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ORIGINAL ARTICLE

Long non-coding RNA XIST functions as an oncogene in human colorectal cancer by targeting miR-132-3p

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Summary

Purpose: *Colorectal cancer (CRC) is a frequent malignant tumor with high death rate and poor prognosis. Few reports have explored the association between CRC development and long non-coding (lnc) RNAs expression. This study focused on the important role of a novel lncRNA XIST in the development of CRC.*

Methods: *To investigate the function of long lncRNA XIST in CRC, its expression level was monitored in both CRC cells and 50 pairs of human CRC tissues and adjacent tissues by RT-qPCR. Moreover, the associations between lncRNA XIST expression level and clinicopathological characteristics and 5-year survival rate of CRC patients were evaluated. Furthermore, function assays containing cell proliferation assay, flow cytometry and colony formation were conducted to investigate the role of lncRNA XIST in CRC. Western blotting assay and luciferase assay were used to explore the regulation mechanism of lncRNA XIST in the development.*

Results: *The expression level of XIST was significantly increased in both CRC tissues sample and CRC cells. XIST promoted CRC cell proliferation by affecting the cell cycle. In addition, XIST and miR-132-3p were inhibited by each other reciprocally. MAPK1 was proved to be a direct target spot of miR-132-3p. We claim that XIST was responsible for CRC cell proliferation working by the miR-132-3p/MAPK1 axis.*

Conclusion: *The present study suggests that lncRNA XIST is a potential oncogene in CRC, and could promote CRC cell proliferation through inhibiting miR-132-3p, which may provide a new therapeutic target of CRC.*

Key words: *colorectal cancer, lncRNA, proliferation, XIST*

Introduction

In American Cancer Statistics in 2015, CRC ranked third among all kinds of cancer [1]. The incidence rate of CRC remains high both in males and in females worldwide [2]. In the last decades, due to the development in therapeutic methods and strategies, individualized treatment is available for CRC patients. However, poor prognosis is a major concern for CRC patients, especially in those with advanced stages [3]. It is well known that aberrations of oncogenes as well as tumor suppressor genes play a critical role in the CRC progression [4,5] and therefore, further exploring the mechanisms of these genomic changes in CRC is urgently required.

Non-coding RNAs are RNAs without the capacity of protein-coding, including small non-coding RNAs and long non-coding RNAs. Recently, a considerable number of studies have revealed that dysregulated non-coding RNAs are identified in many kinds of cancers and probably associated with many known oncogenes [6-8]. Larger than 200 bps nucleotides in length, long non-coding RNAs (lncRNAs) have been reported to function in tumor initiation, proliferation and metastasis in various cancers. Latest studies discovered that lncRNA XIST, located in X chromosome, is overex-

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pressed in several malignant tumors and plays an important role in tumor progression. For example, lncRNA XIST promotes the proliferation and invasion of cells in non-small cell lung cancer and correlates with clinical outcomes [9]. Moreover, lncRNA XIST functions as a critical regulator in osteosarcoma [10]. Besides, according to a recent study, the expression level of lncRNA XIST in CRC tissues is significantly higher when compared to the paired adjacent normal tissues [11]. However, the significant role of lncRNA XIST in CRC and the potential mechanisms remain largely unknown.

Our present study revealed that lncRNA XIST was upregulated in CRC tissues and promoted the proliferation of CRC cells. In addition, we further explored the interaction between lncRNA XIST and miR-132-3p along with the possible mechanism.

Methods

Patients and specimens

Human tissues were acquired from 50 CRC patients who were operated at Beijing Anzhen Hospital. No patient received radiotherapy or chemotherapy before operation. All CRC tissues were stored at –80°C. An experienced pathologist assessed the data. Written informed consent was provided by the patients and the study was approved by the Ethics Committee of Beijing Anzhen Hospital.

Cell culture and culture condition

The American Type Culture Collection (ATCC) provided us LOVO, HCT116, SW620, SW480 and HT29 CRC cell lines, normal human colonic epithelial cells (NCM460) and 293T embryonic kidney cell line. Culture medium was consist of 100 U/ml penicillin (Invitrogen Life Technologies, Carlsbad, CA, USA), 10% fetal bovine serum (FBS; Invitrogen Life Technologies) and RPMI–1640 medium (Gibco:Thermo Fisher Scientific, Inc., Waltham, MA, USA). Besides, humidified incubator was maintained at 37°C with 5% CO_2. Lentiviral small hairpin RNA (shRNA) targeting XIST was synthesized and then cloned into the pLenti-EF1a-EGFP-F2A-Puro vector (Biosettia Inc., San Diego, CA, USA). Then, 293T cells were used for packaging the viruses, the XIST lentiviruses (sh-XIST) and the empty vector (sh-ctrl). The miRNA mimics and inhibitor provided by Genepharma Co., Ltd. (Shanghai, China) were used for transfection of CRC cells. Negative control was transfected with non-specific siRNA.

