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Long noncoding RNA MNX1-AS1 overexpression promotes the invasion and metastasis of gastric cancer through repressing CDKN1A

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Abstract. – OBJECTIVE: Recently, long non-coding RNAs (lncRNAs) have attracted much attention for their roles in tumor progression. The aim of this study was to investigate the specific role of lncRNA MNX1-AS1 in the development of gastric cancer (GC), and to explore the underlying mechanism.

PATIENTS AND METHODS: MNX1-AS1 expression in both GC cells and tissue samples was detected by Real Time-quantitative Polymerase Chain Reaction (RT-qPCR). Moreover, the relationship between MNX1-AS1 expression and the overall survival rate of GC patient explored. Furthermore, wound healing and transwell assay were conducted. In tion, the underlying mechanism of MNX1-AS GC was explored by performing RT-qPCR Western blot assay.

RESULTS: MNX1-AS1 expre GC sam ples was significantly highe n tha the corresponding normal tissu Meanwh MNX1-AS1 expression was ass d wi survival time of GC pa nt. the tion and invasion of cells w arkedly promoted after MNX 1 overexp n *in vitro*. The mRNA and expression DKN1A were remark de egulated a r MNX1-AS1 overe ression. rmore, the expression leve DKN1A wa tively correlated with th pression of MNX in GC tissues.

CO USION: Our results suggested that MN S1 co enhance the metastasis and inva of cells suppressing CDKN1A. Furthe MNX1 might be a potential peu get GC.

Key rds:
non-coding RNA, MNX1-AS1, Gastric cancer

Introduction

Gastric cancer (GC) is a huge threat to public health. It ranks the fourth most prevalent malignancy and the nd leading ancer-related deaths ide[1]. In the ecades, the diagnosis trea t of GC ha e been developed greatly. Howev e prognosis of GC patient undesirable roximately 951,600 G ases were newly di gnosed and 723,100 nts died of G in 2012[2]. Due to atypical or nt symptom the early stage, GC is often d sed at a stage in most cases. GC is cha ized extensive invasion, malignant prolife d distant metastasis. Therefore, there is an urgent need to identify novel biomark- therapeutic targets for GC patients.

Long non-coding RNAs (lncRNAs) are known as a cluster of non-coding transcripts. Recent studies have indicated that lncRNAs are emerging with numerous heterogeneous molecular actions, including tumorigenesis. For example, lncRNA AF147447 depresses the proliferation and invasion of GC cells infected with Helicobacter pylori by regulating miR-34c expression and targeting MUC2[3]. LncRNA UICLM enhances the liver metastasis of colorectal cancer by functioning as a ceRNA to microRNA-215, which modulates the expression of ZEB2[4]. LncRNA 91H has been shown to increase the aggressive phenotype of breast cancer cells. Meanwhile, it exerts oncogenic properties by up-regulating the expression of H19/IGF2[5]. Furthermore, lncRNA MALAT-1 acts as an oncogene in non-small cell lung cancer and tongue cancer by promoting cell proliferation and migration[6,7]. However, the exact function of MNX1-AS1 in GC has not been elucidated so far.

In this work, we found that the expression of MNX1-AS1 was remarkably up-regulated in GC tissues. Moreover, MNX1-AS1 significantly promoted the migration and invasion of GC cells *in vitro*. Our further experiments explored the possible underlying mechanism of MNX1-AS1 function in GC development.

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Patients and Methods

Cell Lines and Clinical Samples

52 GC patients who received surgery at the Gansu Provincial Hospital from July 2015 to December 2017 were enrolled in this study. Before the operation, written informed consent was obtained from each subject. No radiotherapy or chemotherapy was performed for any patients before operation. Tissue samples collected from surgery were stored immediately at -80°C for subsequent use. All tissues were analyzed and confirmed by an experienced pathologist. This study was approved by the Ethics Committee of the Gansu Provincial Hospital.

Four human GC cell lines (HGC-27, MKN-45, SGC-7901, BGC-823) and one normal human gastric epithelial cell line (GES) were purchased from the Institute of Biochemistry and Cell Biology, Chinese Academy of Science (Shanghai, China). All cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Grand Island, NY, USA) consisting of 10% fetal bovine serum (FBS; Life Technologies, Gaithersburg, MD, USA) and penicillin, and maint d in an incubator with 5% CO_2 at 37°C.

