

Original Article

Decreased expression of long noncoding RNA MT1JP may be a novel diagnostic and predictive biomarker in gastric cancer

Jian Yang¹, Yongbin Zhang², Piao Liu³, Hailong Yan¹, Jichun Ma³, Mingxu Da²

¹Clinical Medical College, Ningxia Medical University, Yinchuan 750004, P. R. China; ²Department of Surgical Oncology, Gansu Province People's Hospital Lanzhou, Gansu, P. R. China; ³Clinical Medical College, Gansu University of Traditional Chinese Medicine, Lanzhou 730000, P. R. China

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Abstract: Growing evidence suggests that long non-coding RNAs (lncRNAs) may play critical roles in epithelial-to-mesenchymal transition (EMT) progress. However, the complex molecular mechanisms remain largely unclear. In this study, the expression levels of lncRNAs and their target genes in EMT pathway-focused was measured by lncPath™ microarray. Levels of representative lncRNA-metlothionein 1J, pseudogene (MT1JP) and mRNA-metlothionein 1M (MT1M) were then further verified by real-time quantitative reverse transcription PCR (qRT-PCR). The relationship of their levels with clinicopathological factors was evaluated in patients with gastric cancer (GC). Receiver operating characteristic curve was used analyses potential diagnostic values. MT1JP and MT1M were downregulated in GCs tissues from microarray analysis results, which were further confirmed by qRT-PCR. The areas under ROC curves were 0.710 and 0.657 for MT1JP and MT1M, respectively. The level of MT1JP in normal tissue is significantly higher than that in precancerous lesions and early cancers. lncRNA Microarrays provide comprehensive insights into the expressional relationship between lncRNAs and their target genes, which will be helpful for establishing rapid connections between the lncRNA regulatory mechanisms and their biological functions. The reduced expression of MT1JP and MT1M in gastric cancer tissues, its associations with tumor diameter, differentiation, lymphatic metastasis, distal metastasis, invasion and tumor-node-metastasis (TNM) stage, and its aberrant expression in early cancer and precancerous lesions suggest that they may be a potential biomarker for the diagnosis of GC.

Keywords: Gastric cancer (GC), biomarker, long non-coding RNA (lncRNA), epithelial-to-mesenchymal transition (EMT)

Introduction

Gastric cancer is one of the most common and aggressive human malignancies in the world, with a constantly increasing frequency, especially in China [1, 2]. Although recent advances in clinical and experimental oncology, have significantly improved outcomes for patients with GC, long-term prognosis of GC remains poor, due to late detection of disease, frequent cancer metastasis, high recurrence rate, and lack of effective therapeutic intervention for terminally staged tumors [3, 4]. Unfortunately, the complex molecular mechanisms underlying its carcinogenesis and progression remain largely unclear. Therefore, there is an urgent need to identify novel oncogenes and clinically applica-

ble molecular targets for its early diagnosis, effective therapy, and prognosis evaluation.

Long noncoding RNAs (lncRNAs) are a class of transcripts longer than 200 nucleotides with non-protein-coding potential [5]. Recently, many studies have shown that lncRNAs are abnormal expression in carcinomas and they are emerging as potent regulators modulate tumor progression and metastasis [6, 7]. Metastasis is complex process that involves aberrant proliferation and changes the migratory properties of tumor cells, it is a key process in tumor progression [8, 9]. Epithelial-to-mesenchymal transition (EMT) has been shown to be of critical importance in the early events of tumor cell metastatic dissemination by endowing epitheli-