RNA extraction and qRT-PCR

According to the manufacturer's instructions, TRIzol reagent (Invitrogen, USA) was utilized for extracting the total RNA in CRC cells, normal human colonic epithelial cells (NCM460) and 293T embryonic kidney cells. cDNAs were synthesized via reverse the Transcription Kit (Takara Biotechnology Co., Ltd., Dalian, China) and were quantified by SYBR Green real-time PCR. Then, lncRNA XIST mRNA level was detected using the primers below: lncRNA XIST, forward 5'-CAGACGTGTGCTCTTC-3' and reverse 5'-CGATCTGTAAGTCCACCA-3'; GAPDH, forward 5'-CCACATCGCTCAGACACCAT-3' and reverse 5'-ACCAGGCGCCCAATACG-3'; miR-132-3p, forward 5'-GCGCGCGTAACAGTCTACAGG-3'and reverse 5'-GTCGTATCCAGTGCAGGGTCC-3'; U6, forward 5'-CTCGCTTCGGCAGCACATATACT-3' and reverse 5'-CGCTTCACGAATTTGCGTGT-3. The performance of RT- qPCR was conducted on ABI 7500 system (Applied Biosystems, CA, USA). Then, the thermal cycle followed: 95°C for 30 sec, 95°C for 5 sec for 40 cycles, and 60°C for 35 sec.

Cell counting kit-8 (CCK8) assay

Cell viability of the treated cells in 96-well plates was monitored every 24 hrs by CCK8 assay following the protocol (Dojindo Molecular Technologies, Inc., Kumamoto, Japan. The absorbance was then spectrophotometrically measured at 450 nm (Thermo Scientific, Rockford, IL, USA).

Colony formation assay

About 200 cells were cultured in a 6-well plate containing 10% FBS for 14 days, and the medium was replaced every 5 days. Then, cells were stained with 0.1% crystal violet after being fixed with methanol. For comparison, the number of colonies were counted.

Cell cycle analysis

To further explore the mechanism, cell cycle staining Kit (MultiSciences Biotech Co., Ltd, Hangzhou, China) was used for detecting the cell cycle according to the protocol. More specifically, cells (2×10^5/ml) stayed overnight in 75% ice-cold ethanol diluted by RNase A. Then, they were washed in PBS twice and finally stained successively for 30 min along with 50 mg/mL propidium iodide (PI) at 4°C in the dark. Flow cytometer (FACScan, BD Bioscience, CA, USA) was then used to monitor the distribution of cell cycle.

Western blotting analysis

A protein assay (bicinchoninic acid method;Beyotime) was utilized for quantifying the total protein expression. The target proteins were replaced to the polyvinylidene fluoride (PVDF) membrane, which was then blocked in 5% dry milk at 37°C for 1 hr after being fractionated by SDS-PAGE. Immunostaining followed with antibodies (Cell Signaling Technology, CST, CA, USA) overnight at 4°C: 1:1000 rabbit anti-MAPK1 and 1:5000 rabbit anti-GAPDH. PBS supplement with 0.1% Tween 20 was utilized 4 times to wash the membranes, which were then cultured within a goat anti-rabbit sec-