Cell Transfection

The plasmids of pcDNA3.1-Control (pcDN Control) and pcDNA3.1-MNX (pcDNA MNX1-AS1) were purchased Pharma (Shanghai, China). Cell fection as performed according to the ctions amine 2000 Reagent vit sbad, USA). 48 h later, th pressio l of MNX1-AS1 in transfecte ls was dete sing Real Time-quantitat erase Cha Reaction (RT-qPCR).

RNA E action and Rea Tim uanti ve Polymerase Ch Reac n

Tot n tiss nd cells was extracted g T reage nvitrogen, Carlsbad, CA, Subs extracted total RNA was reve ranscri o complementary deoxyribose nu ids (cDNAs) in strict accordance with K ranscription Kit (TaKaRa Biotechology Co., Ltd., Dalian, China). Primers used for PCR were as follows: MNX1-AS1 primers fo d: 5′-GTGACTTCGCCTGTGATGGA-3′, reverse: 5′-GGCCTCTATCTGTACCTTTATTCC-3′; β-actin primers forward: 5'-GATGGAAATCGTCAGAGGCT-3' and reverse: 5'-TGGCACTTAGTTGGAAATGC-3'. Thermal cycle was as follows: 30 sec at 95°C, 5 sec for 40 cycles at 95°C, and 35 sec at 60°C.

Western Blot Analysis

Reagent radio-immunoprecipitati ssay (RI PA; Beyotime, Shanghai, China) utilized to extract protein in cells. Subsequently, ncentration of extracted proteins wa easure e bicinchoninic acid (BCA) pr assay kit (Dalian, China). Target n ns we separat sodium dodecyl sulph olyacr ide gel electrophoresis (SDS-PA E) t erred o poly-vinylidene difluo (PVD embra Roche, Basel, Switze). Then, t nes were incubated w ry and sec antibodies. Cell Sign g Te ogy (CST, Danvers, MA, USA) provided us wit it anti-β-actin and rabbit anti A, as well as nti-rabbit secondary a dy. The chemilumine cent film was applied t ssess the exp sion of proteins with Image J ware (NIH, B esda, MD, USA).

W He g Assay

A to ×10^5 cells were first seeded into 6-well plates, followed by culture in DMEM overnight. After scratched with a plas- the cells were cultured in serum-free DMEM. Wound closure was viewed at different time points. Each assay was repeated in triplicate independently. Images (10 × 10) were captured using the Olympus microscope (Tokyo, Japan). Software IPP Image-Pro Plus 6.0 (Silver Springs, MD, USA) was applied for data analysis.

Cell Migration and Invasion Assays In Vitro

Cell migration and invasion were measured using transwell chambers. First, cells (5×10^4) in 200 μL of serum-free DMEM were seeded to the upper chamber of an 8μm pore size insert (Corning, Corning, NY, USA) coated with 50 μg Matrigel (BD Biosciences, Franklin Lakes, NJ, USA). Meanwhile, the lower chamber was added with DMEM and FBS. 48 h later, after being wiped by a cotton swab, the upper surface of chambers was immersed with pre-cooled methanol for 10 min. Then, the cells were stained with crystal violet for 30 min. The number of invading cells was calculated under a microscope (40 × 10) to evaluate the migration and invasion abilities. Five random fields of view were randomly selected in each chamber. All experiments were performed in triplicate.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 18.0 (SPSS, Chicago, IL, USA) was used for all statistical analysis. Data were presented as mean ± SD (Standard Deviation). Chi-square test, Student's t-test and Kaplan-Meier method were selected when appropriate. $p<0.05$ was considered statistically significant.

Results

MNX1-AS1 Expression in GC Tissues and its Association With Overall Survival of GC Patients

First, RT-qPCR was conducted to detect the MNX1-AS1 expression in 52 GC patients' tissues. As a result, MNX1-AS1 was significantly up-regulated in GC samples (Figure 1A). The survival of GC patients after surgery was analyzed through the Kaplan-Meier method. Subsequently, 52 GC patients were divided into two groups via median expression, including high-MNX1-AS1 group and low-MNX1-AS1 group. Kaplan-Meier analysis showed that the o[verall] survival of GC patients with higher MN[X1-AS1] level was significantly worse than those w[ith] a lower level (Figure 1B).

Expression of MNX1-AS1 i[n] Cell Line

The expression of MNX1[-AS1] [in] GC cell lines was detected as wel[l. Th]e resu[lts] showed that MNX1-AS1 level [in] GC-27[, GC-9]01 and BGC-823 GC c[ell] lin[es] [was] significantly

higher than that of GES cells (normal human gastric epithelial cell line) (Figure 2). Subsequently, HGC-27 GC cells were chosen for transfection of pcDNA3.1-Control (pcDNA/Control) and pcDNA3.1- MNX1-AS1 (pcDNA/MNX1-AS1).

