

Original Article

Expression of long non-coding RNA CCAT2 has a predictive value for patient survival and tumor metastasis in gastric cancer

Yuanzhi Lan, Fan Zhang, Yan Shi, Yongliang Zhao, Feng Qian, Bo Tang, Yingxue Hao, Peiwu Yu

Department of General Surgery and Center of Minimal Invasive Gastrointestinal Surgery, Southwest Hospital, The Third Military Medical University, Chongqing, China

Received November 1, 2015; Accepted December 26, 2015; Epub February 1, 2016; Published February 15, 2016

Abstract: Objective: To explore the predictive value of CCAT2 expression for patients survival and tumor metastasis in Gastric Cancer (GC). Methods: In this study, we measured the levels of CCAT2 mRNA expression in gastric cancer tissue samples by RT-qPCR assay. The correlation among CCAT2 levels, pathological types, and survivals of GC patients were also explored using the Kaplan-Meier method. We also researched the effects of CCAT2 on GC cell migration/invasion and the molecular mechanisms were also explored primarily. At the same time, the predictive value of CCAT2 on metastasis of GC was detected by ROC curve. Results: The levels of CCAT2 expression in gastric cancer patients are higher than the normal ones ($P<0.01$). Thus, the CCAT2 silencing inhibited the cell migration and invasion in three gastric cancer cell lines ($P<0.01$). After that, the result of test for the predictive value showed that the levels of CCAT2 expression have an effective value ($P<0.05$). Conclusion: Level of CCAT2 expression is higher in GC patients and overexpression of CCAT2 promotes gastric cancer cell migration and invasion by regulating the MMPs and key EMT markers. In addition, the level has a predictive value on the diagnosis of GC metastasis.

Keywords: Colon cancer-associated transcript 2 (CCAT2), gastric cancer, tumor metastasis, prognosis, migration, invasion

Introduction

Gastric cancer (GC) is a frequent malignant disease all over the world and it is a public health problem because it results in high mortality [1]. Although the digestive endoscopy technologies develop quickly in early diagnosis of gastric cancer, a large portion of patients is diagnosed in advanced period which leads to a low survival rate [2].

Lacking of reliable molecular biomarker, which leads to lots of gastric cancer patients missing the early diagnosis, is responsible for the unsatisfactory survival of gastric cancer patients. The TNM staging system and pathological type is the most two vital predictive factors for prognosis and survival of gastric cancer patients. Thus, it is necessary to find novel predictive biomarkers of prognosis and survival for gastric cancer [3, 4].

Long non-coding RNAs (lncRNAs) are RNAs longer than 200 nucleotides in length. The non-

coding RNAs (ncRNAs) were sought to be useless in the cellular activities. However, evidence has showed that lots of lncRNAs have meaningful secondary structures, and it is important for specific binding. To date, more and more lncRNAs with biological functions have been reported [5-9]. These researches of new functions in the ncRNAs are causing a paradigm change in our understanding of gene regulation and its role in cancer development. However, recent researches had greatly changed our understanding to lncRNAs. Many evidences had showed that lncRNAs participate in many cellular activities including the tumor metastasis.

For example, knockdown of HOTAIR expression in the three highly metastatic epithelial ovarian cancer cell lines (SKOV3.ip1, HO8910-PM, and HEY-A8) significantly reduced cell migration and invasion which mediated by the regulation of certain matrix metalloproteinases (MMPs) and epithelial-to-mesenchymal transition (EMT)-

The predictive value of CCAT2 in gastric cancer

related genes [10]. Recently, Li et al revealed that the lncRNA H19 was up-expressed in gastric cancer samples than normal tissues. They also showed that the effect of H19 in gastric cancer is mediated by upregulation of ISM1 and the suppression of CALN1 expression via miR-675 [8].

CCAT2 is an lncRNA that locates the highly conserved 8q24 region and involved in different cancer cell developments. Ling et al reported that CCAT2 overexpressed in colorectal cancer and promotes tumor growth, metastasis [11, 12].

However, there is few study reporting the relationship between CCAT2 expression and gastric cancer.

In our research, we measured the levels of CCAT2 in gastric cancer tissue samples. The correlation between CCAT2 levels, pathological types, and survivals of GC patients were also explored. We also researched the effects of CCAT2 on GC cell migration and invasion and the molecular mechanisms were also explored primarily. At the same time, the predictive value of CCAT2 on metastasis of GC was detected by ROC curve.

