Long non coding RNA CCND2-AS1 promotes gastric cancer growth through Akt signaling

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Abstract: Gastric cancer (GC) is still the second second lethal malignancy worldwide. Mounting evidence indicates that IncRNAs (long non coding RNAs) play vital roles in tumorigenesis. But, the role of IncRNAs in GC is still largely unclear. Firstly, QRT-PCR were performed to assess lncRNA CCND2-AS1 expression 64 pairs of gastric cancer tissues (case group) and matched non-tumor tissues (control group). Both loss- and gain-functions assays were conducted by transfection. The cell viability, proliferation and cell cycle status after transfection were assessed by CCK-8 assay, clone formation assay, and FCM. Our results firstly show that LncRNA CCND2-AS1 is upregulated in GC tissues and cell lines. Moreover, CCND2-AS1 can promote GC cell proliferation and growth through Akt signaling. We demonstrate that CCND2-AS1 promoted GC cell growth via affecting Akt signal pathway and which suggest CCND2-AS1 might act as a potential therapeutic target in GC.

Keywords: Gastric cancer, lncRNA, CCND2-AS1, Akt signaling, cell proliferation

Introduction

Gastric cancer (GC) ranks the fourth most commonly diagnosed cancer and the second lethal malignancy worldwide, seventy percent cases occur in developing countries, with half of all cases occurring in Eastern Asia alone (predominantly in China) [1-3]. Despite efforts in multiple fields, little progress had been made in improving the 5-years overall survival rate of GC. Advances in therapy for the purpose of increasing survival rate have been limited for the pathophysiological mechanisms causing gastric cancer development are still largely unknown [4]. Therefore, revealing the molecular mechanism of gastric cancer development is indispensable for developing effective therapies.

As molecular research has progressed, IncRNAs have been demonstrated to be involved in tumor development and metastasis, which has drawn extensive attention from the scientific and medical community [5]. LncRNA (long non coding RNAs) is a class of transcripts longer than 200 nt with no protein-coding potential [6]. LncRNAs involve in multiple cellular biological processes and in tumorigenesis, and attract more and more researchers’ attention [6-9]. So many studies have shown that IncRNA molecules are abnormally expressed in lung, prostate, bladder, breast, liver, and ovarian cancers, to name a few [5, 10-13]. Importantly, the dysfunction of some IncRNAs have been considered as potential diagnostic cancer biomarkers [8, 14, 15]. However, the relationship between IncRNAs and GC has only recently begun to be studied in detail. LncRNA HOTAIR have been reported linked to GC invasion via reversing EMT [14]. T.DU et al. reported that LncRNA WT1-AS could promote GC proliferation and invasion [16]. H. Endo and T. Shiroki reported that HOTAIR was associated with the development of gastric cancer [17].

CCND2 antisense RNA 1 (CCND2-AS1), is an IncRNA with length of 553 bp. The abnormal expression of CCND2-AS1 has only been observed in glioma and plays oncogenic role for promoting glioma cells proliferation via Wnt/β-catenin signal pathway [18]. To our knowledge, there was no research on the relationship...
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between CCND2-AS1 and GC. Therefore, we hypothesized that CCND2-AS1 up-regulation might be related to the development of GC.

To explore the potential function of lncRNAs in the carcinogenesis of GC, we found one lncRNA, CCND2-AS1, was overexpressed in GC. Then, we found CCND2-AS1 can promote GC progression by performing functional experiments. These results provide a strategy and facilitate the development of lncRNA-directed diagnostics and therapeutics against GC.

Materials and methods

Clinical specimens

All gastric cancer tissue specimens and the corresponding adjacent noncancerous tissues samples were obtained via surgical resection from Tai’an City Central Hospital. These tumors were exclusively primary site cancers that had not been treated with either chemotherapy or radiation. The study protocol was approved by the Ethics Committee of Tai’an City Central Hospital, and the written informed consent was obtained from all patients.