Overexpression of MNX1-AS1 [Re]pressed the Migration and Invas[ion] of C[ancer] Cells

RT-qPCR was then util[ized] to detect [MNX1-]AS1 expression in tran[sfect]ed HGC-27 GC cells (Figure 3A). Wound [heal]ing as[say] revealed that after MNX1-AS1 [over]expression, the m[igr]ation ability of GC [cells] was markedly [enh]anced (Figure 3B). [Subs]equent t[ranswell] assay also demonstrat[ed that] after MNX1-[AS1] was overexpressed in GC cells, the number of migrated and invaded cells was re[markab]ly increased (Figure 3C, D).

Interaction Between CDKN1A and MNX1-AS1 in GC

qPCR res[ult] showed that the expression leve[l of] CDKN1A was significantly down-regulated in GC cells of pcDNA3.1- MNX1-AS1 (pcDNA/MNX1-AS1) group when compared with that in pcDNA3.1-Control (pcDNA/Control) group (Figure 4A). Western blot assay showed that after MNX1-AS1 overexpression, the protein expression level of CDKN1A was remarkably down-regulated (Figure 4B). Furthermore, we found that CDKN1A expression in GC tissues was markedly down-regulated when compared with that of adjacent tissues (Figure 4C). Correla-

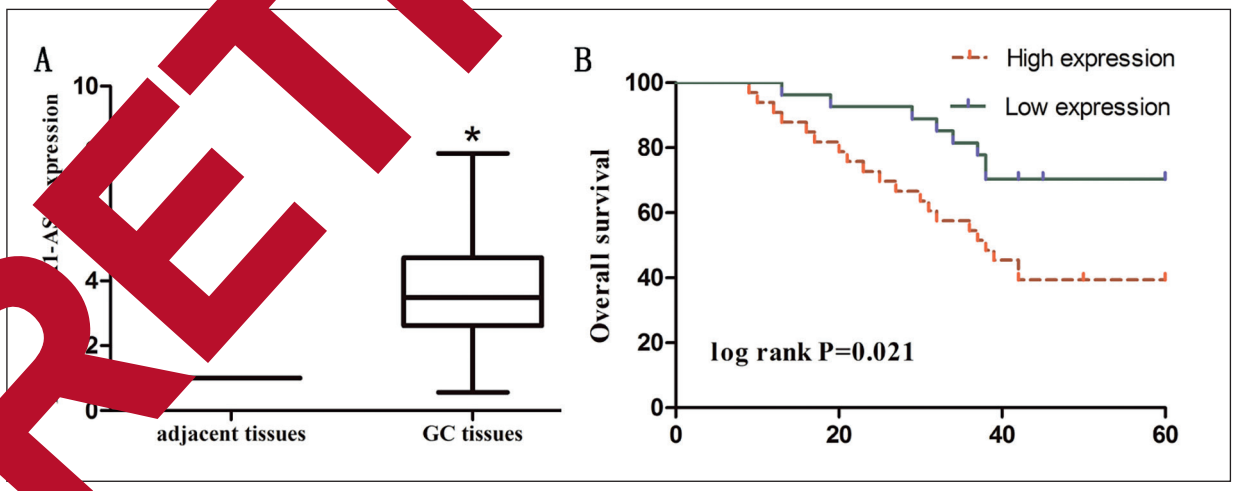


Figure 1. Expression level of MNX1-AS1 was significantly increased in GC tissues, which was associated with worse overall survival of GC patients. ***A***, MNX1-AS1 expression was markedly increased in GC tissues when compared with adjacent tissues. ***B***, Higher level of MNX1-AS1 was associated with worse overall survival of GC patients. Data were presented as mean ± standard error of the mean. *$p<0.05$.