Materials and methods

Patients and tissue samples

Sixty-two human tissue samples of gastric tumors and 30 vicinal non-tumor samples were acquired at the Department of General Surgery, XX Hospital during XX to XX. Tissue samples were frozen immediately in liquid nitrogen after operations and stored at -80°C until experiment. The 62 tumor tissue samples were acquired from patients diagnosed gastric cancer and accepted radical gastrectomy for cancer. All the patients included had not accepted preoperative radiotherapy or neoadjuvant chemotherapy. Thirty non-tumor tissue samples were gathered from patients diagnosed with gastric ulcer who suffered subtotal gastrectomy. The histopathological type, grade and stage of gastric cancer were confirmed according to the criteria of the World Health Organization (WHO). Furthermore, the nodal status regarding lymphatic metastasis was obtained from the histopathological reports. The clinical data of following up was acquired by our clinical follow-

up chats. Our research was approved by the Ethics Committee of XX University, China.

Cell lines and transfection

Cell lines (MKN28, AGS and MKN45) were obtained from XXX. The MKN28, AGS and MKN45 respectively represent well-differentiated, middle-differentiated and low-differentiated gastric cancer. MKN28, AGS and MKN45 cell lines were cultured in RPMI 1640 supplemented with 10% FBS. All cells were incubated in a 37°C atmosphere of 5% CO₂.

The transfections were carried out using Lipofectamine™ 2000 (Invitrogen, Shanghai, China) in direction of the manufacturer's instructions. Two different CCAT2-siRNA and negative control plasmid were transfected into all cell lines. The siRNA sequences were as follows: 5'-UUAACCUCUCCUAUCUCATT-3' (sense) and 5'-UGAGAUAGGAAGAGGUUAATT-3' (antisense) for siRNA1; 5'-AGGUGUAGCCAGAGUUAUTT-3' (sense) and 5'-AUUAACUCUGGCUACACCUTT-3' (antisense) for siRNA2.

RNA isolation and RT-qPCR

Total RNA of all cell lines and tissue specimens were extracted using the Trizol reagent (Invitrogen, USA) in direction of the manufacturer's protocols. The PrimeScript RT Master Mix (Takara, Dalian, China) was used to reverse transcribe RNA into cDNA. Then, the quantitative real-time PCR were carried out using the ABI 7900 system (Applied Biosystems, USA) and the SYBR Green PCR Master Mix (Takara, Dalian, China). The relative mRNA expressions of CCAT2 gene were detecting using real-time PCR with specific primers. Primers used are listed as below. GAPDH was used as an internal control for all the cell lines and tissue specimens. The used sequences of primers were as follow: 5'-CCACATCGCTCAGACACCAT-3' (sense) and 5'-ACCAGGCGCCCAATACG-3' (antisense) for GAPDH and 5'-CCCTGGTCAAATTGCTTAACCT-3' (sense) and 5'-TTATTCGTCCCTCTGTTTATGGAT-3' (antisense) for CCAT2.

Wound healing assay

The wound healing assay was used to detect the ability of cell migration and performed as previous paper described [4, 13]. Forty-eight hours after transfection, after cells reaching