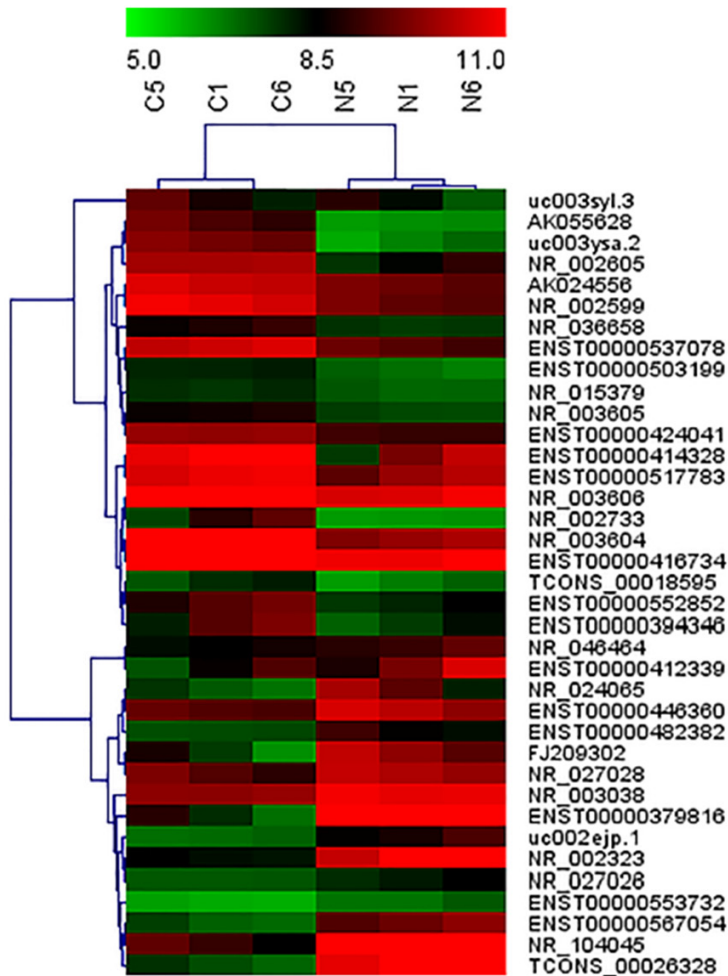


Figure 1. Alterations in lncRNA expression profiles between gastric carcinoma tissues and non-tumorous tissues in lncRNA microarray. Hierarchical clustering analysis showed the 39 significantly dysregulated lncRNAs among samples. The normalized expression values are represented in shades of red and blue, indicating expression above (blue) and below (red) the median expression value across the 6 samples.

um-derived cancer cells with a more motile, invasive potential [10].

EMT seems to drive the initiation of metastasis. Although several lncRNAs have been reported to modulate tumor metastases [11, 12], the specific roles of lncRNAs in regulating EMT are not well studied.

In this study, we investigated the expression level of lncRNAs and corresponding target genes related to EMT signaling pathways in Gastric cancer. lncRNA-MT1JP (uc002ejp.1), at band of 16q13 of chromosome 16, locate 1776 bp away from the nearest pathway genes

MT1M, the expression of MT1JP and MT1M in GC tissues remains unreported. We investigated the potential relationship between the expression of MT1JP/MT1M and clinicopathological features of patients with GC. Our results suggest the potential of lncRNA-MT1JP as a novel diagnostic biomarker and a treatment target for GC.

Materials and methods

Patients and specimens

During February 2013 to June 2015, we collected tissue samples from surgical or biopsy specimens at Gansu Province People Hospital. Ninety-three paired gastric cancer tissues and corresponding adjacent non-tumorous tissues, thirteen precancerous lesion tissues, and ten early cancer tissues were collected in consecutive cases that underwent surgery or endoscopic biopsy. All tissues were preserved in liquid nitrogen immediately after resection. RNA was extracted from these samples and stored at -80°C refrigerator. Each tissue sample was histopathologically confirmed by at least two pathologists. Tumor-node-metastasis (TNM) stage

and histological grade were in accordance with the guidelines of the American Joint Committee on Cancer (ACJJ, 7th Ed) and the World Health Organization digestive system cancer guidelines. All patients received no treatment prior to resection. In this study, all individual participants signed informed consent. The Ethics Committee of Gansu Province People Hospital approved this study.