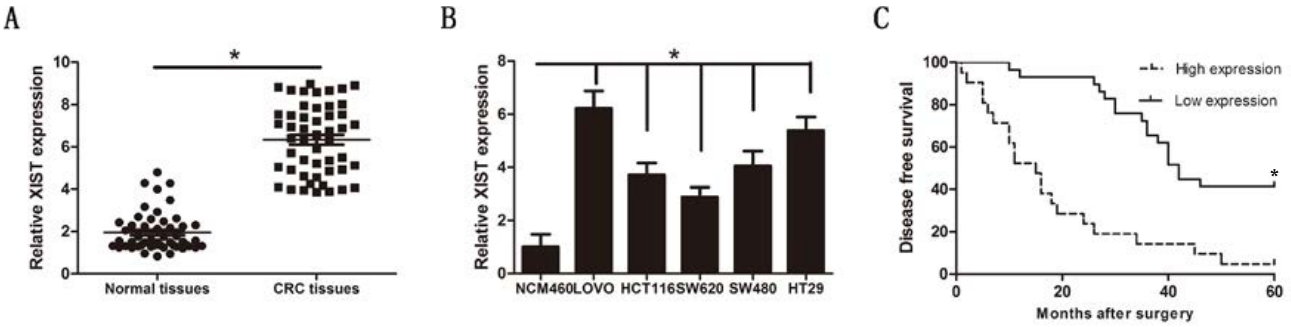

Figure 1. Expression levels of XIST were increased in CRC and associated with short disease-free survival of CRC patients. **(A)**: XIST expression was significantly increased in the CRC tissues compared with adjacent normal tissues (*p<0.05 compared with normal tissues). **(B)**: Expression levels of XIST relative to GAPDH were determined in the human CRC cell lines (LOVO, HCT116, SW620, SW480, HT29) and normal colonic epithelial cells (NCM 460) by RT-qPCR (*p<0.05 compared with NCM460). **(C):** High level of XIST was associated with significantly short disease-free survival of patients with CRC (*p<0.05 compared with lower expression of XIST). Data are presented as mean ± standard error of the mean.

Table 1. Correlation between lncRNA XIST expression and clinicopathological characteristics in CRC patients

Characteristics	*Patients, n*	*Expression of lncRNA XIST*		*p value*
		High expression	*Low expression*	
Total	50	21	29	
Age（years）				0.349
≤60	20	10	10	
>60	30	11	19	
Gender				0.230
Male	36	17	19	
Female	14	4	10	
Tumor differentiation				0.598
Good/moderate	24	11	13	
Poor	26	10	16	
TNM stage				0.034
I-II	18	4	14	
III-IV	32	17	15	
Tumor size (cm)				0.013
<5	27	7	20	
>5	23	14	9	
Distant metastasis				0.390
No	25	9	16	
Yes	25	12	13	

p<0.05 was considered to indicate a statistically significant difference

ondary antibody (1:1000) for 1 hr. After that, PBS was again used to wash the membranes 3 times for 15 min. The comparison between relevant protein levels was conducted by Image J software (Rawak Software Inc., Mannheim, Germany).

Luciferase assays

For the luciferase reporter assay, the 3′-UTR of MAPK1 or XIST were cloned into the pGL3 vector (Promega), which was identified as wild-type (WT) 3′-UTR. Quick-change site-directed mutagenesis kit (Stratagene, Cedar Creek, USA) was used for site-directed mutagenesis of the miR-132-3p binding site in MAPK1 or XIST 3′-UTR, which was named as mutant (MUT) 3′-UTR. Cells were transfected with WT-3′-UTR or MUT-3′-UTR and miR-ctrl or miR-132-3p for 48 hrs. Then, the luciferase assay was conducted on the dual Luciferase reporter assay system (Promega).

Statistics

Data analysis was performed using SPSS 18.0 (SPSS Inc., Chicago, IL, USA). Graph PAD 4.0 (GraphPad Software, Inc., La Jolla, CA, USA) helped presenting these consequences. Quantitative data was presented as mean ± SD. The method of $2^{-\Delta\Delta CT}$ was used to measure the relative expression of mRNA [12]. Percentage (%) was used to express the enumeration data and x^2 test was used for data analysis. The non-parametric total rank of independent samples was used to test grade data. The independent samples t-test was chosen as the method of statistical analysis. A p value <0.05 was considered as statistically significant.

Results

Overexpressed XIST in CRC tissues and cell lines

First, RT-qPCR was performed in 60 pairs of human CRC tissues and adjacent tissues to determine the expression level of XIST. The results showed higher XIST levels in CRC tissues (Figure 1A). It was further found that the LOVO, HCT116, SW620, SW480 and HT29 CRC cell lines showed increased expression of XIST compared with normal colonic epithelial cells (NCM460) (Figure 1B). Subsequently, the correlation between XIST expression level and clinicopathological data was investigated (Table 1). Although XIST expression was not associated with age, gender, differentiation and distant metastasis, high XIST expression was positively associated with tumor size as well as TNM stage in CRC. Kaplan–Meier analysis demonstrated that high XIST level correlated with a shorter disease-free survival in CRC patients (Figure 1C).