The predictive value of CCAT2 in gastric cancer

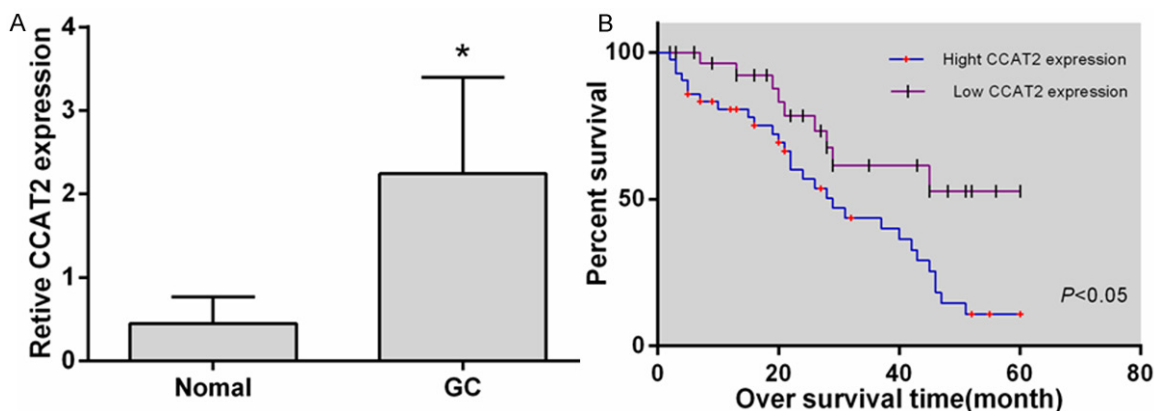


Figure 1. Relative CCAT2 expressions in tissue samples and their predictive effects. A. Relative CCAT2 expressions in gastric cancer samples compared to normal tissue samples. B. The overall survival of gastric cancer patients with different level of CCAT2 expression. * $P < 0.01$.

60% density, the plastic micropipette tip was used to make a cell-blank scratch. Then, the cells were washed using PBS in order to wipe off the trashy cells and then cultured in medium for another 24 h. The gaps of the wounds were measured 24 h later. The distances that the cells moved were observed and recorded.

Transwell assay

The transwell assay was carried out to detect the invasion ability of gastric cancer cells [14]. Twenty-four hours post-infection, the MNK28, AGS and MNK45 cell lines were gathered after infection 24 h later. Cell suspensions (1×10^5 cell/well) were then plated in the upper chamber of Transwell inserts (BD, USA). The inserts were then placed into the bottom chambers were filled in RPMI1640. examined, counted, and imaged using digital microscopy. After incubation at 37°C in a 5% CO_2 incubator for 24 h, the transwell inserts was wiped using tampons to sweep away the useless cells. After that, the polycarbonate membranes were washed using PBS for twice. Cells that cross through the membranes were strained with trypan flue. The cell number in 8 random fields was calculated and analyzed.

Protein extraction and Western blotting

Western blotting assays were used to verify the downstream expressions of CCAT2 gene. All cells were lysed in lysis buffer (2% deoxycholic acid, 5 mM EDTA, 150 mM NaCl, 0.1% SDS, 0.5% Triton X-100, 10 mM Tris-HCl, pH 7.2). The lysates were then separated on SDS-PAGE gels.

After that, proteins were transferred onto nitrocellulose membrane. The membranes were blocked with 5% BSP and incubated with primary antibodies at 4°C for a night. The primary antibodies used in the western blots were as follow: MMP-2 (1:1000 dilution), MMP-9 (1:1000 dilution), E-cadherin (1:1000 dilution) and GAPDH (1:1000 dilution). This was followed by incubation with secondary antibody (1 hour at room temperature). The relative densities of the target proteins to GAPDH bands was recorded and analyzed.

Statistical analysis

The GraphPad Prism 6 (GraphPad Software Inc., USA) was used for statistical analysis and drawing. The data was presented as the mean \pm SD. The one-way ANOVA as used for multiple comparisons, while the independent t test was used to test the differences between two groups and chi-square test was performed to analyze the categorical data. Over survival curve was drew according to the Kaplan-Meier method. $P < 0.05$ was considered as a statistically significant result.

Results

Up-expression of CCAT2 is associated with the poor prognosis of gastric cancer patients

RT-qPCR assay was performed to explore the CCAT2 expressions in sixty-two gastric cancer and twenty-night normal tissue specimens. As we can see in **Figure 1**, the levels of CCAT2 expression in gastric cancer patients are higher than the normal ones ($P < 0.01$, **Figure 1**).