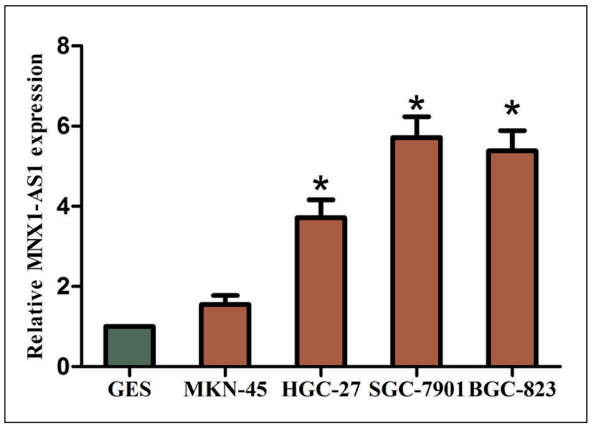
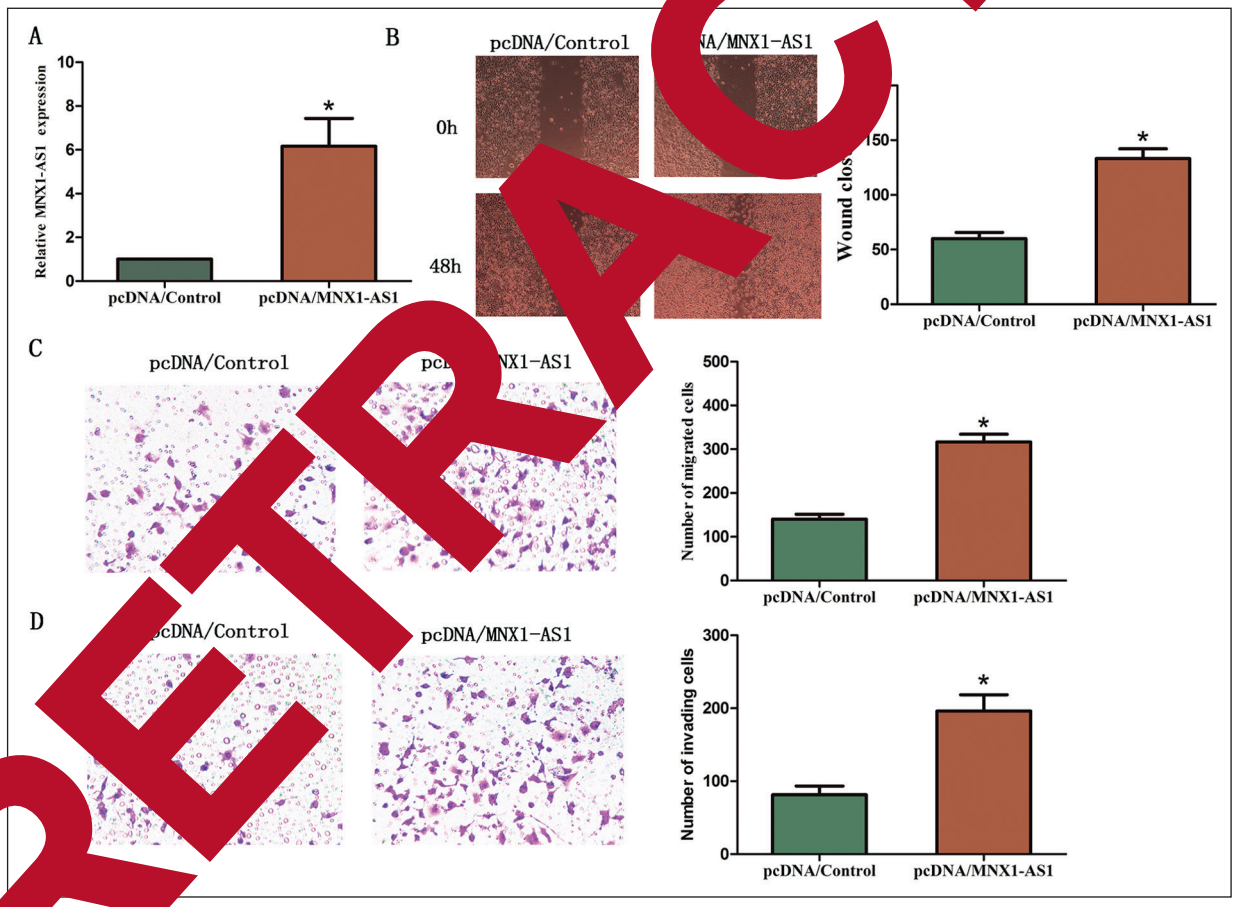


Figure 2. Expression of MNX1-AS1 in GC cell lines. Expression level of MNX1-AS1 relative to β-actin in human GC cell lines and GES (normal human gastric epithelial cell line) was determined by RT-qPCR. Data were presented as mean ± standard error of the mean. *p<0.05.

tion analysis demonstrated that CDKN1A expression was negatively correlated with MNX1-AS1 expression in GC tissues (Figure 4D).