Cell culture

All human gastric cancer cell lines (MGC80-3, BGC-823, SGC-7901, AGS, and NCI-N87) and normal gastric epithelium cell line (GES-1) were obtained from Chinese Academy of Sciences Committee on Type Culture Collection cell bank. All cell lines were cultured in DMEM supplemented with 10% fetal bovine serum (Gibco, USA) and 1% (vol/vol) penicillin/streptomycin (P/S).

Cell transfection

Protocols of cell transfection were used as reported by Zhang H et al. [18]. Gastric cancer cells were transfected with 100 nM of control siRNA or CCND2-AS1 siRNA I or CCND2-AS1 siRNA II purchased from Sigma Aldrich by using Lipofectamine 2000 (Invitrogen). Vectors containing the full length of CCND2-AS1 were constructed into pcDNA3.1 to up-regulate CCND2-AS1. To obtain IncRNA CCND2-AS1 stably overexpressed cells, pcDNA3.1-CCND2-AS1 or pcDNA3.1 was transfected into cells. Then, the cells were selected with 800 μg/ml neomycin for 4 weeks. The change of CCND2-AS1 were confirmed by qPCR. The target sequence of shRNAs were described in Table 1.

RNA extraction and RT-PCR analyses

RNA was extracted using Trizol reagent (Invitrogen, USA), then treated with RNase-free DNase I (Qiagen, Valencia, CA). 1 μg RNA was reverse transcribed to cDNA by using PrimeScript RT reagent kit (Takara, Da Lian, China) and quantitative real time PCR performed by the ABI7500 system (Applied Biosystems, USA). Expression of CCND2-AS1 or coding protein genes was normalized to that of GAPDH. Primers were described in Table 1.

Colony formation assay

After transfection, cells were seeded into six-well plates (10³ cells/well) and cultured for 14 d. Then, the cells were fixed and stained with 0.1% crystal violet. The number of visible colonies was counted. All samples were assayed in triplicate.

Cell cycle analysis

Cells were collected directly or 48 hours after transfection and washed three times with phosphate-buffered saline (PBS), and fixed with 75% ethanol overnight at 4°C. Fixed cells were rehydrated in PBS for 10 minutes and incubated in RNase A (1 mg/ml) for 30 min at 37°C, then the cells were stained with propidium iodide for 30 min followed by flow cytometric analysis. Data were expressed as percentage distribution of cells in G0/G1, S and G2/M phases of the cell cycle. All samples were assayed in triplicate.

Western blots

Whole proteins were extracted by RIPA reagent (Beyotime, China) and protein concentrations

<table>
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<th>Table 1. Sequence of primers and sh RNAs</th>
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<tr>
<td>Name</td>
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| CCND2-AS1 primer | F: GAGGACGTGCTGGGGCT  
R: CTGAGTCCATGTCCCGAATC |
| GAPDH primer | F: AGCCTCAAGATCAGCAATGCC  
R: TGTGGTCAGTCGTCCTCCACGAT |
| Sh-#1 target | CTGTGCCATGCTCTTAA |
| Sh-#2 target | TTGAGAGAAATTACCTGATT |

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were determined by DC protein assay (Bio-Rad). Denatured samples were fractionated by SDS-PAGE gel and transferred to PVDF membranes. Membranes were blocked by skim milk for 1 h at room temperature. Primary antibodies, Akt/pAkt (Cell signaling technology), and GAPDH (Proteintech) were incubated overnight at 4°C. Fluorescent secondary antibodies were incubated at room temperature for 1 h and visualized with the ECL kit (Beyotime, China).

Statistical analysis

Independent-samples t test, paired t test and one-way ANOVA test were used to analyze quantitative data. P-value <0.05 was considered as statistical significant (*: P<0.05; **: P<0.01). The error bars in all figures represented standard deviation. The Kaplan-Meier method and log-rank test were used for survival analysis. All tests were performed by SPSS 15.0 software (SPSS Inc.) while the survival analysis performed by GraphPad 6.0 software.