The predictive value of CCAT2 in gastric cancer

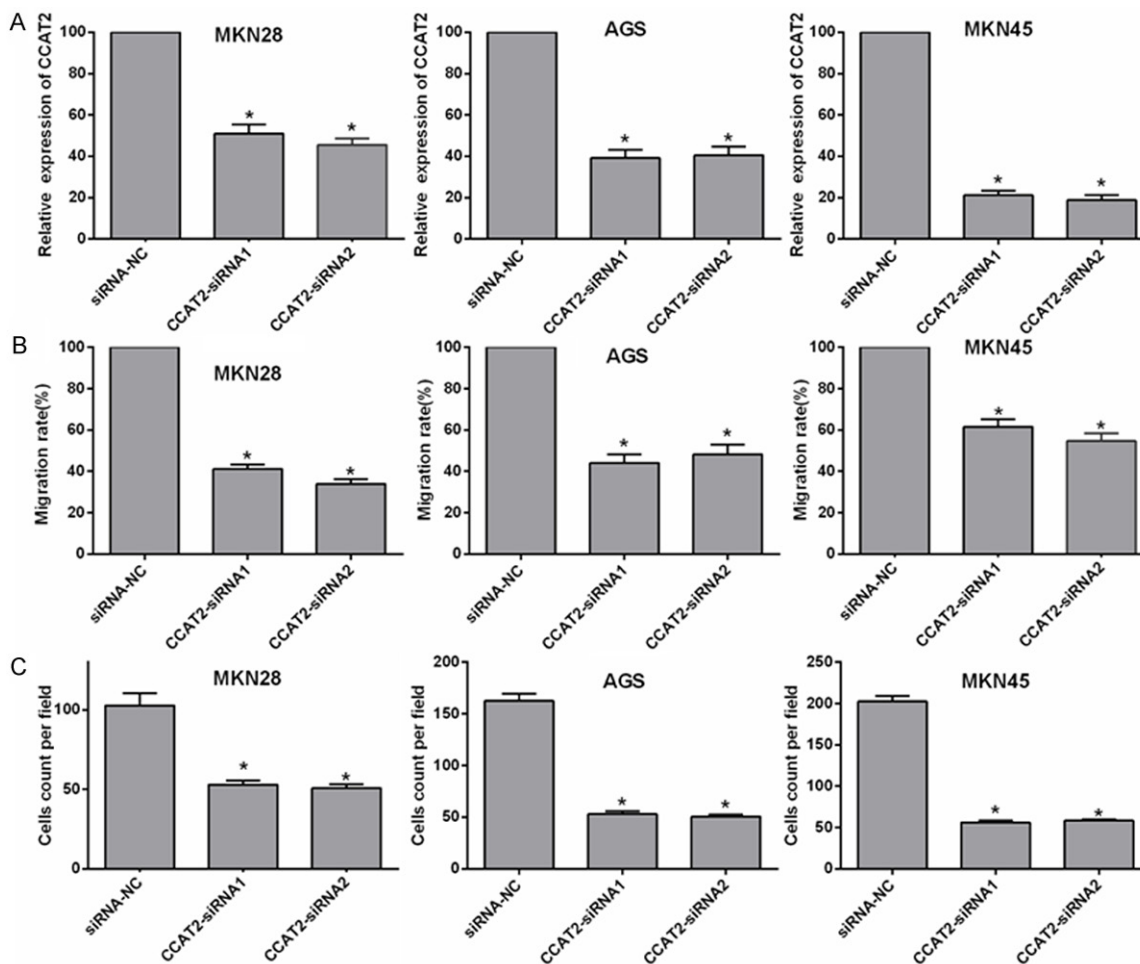


Figure 2. Effects of CCAT2 on cells migration and invasion in MKN28, AGS, MKN45 cell lines by means of wound healing assay transwell assay. The cells number cross through the membrane were counted and recorded. * $P < 0.01$.

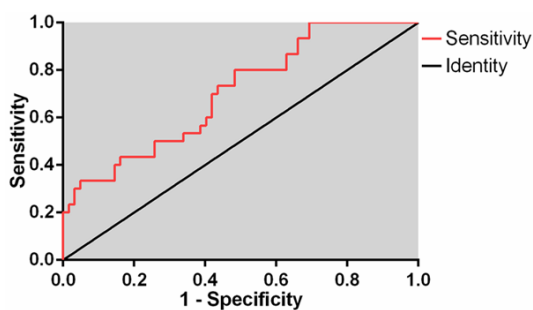


Figure 3. The ROC curves of the test for predictive efficiency of CCAT2.

Next, the correlations between CCAT2 expression and the prognosis of the patient with gastric cancer were discussed. The overall survival curve was drawn according to the Kaplan-Meier method. The CCAT2 expressions were related to the overall survival. **Figure 2** displays that the patients with high CCAT2 expressions have

poor prognosis. ($P < 0.05$, **Figure 2**). Multivariate Cox regression analysis was also performed and reveals that the level of CCAT2 expression ($P < 0.01$), TNM-stage ($P < 0.05$), and lymph node metastasis ($P < 0.05$) were influential factors of overall survival.

Our results reveal that up-expression of CCAT2 has a correlation with a poor prognosis of gastric patient. Furthermore, the levels of CCAT2 expression may have a predictive value for the prognosis of gastric cancer patients.