Total RNA preparation and cDNA synthesis

Total RNAs was isolated from 100 mg tissue using Trizol reagent (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instruc-

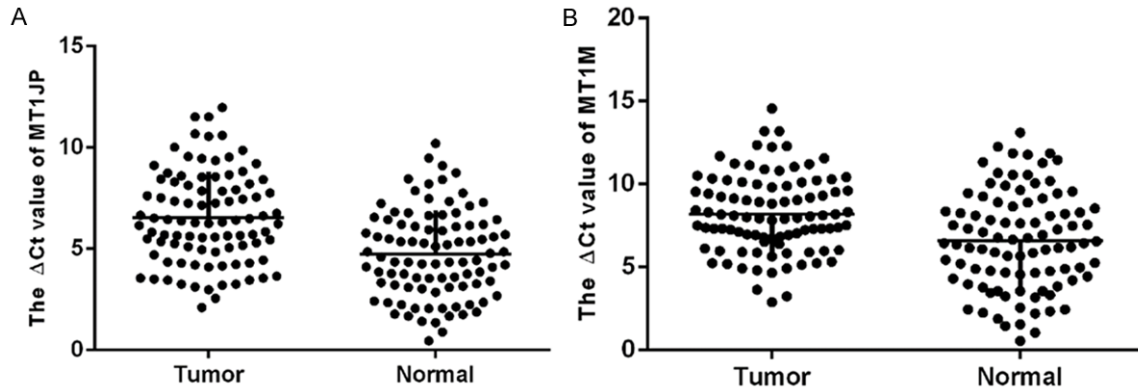


Figure 2. MT1JPA and MT1M were down-regulated in gastric cancer tissues. Real-time qRT-PCR was used to determine the expression level of MT1JPA and MT1M. The Δ Ct value was determined by subtracting the β -actin Ct value from the target lncRNA Ct value. Smaller Δ Ct value indicates higher expression.

tions. Genomic DNA contamination was removed by on-column DNase treatment. The purity and concentration of the RNA was determined using the Nanodrop ND-1000 (Nanodrop technologies). At least 100 ng RNA was reverse-transcribed into cDNA using the iScript cDNA synthesis kit (BioRad) according to the supplier's protocol. First-strand cDNA was generated using the Reverse Transcription System Kit (Takara, Dalian, China).

LncRNAs microarray assay

The LncPath™ Human EMT Pathway LncRNA Microarray (Arraystar Inc., MD, USA) were used in this study, which profile 390 LncRNAs and 219 their target genes simultaneously in EMT pathway-focused from the authoritative data sources. The sample preparation and microarray hybridization were performed based on the manufacturer's standard protocols. For microarray analysis, Agilent Array platform was employed.

QRT-PCR detection of MT1JP and MT1M

Real-time quantitative PCR (qPCR) was performed on a LightCycler® Nano (Roche) using the 2× IQ SYBR Green PCR mix (BioRad) and 400 nM of the forward and reverse primer. The data were analyzed by the Δ Ct method [13]. All results were expressed as the Means \pm SD of three independent experiments. The sequences of the PCR primers for MT1JP, MT1M, and β -actin were as follows: MT1JP 5'-GAAATGG-ACCCAACTACTCC-3' (sense); 5'-GTTCCACATCAGGCACAG-3' (antisense) MT1M; 5'-GCAGC-WCTTCTTGCAGCAGG-3' (sense); 5'-CCGTCGG-

GGTGGGGCCTA-3' (antisense); β -actin 5'-CCA-CGGCTGCTTCCAGCTCC-3' (sense); 5'-GGACT-CCATGCCAGGAAGGAA-3' (antisense).

Serological tumor marker analysis

Using an AU5400 Automation Chemistry System Instrument (Olympus, Tokyo, Japan) measure serum carcinoembryonic antigen (CEA) and carbohydrate antigen 19-9 (CA19-9). The cutoff values for CEA and CA19-9 were respectively 5 ng/ml and 35 U/ml.

Statistical analysis

Statistical analysis was performed using SPSS 23.0 software (SPSS, Chicago, IL) and GraphPad Prism 6.0 (GraphPad Software, La Jolla, CA). Data were summarized as mean \pm SD. Statistical analysis of group differences was performed using the one-way analysis of variance test, two-tailed Student's t-test and rank-sum test in this work. A receiver operating characteristic (ROC) curve was established to evaluate the diagnostic value. $P < 0.05$ was considered statistically significant.