Results

Intracellular RNA CCND2-AS1 overexpressed in GC and confers a poor prognosis

To explore the function of CCND2-AS1 in GC, qRT-PCR were firstly performed to measure the level of CCND2-AS1 in 64 GC tissues. Comparing with matched normal tissues, CCND2-AS1 level was significantly increased in GC tissues.
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Figure 2. Knockdown IncRNA CCND2-AS1 inhibits gastric cancer cell proliferation and growth. (A, B) The expression of IncRNA CCND2-AS1 is significantly down-regulated in indicated SGC-7901 (A) and BGC-823 (B) cell lines after...
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Up-regulation of CCND2-AS1 was shown in 43 cases out of 64 (67%), in which unchanged was revealed in 9 (14%) and down-regulation in 12 (19%) (Figure 1B).

Additionally, Kaplan-Meier method analysis (log-rank test) determined that patients with high level of CCND2-AS1 had a significantly shorter overall survival than those with low expression. (P<0.01, Figure 1A). Up-regulation of CCND2-AS1 was shown in 43 cases out of 64 (67%), in which unchanged was revealed in 9 (14%) and down-regulation in 12 (19%) (Figure 1B).
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level (Figure 1C, P=0.036). Overall, our results indicate that high levels of CCND2-AS1 are associated with overall survival in patients with gastric cancer significantly. Moreover, CCND2-AS1 in 5 human gastric cancer cells line and GES-1 (one immortalized gastric cell lines) were checked. We found that CCND2-AS1 expressed lower in GES-1 cell lines but overexpressed in SGC-7901, BGC-823, MGC80-3, AGS, and NCI-N87 cell lines (Figure 1D). These data together indicated that CCND2-AS1 might be involved in the progression of GC.

Silenced CCND2-AS1 suppresses GC cell proliferation

To determine the biological function of CCND2-AS1 in GC, we evaluated the effects of CCND2-AS1 on the cell proliferation, colony formation and cell cycle. SGC-7901 and BGC-823 cells which express relative high level of CCND2-AS1 were selected and transfected with CCND2-AS1 specific siRNA. The silence efficiency of CCND2-AS1 was shown in Figure 2A and 2B. Our results showed that CCND2-AS1 knockdown inhibited the proliferation (Figure 2C and 2D) and colony formation (Figure 2E, 2F). To determine CCND2-AS1 pro-proliferation mechanism, cell cycle distribution was analyzed by FCM. As showed in Figure 2E, 2F, knockdown of CCND2-AS1 caused G0/G1 arrest significantly. These investigations proved the oncogene effect of CCND2-AS1 on GC cells and such function was attributed to its influence on cell cycle.

Up-regulated CCND2-AS1 promotes cell growth in NCI-N87

To further investigate the oncogene effect of CCND2-AS1 in GC, stable overexpressed cell line was constructed in NCI-N87. The expression of CCND2-AS1 was increased about 6 folds (Figure 3A). Using this stable cell lines, CCK-8 assay and clone formation assay were performed to examine whether CCND2-AS1...
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Indeed, participated in the GC cells proliferation. The proliferation rates were significantly higher in stable NCI-N87 cells (Lv-CCND2-AS1) compared to that of the control cells (Lv-NC) from day 2 to day 4 (Figure 3B), and this expression status can increase the ratio of cells in S phase (Figure 3C) and colony number (Figure 3D).

**CCND2-AS1 regulates AKT signal pathway in gastric cancer cells**

As AKT signaling could regulate cell proliferation and growth in cancers, CCND2-AS1 may regulate Akt signaling, and subsequently affects cell proliferation. Western blot results revealed that silencing CCND2-AS1 reduces phosphorylation of Akt and decreases the ratio of pAkt in SGC-7901 (Figure 4A) and BGC-823 (Figure 4B) cell lines. Thus depletion of CCND2-AS1 reduced Akt signaling significantly. When overexpression of CCND2-AS1, accelerates phosphorylation of Akt, as shown in Figure 4C. Moreover, we then used an inhibitor of Akt, MK-2206 2HCl, to treat NCI-N87 stable cell lines. As shown in Figure 4D, MK-2206 2HCl could partially rescued CCND2-AS1 induced cell growth. These results together indicated that overexpressed CCND2-AS1 contributes to oncogenesis by affect AKT signal pathway in GC.