Knocking down of CCAT2 expression weakens gastric cancer cell migration and invasion

The results showed that the migration distances in CCAT2 silencing groups were significantly decreased than the negative control group ($P < 0.01$, $P < 0.01$; **Figure 2**). Then, the transwell assays were carried out to explore the effects

The predictive value of CCAT2 in gastric cancer

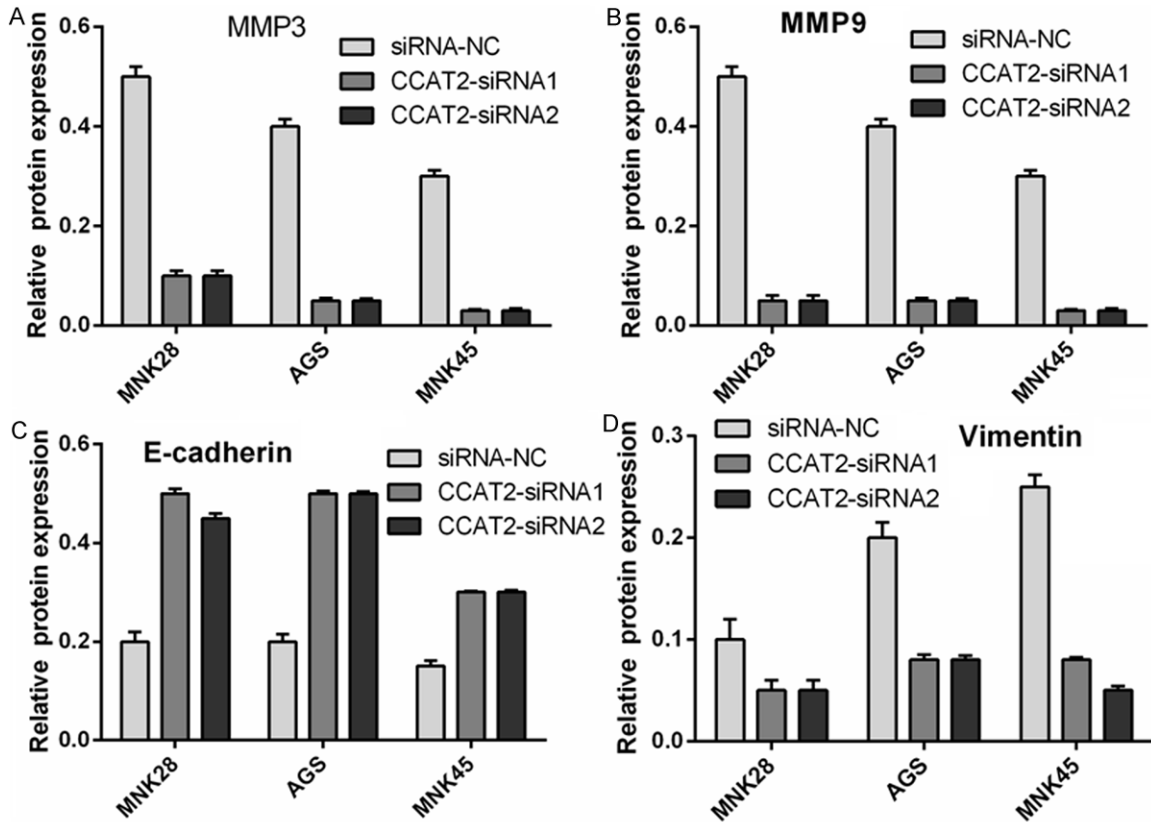


Figure 4. The protein expression of MMP family and key EMT markers. Detection of MMP3, MMP9, E-cadherin, vimentin were carried out by means of western bolt in MNK28, AGS, and MNK45 cell lines which being transfected using CCAT2-siRNAs or siRNA negative control. (A-D) Relative expression of MMP family and key EMT markers.

