

Original Article

Overexpression of lncRNA-422 inhibits proliferation and invasion in colorectal cancer

Hui Yao, Dong Xia, Mingming Wang, Wangsheng Chen, Lei Ren, Liang Xu

Department of Gastrointestinal Surgery, The Affiliated Hospital of Southwest Medical University, Luzhou, Sichuan, China

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Abstract: Long non-coding RNA-422 (lncRNA-422) has been recently reported to be involved in the progression of colorectal cancer (CRC). But its effects on CRC cell phenotypes remain elusive. In the present study, we assessed the function of lncRNA-422 on the aggressive phenotypes of CRC cells *in vitro*. SW1116 cell was transfected with specific recombinant plasmid vector to enhance lncRNA-422 expression. The cell proliferation, invasion and migration were investigated and compared between overexpression group and control group. The apoptosis related protein levels in transfected CRC cells were also detected by western blot analysis. We found that up-regulation of lncRNA-422 inhibited the proliferation and invasion of CRC cells, and activated the apoptosis-related proteins, including JNK, p38, ERK, P-p53, PARP, caspase-3. In conclusion, our findings suggested that lncRNA-422 was implicated in the progression of CRC, which might provide the potential therapeutic target for this fatal cancer.

Keywords: lncRNA-422, proliferation, invasion, apoptosis, colorectal cancer

Introduction

Colorectal cancer is the third most prevailing cancer in the world with high mortality [1]. According to recent statistical data, the number of new patients with CRC is more than one million annually, and it remains the second leading cause of cancer-related death in the USA [2]. The occurrence rate of CRC in China has also obviously increased in recent years [3]. Therefore, it is critically important to elucidate the molecular mechanisms involved in the development of CRC [4, 5].

Long non-coding RNAs (lncRNAs) are described to be longer than 200 nt [6], and a rising number of studies have reported their biological roles as regulatory RNAs in a variety of human diseases including oncogenesis [7-9], and deregulation of lncRNAs is also implicated in the pathogenesis of CRC [10, 11]. Kogo reported that high expression of HOTAIR is associated with a poor prognosis of CRC [12]. Ling revealed that a novel lncRNA CCAT2 (colon cancer associated transcript 2) is significantly overexpressed in microsatellite-stable CRC and underlies metastatic progression and chromosomal instability in colon cancer [13]. Yan and Madamanchi

demonstrated decreased expression of lncRNA ncRUPAR can down-regulate protease-activated receptor-1 (PAR-1), thus inhibits CRC progression [14, 15]. Accordingly, lncRNAs may function as oncogenes or tumor suppressors, add a novel layer to our understanding of the complexity of CRC development [16]. It has already been reported that lncRNA-422 was decreased in CRC [17], while detailed cellular functions and mechanisms of lncRNA-422 in CRC still remain largely elusive.

Therefore, in the present study, we investigated the role of lncRNA-422 in CRC progression. We found that elevated expression of lncRNA-422 could induce CRC cell apoptosis, and inhibit CRC cell migration and invasion capacities. Furthermore, the underlying mechanisms of lncRNA-422 in regulating CRC cell apoptosis were also determined.

Materials and methods

Cell culture and reagents

The human CRC cell lines (SW1116) were obtained from the Shanghai Institutes for Biological Sciences (Shanghai, China), and cultured