**Discussion**

LncRNA CCND2-AS1 involved in proliferation and invasion of cancers. For example, CCND2-AS1 upregulated in glioma and promoted glioma proliferation through affecting Wnt/β-catenin signaling [18]. In present study, we found that the expression level of CCND2-AS1 in GC tissues was increased significantly compared with corresponding normal tissues and the high expression level of CCND2-AS1 in GC patients was associated with poor prognosis. Moreover, upregulated CCND2-AS1 promoted cell proliferation via Akt signaling. Thus, our findings indicated that CCND2-AS1 acted as an oncogene in GC progression.

It is generally acknowledged that mammalian genomes encode thousands of IncRNAs [6]. In addition to microRNAs, IncRNAs are emerging as important players in cell biology. To date, increasing evidences showed dysregulation of IncRNAs related to diverse human diseases including tumors [5, 11]. In previous study, many IncRNAs have been reported as tumor oncogenes and prognosis biomarkers [7, 10]. Many IncRNAs have been reported to involve in gastric tumorigenesis and progression, such as HOTAIR, H19, MEG3, HOXA-AS2, ZDAS-1 and KRT7-AS [16, 17, 19-27]. Emerging evidences have demonstrated that CCND2-AS1 could act as an oncogene in malignancies and its up-regulated expression may result in proliferation of tumor cells. Zhang H et al. firstly identified the abnormal expression of CCND2-AS1 in glioma tissues, which showed that high expression of CCND2-AS1 in glioma tissues was associated with poor overall survival [18]. There was no report about the role of CCND2-AS1 in other tumors, so as in GC. In this study, we firstly reported that CCND2-AS1 highly expressed in GC and this expression pattern of CCND2-AS1 was associated with poor prognosis. We further found that silencing CCND2-AS1 significantly inhibits cell proliferation and growth, reversed phenomenon was observed while overexpression of CCND2-AS1. These findings indicate that CCND2-AS1 promotes GC cells growth.

LncRNAs involved in several tumor signaling pathways such as Notch, Akt, mTOR, NF-κb, and Wnt to manipulate cell proliferation, apoptosis, migration, invasion, metastasis and cell cycle [14, 28, 29]. Akt signal pathway have been reported activated in various tumors and plays a pivotal role in regulating cancer cell proliferation, growth and survival, so is in GC [30, 31]. Studies to reveal the mechanisms underlying CCND2-AS1 regulation on tumor development has been reported by Zhang H et al. and the results demonstrate that CCND2-AS1 plays a critical role in the migration and proliferation of cancer cell via Wnt/β-catenin signaling [18]. However, it is far from enough to illuminate the molecular mechanisms of CCND2-AS1 on cancers. Our in vitro results found that the expression level of pAKT protein were significantly decreased by slicing CCND2-AS1 in BGC-823 and SGC-7901 gastric cancer cells, while overexpression CCND2-AS1 promoted the pAKT expression and increased the ration of pAKT in NCI-N87 cell line. In addition, we found AKT inhibitor MK-2206 2HCl rescues the promotive proliferation effects of CCND2-AS1 in NCI-N87 overexpression stable cell lines. These findings implied that AKT/pAKT as downstream target of CCND2-AS1 was involved in the regulation of...
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gastric cancer development. Our findings shed the light on the mechanism that CCND2-AS1 regulates Akt signaling to promote cell growth. However, the underlying mechanism of CCND2-AS1 in regulating Akt signaling remains unclear, and its mechanism should be investigated in further studies.

In summary, our study first demonstrated CCND2-AS1 upregulated in GC. Moreover, we first reported that CCND2-AS1 playing oncogenic role in the development of GC via AKT signaling. Our results indicate that CCND2-AS1 serve as oncogene in GC and may be a promising target in GC treatment.

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Disclosure of conflict of interest

None.

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