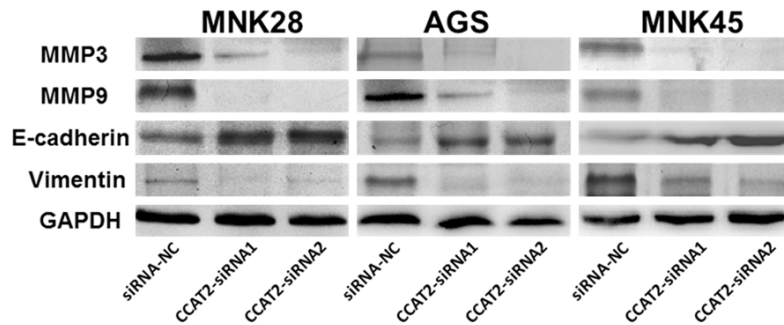


Figure 5. The results of western blotting.

of CCAT2 expressions on invasive ability of gastric cancer cells. Similarly, the results showed that the numbers of invasion cells in CCAT2 silencing w groups were decreased than the negative control (Figure 3). Our results showed that the CCAT2 silencing could inhibit migration and invasion of the gastric cancer cells. These results revealed that the levels of CCAT2 expression may have a predictive value for gastric cancer metastasis.

CCAT2 levels have a predictive value of a biomarker for gastric cancer metastasis

Although our results suggested that the levels of CCAT2 expression may have a predictive value for gastric cancer metastasis, the predictive efficiency remains unclear. Receiver operating characteristics (ROC) curve was used to

explore the predictive efficacy of CCAT2. As we can see in Figure 4, the levels of CCAT2 expression have a effective value for the prediction.

CCAT2 impresses gastric cancer cell migration and invasion via MMP family and key EMT markers

Our results above showed that the up-regulated expression of CCAT2 could promote the migration and invasion of gastric cancer cell.

The predictive value of CCAT2 in gastric cancer

However, the molecular mechanisms were not clear. Thus, to explore the molecular mechanisms of the promoting effects of CCAT2 expression for gastric cancer cell migration and invasion, the western blot analysis was carried (Figure 5). Western blot analysis has showed that the CCAT2 silencing groups have a decreased expression in MMP-3 and MMP-9 protein. The results suggested that the CCAT2 promote the gastric cancer cell migration and invasion via MMPs family protein (Figure 4A, 4B). We also explore the key EMT makers which important role in cancer cell migration and invasion. Our results showed that knockdown of CCAT2 lead to increased expressions of E-cadherin (Figure 4C, 4D). At the same time, decreased expressions of vimentin were observed in CCAT2 silencing groups (Figure 4).

Discussion

The early diagnosis of gastric cancer remains a problem worldwide, which leads to quiet a low 5-year overall survival rates (OS) [15]. The overall survival are no more than 25% though the advance in therapeutic techniques for GC recently [16]. So, we attempted to find out a novel biomarker that can predict the prognosis of GC. The biomarker may be a risk factor of the poor prognosis of GC patients.

Most of the previous studies focused on the encoding genes which encode proteins [6]. The non-coding RNAs (ncRNAs) were sought to be useless in the cellular activities. To date, more and more lncRNAs with biological functions have been reported. These researches of new functions in the ncRNAs are causing a paradigm change in our understanding of gene regulation and its role in cancer development [17]. Up to now, well-known discovered ncRNAs are the rRNAs, tRNAs, piRNAs, miRNAs and lncRNAs. There are many studies revealing the relationship between miRNAs and cancer developments [18-21]. It makes us to wonder if the lncRNA also correlate with the cancer diagnosis and prognosis. As we had expected, recent evidences has revealed that lncRNAs also play important roles in cancer development and metastasis. For example, Qiu et al examined the HOTAIR expressions in epithelial ovarian cancer tissue samples. Their results suggested that HOTAIR plays a vital role in EOC metastasis and could represent a novel prognostic marker and potential therapeutic target

in patients with epithelial ovarian cancer tissue samples [22]. However, the predictive application of lncRNAs on GC metastasis and prognosis needs further research.

In our research, we measured the levels of CCAT2 in gastric cancer tissue samples. The levels of CCAT2 was higher in GC tissue samples compared to normal ones ($P < 0.01$). The correlation between CCAT2 levels, pathological types, and survivals of GC patients were also explored. Our results reveal that up-expression of CCAT2 has a correlation with a poor prognosis of gastric patient. Furthermore, the levels of CCAT2 expression may have a predictive value for the prognosis of gastric cancer patients. We also researched the effects of CCAT2 on GC cell migration and invasion and the molecular mechanisms were also explored primarily. The CCAT2 silencing could inhibit migration and invasion of the gastric cancer cells. These results revealed that the levels of CCAT2 expression may have a predictive value for gastric cancer metastasis. At the same time; the predictive value of CCAT2 on metastasis of GC was detected by ROC curve. Our results showed that the levels of CCAT2 expression have an effective value for the prediction.