Discussion

Numerous studies have revealed ncRNAs are emerging as critical re tors pro-gression. For example, ln A XIST is pressed in GC, which i o repo d asso with the phenotypes gressi mor and the prognosis of patien[8]. HG5 es as an important ant coge e pro sion of GC by trappi TA2 in th LncRNA DANCR f as a progn ndicator in GC, whic cilita e proliferation and tumorigenesis of GC[10]. By ating vasculogenic an-



igure 3. Overexpression of MNX1-AS1 promoted GC cell migration. ***A,*** MNX1-AS1 expression in GC cells transduced with NA3.1- MNX1-AS1 (pcDNA/MNX1-AS1) and pcDNA3.1-Control (pcDNA/Control) was detected by RT-qPCR. β-actin was an internal control. ***B,*** Wound healing assay showed that the overexpression of MNX1-AS1 markedly increased the mig on of GC cells (magnification: 10×). ***C,*** The transwell assay showed that the number of migrated cells was remarkably increased via overexpression of MNX1-AS1 *in vitro* (magnification: 40×). ***D,*** The transwell assay showed that the number of invaded cells was significantly increased after overexpression of MNX1-AS1 *in vitro* (magnification: 40×). The results represented the average of three independent experiments (mean ± standard error of the mean). *p<0.05, compared with control cells.