XIST knockdown inhibited CRC cell proliferation

Since XIST expression was the highest in the LOVO CRC cell line, we chose LOVO cells for knockdown of XIST which was called sh-XIST. Besides, sh-ctrl referred to the cells transfected

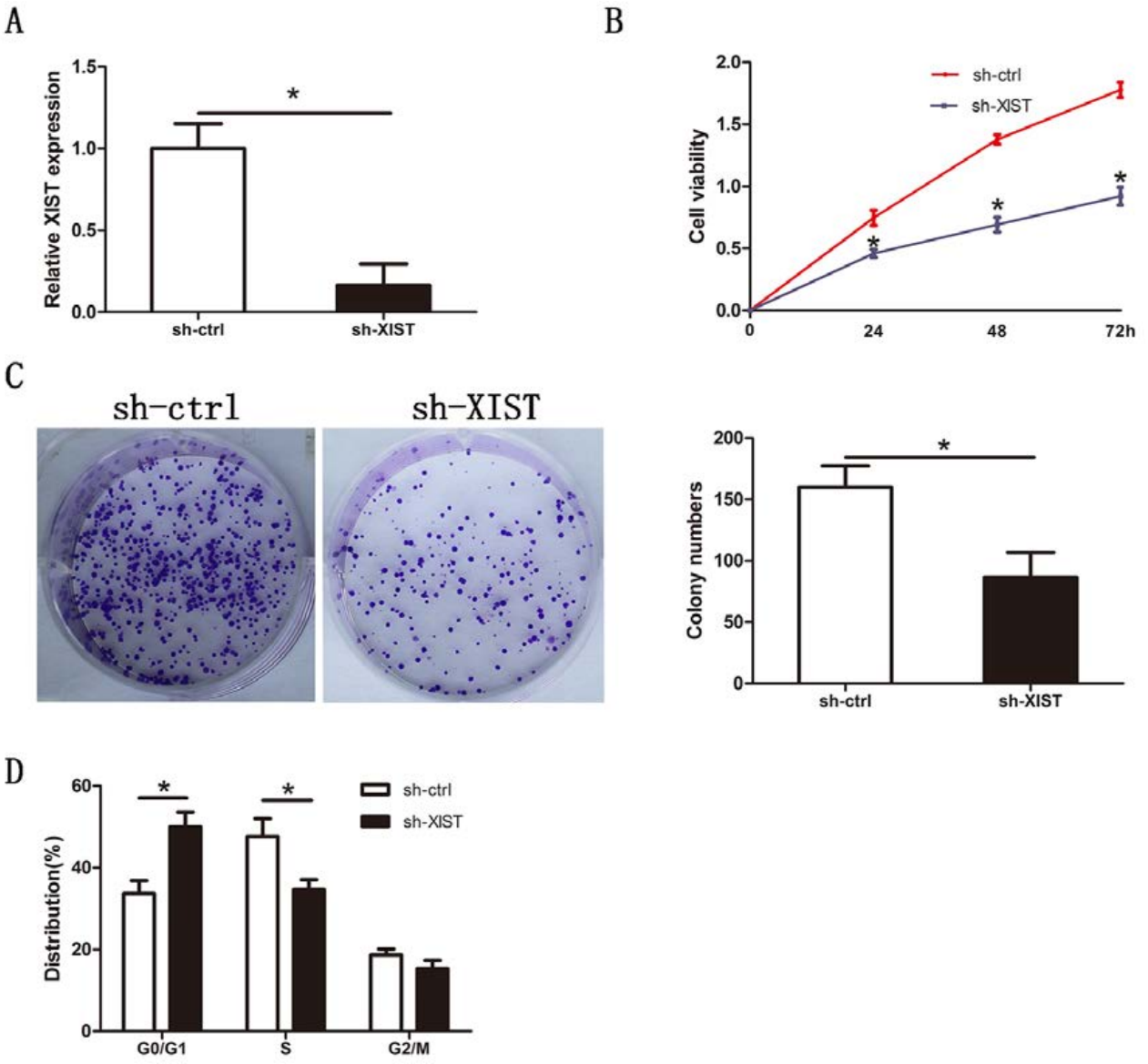

Figure 2. XIST inhibition decreased CRC cell proliferation and altered the distribution of cell cycle. **(A):** XIST expression in LOVO transduced with control shRNA vector (sh-ctrl) or XIST shRNA (sh-XIST) was detected by RT-qPCR. GAPDH was used as an internal control. **(B)**: CCK8 assay showed that knockdown of XIST significantly decreased cell proliferation in sh-XIST cells compared with sh-ctrl cells. **(C):** Colony formation assay demonstrated that oncogenic survival was significantly decreased in sh-XIST cells compared with sh-ctrl cells. **(D):** Flow cytometric analysis of the cell cycle distribution in sh-XIST or sh-ctrl cells. The results represent the average of three independent experiments (mean ± standard error of the mean). *p<0.05, as compared with the control cells.