Previous researches had reported that lncRNAs promote tumor developments by encoding genes. Preceding evidences attempted us to explore whether CCAT2 affects GC metastasis by dysregulating the proteins related metastasis [6, 11, 22-24]. Thus, the western blotting assays were used to verify the metastasis-related gene by which CCAT2 effect the GC metastasis. Our results showed that knockdown of CCAT2 lead to increased expressions of E-cadherin. At the same time, decreased expressions of vimentin were observed in CCAT2 silencing groups.

In summary, the level of CCAT2 expression is high in GC patients and overexpression of CCAT2 promotes gastric cancer cell migration and invasion by regulating the MMPs and key EMT markers. In addition, the level has a predictive value on the diagnosis of GC metastasis.

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Peiwu Yu, Department of General Surgery and Center of Minimal

The predictive value of CCAT2 in gastric cancer

Invasive Gastrointestinal Surgery, Southwest Hospital, Third Military Medical University, 30 Gaotanyan Street, Shapingba District, Chongqing 400038, China. Tel: +86 23 68754167; E-mail: Yupeiwu01@sina.com

References

- [1] Cai H, Ye X, He B, Li Q, Li Y, Gao Y. LncRNA-APO01631.9 promotes cell migration in gastric cancer. *Int J Clin Exp Pathol* 2015; 8: 6235-44.
- [2] Du M, Wang W, Jin H, Wang Q, Ge Y, Lu J, Ma G, Chu H, Tong N, Zhu H, Wang M, Qiang F, Zhang Z. The association analysis of lncRNA HOTAIR genetic variants and gastric cancer risk in a Chinese population. *Oncotarget* 2015; 6: 31255-62.
- [3] Shao Y, Chen H, Jiang X, Chen S, Li P, Ye M, Li Q, Sun W, Guo J. Low expression of lncRNA-HMlincRNA717 in human gastric cancer and its clinical significances. *Tumour Biol* 2014; 35: 9591-5.
- [4] Zhou X, Ye F, Yin C, Zhuang Y, Yue G, Zhang G. The Interaction Between MiR-141 and lncRNA-H19 in Regulating Cell Proliferation and Migration in Gastric Cancer. *Cell Physiol Biochem* 2015; 36: 1440-52.
- [5] Sun M, Nie FQ, Wang ZX, De W. Involvement of lncRNA dysregulation in gastric cancer. *Histol Histopathol* 2016; 31: 33-9.
- [6] Wang CY, Hua L, Yao KH, Chen JT, Zhang JJ, Hu JH. Long non-coding RNA CCAT2 is up-regulated in gastric cancer and associated with poor prognosis. *Int J Clin Exp Pathol* 2015; 8: 779-85.
- [7] Xu C, Shao Y, Xia T, Yang Y, Dai J, Luo L, Zhang X, Sun W, Song H, Xiao B, Guo J. lncRNA-AC130710 targeting by miR-129-5p is up-regulated in gastric cancer and associates with poor prognosis. *Tumour Biol* 2014; 35: 9701-6.
- [8] Yang F, Bi J, Xue X, Zheng L, Zhi K, Hua J, Fang G. Up-regulated long non-coding RNA H19 contributes to proliferation of gastric cancer cells. *FEBS J* 2012; 279: 3159-65.
- [9] Yang Y, Shao Y, Zhu M, Li Q, Yang F, Lu X, Xu C, Xiao B, Sun Y, Guo J. Using gastric juice lncRNA-ABHD11-AS1 as a novel type of biomarker in the screening of gastric cancer. *Tumour Biol* 2015; [Epub ahead of print].
- [10] Qiu JJ, Lin YY, Ye LC, Ding JX, Feng WW, Jin HY, Zhang Y, Li Q, Hua KQ. Overexpression of long non-coding RNA HOTAIR predicts poor patient prognosis and promotes tumor metastasis in epithelial ovarian cancer. *Gynecol Oncol* 2014; 134: 121-8.