LncRNA-422 plays an anti-oncogenic role in CRC

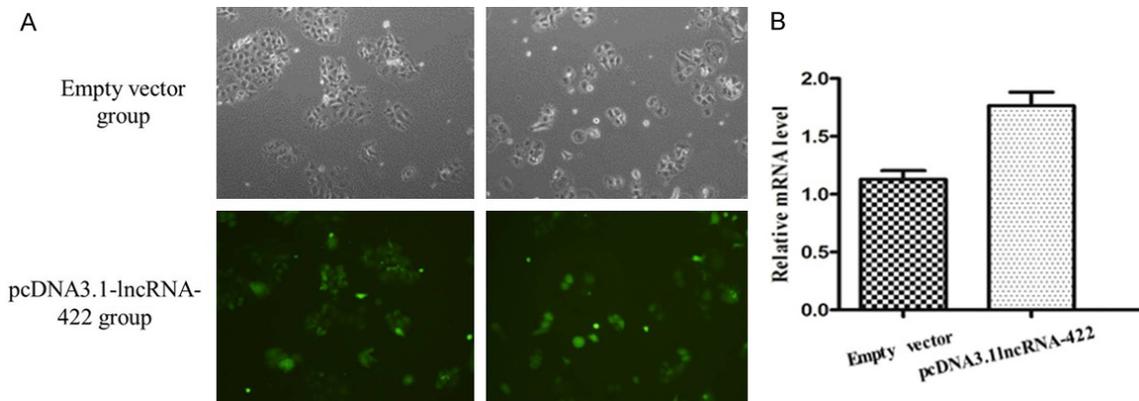


Figure 1. Overexpression of lncRNA-422 in SW1116 cells by transfection with pcDNA3.1-RNA-422. A: Representative cell proliferation images of SW1116 cells by transfecting with empty vectors and pcDNA3.1-lncRNA-422. B: Real-time PCR analysis of lncRNA-422 levels in SW1116 cells by transfecting with empty vectors and pcDNA3.1-lncRNA-422.

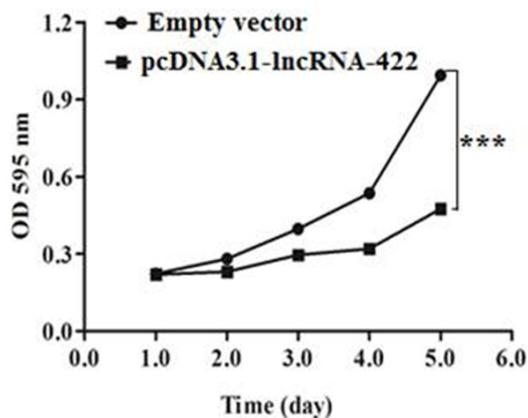


Figure 2. Overexpression of lncRNA-422 inhibited proliferation of SW1116 cells transfected with pcDNA3.1-RNA-422. *** $P < 0.001$.

in RPMI-1640 containing 10% fetal bovine serum (FBS) (Gibco, Grand Island, NY, USA), 100 U/ml of penicillin sodium (Sigma, USA) at 37°C in a humidified environment by containing 5% CO₂.

Construction of lncRNA422 plasmid and transfection

To generate plasmid particles with lncRNA-422 overexpression, ligation reactions of the lncRNA-422 gene fragments and pcDNA3.1 vectors were conducted using T4 DNA ligase Kit (TaKaRa), and the products were transformed into *E. coli* JM109 competent bacteria. Monoclonal colonies were picked, and positive recombinants were identified. Subsequently, SW-1116 cells were transfected with pcDNA3.1-

lncRNA-422 and empty vectors, respectively. Successful transfections were confirmed by counting the number of green fluorescent protein (GFP) positive cells under a fluorescence microscope.

Reverse transcription-quantitative polymerase chain reaction analysis

After 5-day and 6-day infection, SW1116 cells were lysed to extract total RNA. Complementary DNA (cDNA) was then synthesized from 1 µg RNA, and the primers were synthesized by TaKaRa. The sequences are as follows: forward: 5'-CC AAG CTT GTC TTT TTC AGT CTG AAG TCT TTA TTT GG-3', and reverse: 5'-CTC TCT CTC GAG TAA ACC ACC AAA TTT TTA TTA AGA T-3'. GAPDH (forward primer: 5'-GGGAGCCAAAAGGGT-CAT-3'; and reverse primer: 5'-GAGTCCTCCAC-GATACCAA-3') was used as an internal control. Then RT-qPCR was performed on BioRad Connet Real-Time PCR platform with prepared 20 µl PCR reaction mixture (10 µl 2×SYBR premix ex taq, 0.8 µl primers, 4.2 µl ddH₂O and 5.0 µl cDNA) using the following amplified procedure: pre-denaturation at 95°C for 1 min, followed by 40 cycles consisting of 5 s denaturation at 95°C and 20 s annealing extension at 60°C. The relative gene expression was analyzed using 2^{-ΔΔCt} method.