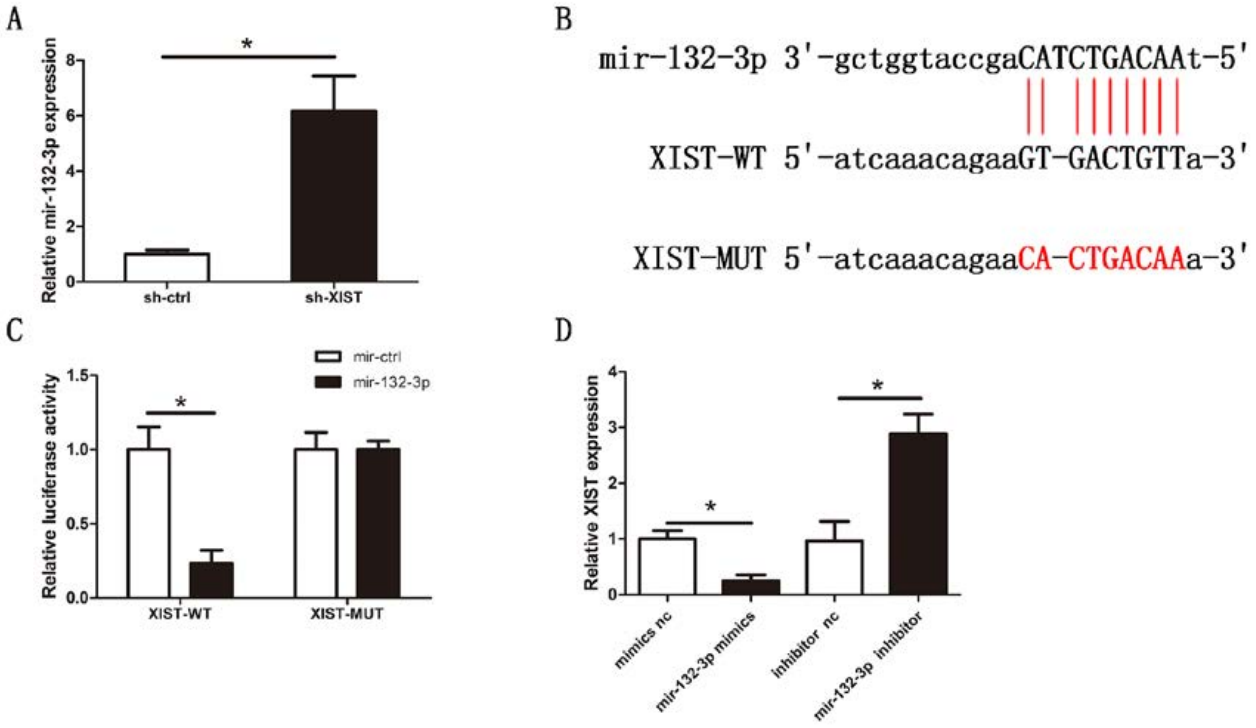

Figure 3. Reciprocal repression between XIST and mir-132-3p. **(A):** Mir-132-3p expression was increased in sh-XIST group compared with sh-ctrl group. **(B):** The binding sites of mir-132-3p on XIST. **(C):** Co-transfection of mir-132-3p and XIST-WT strongly decreased the luciferase activity, while co-transfection of mir-control and XIST-WT did not change the luciferase activity, and co-transfection of mir-132-3p and XIST-MUT did not change the luciferase activity either. **(D):** Mir-132-3p negatively regulated XIST expression. The results represent the average of three independent experiments. Data are presented as mean ± standard error of the mean. *p<0.05.

by a negative control (Figure 2A). Knockdown of XIST decreased cell proliferation in sh-XIST cells compared with sh-ctrl cells (Figure 2B). In parallel, colony formation assay demonstrated that the number of colonies was significantly decreased in sh-XIST cells compared with sh-ctrl cells (Figure 2C). Next, flow cytometry analysis was performed to examine whether XIST promoted CRC cell proliferation through altering cell cycle. The results showed that sh-XIST cells led to cell cycle arrest at the G0/G1 phase (Figure 2D). Taken together, the results above indicated that XIST promoted CRC cell proliferation by affecting the cell cycle.