- [11] Ling H, Spizzo R, Atlasi Y, Nicoloso M, Shimizu M, Redis RS, Nishida N, Gafa R, Song J, Guo Z, Ivan C, Barbarotto E, De Vries I, Zhang X, Ferracin M, Churchman M, van Galen JF, Beverloo BH, Shariati M, Haderk F, Estecio MR, Garcia-Manero G, Patijn GA, Gotley DC, Bhardwaj V, Shureiqi I, Sen S, Multani AS, Welsh J, Yamamoto K, Taniguchi I, Song MA, Gallinger S, Casey G, Thibodeau SN, Le Marchand L, Tiirikainen M, Mani SA, Zhang W, Davuluri RV, Mimmori K, Mori M, Sieuwerts AM, Martens JW, Tomlinson I, Negrini M, Berindan-Neagoe I, Foekens JA, Hamilton SR, Lanza G, Kopetz S, Fodde R, Calin GA. CCAT2, a novel noncoding RNA mapping to 8q24, underlies metastatic progression and chromosomal instability in colon cancer. *Genome Res* 2013; 23: 1446-61.
- [12] Pan W, Liu L, Wei J, Ge Y, Zhang J, Chen H, Zhou L, Yuan Q, Zhou C, Yang M. A functional lncRNA HOTAIR genetic variant contributes to gastric cancer susceptibility. *Mol Carcinog* 2016; 55: 90-6.
- [13] Li L, Zhang L, Zhang Y, Zhou F. Increased expression of lncRNA BANCR is associated with clinical progression and poor prognosis in gastric cancer. *Biomed Pharmacother* 2015; 72: 109-12.
- [14] Li H, Yu B, Li J, Su L, Yan M, Zhu Z, Liu B. Overexpression of lncRNA H19 enhances carcinogenesis and metastasis of gastric cancer. *Oncotarget* 2014; 5: 2318-29.
- [15] Gatenby P, Shaw C, Hine C, Scholtes S, Koutra M, Andrew H, Hacking M, Allum WH. Retrospective cohort study of an enhanced recovery programme in oesophageal and gastric cancer surgery. *Ann R Coll Surg Engl* 2015; 97: 502-7.
- [16] Oholendt AL, Zadlo JL. Ramucirumab: A New Therapy for Advanced Gastric Cancer. *J Adv Pract Oncol* 2015; 6: 71-5.
- [17] Zhang A, Xu M, Mo YY. Role of the lncRNA-p53 regulatory network in cancer. *J Mol Cell Biol* 2014; 6: 181-91.
- [18] Ayers D, Baron B, Hunter T. miRNA Influences in NRF2 Pathway Interactions within Cancer Models. *J Nucleic Acids* 2015; 2015: 143636.
- [19] Doldi V, Callari M, Giannoni E, D'Aiuto F, Maffezzini M, Valdagni R, Chiarugi P, Gandellini P, Zaffaroni N. Integrated gene and miRNA expression analysis of prostate cancer associated fibroblasts supports a prominent role for interleukin-6 in fibroblast activation. *Oncotarget* 2015; 6: 31441-60.
- [20] Shu D, Li H, Shu Y, Xiong G, Carson WE, 3rd, Haque F, Xu R, Guo P. Systemic Delivery of Anti-miRNA for Suppression of Triple Negative Breast Cancer Utilizing RNA Nanotechnology. *ACS Nano* 2015; 9: 9731-40.
- [21] Zhao X, Li J, Huang S, Wan X, Luo H, Wu D. MiRNA-29c regulates cell growth and invasion by targeting CDK6 in bladder cancer. *Am J Transl Res* 2015; 7: 1382-9.

The predictive value of CCAT2 in gastric cancer

- [22] Zhang X, Xu Y, He C, Guo X, Zhang J, He C, Zhang L, Kong M, Chen B, Zhu C. Elevated expression of CCAT2 is associated with poor prognosis in esophageal squamous cell carcinoma. *J Surg Oncol* 2015; 111: 834-9.
- [23] Qiu M, Xu Y, Yang X, Wang J, Hu J, Xu L, Yin R. CCAT2 is a lung adenocarcinoma-specific long non-coding RNA and promotes invasion of non-small cell lung cancer. *Tumour Biol* 2014; 35: 5375-80.
- [24] Redis RS, Sieuwerts AM, Look MP, Tudoran O, Ivan C, Spizzo R, Zhang X, de Weerd V, Shimizu M, Ling H, Buiga R, Pop V, Irimie A, Fodde R, Bedrosian I, Martens JW, Foekens JA, Berindan-Neagoe I, Calin GA. CCAT2, a novel long non-coding RNA in breast cancer: expression study and clinical correlations. *Oncotarget* 2013; 4: 1748-62.