MTT assay

SW1116 Cells (4×10³/well) were plated onto 96-well plates after infection for 3 days. MTT assay was used to determine cell viability. Briefly, total 20 µl MTT solution was added to

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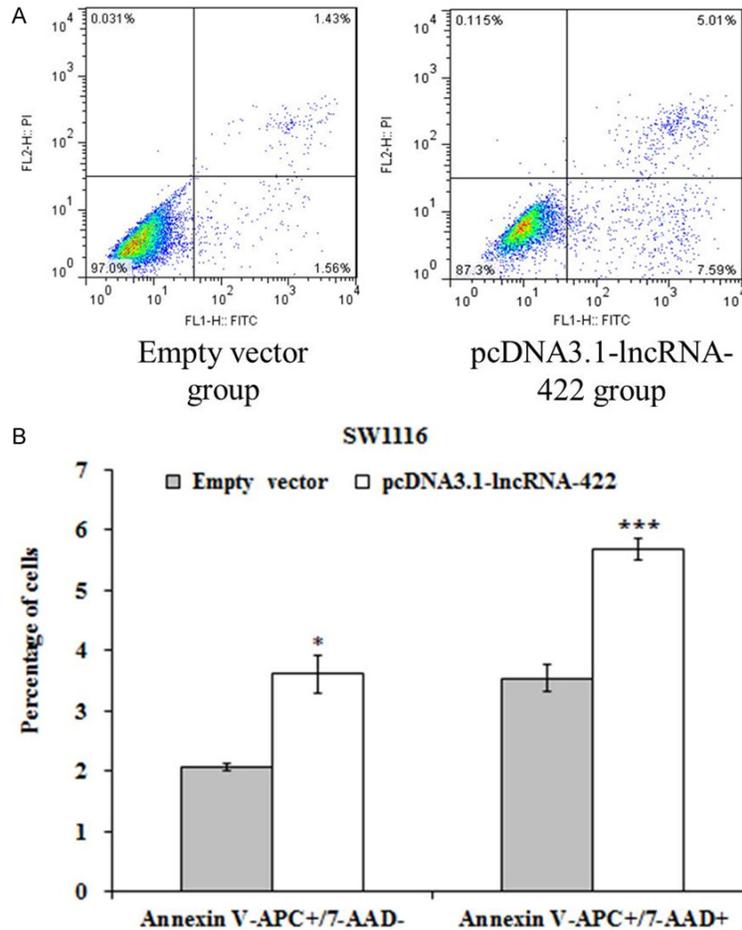


Figure 3. Overexpression of LncRNA-422 induced apoptosis in SW1116 cells. A: Cell apoptosis of SW1116 cells was determined by flow cytometry in transfecting with pcDNA3.1-RNA-422 and empty vector. B: Quantification of apoptotic cells in SW1116 cells by flow cytometry. Annexin V+/7-AAD⁻, early apoptotic cells; annexin V+/7-AAD⁺, late apoptotic cells. * $P < 0.05$, *** $P < 0.001$.

each well at day 1, 2, 3, 4 and 5 and incubated at 37°C for 4 h. Then the samples were dissolved in 100 μ l acidic isopropanol containing 5% isopropanol, 10% SDS and 0.01 mol/L HCl and the optical density of each well was measured by a microplate reader at 595 nm. Each test was conducted in triplicate.

Annexin V/7-AAD staining

After 24 h, the transfected cells were harvested by trypsinization and Annexin V-APC/7-AAD Apoptosis Kit (KA3808, Abnova) was used to evaluate SW1116 cells apoptosis according to the manufacturer's instruction. Percentages of cells stained with Annexin V-APC and/or 7-AAD relative to total cell numbers were measured

with Becton-Dickinson FAC-alibur system.