The interaction between miR-132-3p and lncRNA XIST in CRC

Starbase v2.0 (http://starbase.sysu.edu.cn/mirLncRNA.php) was used to find the miRNAs that contained complementary base with XIST. Because miR-132-3p was a tumor suppressor and was able to suppress cancer cell proliferation, we focused on this miR among the miRNAs that predicted possible interaction with XIST. Indeed, the RT-PCR assay showed that the expression of miR-132-3p was higher in sh-XIST cells compared with sh-ctrl cells (Figure 3A). So, XIST was predicted to harbor miR-132-3p binding sites (Figure 3B). Furthermore, the luciferase assay revealed that co-transfection of XIST-WT and miR-132-3p largely decreased the luciferase activity, while co-transfection of XIST-MUT and miR-132-3p had no effect on the luciferase activity either (Figure 3C). Meanwhile, XIST expression of miR-132-3p mimics cells was lower, while the XIST expression of the miR-132-3p inhibitor cells was higher (Figure 3D). In summary, these data demonstrated that miR-132-3p could negatively regulate XIST expression by directly binding to XIST.

The influence of XIST on cell growth was mediated by miR-132-3p

MicroRNAs participate in biological process through their downstream targets. MAPK1, as a downstream target of miR-132-3p, was reported to be a key regulator of cell growth in cancer development (Figure 4A). The luciferase assay revealed that the group co-transfected with miR-132-3p and WT-MAPK1-3'-UTR had less luciferase activity than other groups (Figure 4B). Western blot assay showed that MAPK1 could be suppressed