Results

Aberrantly expression of LncRNAs associated with EMT in gastric carcinoma

LncPath™ Human EMT Pathway LncRNA Microarray was used to analyze lncRNA expression profiles 3 GC tissues and their paired adjacent non-neoplastic gastric tissues, the expression levels of 390 LncRNAs and 219 target genes related to epithelial mesenchymal transition were detected. Total of 39 lncRNAs, which

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Table 1. Correlation of clinicopathological factors with MT1JP and MT1M expression in gastric cancer

Characteristics	No. of patients (%)	MT1JP		MT1M	
		Mean ± SD	P value	Mean ± SD	P value
Age (y)					
≥ 60	31 (33.3)	6.42±2.43	0.780	8.04±2.56	0.665
< 60	62 (66.7)	6.57±2.21		8.27±2.26	
Gender					
Male	41 (44.1)	6.62±2.17	0.730	8.25±2.28	0.097
Female	52 (55.9)	6.45±2.37		8.15±2.24	
Diameter (cm)					
≥ 5	64 (68.8)	6.86±2.32	0.036	8.55±2.38	0.031
< 5	29 (31.2)	5.80±2.02		7.42±2.13	
CEA					
Positive	66 (71.0)	6.11±1.97	0.280	8.32±2.39	0.410
Negative	27 (29.0)	6.69±2.37		7.88±2.26	
CA19-9					
Positive	60 (64.5)	6.07±2.01	0.150	8.46±2.40	0.142
Negative	33 (35.5)	6.78±2.38		7.71±2.21	
Differentiation					
Well	7 (7.5)	4.20±1.14	0.010	5.76±1.43	0.010
Moderate	32 (34.4)	6.02±1.78		7.64±1.92	
Poor	54 (58.1)	7.13±2.39		8.84±2.41	
Lymphatic metastasis					
N0	33 (35.5)	5.77±2.09	0.016	7.35±2.25	0.009
N1&N2&N3	60 (64.5)	6.94±2.23		8.66±2.29	
Distal metastasis					
M0	82 (88.2)	6.36±2.28	0.048	8.02±2.37	0.053
M1	11 (11.8)	7.80±1.81		9.48±1.81	
Invasion					
Tis&T1-T3	19 (20.4)	5.48±1.84	0.023	7.04±2.03	0.016
T4	74 (79.6)	6.80±2.31		8.49±2.35	
TNM stage					
0&I&II	24 (25.8)	5.65±1.86	0.027	7.24±2.02	0.020
III&IV	69 (74.2)	6.83±2.33		8.53±2.38	

expression change was more than 1.5 fold and $P < 0.05$. Among them, 22 up expressed and 17 down expressed (**Figure 1**). MT1JP expression was downregulated (mean fold change 3.43, $P < 0.05$). MT1JP was annotated with the coding gene MT1M (mean fold change 4.42, $P < 0.05$), potential regulatory mechanisms “neighboring lncRNA”. We chose MT1JP and MT1M for further research.

QRT-PCR validation for MT1JP and MT1M expression were downregulated in GCs tissues

we examined MT1JP and MT1M expression in gastric cancer tissue samples and paired adja-

cent non-neoplastic tissue samples. The results showed that MT1JP and MT1M were downregulated in 86.02% (13/93) and 80.64% (18/93) of GC tissues relative to that in the non-neoplastic tissues ($P < 0.01$, **Figure 2**). Taken together, these data indicate that MT1JP and MT1M are downregulated in human GC.

Correlation of clinicopathological factors with MT1JP and MT1M expression of GC patients

To evaluate the role of MT1JP and MT1M in gastric cancer progression, the correlation between MT1JP and MT1M expression and clinicopathological features of patients was analyzed. As summarized in **Table 1**, there were no significant correlations between the expression level of MT1JP and patient age, gender, CEA and CA19-9 in patients with gastric cancer. However, there was a close relationship between the positive rate of MT1JP and tumor diameter ($P = 0.036$), degree of histological differentiation ($P = 0.01$) lymphatic metastasis ($P =$

0.016), distal metastasis ($P = 0.048$), tumor invasion ($P = 0.023$), and TNM stage ($P = 0.012$). Expression of MT1M in the gastric cancer tissue was correlated with tumor diameter ($P = 0.031$), degree of histological differentiation ($P = 0.01$) lymphatic metastasis ($P = 0.009$), distal metastasis ($P = 0.053$), tumor invasion ($P = 0.016$), and TNM stage ($P = 0.020$), but not with age, gender, CEA and CA19-9 ($P > 0.05$).