Transwell invasion assays

Transwell assays were performed using Boyden's chambers. Cells were planted in the upper chamber consisting of 8-mm membrane filter inserts coated with (for invasion assay) or without (for migration assay) Matrigel (BD Biosciences). The chemoattractant in the lower chamber was supplemented with medium containing 10% FBS. Cells on the upper surface were removed after 24 h, and those attached on the lower side of the membrane were fixed and stained with crystal violet before counting under a microscope in five randomly selected fields. At least three chambers from three different experiments were analyzed.

Wound healing assay

Cultured plates were seeded on the back of the line before the label, cell digestion after access to 12-well plate, perpendicular to the orifice to create cell scratches. Absorb the cell culture medium, rinse the orifice plate three times with

PBS, and wash away the scratches generated cell debris. Add serum-free medium, the culture plate into the incubator culture, every 4-6 hours to take pictures. Analyze the experimental results based on the collected image data.

Western blot assay

SW1116 cells were lysed with RIPA protein extraction reagent (Beyotime, Beijing, China) supplemented with a protease inhibitor cocktail (Roche, CA, USA). Protein extracts (40 μ g) were separated by 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to 0.22- μ m nitrocellulose membranes (Sigma), following incubation with appropriated primary and secondary antibodies. GAPDH was

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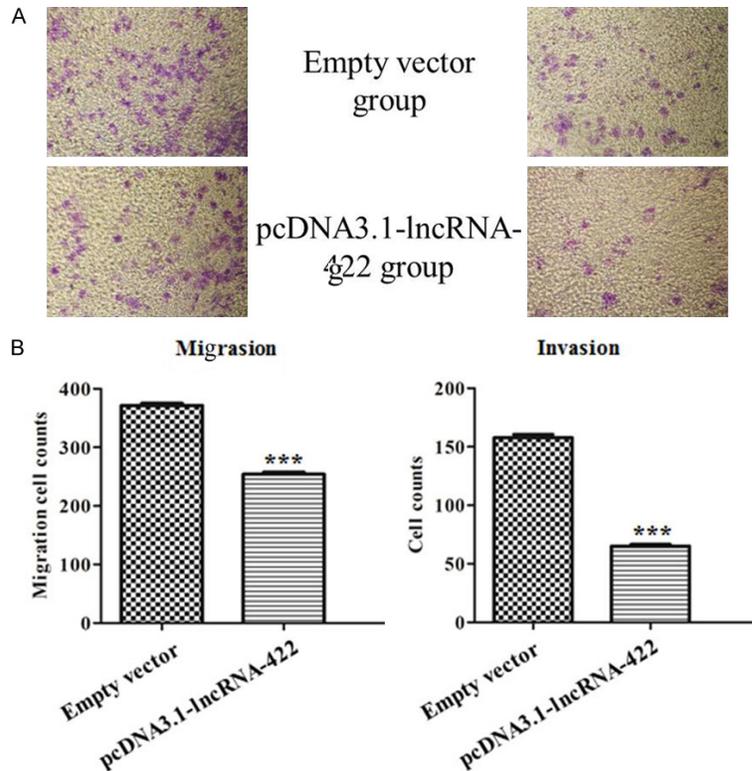


Figure 4. Overexpression of lncRNA-422 obviously decreased cell migration and invasion capacities. A: Wound Healing and Transwell assays were performed to detect cell migration and invasion capacities in SW1116 cells by transfecting with pcDNA3.1-RNA-422 and empty vector. B: Quantification of Wound Healing and Transwell assays results. *** $P < 0.001$.

used as a control. ECL chromogenic substrate was used, and signals were quantified by densitometry (Quantity One software, BioRad).

Statistical analysis

The data were presented as mean \pm standard deviation (SD). Statistical analysis was performed using the SPSS version 15.0 software (SPSS, Chicago, IL, USA). Statistical analysis was conducted using a Student's *t*-test. Differences at the level of $P < 0.05$ were considered to be statistically significant.