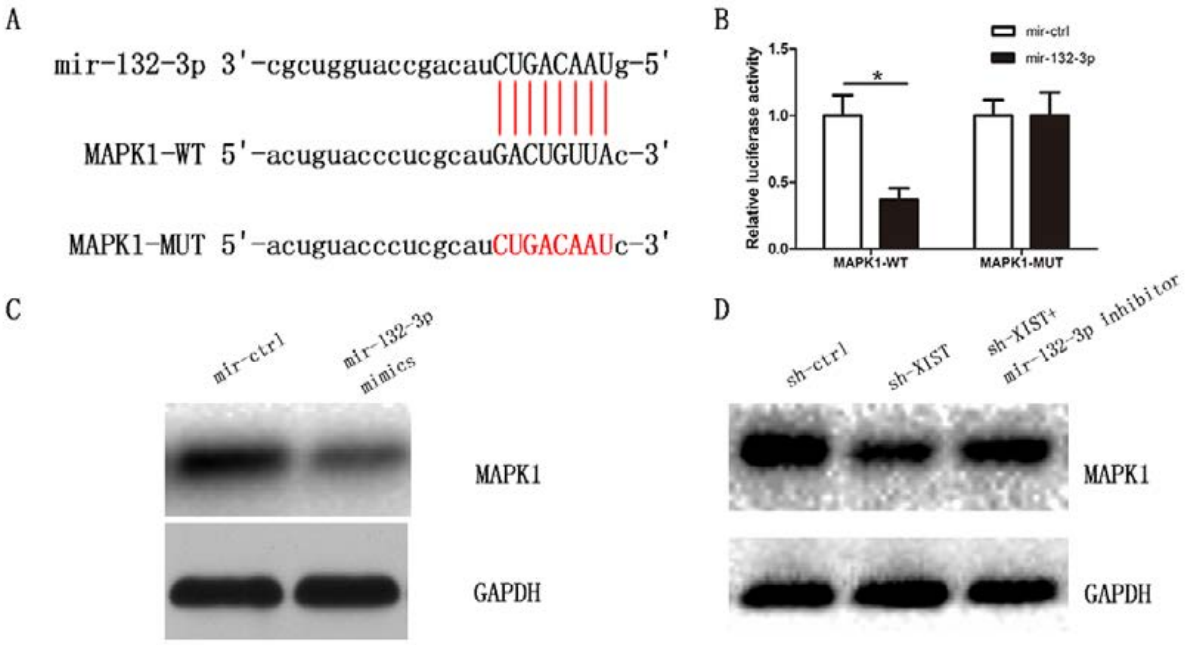

Figure 4. Mir-132-3p/MAPK1 axis mediated the effect of XIST on cell growth. **(A):** The binding sites of mir-132-3p on MAPK1. **(B)**: The luciferase assay showed that cells transfected with mir-132-3p had less luciferase activity than those transfected with mir-ctrl. **(C):** Mir-132-3p mimics repressed MAPK1 protein expression in CRC cells. **(D)**: Mir-132-3p inhibitor led to the restoration of MAPK1 in sh-XIST cells. *p<0.05.

at protein level by miR-132-3p (Figure 4C). Besides, the protein level of MAPK1 was lower in sh-XIST cells than that in sh-ctrl cells. However, miR-132-3p inhibitor treatment led to restoration of MAPK1 in sh-XIST cells (Figure 4D). To sum up, these results indicated that the miR-132-3p/MAPK1 axis had a fundamental role in CRC cell growth.

Discussion

CRC is characterized by high prevalence and mortality around the world [13]. Despite the successful surgical intervention used in early stage, many patients still develop metastasis. To help improve the CRC survival rate, it is urgent to thoroughly understand the mechanisms of CRC development and metastasis. Evidence has proved that lncRNAs may be used as effective therapeutic targets for cancer therapy. Some authors [13] have shown that several lncRNAs can be considered as potential prognostic indicators for CRC, including XIST. In this study, XIST expression was found upregulated in both CRC tissue samples and cells. More interestingly, XIST expression was tightly correlated with TNM stage and the size of tumor. Furthermore, cell proliferation was inhibited in CRC cells after XIST was knocked down. Besides, we found that XIST knockdown caused cell cycle arrest at G0/G1. The above data suggests that XIST might serve as an oncogene and promote cell proliferation in CRC.

Latest studies reveal that lncRNAs function in tumor progression by binding to miRNAs. For example, silenced lncRNA MALAT1 can remarkably inhibit the growth , invasion capability and metastasis of esophageal squamous cell carcinoma cells by miR-101 and miR-217 [14]. Meanwhile, lncRNA MEG3 suppresses the cell proliferation and promotes cell apoptosis in cervical cancer by regulating miR-21-5p [15]. Served as a competing endogenous RNA for miR-138, lncRNA H19 promotes epithelial-mesenchymal transition (EMT) and accelerates the growth of colon cancer [16]. Previously, lncRNA XIST was found to act as an oncogene in nasopharyngeal carcinoma by aiming at miR-34a-5p [17]. In our study we performed bioinformatic analysis to demonstrate whether XIST acted as an endogenous miRNA sponge in CRC. We discovered that XIST contained several binding sites fitting to miRNAs, among which miR-132-3p has been reported to suppress the progression of CRC cell lines and correlate with prognosis in CRC patients [18,19]. In the present study, we found that miR-132-3p could directly bind to XIST through luciferase assay. In addition, XIST expression could be suppressed by upregulated miR-132-3p, while downregulated miR-132-3p expression could induce a reverse outcome. On the other hand, miR-132-3p expression could

be upregulated after XIST knockdown. All these results suggest that XIST and miR-132-3p might inhibit the expression of each other.

In our study, it has been proved that MAPK1 is a possible target spot of miR-132-3p. What's more, the expression of MAPK1 in CRC was negatively regulated by the miR-132-3p. Furthermore, MAPK1 is a crucial regulator of cell growth, metastasis and apoptosis through a few important signal transduction pathways [20,21]. In addition, we found that XIST knockdown suppressed the expression of MAPK1, which could be reversed by miR-132-3p inhibition. These results suggest that XIST may realize its function by means of the miR-132-3p/MAPK1 axis. Consequently, we put forward the idea that XIST promotes cell proliferation and it works by the miR-132-3p/MAPK1 axis in CRC.

In conclusion, the current study demonstrated that XIST was overexpressed in CRC tissue samples and the higher expression was correlated with worse disease free survival of CRC patients. Besides, XIST could promote cell proliferation through the miR-132-3p/MAPK1 axis. These findings implied that lncRNA XIST can act as a prospective therapeutic target for CRC.

Conflict of interests

The authors declare no confict of interests.

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