Diagnostic values of MT1JP and MT1M for GC

Receiver operating characteristic curve analyses indicated that MT1JP and MT1M have some

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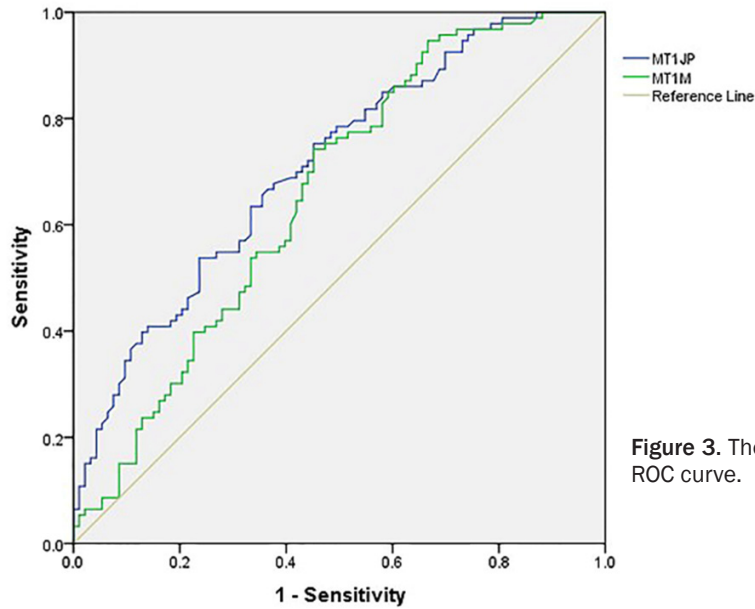


Figure 3. The ROC curve.

capability for distinguishing GC tissues from benign tissues. The areas under ROC curve values were 0.710 and 0.657 for MT1JP and MT1M, respectively (**Figure 3**). The positive detection rates of MT1JP and MT1M are 86.0% and 80.9%. Obviously, both of them are higher than gastric cancer biomarker CEA (71.0%) and CA19-9 (64.5%) (**Table 1**).

Expression of MT1JP was aberrant in precancerous lesions and precancerous

We measured the expression level of MT1JP in early cancer and precancerous lesions to search the early diagnostic values. We were very surprised to find that the level was conspicuous lower in these precancerous lesions compared with those of corresponding adjacent non-tumorous tissues (**Figure 4A**). The level of MT1JP in normal tissues was remarkable higher than that in precancerous lesions and early cancer tissues. Besides, the expression level in precancerous lesions tissues was significantly higher than the early cancer tissues (**Figure 4B**).

Discussion

Gastric cancer patients currently have a poor prognosis, and early detection and treatment could significantly increase their chances of survival. Recently, lncRNAs have shown as potent regulators of tumor progression and

metastasis [14, 15]. Some lncRNAs, such as HOTAIR [16], H19 [17], GAPLINC [18], HULC [19] and SPRY4-IT1 [20], were found to be significantly elevated in GC, and as regulators of cell invasion and metastasis in gastric cancer. However, BM742401 [21], FENDRR [22] and MEG3 [23] lncRNAs were downregulated in gastric cancer, which could inhibit cell-cycle progression and promote apoptosis of gastric cancer cells. Nonetheless, the current understanding of lncRNA biology is far behind the information available on the functions of the protein-coding transcriptome [14].