Results

LncRNA-422 was upregulated after transfection in SW1116

To assess the role of lncRNA-422 in CRC, we established lncRNA-422 overexpressed SW1116 cells by infecting with pcDNA3.1-lncRNA-422. Controls were transfected with empty vec-

tors. As depicted in **Figure 1A**, there were more than 80% cells infected with plasmid vectors as indicated by GFP positive cells under green fluorescence, indicating a successful infection. To further confirm the transfection efficiency, lncRNA-422 expression levels were examined using qRT-PCR. As shown in **Figure 1B**, lncRNA-422 expression levels were increased after transfection with pcDNA3.1-lncRNA-422. These results suggested that lncRNA-422 was up-regulated stably in CRC cells after transfection.

Overexpression of lncRNA-422 inhibited SW1116 cell proliferation

To confirm the effect of lncRNA-422 on CRC cell proliferation, MTT assay was performed. As shown in **Figure 2**, SW1116 cell growth was suppressed due to overexpression of lncRNA-422 ($P < 0.001$). Furthermore, 5 days after transfection, the viability of SW1116 cells with lncRNA-422 overexpression was over 2-fold lower than that in control group.

Overexpression of lncRNA-422 induced SW1116 cell apoptosis

For further confirm whether overexpression lncRNA-422 caused SW1116 cell apoptosis, the quantitative analysis of SW1116 cell apoptosis was monitored by using Annexin V-APC/7-AAD Apoptosis Kit. As depicted in **Figure 3**, the apoptotic rates, including early apoptosis (Annexin V+/7-AAD-) and late apoptosis (Annexin V+/7-AAD+), in SW1116 cells with lncRNA-422 overexpression were all significantly increased ($P < 0.05$).

Overexpression lncRNA-422 suppresses SW1116 cell invasion

The above findings indicated that overexpressed lncRNA-422 inhibited the proliferation of SW1116 cells. Thus, we detected the migration

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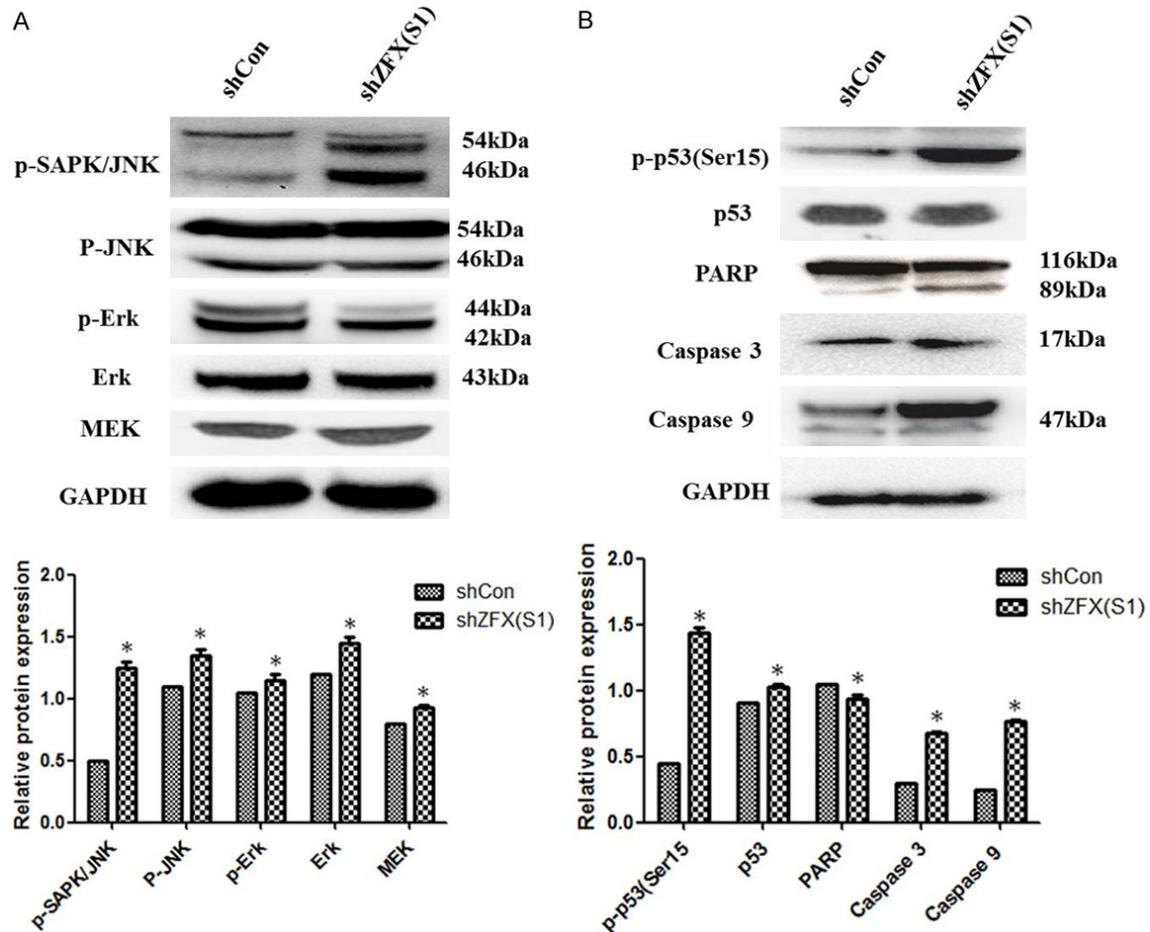