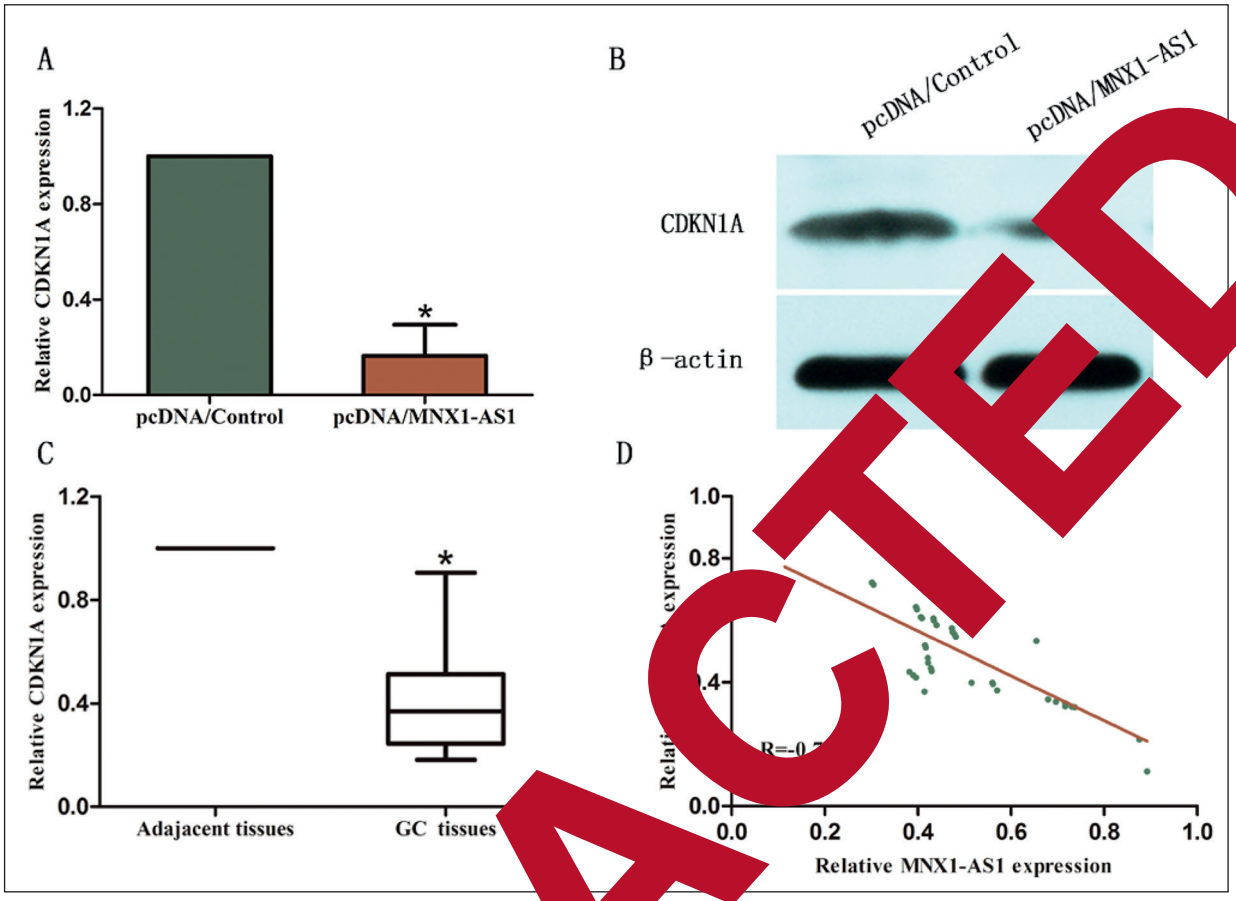


Figure 4. Interaction between MNX1-AS1 and CDKN1A. ...-PCR results showed that CDKN1A expression was markedly lower in pcDNA3.1- MNX1-AS1 (pc...X1-AS1) g... compared with pcDNA3.1-Control (pcDNA/Control) group. ***B,*** Western blot assay revealed tha... pro... xpression ...decreased in pcDNA3.1- MNX1-AS1 (pcDNA/MNX1-AS1) compared with pcDNA3.1-Cont... cDNA/C...ol). β-acti... as used as an internal control. ***C,*** CDKN1A was significantly down-regulated in GC tissues ... compa... diacent tissues. ***D,*** Linear correlation between the expression level of CDKN1A and MNX1-AS1 ... es... esented the average of three independent experiments. Data were presented as mean ± stan... error... an. *$p<0.05$.

giogenesis, ln...NA M...T1 has been demonstrated to ...note tumori...ity and metastasis of GC[11]. ...ddition, lncRNA ...00261 functions as a ...or sup...sor in GC by depressing the sta... of S... protein and inhibiting epithelial-me... trans... (EMT). Furthermore, ...may ...litate ...process of searching for ...therap...tegies[12].

...ently, a ...el lncRNA, MNX1-AS1, has be...ted to promote the malignancy of some ...cers. ...stance, by altering the expressions of ...K4, cyclin D, Bax, and Bcl-2, lncRNA MNX1-... functions as an oncogene in ovarian cancer[13]. L...A MNX1-AS1 facilitates the development of cervical cancer by activating the MAPK pathway[14]. In addition, lncRNA MNX1-AS1 participates in the MNX1-AS1-miR-218-5p-SEC61A1 feedback loop mediated by E2F1, eventually promoting the progression of colon adenocarcinoma[15]. Nevertheless, the specific role of MNX1-AS1 in GC has not been clearly elucidated.

In this work, we found that MNX1-AS1 was significantly up-regulated both in GC tissues and cells. Besides, the close relationship was observed between patients' prognosis and expression level of MNX1-AS1. Furthermore, after MNX1-AS1 overexpression, the migration and invasion of GC cells were remarkably promoted. The above results indicated that MNX1-AS1 promoted the tumorigenesis of GC, which might act as an oncogene.

Cyclin-dependent kinase inhibitors, especially CDKN1A ($p21^{Cip1}$), are canonical polycomb target genes and tumor suppressors[16]. For exam-

ple, by suppressing CDKN1A (a positive feedback loop), EZH2 controls the proliferation of germinal centers B cell and enables cell cycle progression[17]. LRH-1 inhibits the proliferation of breast cancer cells by regulating CDKN1A transcription expression. This may provide an attractive targeted therapy for breast cancer[18]. Meanwhile, CDKN1A enhanced the response of cutaneous tumors to radiotherapy by manipulating langerhans cell survival and promoting Treg cell generation[19]. In addition, CDKN1A expression is significantly correlated with the prognosis of patients with gastric adenocarcinoma resection[20].

In the present study, CDKN1A expression was markedly down-regulated after the overexpression of MNX1-AS1. Moreover, CDKN1A expression in GC tissues was negatively correlated with MNX1-AS1 expression. All the results above suggested that MNX1-AS1 might promote tumorigenesis of GC via targeting CDKN1A.

Conclusions

We identified that MNX1-AS1 was rem up-regulated in GC tissues and cells. Mean e, MNX1-AS1 expression was negatively corre with the overall survival of GC patients. Besid MNX1-AS1 could significantly e the m gration and invasion of GC rgeting CDKN1A. These findings gested t MNX1-AS1 might contribute t apy f candidate target.

Conflict of In

The Authors dec that th e no conflict of interests.

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