is crucial. To the best of our knowledge, in the current study, it was demonstrated for the first time that circCTDP1 is associated with the progression of NPC.

Previous studies have demonstrated that circRNAs interact with miRNAs by acting as ceRNA to regulate the proliferation and migration of tumor cells (36-38). Recently, Cao et al (39) reported that circ0001429 promoted the progression of bladder cancer through sponging miR-205-3p and upregulating expression of VEGFA. Regarding NPC, Ke et al reported that circHIPK3 acts as a ceRNA to upregulate E74 like ETS transcription factor 3 by sponging miR-4288 (40). In the present study, circCTDP1 was found to directly interact with miR-320b, and to inhibit its expression through bioinformatic analysis and luciferase reporter assay. Furthermore, miR-320b inhibitor could significantly abolish the inhibitory effect of shcircCTDP1 on NPC phenotypes.

HOX genes are divided into 4 groups (HOXA, HOXB, HOXC and HOXD) encode transcription factors involved in the control of cell growth (41,42). It has been reported that HOXA10 is associated with cell migration, proliferation and survival in various types of cancer (43,44). In the current study, it was demonstrated that HOXA10 was a direct downstream target of miR-320b through bioinformatic prediction and in vitro experiments, and overexpression of HOXA10 was found to promote the progression and development of NPC cells. Moreover, expression of HOXA10 was positively correlated with that of circCTDP1, but negatively correlated with miR-320b expression. These results indicated that miR-320b may inhibit the proliferation, migration and invasion of NPC cells by downregulating HOXA10 expression.

HOXA10 has been reported to increase the levels of TGFβ2 in pancreatic cancer cells (45). Therefore, it was hypothesized that HOXA10 may exert its role in NPC by regulating the expression of TGFβ2. The findings of the present study demonstrated that upregulation of HOXA10 increased the expression of TGFβ2 and downregulation of HOXA10 decreased the expression of TGFβ2.

In the current study, it was demonstrated that circCTDP1 promotes proliferation, migration and invasion of NPC cells through a miR-320b/HOXA10 axis. This study offers an improved understanding of the pathogenesis of NPC, and the circCTDP1, miR-320b, HOXA10 and TGFβ2 may have potential as therapeutic targets for NPC.

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Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author upon reasonable request.

Authors’ contributions

HL, CC and XT designed the study. JY and HX performed experiments. HL and CC analyzed the data and wrote the
manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Ethics Committee of the Third Affiliated Hospital of Soochow University. Written informed consent was obtained from all patients prior to the study start. All animal experiments were conducted with the approval of the Third Affiliated Hospital of Soochow University.

Patient consent for publication

Not applicable.

Competing interests

The authors declare no that they have no competing interests.

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