Figure 5. Markers of cell apoptosis pathway were determined in lncRNA-422-overexpressed cells. A: Downstream protein expression levels of DUSP4 and DUSP6, including JNK, p38, Erk, were increased in lncRNA-422-overexpressed SW1116 cells. B: Cell apoptosis-related proteins, including p-p53, PARP, caspase-3, were increased in lncRNA-422-overexpressed SW1116 cells. * $P < 0.05$.

and invasion of SW1116 cells following pcDNA3.1-lncRNA-422 transfection. Wound healing assay was demonstrated that overexpression of lncRNA-422 inhibited the migration of SW1116 cells by comparing with the control empty vector-transfected cells (Figure 4A). In analogical results observed in transwell assay, the SW1116 cells with lncRNA-422 overexpression showed decreased invasion capacities in comparison with control cells (Figure 4B).

Overexpression of lncRNA-422 induces SW1116 apoptosis

To explore the mechanisms of lncRNA-422 in regulating tumorigenesis, we then investigated whether lncRNA-422 regulates cell apoptosis-related signals. The expression of apoptosis-related proteins was detected by western blot

analysis. As shown in Figure 5A, up-regulated lncRNA-422 resulted in an increase in the phosphorylation levels of p-38, SAPK/JNK (p54 and p46) and MEK. Furthermore, cell apoptosis-related proteins, including p-p53, PARP, caspase-3 were also activated in the SW1116 cells with lncRNA-422 overexpression by comparing with control cells (Figure 5B).

Discussion

As research on human cancer was developing rapidly, lncRNAs had been regarded as crucial regulators in various biological processes [18, 19]. lncRNAs played important roles in carcinogenesis and cancer metastasis in CRC, a serious disease that was complex and heterogeneous [19, 20]. Wu found that lncRNAs might function as oncogenes or tumor suppressors in

the cancer initiator [21]. In 2003, MALAT1 was first found to be highly expressed in non-small cell lung cancer by Ji [22]. Ji's study showed that the high expression of MALAT1 was associated with metastasis and poor prognosis. Finally in 2011, Xu identified the functional motif of MALAT-1 in CRC. They found a motif of the 3' end MALAT-1 gene layered an important role in the biological processes of human colorectal malignancies [23].

In addition, Zheng conducted an important experiment to assess MALAT-1 expression in 146 CRC patients (stage II/III) and 23 paired normal colonic mucosa samples [24]. Results presented that the expression of MALAT-1 was up-regulated in CRC tissues, and the over-expression of MALAT-1 might serve as a negative prognostic marker in stage II/III CRC patients. In recent years, HOTAIR was found significantly overexpressed in many cancers, such as breast cancer, hepatocellular cancer and laryngeal squamous cell carcinoma [25, 26]. Kogo's group found that the expression levels of HOTAIR were higher in cancerous tissues than in noncancerous tissues, they also indicated that expression of HOTAIR and members of the PRC2 complex (SUZ12, EZH2, and H3K27me3) had a close correlation [12].