Growing evidence suggests that lncRNAs may play critical roles in EMT progress not only in GC [16, 20] but also in other cancers [24, 25]. To study the potential biological functions of lncRNAs in GC, we firstly assessed the lncRNAs and their target genes expression profile simultaneously in EMT pathway-focused in human GC through LncPath™ Human EMT Pathway lncRNA Microarray. We focused on lncRNA-MT1JP and mRNA-MT1M, further validated expression levels by qPCR. We found that MT1JP and MT1M expression in GC were significantly downregulated (**Figure 2**). More importantly, the expression levels of MT1JP and MT1M were significantly correlated with lymph node metastasis, distant metastasis, TNM stages, and differentiation. These results suggest that MT1JP and MT1M may as an important player in inhibiting the development of gastric cancer.

Recent studies showed that lncRNAs can guide changes in gene expression either in *cis* (on neighboring genes) or in *Trans* (distantly located genes) manner [26, 27]. MT1JP may exert its functional role by regulating the neighboring gene MT1M at transcriptional or posttranscriptional level. MT1M, encodes a member of the metallothionein superfamily, is located next to MT1JP. MT1M expression markedly decreased in human HCC specimens and identified as a suppressor of hepatocellular carcinoma [28]. MT1M promoter methylation status was high

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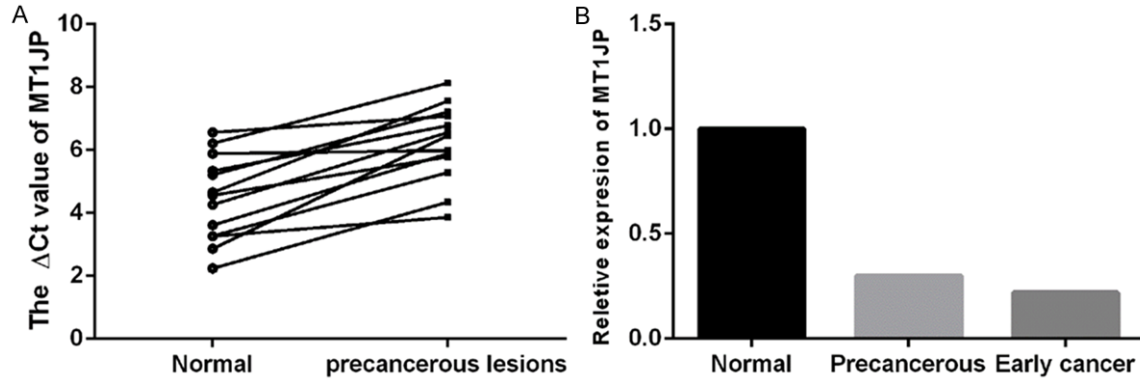


Figure 4. MT1JP expression in early cancer and precancerous lesions. The level of MT1JP in early pathological change tissue is lower than that in paired normal tissue ($n = 13$, $P = 0.0018$; A). The level of MT1JP in normal tissue is significantly higher than that in precancerous lesions and early cancers $P < 0.05$ (B).

expression in hepatocellular carcinoma [29]. Based on these findings, we speculate these that lncRNA MT1JP regulate EMT by guide MT1M promoter methylation status change in gastric cancer. However, a limitation current study is that we did not clearly demonstrate this molecular function which should be further explored.

Molecular tumor biomarkers are vital diagnostic and prognostic tools in the early stage to reduce GC mortality rate. Our data show that the expression of MT1JP was aberrant in early gastric cancer and gastric precancerous lesions (Figure 4A). And the extraordinary changes maybe appear in the precancerous lesions (Figure 4B). This investigation indicates that MT1JP may be a candidate biomarker of gastric cancer.

In summary, we depict lncRNAs and their target genes simultaneously in EMT pathway-focused expression that associated with gastric cancer. The reduced expression of lncRNA-MT1JP and mRNA-MT1M suggest that they may be participated in gastric cancer. lncRNA MT1JP may be a regulator of GC and may have potential as a novel biomarker and treatment target for this type of cancer.

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Disclosure of conflict of interest

None.

Address correspondence to: Dr. Mingxu Da, Department of Surgical Oncology, Gansu Province People's Hospital, 204 Donggang West Road, Lanzhou 730000, P. R. China. E-mail: hxdamingxu@hotmail.com

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