LncRNA-422 might be implicated in carcinogenesis through regulating protein coding genes involved in special biological process relevant to cancer [17]. In the present study, we demonstrated that lncRNA-422 could inhibit CRC cell proliferation and invasiveness. Overexpression lncRNA-422 SW1116 cell line was generated, and functional experiments showed that up-regulated lncRNA422 expression could induce SW1116 cell apoptosis, and the ability of migration and invasion were also obviously decreased after transfection with pcDNA3.1-lncRNA-422. We further investigated the markers of cell apoptosis pathway in lncRNA-422-overexpressed cells by western blot. Results indicated that lncRNA-422 could promote the activities of JNK, p38, Erk. And up-regulated the expression of lncRNA-422 could induce the SW1116 cells apoptosis through caspase pathway.

In conclusion, our results suggested that lncRNA-422 was involved in the progression of CRC and might provide evidence for lncRNA-422 being as a potential target for therapy of this disease in the near future.

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Liang Xu, Department of Gastrointestinal Surgery, The Affiliated Hospital of Southwest Medical University, Taiping Road 25, Luzhou 646000, Sichuan, China. Tel: +86-13708289307; E-mail: yhui618@126.com

References

- [1] Jemal A, Bray F, Center MM, Ferlay J, Ward E and Forman D. Global cancer statistics. *CA Cancer J Clin* 2011; 61: 69-90.
- [2] Siegel RL, Miller KD and Jemal A. Cancer statistics, 2015. *CA Cancer J Clin* 2015; 65: 5-29.
- [3] Sung JJ, Lau JY, Goh KL, Leung WK; Asia Pacific Working Group on Colorectal Cancer. Increasing incidence of colorectal cancer in Asia: implications for screening. *Lancet Oncol* 2005; 6: 871-876.
- [4] Vaiopoulos AG, Athanasoula K and Papavassiliou AG. Epigenetic modifications in colorectal cancer: molecular insights and therapeutic challenges. *Biochim Biophys Acta* 2014; 1842: 971-980.
- [5] Colussi D, Brandi G, Bazzoli F and Ricciardiello L. Molecular pathways involved in colorectal cancer: implications for disease behavior and prevention. *Int J Mol Sci* 2013; 14: 16365-16385.
- [6] Mercer TR, Dinger ME and Mattick JS. Long non-coding RNAs: insights into functions. *Nat Rev Genet* 2009; 10: 155-159.
- [7] Wilusz JE, Sunwoo H and Spector DL. Long noncoding RNAs: functional surprises from the RNA world. *Genes Dev* 2009; 23: 1494-1504.
- [8] Whitehead J, Pandey GK and Kanduri C. Regulation of the mammalian epigenome by long noncoding RNAs. *Biochim Biophys Acta* 2009; 1790: 936-947.
- [9] Wapinski O and Chang HY. Long noncoding RNAs and human disease. *Trends Cell Biol* 2011; 21: 354-361.
- [10] Qi P, Xu MD, Ni SJ, Huang D, Wei P, Tan C, Zhou XY and Du X. Low expression of LOC285194 is associated with poor prognosis in colorectal cancer. *J Transl Med* 2013; 11: 122.
- [11] Ge X, Chen Y, Liao X, Liu D, Li F, Ruan H and Jia W. Overexpression of long noncoding RNA PCAT-1 is a novel biomarker of poor prognosis in patients with colorectal cancer. *Med Oncol* 2013; 30: 588.
- [12] Kogo R, Shimamura T, Mimori K, Kawahara K, Imoto S, Sudo T, Tanaka F, Shibata K, Suzuki A, Komune S, Miyano S and Mori M. Long non-coding RNA HOTAIR regulates polycomb-dependent chromatin modification and is associ-

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- ated with poor prognosis in colorectal cancers. *Cancer Res* 2011; 71: 6320-6326.
- [13] 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, Mimori K, Mori M, Sieuwerts AM, Martens JW, Tomlinson I, Negrini M, Berindan-Neagoe I, Foekens JA, Hamilton SR, Lanza G, Kopetz S, Fodde R and Calin GA. CCAT2, a novel noncoding RNA mapping to 8q24, underlies metastatic progression and chromosomal instability in colon cancer. *Genome Res* 2013; 23: 1446-1461.
- [14] Yan B, Gu W, Yang Z, Gu Z, Yue X, Gu Q and Liu L. Downregulation of a long noncoding RNA-ncRuPAR contributes to tumor inhibition in colorectal cancer. *Tumour Biol* 2014; 35: 11329-11335.
- [15] Madamanchi NR, Hu ZY, Li F, Horaist C, Moon SK, Patterson C, Runge MS, Ruef J, Fritz PH and Aaron J. A noncoding RNA regulates human protease-activated receptor-1 gene during embryogenesis. *Biochim Biophys Acta* 2002; 1576: 237-245.
- [16] Lian Y, Ding J, Zhang Z, Shi Y, Zhu Y, Li J, Peng P, Wang J, Fan Y, De W and Wang K. The long noncoding RNA HOXA transcript at the distal tip promotes colorectal cancer growth partially via silencing of p21 expression. *Tumour Biol* 2016; 37: 7431-7440.
- [17] Xue Y, Ma G, Gu D, Zhu L, Hua Q, Du M, Chu H, Tong N, Chen J, Zhang Z and Wang M. Genome-wide analysis of long noncoding RNA signature in human colorectal cancer. *Gene* 2015; 556: 227-234.
- [18] Xu MD, Qi P and Du X. Long non-coding RNAs in colorectal cancer: implications for pathogenesis and clinical application. *Mod Pathol* 2014; 27: 1310-1320.
- [19] Prensner JR and Chinnaiyan AM. The emergence of lncRNAs in cancer biology. *Cancer Discov* 2011; 1: 391-407.
- [20] Gutschner T and Diederichs S. The hallmarks of cancer: a long non-coding RNA point of view. *RNA Biol* 2012; 9: 703-719.
- [21] Wu W and Chan JA. Understanding the role of long noncoding RNAs in the cancer genome. *Journal of the Mathematical Society of Japan* 2014; 19: 30-31.
- [22] Ji P, Diederichs S, Wang W, Boing S, Metzger R, Schneider PM, Tidow N, Brandt B, Buerger H, Bulk E, Thomas M, Berdel WE, Serve H and Muller-Tidow C. MALAT-1, a novel noncoding RNA, and thymosin beta4 predict metastasis and survival in early-stage non-small cell lung cancer. *Oncogene* 2003; 22: 8031-8041.
- [23] Xu C, Yang M, Tian J, Wang X and Li Z. MALAT-1: a long non-coding RNA and its important 3' end functional motif in colorectal cancer metastasis. *Int J Oncol* 2011; 39: 169-175.
- [24] Zheng HT, Shi DB, Wang YW, Li XX, Xu Y, Tripathi P, Gu WL, Cai GX and Cai SJ. High expression of lncRNA MALAT1 suggests a biomarker of poor prognosis in colorectal cancer. *Int J Clin Exp Pathol* 2014; 7: 3174-3181.
- [25] Chisholm KM, Wan Y, Li R, Montgomery KD, Chang HY and West RB. Detection of long non-coding RNA in archival tissue: correlation with polycomb protein expression in primary and metastatic breast carcinoma. *PLoS One* 2012; 7: e47998.
- [26] Li D, Feng J, Wu T, Wang Y, Sun Y, Ren J and Liu M. Long intergenic noncoding RNA HOTAIR is overexpressed and regulates PTEN methylation in laryngeal squamous cell carcinoma. *Am J Pathol* 2013; 182: 64-70.